Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted October - December 2023 that require a retrospective assessment

Contents

1. Growth, development, ageing and repair of striated muscle and associated tissues

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Sarcopenia, Cardiomyopathy, striated muscle, skeleton, nervous system

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult, aged
Medaka (Oryzias latipes)	embryo, neonate, juvenile, adult
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	neonate, juvenile, adult, embryo, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description 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 molecular mechanisms of heart and skeletal muscle structure and growth, the decline of nerve/muscle/skeleton function in ageing and what might be done about it.

A retrospective assessment of these aims will be due by 09 April 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

How the growth of striated muscle is controlled is important for the following reasons:

1) Ageing muscle gets weaker and mitigating this will improve quality of life for most members of society.

2) Skeletal muscle genetic diseases such as Duchenne Muscular dystrophy could be prevented or reversed by enhancing growth of ‘corrected’ muscle.

3) Many other common diseases, such as cancer-related muscle wasting, rheumatoid arthritis and various other autoimmmune diseases cause skeletal muscle wasting, the reversal of which would be beneficial to patients.

4) Muscle changes are a major component of metabolic diseases such as obesity and type 2 diabetes. Understanding how muscle character, as well as muscle size, is controlled may permit reversal of pathogenic change.

5) Most heart disease involves either decrease (dilated cardiomyopathy) or increase (hypertrophic cardiomyopathy) in heart muscle size. Reversal or prevention of these changes would be beneficial.

6) Heart attack causes heart muscle cell death. Heart muscle regeneration is desirable, but currently impossible.

7) Muscle growth and character are important in sport.

8) Muscle growth rate and quality are major factors in livestock rearing and aquaculture.

Our MRC- and EU-funded projects aim to understand, reverse or prevent damaging changes in muscle occurring across the life course.

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

Increased understanding of vertebrate muscle structure and muscle growth control that will benefit medicine, meat production (including cellular agri/aquaculture), sports science and advance fundamental knowledge of how tissues respond to forces.

Peer-reviewed publications

Vertebrate animal models of human disease conditions that may facilitate trials of therapies

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

Short term (< 1 year)

- Continued scientific output to bring work from current Project Licence to publication.

- Researchers and students will benefit from training, ability to address important biomedical questions

Medium term (1-5 years)

- Academic research in heart, skeletal muscle, skeletal and neuroscience from increased understanding of muscle structure and growth of muscle and associated tissues through our publications and other communications.

- Academic researchers working in developmental biology, human genetics and physiology of sarcomeric muscle from increased understanding of processes controlling tissue growth and homeostasis from our publications and other communications.

- Sports scientists, agri/aquaculture scientists, myologists, human geneticists from increased understanding of processes controlling musculoskeletal growth and maintenance from our publications and other communications.

Long term (>5 years)

- The ageing human population from improved personalized genetic diagnosis, tailored lifestyle advice, and potential pharmaceutical interventions to treat ageing-related muscle wasting.

- People with muscle diseases such as muscular dystrophies, myasthenia gravis from improved personalized genetic diagnosis, tailored lifestyle advice, and potential pharmaceutical interventions.

- Diabetics and those overweight from improved personalized genetic diagnosis, tailored lifestyle advice, and potential pharmaceutical interventions.

- People with heart disease from improved personalized genetic diagnosis, tailored lifestyle advice, and potential pharmaceutical interventions.

- Various muscle diseases from better understanding of the native sarcomere structure.

- Cellular agriculture industry from understanding of controls of muscle growth in vivo that may help improve muscle growth in vitro.

- Aquaculture industry from understanding of genetic basis of control of muscle growth in vivo in fish.

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

Dissemination of new knowledge: we will continue to publish our work in quality peer-reviewed publications, discuss it in reviews, disseminate our new methods in appropriate online and print media, attend and speak at conferences. In conformity with UKRI/MRC policy, publications will be Gold Open Access.

We will engage with the public concerning our animal work through school visits and interaction with the press.

Protection of intellectual property: where appropriate, we will continue to consult with UKRI and institutional licencing departments concerning patenting and thus dissemination of our translational work.

Data without IP implications will be stored in suitable public or institutional repositories where practicable, and as frequently required by journals.

Species and numbers of animals expected to be used

- Mice: 200
- Rats: 200
- Zebra fish (Danio rerio): 40,000
- Medaka (Oryzias latipes): 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.

To understand the growth of skeletal and heart muscle in the context of the whole body, we must examine muscle growth in response to genetic variations and environmental stimuli such as training. This can only be done in the context of the whole organism, where the muscle in naturally activated to nerves, fed by blood vessels and connected to the skeleton.

For scientific (ease of imaging and manipulation) and 3Rs reasons we mainly work with zebrafish. However, we need to retain the capacity to test appropriate hypotheses in rodents, in order to translate our findings to the human context. Moreover, genetic resources, in particular, are sometimes avialble in medaka and are frequently better and/or already available in mice. The use of genetically altered mice or rats, when available, thus reduces the need to replicate work in the zebrafish and avoids certain technical barriers (such as the current inefficiency of precise genome editing by homologous recombination in the zebrafish and the partial genome duplication of zebrafish compared to mammals). Most of our zebrafish work occurs in embryonic/larval stages outside the Act, but to examine important processes of later growth and maintenance we need to employ genetically altered fish at all life stages. Additionally, we wish to examine the ageing process in zebrafish muscle in order to understand its similarities and differences, if any, to human muscle ageing. This can only be done in the context of the whole organism.
We are mainly focusing our studies on the early stages of zebrafish development (before independent feeding) to understand the basic cell and molecular genetic controls of muscle growth. Muscle growth involves a) generation of individual muscle cells from stem cells, b) fusion of individual cells to make muscle fibres, c) increase in fibre size to give each individual muscle its unique size and properties.

Each of these processes is precisely regulated during embryonic development of vertebrates by a combination of genetic and environmental factors that are poorly understood. Each is then maintained/adapted by exercise, nutrition, and other lifestyle and, potentially, genetic factors. These cannot currently be studied in cell or tissue culture due to lack of knowledge of the appropriate chemical and physical environmental factors required to mimic the conditions inside the animal. In important respects, vertebrates all build their muscle similarly, but differently from invertebrates.

Zebrafish have the advantage of excellent genetics and in vivo imaging, that allow analysis of muscle growth and maintenance in unprecedented detail and are therefore a model system of choice for understanding the fundamentals of human muscle biology and medicine.

In order to understand the progression of sarcopenia (ageing-related muscle weakening) and/or muscle diseases it is therefore necessary to grow zebrafish to such age as sarcopenia commences or diseases become apparent in order to examine the effects of genetic or other manipulation on sarcopenia. Again, this cannot currently be credibly be mimicked in vitro.

On occasion, it may be desirable to verify that controls on muscle size discovered in zebrafish also occur in mammals, prior to moving to human trials. This would necessitate the use of rodents of a range of ages. However, this is not a primary focus of the project and would involve low numbers of animals. Work in mice will be facilitated by the wide range of genetic modifications available. Work in rats, a species in which we have extensive prior data from earlier work in our laboratory, may benefit from a) the somewhat closer similarity in muscle composition in rats than mice (for example, mice have essentially no slow muscle fibres in their EDL muscle, whereas rats, like humans, do possess slow fibres in the EDL, b) the more extensive prior published data on certain aspects of rat neuromuscular development, for example the role of electrical activity in the control of muscle fibre type, c) the increasing availability of genetically modified rat strains.

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

There is no single ‘typical’ procedure/set of procedures. Genetically altered animals would be bred and their muscle growth analysed either under anaesthesia (fish) or post-mortem (fish or rodents). More rarely, genetically altered animals (fish or rodents) might be treated with substances (by immersion or injection for fish, by injection for rodents) or exposed to exercise training (fish) or altered environmental regimes (such as shifted day/night cycles) and their muscle growth analysed. Some surgeries may be performed to cut the nerve leading to a small region of muscle or to damage a region of muscle tissue in order to analyse the roles of genes and cells in repair. Some animals (zebrafish) may be subjected to training regimes in a swim tunnel and/or allowed to age to determine how exercise can improve and preserve muscle performance.

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

Animals (fish or rodents) may gain or lose weight due to altered muscle mass lifelong; these effects are unlikely to significantly affect their life in an aquarium/cage environment. Some animals that model muscle ageing (zebrafish) or disease (zebrafish or rodents) would have muscle weakening and possible low-level inflammation, as occurs in similar

human conditions. Some zebrafish may develop changes to heart function with age or under exercise, or changes to regenerative capability, which would generally be monitored for short experimental periods. No tumours or long term pain, and little long term alteration in feeding or change in behaviour are expected in any species, although older fish swim less than young ones.

Small numbers of zebrafish of certain strains may develop lifelong spinal curvature, severe muscle degenerative disease, or fail to grow at the normal rate.

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

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

Fish

Sub-threshold 70%

Mild 20%

Moderate 10%

Non-recovery <1%

Severe <1%

Mice

Sub-threshold 80%

Mild 10%

Moderate 10%

Non-recovery <1%

Severe <1%

Rats

Sub-threshold 80%

Mild 10%

Moderate 10%

Non-recovery <1%

Severe <1%

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

- Killed
- Used in other projects

A retrospective assessment of these predicted harms will be due by 09 April 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

The majority of our work has phased out rodents, and larval zebrafish outside the Act have been used to replace them in part for reasons of replacement and in part for other reasons, such as the ability to observe muscle growth in vivo in live animals and to perform more complex genetic analyses at lower cost.

We are mainly focusing our studies on the early stages of zebrafish development (before independent feeding) to understand the basic cell and molecular genetic controls of muscle growth. As explained above, these cannot be studied in vitro due to lack of knowledge of the appropriate chemical and physical environmental factors required to mimic the in vivo condition.

In order to understand later muscle growth/maintenance, older animals are required for similar reasons. To study the progression of sarcopenia and/or muscle diseases it is necessary to grow zebrafish to such age as sarcopenia commences or diseases become apparent in order to examine the effects of genetic or other manipulation on sarcopenia or disease. Again, this cannot currently credibly be mimicked in vitro at present.

On occasion, it may be desirable to verify that controls on muscle size discovered in zebrafish also occur in mammals prior to moving to human trials, necessitating the use of rodents of a range of ages. However, this is not a primary focus of the project and would involve low numbers of animals. Either mice or rats, depending on the genetic strains already available, may be used for this work to avoid the need to duplicate genetic modifications in zebrafish that have already been generated in rodents.

Additionally, depending upon the specific question to be addressed, it may be advantageous in the light of existing published or unpublished data to perform studies in the rodent species with the most robust prior information or the muscle that best models the human condition.

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

Cell culture, Induced pluripotent stem cells (IPS), Organoids, Human studies, Invertebrate models

We are performing studies using human data from the UK Biobank and other studies to discern genetic variants controlling age-related muscle loss. Our plan is to use animal studies to test such variants for causal roles. Reciprocally, we will determine regulators of animal muscle growth by experimental analyses described herein, and then examine their potential role in humans through existing cohort studies.

Why were they not suitable?

No cell culture, organoid or induced pluripotent stem cell systems exist which develop normal muscles with a normal number of fibres, with the correct number of nuclei in each and with the correct volume of contractile material per nucleus. It is understanding how these characteristics of muscle lead to correct muscle size that is the aim of our work. It can therefore only be investigated in an intact organism in which manipulation of genetic or environmental variables can be tested for their effects on these properties of specific muscles.

A major aim of our work is to understand the role of physical force in muscle growth. Natural levels and patterns of force can only be measured and applied to muscle in the context of the whole animal, where reciprocal effects on all relevant tissues can be analysed in parallel.

Cell culture: no suitable 3D system involving muscle, connective tissue, skeleton and innervation is available.

Organoids/IPS cells: no suitable 3D system involving muscle, connective tissue, skeleton and innervation is available. While there are papers claiming to be able to differentiate hPSCs into a 3D human skeletal muscle organoid (hSkMO), and that this could recapitulate myogenesis and had regenerative capacities after tissue damage (e.g. Shin et al 2022 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9103168/)), careful inspection of the data in such papers reveals the muscle is a) not attached to the skeleton, b) not innervated, c) less mature than that observed in a 2 day old zebrafish embryo or an embryonic day 15 mouse. While such constructs may provide a source of interesting human muscle precursor cells for those wishing to study their properties, they cannot be used to analyse the effects of physical force on the growth of muscle, let alone the decline in muscle size with ageing.

Human studies: we are analysing human data in the UK Biobank but manipulative experiments to test hypotheses arising are unethical in people.

Invertebrates: vertebrates have evolved many fundamentally different ways of regulating growth of both skeletal and cardiac muscle. Therefore any knowledge derived from invertebrates requires checking in a vertebrate before application to people, which is one aspect of our group’s expertise and what our project entails.

A retrospective assessment of replacement will be due by 09 April 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

Based on actual previous usage. Our primary use of animals will be as breeding colonies to provide embryos/larvae with complex genetic characteristics for analyses. Genome editing and other genetic manipulations will be used to create new lines. Fish numbers are large because each breeding produces around 100-300 embryos/larvae, which is one reason for the great power of zebrafish genetics compared to rodent studies. We expect to rear three or four generations of each of 40 fish strains within the 5 year period, requiring 40,000 individuals.

Rodent studies will include breeding of genetically altered lines (we expect to use no more than a few generations (<10 individuals per litter) of a few strains) and possible manipulations to check results from fish analyses.

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

To minimize use, only animals for which there are reasonable grounds to suppose the genetic alteration may affect development, growth or ageing of sarcomeric muscle and associated tissues or enable analysis of the same will be bred. Nevertheless, the aim of the project requires the use of animals and we hope to investigate the function of many genes at a variety of ages.

For experiments involving interventions and quantitative measurements, pilot studies and power calculations will be employed to ensure a high likelihood of detecting a biomedically-relevant effect size with minimal 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?

Lab members routinely share tissue from adult fish for different analyses.
Fish lines will be stored as frozen sperm when not immediately required for experiments, avoiding the need to breed new generations simply to maintain lines.
Zebrafish can live for five years under good husbandry in an aquarium. In order to reduce fish numbers, when animals are being used for ageing studies fish may be bred up to the age of 27 months, which will a) reduce the total number of fish bred, b) reduce the ill effects of in-breeding and c) enable collection of data on whether purposeful breeding benefits the overall health of ageing fish.

A retrospective assessment of reduction will be due by 09 April 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

Which animal models and methods 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 primarily zebrafish or medaka with genetic alterations to analyse muscle growth and maintenance. Almost all animals will experience no pain, suffering, distress, or lasting harm beyond life in an aquarium.
Small numbers of zebrafish will be allowed to age to study the effects of age on muscle tissue.

Rodent breeding studies will experience no pain, suffering, distress, or lasting harm beyond life in a cage.

When manipulations such as muscle injury or denervation are performed (under general anaesthetic), pain and suffering will be similar to that experienced in a minor human surgical operation such as a muscle biopsy; distress and lasting harm are not expected.

Key results may be checked in rodent models before moving to human trials.

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

As explained above, we aim to understand vertebrate muscle growth; we are using teleost fish, which some assume to be among the least sentient vertebrates. Over 95% of our experiments employ early life stages prior to independent feeding, so that older animals are generally only used for breeding, or for specific study of muscle ageing.
Invertebrate models such as Drosophila and C. elegans do not show muscle growth in response to force and do not have muscle stem cells as in vertebrates. They also do not have hearts similar to those in vertebrates, or show regeneration.

We anticipate little use of rodents, but wish to retain the option in case this becomes essential to raise the significance of the work with respect to potential human application. Without studies in a mammalian species it may be hard to convince institutional review boards to permit human trials of interventions. Moreover, certain studies can be done better in rodents, particularly mice. For example, conditional Cre/loxP genetic analyses may be possible with mouse lines already extant, thereby avoiding the need to create such lines de novo in zebrafish, while simultaneously increasing the likelihood of relevance to

humans, given the closer evolutionary relationship of rodents, as compared to teleosts, with people. Rats have some characters in muscle more similar to humans, such as the presence of slow fibres in fast muscles. There are also increasing numbers of genetically altered rat strains that may provide better models than mice. Their use may allow analyses of this functionally- important commonly used fibre type.

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

The vast majority of zebrafish will experience no harms as they are simply used to breed embryos/larvae of appropriate genetic make-up.

Zebrafish can live for five years under good husbandry in an aquarium. In order to reduce fish numbers, when animals are being used for ageing studies fish may be bred up to the age of 27 months, which will a) reduce the chance of females becoming 'egg-bound', b) reduce the ill effects of in-breeding in complex genetic backgrounds and c) enable collection of data on whether purposeful breeding benefits the overall health of ageing fish.

During surgery, particularly myocardial injury in zebrafish, any animals deemed unlikely to recover well will be culled without recovery from anaesthesia.

Any animals undergoing surgical procedures, exposure to substances or exercise regimes will be monitored at least twice daily to ensure endpoints can be captured at a lower severity.

Muscle and neural injuries will be kept to the minimum size compatible with addressing the research goals, both in fish and rodents.

Analgesia will be applied before and/or after surgical procedures as advised by NVS.

Animals will be culled when no longer needed. To this end, GA fish will be routinely stored as frozen sperm, permitting culling and recovery, rather than continued breeding when required for immediate use.

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

We adhere to local AWERB standards and are constantly following published protocols, attending conferences, and make use of sites such as ZFIN.org and https://www.nc3rs.org.uk/ as well as informal discussions with colleagues.
Our analyses conform to ARRIVE guidelines arriveguidelines.org
Our researchers periodically review the RSPCA science.rspca.org.uk/sciencegroup/researchanimals and UFAW www.ufaw.org.uk websites for on-going guidance.
Of particular note are:
Refining procedures for the Administration of substances:
https://doi.org/10.1258/0023677011911345

Guiding principles aseptic surgery:
https://www.lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf

We have obtained a copy of the forthcomng UFAW Handbook comments on the effect of age in zebrafish and are in discussion with the cited authors concerning the health of ageing fish, both to follow evidence-based best practice and to clarify what data needs to be collected.

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

Consultation with animal facility staff, NACWO, NVS, colleagues and the wider zebrafish and medaka communities through conferences, publications and web resources such as www.nc3rs.org.uk/resources and ZFIN.org.

We are in communication with personnel at the Zebrafish International Resource Centre concerning best practice in breeding and maintenance to minimize genetic and environmental health problems, particularly in ageing zebrafish.

Concerning rodents, we are frequent attenders of conferences at which most work presented is on rodents and are known personally to most of the players in the field internationally. Prior to any procedures on rodents we will consult NVS and colleagues concerning current best-practice.

A retrospective assessment of refinement will be due by 09 April 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

2. Neuronal networks underlying learning, memory, and decision-making

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

brain networks, decision-making, learning, prefrontal cortex, temporal lobe

Animal types	Life stages
Rhesus macaques	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Uses non-human primates
- Contains severe procedures

Objectives and benefits

Description of 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 advance scientific understanding of the neuronal level physiological mechanisms operating within and between networks of interacting brain areas that together underlie advanced forms of primate cognition, particularly learning, memory, and decision-making.

A retrospective assessment of these aims will be due by 11 April 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

The human brain is arguably the most complex system known to exist. It has over 80 billion neurons each with tens of thousands of connections. Those neurons are organised into networks of interacting subregions. Ultimately this complex dynamic system of underlies all of behaviour and cognition in human and other animals. Yet we do not yet understand how the dynamic interactions operate at a functional and mechanistic level.

Normal mechanistic interactions at the neuronal level within this network underlie normal human behaviour and cognition. Behavioural and cognitive neuroscience research is therefore crucial to understand how the brain works and functions as a collection of interconnected neurons within a network of interconnected brain areas. In short, if we cannot advance science in this way then we will never understand the brain mechanisms that underlie normal behaviour and cognition.

Moreover, abnormal mechanistic interactions at the neuronal level within this complex network are already known to underlie a large number of significant clinical conditions that adversely afflict very large numbers of people (e.g., amnesia, stroke, Schizophrenia, obsessive compulsive disorder, dementia, and a wide range of other learning and decision-making disorders). If we cannot progress to understand how brain regions interact normally to support normal behaviour and cognition then we cannot proceed to understand what changes to those normal interactions might underlie brain dysfunction and brain disorders.

Hence our neuroscientific investigations aim to elucidate the neuronal level brain mechanisms that underlie normal and abnormal brain function. Understanding of brain anatomy and anatomical connections between brain regions has steadily advanced. Over recent decades neuroscientists have also advanced from a focus on regional functional specialization (i.e. that specific brain regions/circuitries/pathways that appear more closely involved than others in certain cognitive functions) to a greater consideration of distributed function (i.e. that networks of interconnected and dispersed regions of the brain work together in concert to mediate cognition). But large fundamental knowledge gaps exists in that we do not understand much about how neurons within and between brain areas interact at a mechanistic level to shape, guide, and optimise cognition. For example, one useful analogy might be to consider all the known connections between all brain regions as akin to a road-map connecting all the towns and cities in the UK. This ‘map’ informs us of all the possible ‘routes’ information/influence can travel around the brain. But the map is dynamic, not fixed, because the viability of some routes may be dynamically altered by functional processes in the normal brain (and by dysfunctional processes in the abnormal brain). Some basic knowledge exists as to how the viability of routes are changed; for example, some brain regions show periodic oscillatory activities (i.e. cyclic and timed ebbs and flows of activity patterns) at certain frequencies and one influential idea is that if pairs of brain regions oscillate coherently (i.e. the ebbs and flows are closely associated in time) with each other that might act improve the effectiveness of their communication compared to if they oscillate incoherently (i.e. the timings of ebbs and flows occur randomly in one

region compared to another). According to the map analogy some routes on the map are temporarily viable and other temporarily road-blocked. But it is not generally known whether the different frequencies-of-oscillation (i.e. the specific time taken for each cycle of a repeated ebb and flow) are specific to all cognitive processes or brain-regions. Nor is it known how the system is dynamically controlled to optimise and/or control behaviour. According to the map analogy the viable routes are constantly in flux. Finally, we also do not fully know the characteristics of each brain region in terms of the computations and functional transformations each impacts on signals being routed around the brain; we therefore need to record the specifics of neuronal activities in those regions and compare it to that of others. Resorting to the map analogy one final time, we may know information flow needs to route via certain service- station en route but we do not know what specific 'services' are available at all of the different service- stations.

Our research requires recording neurophysiological activity of neurons in multiple areas simultaneously. It falls under the category of basic/physiological research aiming to understand how the brain works normally (and how it may be dysfunctional in abnormal conditions). Our research does not focus on a specific disease or clinical goal. However, our work may nonetheless have indirect secondary impact on future translational work because the brain regions and networks we study are heavily implicated in some key neurological and neuropsychological disorders that are largely without effective treatment.

Other secondary reasons why our work is important is that understanding how complex systems interact to mediate behaviour and cognition has impact on technologies that benefit the public. One obvious example is in Artificial Intelligence which benefits from understanding normal intelligence and how it is instantiated in the brain. Another benefit is for clinical advances such as brain-machine interfaces and other brain implants that can influence cognition. Our work speaks in part to interventions and devices that can influence cognition. Examples may include timed periodic stimulation to specific nodes of the network to encourage network activity to return more towards normality in patients whose abnormal network activities may be linked to impaired cognition; this may be akin to a boost to cognition and such stimulation may achieve 'cognitive enhancement' even in non- patients (e.g., notwithstanding ethical issues, artificially increased task focus/attention would have range of potential applications in education, in medical and legal and many other professions, in the military etc).

In short, the current state of knowledge as to how multiple primate brain regions causally interact with one-another at the neuronal level in the support of learning, memory, and choice behaviour is very limited. This is largely because most behavioural and cognitive neuroscience studies to-date have been limited by technology to investigating neuronal mechanisms in single areas. Our work is important because it seeks to address this significant gap in scientific understanding given it is now becoming increasingly understood that most human psychiatric/neurological/neuropsychological disorders (possibly all) are related to disturbances within extended networks of interacting brain regions, not single regions. Moreover, the technology to investigate multiple interacting areas is increasingly available and we are expert in some of these methodologies so it is important for economic and societal benefits, that the UK remains at the forefront of such pioneering research.

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

Our project's three scientific objectives are described in detail in the Project Plan but may be briefly summarised here as investigation into the nature of, and neuronal mechanisms supporting, interacting brain networks that are centred on: (i) the temporal lobe and perception, memory, and simple value- guided choice (objective 1), (ii) the frontal lobe and higher order value and rule-guided decision-making (objective 2), and (iii) frontal-temporal interactions and more complex/abstract or supra-modal decision-making (objective 3).

New information

- The outputs we will achieve include big data-sets of four main types: (1) millisecond-by- millisecond electrophysiological recordings of neuronal-level activities from many neurons simultaneously divided across combinations of interacting brain regions in networks while NHPs perform cognitive tasks; (2) data on causal influence, primarily from data on effects of interventions on those aforementioned neuronal mechanism with or without the intervention; (3) data-sets of neuroimaging-based assessment of voxel-level activities (voxels are volumes of about 1-4 cubic mm and can contain hundreds of thousands of neurons) that indirectly relate to neuronal activity but are useful for the purposes of targeting electrophysiological neuronal-level recording, and for relating human neuroimaging data with NHP neuronal-level activities so to help bridge the species divide; and

(4) data-sets of behavioural data, including trial-by-trial choices and eye-movements over many hundreds to thousands of trials, which are crucial to study the relationships between behaviour and the underlying neuronal mechanisms.

- Our multi-area neuronal recordings will provide new information to advance scientific understanding about the normal neuronal mechanisms that operate both within and between networked brain areas that underlie normal primate cognition in the areas of learning, memory, and decision-making. This will therefore address all 3 specific objectives.

- Our neuronal recordings in parallel with interventions research will provide new information about causal influences between brain region. The intervention component will also advance scientific understanding about the abnormal neuronal mechanisms that operate both within and between networked brain areas that underlie abnormal primate cognition in the areas of learning, memory, and decision-making. Hence this new information will help understand causal influences in the brain underlying both normal cognitive function and disturbed cognition. Conducting neuronal recordings in parallel with interventions is a more complex endeavour than neuronal recording alone so 'causal' studies may be more limited in scope than recording studies, but to the extent we can intervene in multiple areas we may similarly address all 3 specific objectives by this approach too.

- The new information that arises (i.e. the scientific advances) will be a product of careful analyses of the raw data-sets that the research will provide using a range of complex analysis approaches that aim to extract, characterise, and quantify the dynamic neuronal mechanisms operating real-time within and between brain regions (and so will likely include measures of selectivity, precedence (i.e. activities occurring in a site prior to others), synchrony (i.e. activities repeatedly co-occurring at same time), coherence (i.e. activities related in time, including both at exact same time and after a consistent time-

lag), polychrony (i.e. consistent order of activities across different neurons), and inferred or actual causality etc). Hence this will similarly encompass all objectives.

- The data-sets may be analysed in many ways to answer different questions. We will analyse these data-sets ourselves to provide our own scientific outputs (e.g. publications). In addition, we make the data sets available to other scientists (and the public) so that any new innovative analyses now or in the future may be conducted. The data-sets are expected to be a lasting resource and will be maintained accordingly. This also reduces any future need to repeat the studies and should reduce unnecessary additional animal use in the future.

- Our research focuses on certain types of behaviour (e.g., memory and decision-making) and certain brain networks involved in supporting those cognitive processes (e.g., temporal and frontal lobes) but it is not focussed on a specific defined clinical condition nor in producing a specific clinical benefit. However, the new information we provide from our basic physiological research towards all three objectives may sometimes have clinical implications or impact. As an example, one of our target brain regions exhibits hypoactivity (i.e. lowered activity levels compared to controls) in patients who have obsessive compulsive disorder and in patients who are severely depressed and impaired in social functioning. In investigating the function of this brain region with combined recording and interventions we showed that brief microstimulation can significantly impact behaviour for many trials subsequently in a cognitive enhancing manner; our modelling work suggests this is likely due to modulating the amount of focus on the specific task. This proof of principle in NHP studies may have indirect clinical implications for future therapeutic treatments that consider the brain regions we investigate. At the moment common therapeutic treatments for severe OCD include non-invasive or invasive brain stimulation albeit focused on entirely different brain sites with only a ~50% efficacy that is only temporary. Our work may not only potentially suggest new sites to target therapeutic interventions but may contribute mechanistic understanding of how cognitive functions are mediated therein so to provoke initial consideration of the specific nature of interventions.

- We expect this link between our work and that of clinicians to lead to interactions and collaborations which may give rise to further outputs based directly on our NHP work. For example, previous collaborations have arisen with neurologists and neurosurgeons and we have given our tasks to patients with deep brain stimulation and recording. We have also published several papers documenting neuropsychological testing of patients with memory and decision-making disorders due to brain lesions. We have been invited to present our work at the Grand Round at the local NHS Trust Hospitals (Grand rounds are a methodology of medical education and inpatient care, consisting of presenting the medical problems and treatment of a particular patient to an audience consisting of doctors, pharmacists, residents, and medical students.)

Publications and associated outputs

- The keyway we communicate our new information is via multiple peer-reviewed scientific publications (research papers) in scientific journals. We choose open access policies of journals wherever possible so that scientists and public around the world may freely access the outputs. Over the last 5 years we have published ~25 papers in very high impact journals.

- Other 'outputs' related to dissemination of the results to scientists and clinicians and public from these projects include 'talks', 'seminars', 'posters', and 'conference presentations', that the team of Principal Investigators and all other researchers will make. Typically we send various team-members to represent us, and disseminate our research, at ~3 conferences per year (a mixture of National and International meetings) and the team as a whole typically deliver ~20 talks/posters/presentations per year.

- Evidence of the recognition of our scientific advances and the impact of our work occurs via traditional media but we also consider newer media (e.g., social media references to our work are frequent on 'academic twitter' and 'academic mastodon').

Other, and Indirect outputs (i.e. expected consequential effects, not stated research goals)

- advancing methodological refinements: specifically in non-human primate multi-electrode, multi- area, recording methodologies (e.g., in surgical approaches, in stabilising implants, in developing refined approaches to record from new combinations of brain areas simultaneously etc). These are not published necessarily but are more typically communicated to colleagues at conferences, and when we visit similar labs, or when they visit us. For example, the licence holder has now performed neurosurgical procedures for several other labs around the world (5 different countries to-date). Similarly, we have invited leading international human and animal neurosurgeons to our own laboratory to help discuss and refine our procedures. This interplay and interaction between experts contributes to international refinement of procedures.

- advancing science: the next generation of researchers in primate systems neuroscience, through participating in the research under the guidance of experts, will become experts themselves in methodologies and may eventually become independent investigators pursing new science and their own new ideas to further benefit humankind.

- collaboration based outputs: the work, given its international standing, interest, and pioneering approaches, will foster new academic collaborations between UK and non-UK researchers, including between NHP researchers and non-animal researchers. Three new modelling collaborations include those with a Professor of Computational Neuroscience, a Professor of Mathematics, and a Professor of Computational Modelling.

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

Beneficiaries include:

Basic science researchers working in directly related fields of neuroscience. Major beneficiaries of our specific data outputs are other basic research scientists who continually build on each other's work (attested to by the team having very many thousands of citations to our work to-date), specifically those working in the fields of memory and decision-making, and behavioural and cognitive neurosciences. They will benefit from our data on interacting neural systems. Our 'big data' (simultaneously recorded spikes and field potentials across hundreds of electrodes across several cortical areas during all periods of advanced rule-guided behaviour) will be invaluable for years to come for researchers to scrutinise and analyse with new methods as they emerge. Such data-sets will be available upon request. The PI has developed many collaborations with

leading international laboratories (in four different continents) and we expect new collaborations to arise through our seminars and workshops.

Basic science researchers working in directly related fields of Computational and Cognitive System Modelling. Our across-systems neural data will be important for modelling by researchers in computer science communities, particularly those who are now producing advanced, new, spike-time dependent, models of cognition and who need raw neural data to model. We have new collaborations with two world leading computational modellers and one world leading mathematician.

Basic science researchers working in non-directly related fields of neuroscience: Our research will engage and benefit a broad range of neuroscientists in non-directly related fields because our work is about fundamentals of neural network interactions. We will facilitate this by accepting invitations to give general lectures/seminars to institutions in the UK, Europe, and Internationally (resource implications are covered by the inviting institutions) and by publishing accessible review articles. Our track record is excellent in the most high impact journals. The PI and team have given public lectures in UK and abroad and will continue to do so.

Clinically orientated researchers. Clinicians and clinical researchers (sometimes the same people) are also beneficiaries of our advances in scientific understanding of systems neuroscience. There are many neurological and neuropsychological disorders that are not understood and remain hard or impossible to effectively mitigate. Clinically applied research needs to build upon basic underlying research into physiology and without basic research there may not be clinical advances. Our general papers and review articles target clinicians as well as researchers; indeed clinicians often attend basic science conferences where we present our findings.

They will benefit from a deeper understanding of network activity in frontal and temporal cortex regions; of particular relevance to this group will be the causal influences that regional interventions have upon the function of widespread interconnected. This is because this will help understanding of the mechanisms of disturbances in network function that are increasingly understood to underlie behavioural disturbances in neurological diseases and neuropsychological disorders. The team have collaborated with research clinicians including neurosurgeons and neurologists and neuropsychologists, and have given talks to clinicians, for example, through invited presentations at Grand Round (these are ways of medical education and in-patient care, consisting of presenting the medical problems and treatment of a particular patient to doctors, pharmacists, residents, and medical students).

Our work is not designed to focus upon or create a specific clinical intervention, moreover basic science researchers cannot foresee which element of their research might have the most clinically relevant applications. In our case however we have some intuition, for example, in recent studies we showed cognitive effects of brain stimulation in NHPs via a small implanted neurostimulator to give temporal control of the effect (a form of resistance to distraction during a complex task). Controlling distraction and moderating exploration of alternatives is relevant to understanding clinical conditions including OCD and Depression and Schizophrenia (all of which are known to be associated with changes in the normal function of the brain areas we study); this suggests further clinically relevant research may potentially pave the way to therapeutic interventions or the future in the developing realm

of brain machine interfaces (BMI) and neuro-prostheses (which are already successfully implemented in the motor control realm in patients to relieve some motor disorders). This type of advance in the realm of cognitive disorders is in relative infancy but implanted neural devices in human temporal lobe can already significantly enhance memory. Future devices may be able to control, manipulate, or even enhance decision-related cognitive function, as well as allow interaction with the world with those unable to implement choices directly. These areas of future technology are already a thrust of several major corporations who see this as a profitable future (e.g., Neuralink; Neurolynx etc) even though the underlying neuronal mechanisms are not yet well established (the aim of our work).

University and higher education educators. Our review articles and general book chapters inform educators of science, and neuroscience in particular, and through them students. Our research features in many lectures and courses in Psychology and Neuroscience (and Medicine). Students of Psychology and Neuroscience often proceed to become trained Cognitive Neuropsychologists, Psychiatrists (via medicine), Educational Psychologists, Occupational Psychologists, and all these professions benefit the public in general. The team directly teach medical students as well as teaching students who are Psychologists and Neuroscientists. Our research features heavily in our teaching at our UK university and features in courses in all undergraduate years (and also in Master level courses).

Public through education. Communicating the science behind the project as well as publicising potential applications to the public leads to public benefit and is central to our entire ethos. The public benefit through awareness of biomedical science and its applications, some of which are long-term. And indeed, the public benefit if our research has clinical/translational benefits in the long-term. There are many examples of our animal work leading directly to work and collaborations with clinicians and we have co-published several papers with the neuropsychological patients involved. Throughout the project we will further these benefits by utilising the public engagement structures that are already in place in our institution as well as further expanding our engagement with UK and international communication structures. We will interact with our Press Offices to develop press releases of new high impact papers and new impact developments arising from the work. The public also benefit through our frequent engagement in public events. Our public engagement activities are supported by links to our websites and social media accounts where we will make available summary reports, and links to publications and preprints. Lab members past and present have received interest from national media outlets, including radio and TV appearances, and accrued local and national tabloid coverage; they have benefited the public by delivering public lectures inspired by our science, and have received prizes for science communication writing. We also engage and support public outreach.

Public through indirect health benefits: benefits to health of the nation occur via our collaborations with clinicians including neuropsychologists, neurosurgeons, neurologists. As a result of our animal-based studies, we have been invited to work directly with patients with deep-brain electrode stimulation. We have published several papers on patient neuropsychological studies. We have been invited to speak on our animal work to clinicians and neurologists at NHS Trust Hospital Grand Rounds. We will continue this clinical engagement and plan to expand our collaborations with clinically orientated

research groups. We anticipate that our research in the area of targeted neurostimulation influencing cognition, may be of particular interest to clinicians.

Commerce and industrial benefits: Our research presentations at large international conferences are attended by specialized industrial representatives so we will reach these potential audiences. Our work on distributed brain systems and their efficient interactions is relevant to several specialized industries (e.g., robotic control systems, brain-machine interfaces and neural prosthetics, neural network and artificial intelligence applications). The industries are interested in practical application of our basic science research. The applicant has recently collaborated with and published a paper with an Artificial Intelligence charity whose charitable objectives benefit society.

Basic science researchers working in Artificial Intelligence. A key potential future impact, once we understand principles and mechanisms by which frontal network activity underlies certain memory processes, is the exploitation of this understanding of biological systems to develop intelligent and flexible artificial systems.

Other general UK Economic benefits: Our supervision and mentorship of skilled research assistants on the project that we hire, and graduate students (we provide specific opportunities for them to participate in parts of the research project) will create highly skilled individuals, expert in the most refined animal handling/training methods and expert in innovative behavioural and neuroscientific (e.g., in-vivo recording) and modelling/analysis techniques. This will have a direct impact on their skill sets and career opportunities in academia and/or industry after this project is complete, and therefore enhance UK competitiveness in a key area of economic interest. Any translational work done in the UK that ultimately contributes to further scientific discoveries and innovative technologies will have positive impact on the UK economy.

Educational benefits to public: The research team deliver talks to educational establishments; these include UK and International universities, and UK schools. Team members have delivered talks to the general public to increase awareness of neuroscience. Educating and inspiring the potential next generation of neuroscientist is a key educational aim with undoubtedly positive outcome for the UK both in educational and economic terms.

Timeframe of benefit:

Most direct benefits cannot be realised until we have completed the project, but then the benefits will accumulate over several years afterwards (5-10 years or more) as we analyse more aspect of the data outputs provided and publish more papers. This is evidenced by outputs from previous licences (from 5 and from 10 years ago) which are still leading to periodic new publications, with more planned. It is important to note that advances in scientific knowledge occur gradually in stages.

Moreover, in our case, the work is particularly long and laborious given its nature. This is because when recording from hundreds of electrode channels simultaneously there can be many months of data processing to extract neuronal signals. Analyses of the neuronal signals can only begin after recording is complete and recordings are made during the final stages of all studies. Often analyses continue in the years after the project licence ends (i.e. while we are recording data on the current project licence, we will be analysing data from previous projects, and so on).

Preliminary analyses are different as they may occur anytime during the 5 years of the project (although only after at least some robust neuronal data recorded). These analyses can support some outputs like 'talks', and 'conference posters', and 'seminars' when we disseminate the latest understandings, even if preliminary.

But robust full analyses of a quality for publication (and further adjustments/analyses after peer-review) typically take a couple of years. Consequently these outputs (full 'major' papers) tend to start to occur between 2-3 years after completion of the recording.

The data sets are large ('big data') and may subsequently be analysed in many ways (and in new ways when new analytical approaches are developed) and so each study typically results in multiple publications over a period of 2-10 years after data is recorded.

Our general expectations are that new scientific information and outputs from the work conducted under this PPL will be acquired gradually and continually through the PPL, and well beyond. Formal citations to our work (one way to demonstrate that it is having impact) follow the date of publication. But alternative social media references/likes/tweets/discussion of our work may occur sooner as typically as they also result in response to our preliminary data presentations (posters, conference talks, workshops etc) which we advertise to scientists (and public) by social media such as via our lab Twitter and Mastodon accounts.

Refinements to procedures will most likely arise from direct experiences, and subsequent attempts to improve them, so the expected timeframe would be in the 2nd half of the project.

Several of our previous lab members have gone on to obtain fellowships or have become faculty members in UK and in international institutions and we expect this to continue. The time frame is variable but working on projects in our lab has generally made researchers highly competitive for future jobs in science, hence furthering science in general for the public good.

The work inevitably results in collaborations. In turn these collaborations, in the majority of cases, leads to still further outputs, such as additional publications that we co-author or in which we are acknowledged.. The outputs may include publications directly inspired by our animal work but not including animal data (e.g., human patient or neuroimaging investigations, or modelling studies, using the tasks that we have developed). Whereas other collaborative outputs are based directly upon our data (e.g., joint publications modelling our data, or re-analysing our data, or combining our data with that of other studies to make new contrasts or comparisons).

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

Collaboration

Scientific collaborations are expected throughout the duration of the project. Some have been pre- agreed at the time we wrote the grants that fund this work and those scientists are named collaborators and they will work with us on the project. We selected these collaborators on the basis of the specific expertise they bring that can complement our own. These collaborators are typically investigators studying the same cognitive processes in humans but are unable, in their human subjects, to study mechanisms as we can. Other

collaborators tend to be advanced modellers who can use our data to refine our, and their own, computational models. We also expect new collaborations to develop. We proactively attend workshops and conferences and accept invitations (nationally and internationally) to give seminars and visit labs and we try to engage with those whose work can benefit from appreciation and understanding of our work, and vice versa. This can happen at the level of data (we collaborate with those who can bring different expertise to analysing our data) and at the level of methodologies (we collaborate with those who can help us implement more advanced or refined methodologies). It can also happen in reverse, and we are invited to analyse their data-sets with our analysis approaches or we can help them introduce new methodologies (e.g., teach how to implant or refine specific devices or make lesions/interventions to areas they do not yet have experience in; we have done such procedures for others in multiple countries). All this maximises outputs as all collaborations typically lead to additional publications and helping others refine their procedures contributes to improvements in animal welfare.

Dissemination

- We will aim to continue our excellent track record of impactful publications in well-respected peer- reviewed accessible journals.

- We also strive to make all our research papers openly accessible, and comply with the requirements of our funders in doing so. This increases availability to other scientists and all beneficiaries including the public. It also has animal welfare benefits as it reduces the need for confirmatory or repeat studies by others.

- Additionally, all our research papers are submitted to a local university archive that allow public access to author accepted manuscripts.

- We also strive to make our data-sets freely available in some useful form post-publication so that others may analyse the data-sets in new ways in the future to ask their own specific questions. For example, we have made data-sets of monkey behavioural data and associated code publicly available in github. One leading international Ecologist in another continent is already using these data to test their ecological framework. Similarly we have also used github to make our MRI data sets freely available.

- The Principal Investigators and team of researchers will be encouraged to take up opportunities to disseminate our data at a wide range of national and international meetings, conferences, symposia, and workshops. This is deemed so important that every grant application has a separate section to account for this (with distinct budget heading). We find that actively disseminating data in person (giving talks, presenting posters, chairing panel discussions etc) is most productive, but we also do online presentations and attend online meetings too to maximise exposure (and which may be less expensive, more accessible, and arguably, more environmentally sound). Presenting preliminary data mid project is a keyway to raise awareness of our work and increases awareness of, and traditional citations to, our actual main scientific publications.

- We also have lab Twitter and Mastodon accounts (with many followers) to draw attention to our conference presentations and papers and preprints and allow us to engage with an increasingly online academic community (and general public) via social media.

- In terms of disseminating welfare refinements we join with the community of peers and experts at laboratory animal welfare meetings (e.g., NC3R) and indeed, have presented our own refinements to the community at such national and international meetings.

Publication of less impactful or unsuccessful approaches

We will also strive to publish data from all studies including any whose results may not be as impactful, and if possible those that were unsuccessful. This is important with animal work so that others do not address unproductive avenues in same way and hence have unnecessary use of additional animals.

However it is generally hard to define an unsuccessful study as all our studies typically provide some data. Generally speaking, journals have traditionally been reluctant to publish negative results, but several journals now encourage this. If appropriate, we will submit to those journals but it has not been necessary to-date as rather than generating unsuccessful/negative per se our results of our studies tend to result in publications.

Species and numbers of animals expected to be used

- Rhesus macaques: 17

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 functional-units / building-blocks of cognition in the brain are neurons. Hence to study the mechanisms operating in distributed brain networks that underlie cognition requires studying brain activities at the neuronal level. With electrodes implanted in the brain we can sample activity from individual neurons (as well as populations) and we need to record that activity at a millisecond-by- millisecond temporal resolution. This simply cannot be done with non-invasive brain imaging because that technology does not have anywhere near the spatial or temporal resolution required to record neurons. Current non-invasive neuroimaging (fMRI) provide a spatial resolution at the voxel level (a voxel is a volume which is typically in the order of 1-4 cubic mm) and within such a voxel there could be 10s to 100s of thousands of neurons. Moreover, the temporal resolution of neuroimaging (fMRI) is in the order of several seconds which is orders of magnitude too slow to study neuronal level mechanisms that operate on the millisecond level.

To achieve our aims and objectives we need to studying the activity of neurons, and their influences on other neurons, and this requires invasive electrophysiological recording which requires putting electrodes into the brain. Moreover, if we want to study interactions within and between areas we need multiple electrodes in multiple regions. The only category of humans with many electrodes implanted in their brains are patients with significant clinical/neuropsychological/neuropsychiatric needs (i.e. the condition is profound and its diagnosis or therapeutic relief if possible justifies the invasive approach). Accordingly, these patients are not suitable for our research as they have dysfunctional brain systems where data about normal brain operation and normal brain interactions

could not be obtained. In addition, the targets of their electrodes are solely determined by each patient's specific clinical needs, whereas in our studies the targets of electrodes are determined by experimental needs and the two rarely map onto each other. Hence our research needs the use of invasive techniques and it requires the use of experimental animals.

This work has to be done with non-human primates, specifically adult macaque monkeys, for the following reasons:

1.We use adult macaques because we study normal adult brain function, not juvenile stages when cognition and the brain is still developing.

2.We cannot use rodents because they do not have several of the frontal cortex brain regions that primates have that we need to study and that we know are involved in the brain networks and behaviour we need to study (Preuss TM (1995) Do Rats Have Prefrontal Cortex? The Rose-Woolsey- Akert Program Reconsidered. J Cognitive Neurosci 7:1–24.).

3.There are a few reasons why we need to use macaques and not marmosets. Firstly, there are other major differences in the number of anatomical areas in the highest region of the brain, the cerebral cortex, and the connections between them that provide the basis for complex cognition that make macaques the most appropriate model for understanding complex cognition in humans. Evidence has shown mice have 43 different areas in each hemisphere, marmosets as many as 117, macaque monkeys as many as 161, and humans as many as 180 (see: Buffalo EA, Movshon JA, Wurtz RH (2019) From basic brain research to treating human brain disorders. Proc National Acad Sci 116:26167–26172; Essen DCV, Donahue CJ, Coalson TS, Kennedy H, Hayashi T, Glasser MF (2019) Cerebral cortical folding, parcellation, and connectivity in humans, nonhuman primates, and mice. Proc National Acad Sci 116:26173–26180.). Hence the macaque brain is the most appropriate NHP brain to study for the reason it is the most similar brain to the human brain.

4.Secondly, the frontal regions and networks that we need to study underlie the ability of primates to construct novel, complex, structured sequences of intelligent, goal-directed behaviour which includes the behaviour some of our main objectives are focussed upon. We cannot use animals that do not exhibit the advanced, flexible, rapidly learnt and re-learnt, goal-directed behaviours we need to study. Hence we cannot use rodents, but nor can we use new world monkeys (marmosets), as those species also do not show sufficiently developed cognitive abilities in the kinds of complex tasks we typically employ to investigate these cognitive processes. As an example, marmosets can learn a basic extra- dimensional set shifting task but cannot achieve sufficient shifts/session (sometimes as few as one, and sometimes zero as studies report marmosets require more than one session with so few trials/session possible). Macaque monkeys can perform a far more complex extra-dimensional set- shifting task with hundeds of trials/session and shift >10 times/day on average, sometimes making only a couple of errors to consolidate learning of each shift. Hence we cannot study dynamic rapid set- shifting behaviour in marmosets.

5.Thirdly, another key reason to use macaque monkeys is because all our work builds upon an extensive behavioural, anatomical, and physiological scientific literature that already exists in the macaque; this literature is unavailable to the same extent in any other

species and to shift to a new species now may entail unnecessary replication of additional work that hadn't yet been done in that species.

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

There are 19 steps in the protocol but it is never the case that all will be conducted in any animal. Rather, different combinations are used in different animals. With reference to all the steps we briefly describe what they entail, and how long they'll be used for and how often, and whether the most typical animal would be likely to experience procedures with in them or not, and if so typically which and or how long. After running through the steps we'll end with a broad summary overview.

Step 1: Preliminary training without head-fixation (mandatory)

All animals enter this step. The typical procedures for a typical animal are as follows:

They are gradually acclimatised to what is commonly described as a 'primate chair'; these are commercially available specially-designed restraint-chairs in which animals 'sit' in place for sustained periods of time (and which, according to the NC3R website, if they are as "fully refined as possible will reduce any stress and discomfort to a minimum, facilitating good performance from the monkey and good scientific value"). Hence we use the commonly used phrase 'chair' or 'primate-chair' hereafter to refer to this device. Once acclimatised to the chair, and used to receiving reward in the chair, the animals next become gradually comfortable and familiar with raising their heads for neck-plate closure, and to learning about the basic ways in which highly palatable reward (e.g., smoothie) may be obtained by their interacting appropriately with devices in the lab such as levers and screens. We use predominantly positive reinforcement but sometimes rare use of negative non-reward procedures too (limited to subthreshold procedure such as raising a net, showing an intention to reduce, or temporarily reducing, available cage volume). They are then gradually trained, in stages, to perform behavioural tasks (typically involving choosing between stimuli on a touchscreen for reward) while sat still in a primate chair (a plate is closed around their neck, but they are otherwise free to rotate head and body and can freely move body and limbs. These sessions are short to begin with and build up to an hour. In addition to trial-by-trial reward opportunities positive reinforcement is also provided by opening of a jackpot lunchbox at end-of-session which contains a large proportion of the daily diet of wet mash and fruit and treats. The animals may typically be on this stage for 5 days/week for around 6 months. Water control is never used in this stage so they are never working because they are thirsty.

Step 2: Home-cage training using Mymou (optional)

Some animals enter this step (<50%). The typical procedures for a typical animal are as follows:

Mymou is a touch screen tablet integrated into a sturdy frame which can be hooked/attached to the outside of the home-cage enabling NHPs to voluntarily (i.e., only if and when they choose) interact with the Mymou device to touch the screen (e.g., touching stimuli, or performing tasks) in order to receive reward (i.e., smoothie/juice). It may help some animals learn some aspects of our lab tasks faster, but this needs to be established as we have not used Mymou enough yet and rely on the advice of others who have developed and/or use this NC3R funded device. Regardless, another scientific use of

Mymou will be to pre-expose animals to some stimuli (but not others) in order to manipulate familiarity (e.g. of faces or objects) when familiarity is a parameter we need to manipulate.

Step 3: More advanced behavioural training typically proceeding to training with head-post restraint (optional)

Most animals enter this step (~90%). The typical procedures for a typical animal are as follows:

Animals now proceed with more advanced cognitive training on computer-controlled cognitive tasks. Initially the animal's head is not restrained (by head-post) and we train more complex task using the same motivational tools as described earlier. These sessions are built up gradually to a typical duration 60-90 minutes. Animals typically spend pend 1-2 years on this step. We typically do not need fluid control (neither timed access nor volume control) in this step (<10% expectation of fluid volume control ever being used in a given animal and if it is typically only for a few days; timed access fluid control is used more frequently but still in a minority of animals, estimated <30%). For all or most animals in this step the behavioural training will involve progression eventually to head-fixated training; this requires a head-post and the surgery for this typically occurs halfway through this stage.

Step 4: Fluid control (optional)

A minority of animals require this step (see below). The typical procedures for a typical animal are as follows:

For training in steps 3,8,12 only a minority of animals need fluid control in the context of the tasks we use to provide additional motivation. We estimate <30% need timed-access control (access to water is scheduled, for example water bottle removed from cage a few hours before testing, but volume of water accessible in the day as a whole is not reduced). Far fewer animals require (<10%) require fluid volume control, and in all cases to-date that has been temporary (typically only for a few days). There are detailed monitoring procedures and controls enacted to ensure they remain healthy if ever used in the licence. In summary the procedure is staged so that only the minimum fluid control required is used and it is ceased when no longer required. Most animals on volume control (i.e. the majority of the 10% for which is may be necessary) would not rise to the maximum permissible level of volume control which is no less than 20 ml/kg/day or no less than 50% of their estimated average free access intake per day (whichever is greater). Animals on fluid-volume control will be continued to be fed wet mash and fruits throughout. As a result, no more severe adverse effects than low-moderate dehydration will be experienced and in the very unlikely event that level of dehydration is ever experienced it will be of short duration as water control will be relaxed as soon as detected, due to our control procedures.

Step 5: Food control (optional)

A minority of animals require this step (<15%). The typical procedures for a typical animal are as follows:

Scheduling of food is standard practice in laboratory husbandry as they are fed at certain times of the day on a schedule (no animals have ad lib food) and so we may adjust the

schedule (delay some food by a few hours) so as not to reward/encourage failure (but actual food volume control is rarely used,

<15% animals, and only a temporary small reduction of no more than 50g of wet mash (which is never more than 50% of normal wet mash and often a smaller proportion) but no reduction in fruit may be employed (for no more than 3 consecutive days in a week).

Step 6: Anaesthetised MRI scans (optional)

Most animals require this step (>90%). The typical procedures for a typical animal are as follows:

The animals will be sedated and anesthetised (general) and then have a scan typically lasting ~ 2 hours.

The typical animal may have 2 anaesthetised MRI scans (one for screening the animal's brain for major abnormalities and one for planning the implants including head-post).

Rarely, some of these anaesthetised MRI scans may be preceded by focal ultrasound neuromodulation (FUN) intervention used to investigate network interactions in the brain which are influenced by the FUN intervention. We expect FUN to be employed in a minority of animals. FUN is unlikely to be felt (apart from occasional muscle twitch) and is applied via holding an ultrasound transducer and funnel/cone near the head.

Step 7: Surgery (without craniotomy) for cranial implant/s (optional)

Most animals require this step (>90%). The typical procedures for a typical animal are as follows:

Most animals (>90%) will have a surgery under general anaesthesia to implant a head-post. This will tend to happen in the middle of step 3.

Some animals (~45%) will have one surgery under general anaesthesia to implant one or more chambers. If they do it likely means they'll be entering a path where the neuronal recording will be from chambers and in that case they will not also have lesions. It is anticipated that only a minority of animals, if any (so 0-10%), would have a combination of arrays and chambers.

Step 8: Awake behaving fMRI (optional)

A minority of animals require this step (<10%). The typical procedures for a typical animal if they do are as follows:

For animals that do undergo awake behaving fMRI they first need additional training to acclimatise to the horizontal chair used for fMRI. This is typically done in the context of a mock magnet and it is done in stages to acclimatise animals to the noise and the MRI environment. Sessions (mock and/or real) are typically run 5 times a week for a maximum duration of 3 hours/day, and over a maximum period of 36 months.

Rarely, again, some of these anaesthetised MRI scans may be preceded by focal ultrasound neuromodulation (FUN) intervention as described above.

Step 9: Surgery with craniotomy for implanting arrays (optional)

Some animals (~45%) will have one surgery under general anaesthesia to implant arrays into the brain to record neuronal activities. If they do it likely means they'll be entering a path where the neuronal recording we do will only be from arrays, hence it also likely means they will not also have lesions, and will not also have chambers implanted.

Step 10: Surgery with craniotomy for brain lesions (optional)

Some animals (~10%) will have one surgery under general anaesthesia to make a brain lesion as an intervention. If they do have a brain lesion they will not also have semichronic multi-electrode microdrive and their 3-step surgery for associated chambers. If they do have a brain lesion they are likely to have anaesthetised MRI recordings of brain activity, but are highly unlikely to have either array-recordings or awake behaving MRI (<10% for either, none would have both); regardless, they will have fewer surgeries in total than non-lesioned animals having semichronic multi-electrode microdrive (because that entails a 3-step surgery for associated chambers to be implanted).

Step 11: Surgery to make craniotomy under a chamber (optional)

The ~45% of animals who have had a chamber implanted in a previous surgery will later need a craniotomy under the chamber which is typically done in a separate surgery with general anaesthesia.

Step 12: Behavioural training/testing with neuronal recordings (optional)

About 90% of animals (the ~45% with chambers in whom we'll do chamber-based recording, and the ~45% with arrays in whom we'll do array-based recording) will enter a phase in which they return to the behavioural tasks and complete them as before (max 5 days/week) for the same motivation as before. From the animals' perspective this step is little different from step 3 as the animal is already chair- trained, neck-plate trained, lab-trained, head-post trained, and task-trained for liquid reward (smoothie and/or juice and/or water).

Recording via chronically implanted arrays simply entails screwing the digital head-stage onto the pedestal, and connecting the cable from the head-stage to the recording device, and recording commences (it is akin to 'plug and play'). Recording via semi-chronic multi-electrode microdrive systems is similarly straight-forward, this time the microdrive system is connected to the recording acquisition system and recording commences. This is also 'plug and play' albeit the experimenter reviews the signal from each channel and may adjust the fine depth of any electrode within or between days to better isolate single spiking cells (typically a few out of the 10s to 100s in the chamber/s would be adjusted each day to 'maximize' single unit data yield); whereas in arrays there is no adjustment that can be made.

The precise nature of the tasks will typically be modified as the experiment progresses as guided by our observations of behaviour and/or data on the neuronal responses. Note we still typically do not need fluid control (neither timed access nor volume control) in this step. Similarly, we also do not typically need to vary the standard daily diet to increase motivation.

Typically this stage might last 1-2 years with testing 5 days/week, but sometimes we will employ reversible interventions in this step if we need to see their causal effect upon neuronal recording data and behaviour as required by one of our output categories. Typically will use one of the following methods:

(i)direct electrical stimulation via the already implanted electrodes. This require no further implantations or procedures. The device we plug into the electrode contacts is larger but otherwise there is no difference from the animal's perspective. They animals cannot feel the stimulation, but it can bias choice behaviour in the task temporarily.

(ii)focal ultrasound neurostimulation (FUN); FUN alters neural activity for minutes or hours (so complements as opposed to replaces the milliseconds to seconds alterations expected from direct electrical stimulation via arrays). The animal requires to have a FUN transponder placed next to its head to deliver the FUN prior to the behavioural testing session but it is not invasive and is not painful. They animals are unlikely to feel the stimulation (but there could be muscle twitching); it can similarly bias choice behaviour in the task temporarily.

A third possible method is injecting neuromodulatory drugs for temporary inactivation via the chambers but this is not typical yet.

It is likely that in the ~45% animals with chambers in whom we'll do chamber-based recording, we'll need to make electrolytic markings: towards the end of a recording phase of the experiment we may need, in one or more sessions. The animals do not feel the marking so the experience is just to sit in the chair and they'll be receiving positive reinforcement.

Sometimes the experimental design will seek to compare neuronal activity for choices made in isolation in some sessions versus choices made in the presence of an observer. Sometimes the observer will take turns making choices. In such cases the animals will not be able to reach each other or each other's rewards (they will only be able to reach different parts of horizontal screen) and we will only use compatible animals.

Step 13: Setting/refitting/adjusting/removing semichronic microdrive systems (optional)

In the ~45% animals with chambers in whom we'll do chamber-based recording, we'll need to do some adjustments to the semichronic microdrive system. All animals will need the system inserting (electrodes lowered into the brain). Typically animals may have one or two more adjustment steps (lowering or raising electrodes).

Step 14: Chamber/Implant cleaning (optional)

In the ~45% animals with chambers in we'll perform periodic cleanings of the chamber margins. In the 90% of animals with a head-post we'll similarly perform periodic skin margin inspections and cleaning if required. Typically (~70% animals) this may occur around once per week (~20% will be less frequent;

~10% more frequent) and will include restraint by physical means in primate chair and likely by head- post fixation if present (only in rare circumstances will the animal need to

be sedated). For animals on a daily testing schedule this step may be contiguous with the testing.

Step 15: Implant repairs (optional)

It is not typical to need to make any repairs but we have the option to do so if we need to.

Step 16: Dural scrape (optional)

It is not typical to need to make any dural scrapes (thinning the tissue/dura if electrodes becoming difficulty to penetrate into the brain) but we have the option to do so if we need to.

Step 17: X-rays to check electrode condition (optional)

In the ~45% animals with chambers in we'll perform x-rays to check and document electrode conditions and locations. They'll be chaired and may or may not be sedated. It will only take around 30 minutes.

Step 18: Fitting a protective mesh head cap (optional)

This is protective mesh head-cap device that can be attached to the head-post if it may help reduce wound picking and increase the rate of success of wound healing. In our experience we have rarely needed it and so it is not typical but is available if we need it.

Step 19: Perfusion or Schedule 1 method of killing (mandatory)

All animals will eventually be perfused or Schedule 1 killed but in some cases we expect this to be on the successor licence due to transfer authority obtained at the time. Animals are staggered and grants are staggered (but licences are not, they have a rigid 5-year duration) so this is necessary.

Summary overview:

For all animals the vast majority of the typical experience in numbers of days includes training/testing days (some of those days may have recording or stimulation or inactivation during testing depending on the design). The animals will have daily restraint of up to 2-3 hours/day for up to 5/days/week in the primate chair while training/testing/recording. These days are interspersed with rare surgical procedure days under GA. The testing sessions provide opportunity for reward and are generally positive experiences although restraint is required they tolerate it and voluntarily enter the chair. Animals obtain the majority of their daily diet after these testing session which provides additional motivation to complete them efficiently; hence all animals have scheduled-access food (only ~15% ever have food-volume reduction and if so, it is a modest reduction of mash, not fruit, as described and is for no more than 3 days/week, and is used rarely even in any animal that need it). Most animals will also not have fluid control because in our experience in using our refined approach for behavioural training over the entirety of the previous licence (including end-of-session jackpot lunchbox and smoothie trial-by-trial reward opportunity) for the kinds of tasks we use, and for the numbers of trials we need per session, a majority of animals are sufficiently motivated most of the time without fluid control. However, not all animals are identical in temperament and some animals do need additional motivation provided by fluid

control else they could not complete the testing and the experiments would not complete. There are two categories of fluid control which are timed-access control and fluid-volume restriction. Typically<30% of animals need time-access control and those that do have ad lib access to water the rest of the day and night and so cannot dehydrate. Around 10% of animals to-date have needed fluid-volume control; in these rare cases it is often used temporarily and if ever used it is always introduced in stages so that the minimal level required is implemented (and also ceased when no longer necessary). Sometimes these animals only need fluid control in later stages (e.g. after 1-3 years, and/or after implantation, and/or after recording has commenced, after which the long and expensive and invasive studies need to complete) so it is not possible to start with 10% more animals and screen out animals that do need fluid-volume control, rather we adopt the refined approach described to minimise use and extent of fluid control. No animal will have fluid and food control simultaneously.

The two most typical routes through the steps are:

(i) Neuronal recording and stimulation using semichronic microdrive chambers: Typically animals experience: 4 surgeries with general anaesthesia (one for head-post, one for chambers, one for craniotomies under chambers, one for inserting/setting microdrive systems); only one of those surgeries involves craniotomies and exposes the brain; they will likely have one or two anaesthetised MRI scans for implant design and target localization; the implant and skin margins will be cleaned if required; they will have x-rays, after about 4-5 years they will be perfused; they will typically have had no fluid control, no lesions, and no awake fMRI.

(ii) Neuronal recording and stimulation using arrays: Typically animals experience: 2 surgeries with general anaesthesia (one for head-post, one for arrays); only one of those surgeries involves craniotomies and exposes the brain; they will likely have one or two anaesthetised MRI scans for implant design and target localization; the implant and skin margins will be cleaned if required; after about 4-5 years they will be perfused; they will typically have had no fluid control, no lesions, and no awake fMRI, and no chambers (so no chamber maintenance, no dural scrapes, no chamber craniotomies, no chamber microdrives nor other devices), and no x-rays.

The worst case scenarios for those two most typical routes through the steps would include these additional steps:

(i) Neuronal recording and stimulation using semichronic microdrive chambers: Additionally, in worst case scenario they may have the additional maximum of three surgeries with general anaesthesia for significant implant repairs or adjustments (we estimate <25% animals need one such repair, <15% animals need two such repairs, and <10% animals need three such repairs). Worst case scenario animals may also require a maximum of one craniotomy revision (estimated <5% animals). Worst case scenario animals may also require a dural scrape under general anaesthesia up to every 6 months (this worst case is extremely unlikely as it does not apply to array-recording animals or lesion- study animal, it only applies to chamber-recorded animals, and nowadays we expect only <30% of chamber-recorded animal (so <15% of all animals) to require dural scrapes once or twice in totality). Worst case scenario animals may also require fluid-volume control as discussed to the maximum level as outlined above (but as described above is very rare). Worst case scenario animals may also require additional MRI sessions

under general anaesthesia for additional targeting or additional focal ultrasound interventions associated with MRI (up to 6 more anaesthesia's: 1 or more expected in <30% animals, but 6 more expected rarely, in <10% animals).

(ii) Neuronal recording and stimulation using arrays: Additionally, in worst case scenario they may have the additional maximum of three surgeries with general anaesthesia for significant implant repairs or adjustments (we estimate <25% animals need one such repair, <15% animals need two such repairs, and <10% animals need three such repairs). Worst case scenario animals may also require a maximum of one craniotomy revision/re-opening for array adjustment/replacement (estimated <5% animals; not been needed to-date). Worst case scenario animals may also require fluid-volume control as discussed to the maximum level as outlined above (but as described above is very rare). Worst case scenario animals may also require additional MRI sessions under general anaesthesia for additional targeting or additional focal ultrasound interventions associated with MRI (up to 6 more anaesthesia's: 1 or more expected in <30% animals, but 6 more expected rarely, in <10% animals).

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

- Collar pulling: There may be temporary redness from collar pulling if animals that have a collar manipulate/pull upon it themselves too much (<5% animals have collar and few who do show the adverse effect which would be temporary as collar could be removed).

- Stress: all animals may experience periods of mild stress, particularly during the initial stages of chair training, neck-plate training, or when tasks increase in complexity; however moderate and severe stress are both likely to be rare occurrences due to our management

- Frustration/Boredom: it is expected that some animals may sometimes experience some degree of frustration/boredom associated with prolonged periods of behavioural testing given hundreds of trials may be required. However they have continual opportunities to obtain reward and that and the end of task jackpot lunchbox with food and treats motivates performance.

- Home-cage disturbances: As with any addition to a home-cage Mymou could potentially affect changes in behaviour such as disturbed social group interactions (e.g., stressful/physical competition) or disturbed sleep patterns. This would be temporary as Mymou could be removed.

- Discomfort: it is expected that all animals may experience mild discomfort associated with prolonged periods of head-restraint.

- Superficial lesion: there is a risk that with repeated application of FUN to the same region that the skin might acquire a very superficial lesion ('crusting') that does not seem to cause distress and which typically resolves spontaneously.

- Dehydration: As fluid volume control is rare (<10% animals) there is only expected to be low levels of mild dehydration in few animals. Higher levels of dehydration are

extremely unlikely to occur due to our management, they would never be severe and so moderate for only for a short period (as fluid control can be immediately relaxed).

- Temporary hunger: An animal may be hungry for a few hours prior to when we schedule food, as all are fed their main daily diet on a daily schedule as per standard husbandry. Implementation of minor reductions in food volume control are rare (<15% animals) and are infrequent (and never more than 3 days in every 14 days) so the effects are without effect on ongoing health and any additional temporary hunger will not be the normal daily experience. All animals will still get a nutritious and balanced diet every day though in rare cases it may be delayed (some portion of end-of-testing-session lunchbox delayed a few hours so as not to reward failure).

- Post-recovery limb weakness and/or stiffness: due to prolonged immobility during MRI (likely incidence <15%)

- Sores over the course of long MRI sessions: likely incidence rare to the body, but incidence greater below eyes when using restraining device with eye-bars, or around mouth when using restraining devices with mouth bar (likely incidence below eyes and around mouth ~30%).

- Eye Irritation: When anaesthetised scans for localisation require eyes open (in order to show visual stimuli) the eyes are taped open for typically 90 minutes. Irritation of the eye may result if eye gets too dry (<20%) that can be immediately treated with drops (and is mitigated by frequent drops during the procedure).

- Recovery and temporary disability post-surgery: all animals experience a period of reduced activity during waking and immediately after general anaesthesia. The duration depends on the nature of the surgical approach. Typically, animals are expected to recover fully from reduced activity by showing signs of returning to normal behaviour (e.g., be brighter, more alert and responsive, and more willing to interact with other monkeys and humans) during the 24-48 hours post-surgery period.

- Pain: there may be mild pain/soreness associated with general post-operative recovery process evidenced by an animals changed demeanour (e.g., lethargy, and/or excessive picking, pulling, etc) differing from normality for that animal; this is expected to reduce in severity over 72 hours.

- Gastrointestinal discomfort: (likely incidence <10%) caused by prolonged anaesthesia; could include nausea/ vomiting, reduced appetite and constipation, or changes in faecal output/consistency.

- Intubation related: animals are intubated and mechanically ventilated for a number of elective procedures (e.g., surgeries and anaesthetised MRI etc) on this PPL. In rare cases however, transient clinical signs of upper respiratory tract (URT) irritation (laryngeal noise) due to intubation could occur (expected frequency <5%).

- Low and moderate grade peri/post-operative infection - low grade infection may be ubiquitous and impossible to entirely preclude at any level despite surgical procedures are sterile (the wound is not dressed post-operatively and the home cage environment is not sterile). There may be mild swelling, redness, and possible irritation (~50%).

Expected trajectory to recovery: approx. 14 days during which time animals may exhibit signs of transient discomfort such as picking implant and/or surrounding tissue or head-holding. An infection progressing to moderate levels where it produces more marked pain/discomfort is only likely in a minority of animals (estimated ~ 20%) and is kept low by our procedures for aseptic surgery. Animals may have more severe inflammation and redness, and/or excessive picking, pulling, erythema of wound site, mild lethargy, less than normal willingness to eat or drink. Expected trajectory to recovery with treatment is approx. 21 days which time animals may exhibit signs of transient discomfort such as picking implant and/or surrounding tissue or head-holding.

- Deep infection: deeper localised infection to the bone (acute osteomyelitis) is highly unlikely (estimated <5%) and would be expected to be associated with moderate pain/discomfort evidenced by an animal's changed demeanour (e.g., repeated head-holding, and lethargy clearly different from normality for that animal). More widespread chronic deep bone infection is even rarer (<1%) and expected to cause severe pain evidenced by severe lethargy (sustained period of remaining still from which cannot be easily roused). However, such clinical signs of widespread chronic bone infection are unlikely to appear suddenly (may appear and increase gradually over several days to weeks). Therefore the most severe clinical signs (animal immobile and/or unable to eat/drink or in severe pain) if they ever occur will be transient (<24 hours due to endpoint implementation if treatment unsuccessful). No animal has ever had deep infection of the brain.

- Wound healing related: Low-grade inflammation of the surgical site (e.g., swelling, redness) is expected in all animals for 10-14 days post-operation. In addition, some animals develop a localised seroma around the surgical site. The majority of animals will experience mild inflammation / redness / swelling / scabbing to the skin just ventral of the eyes, due to positioning in the head-holder, and/or, occasionally around the mouth due to the mouth bar, for a few days post-operation, that can cause scarring. Sometimes (~75% in complex/multiple implants but lower, ~35% with head-post alone implants) there is some wound dehiscence over time around implants though most to-date have not required skin revision surgery upon veterinary advice.

- Mild pain/discomfort/itchiness are potential occasional implant associated adverse effects (e.g., increased picking/pulling/implant-attention).

- Fear of loud noise: the adverse effect is fear from exposure to loud sounds from MRI and this is not expected to me more than mild. The noise levels generated by the scanner are unlikely to result in pain as they are significantly below the level known to cause pain. They are gradually acclimatized to increasing noise in a mock magnet to reduce fear.

- Midline retraction: ~45% of animals are expected to have array surgery and of those animals only about a fifth may require midline retraction of brain during the surgery which occasionally resulting in temporary weakness in one or both limbs (estimated occurrence in <10% of those needing midline retraction procedures). Midline retraction is required in ~25% of lesion surgeries too but lesion surgeries are rare (estimated <10% animals, and similarly 10% likely occurrence in any one of those animals). Weakness can be apparent immediately or after several hours post operation and may

get worse for up to 72 hours post-surgery after which gradual improvement is expected (and observed in all previous animals) over the course of up to 10 days at which point the animals are expected (given past experience) to have made a full recovery. The weakness does not hamper the ability to feed or drink; animals use their weaker limbs less; they are mobile but their gait is altered during this time. They may be clumsy in climbing if have to compensate for having weaker limbs.

- Removal of zygomatic arch: is required for access to some ventral regions of the temporal lobe (estimated in ~30% of array implantation surgeries). The temporal muscles are retracted in order to remove the zygomatic arch which can typically result in inflammation and swelling for up to 10- 14 days post-operation while the wounds are healing. Zygomatic arch removal does not result in any difficulty in chewing or eating (in the immediate recovery period they will be given soft food – see below).

- Cerebral oedema: Mild (common) to moderate (<20%) oedema is expected to result from surgeries that manipulate the brain. Cerebral oedema can occur immediately, within several hours or during the subsequent post-surgery days. Appropriate medication will be administered peri-operatively on the advice of the NVS to try to prevent clinical signs arising from the oedema. The expected clinical signs of mild oedema are drowsiness, lethargy, apathy, and in moderate oedema there may additionally be mild limb weakness/paresis, mild akinesia, or mild ataxia, and very occasionally seizures. Typically the clinical signs of oedema last between 24-48 hours and are not expected to extend beyond 72 hours. The extent of the disability in most animals is expected to be mild to moderate and always temporary. Only in rare cases (<1%) may animals show weakness/ataxia/paresis/ataxia to an extent that they are unable to stand or support their own weight in a sitting position without an extraneous form of support; this too will be temporary with gradual improvement seen over 48 hours. They typically sleep for extended periods of time. They may also not be able to eat and drink on their own in which case food must be held for them and fluids provided via a syringe directly into their mouth (<1%); this too will be temporary with gradual improvement seen over 48 hours. They may lapse into periods of unresponsiveness (sleeping and drowsiness), may have reduced interest in their environment, lack of ability to focus on objects, and lack of resistance to being handled. In our experience all animals will make a full recovery with no lasting disability.

- Seizures: Full range of seizures (simple, or complex partial, or general seizures such as absence seizures, clonic, tonic- clonic seizures) are possible after manipulating the brain but it is rare (<5%). In these cases seizures are typically expected to occur within the first 48 hours of surgery, be single (or a few in number) episodes, transient, resolve spontaneously and/ or respond (i.e. stop) immediately to an appropriate medication.

- Neurotoxic brain lesion: Currently we estimate a minority of animal would have lesions and if they did then ~70% likelihood we'd use this neurotoxic technique; it may cause localised intracranial inflammation resulting in mild cytotoxic oedema. Mild to moderate clinical signs of oedema may be expected in <30% of cases but severe clinical signs of oedema in <1% cases (see section above for clinical signs of oedema). After lesions some animals (<30%) may be quiet, withdrawn or slower to respond/come forward, and be less interested in drinking and food/ treats; typical

duration of clinical signs is between 24-48 hours and is not expected to extend beyond 72 hours (sometimes with neurotoxic lesions, <15%, oedema may have delayed onset with symptoms first noticeable after 24-48 hours post-op, but are similarly not expected to last more than 72 hours from onset). Extent of disability: animals are able to eat and drink unaided (typically offered fluid within 60 mins and food within 90 mins of recovery from anaesthesia), they are mobile but may exhibit limb weakness, aware of their surroundings and objects in their environment. Animals will make a full recovery with no lasting disability.

- Surgical excision/ aspiration/ cautery to induce a brain lesion: This approach to making lesion (~30% likelihood we'd use this technique in any animal having lesions) is associated with clinical signs that typically less extensive and less severe than those caused by neurotoxins/ neurochemicals (above). Mild to moderate clinical signs of oedema may be expected in <20% of cases but severe clinical signs of oedema in <1% cases (see section above for clinical signs of oedema). After lesions some animals (<30%) may be quiet, withdrawn, or slower to respond/come forward, and be less interested in drinking and food/ treats; typical duration of clinical signs is between 24-48 hours and is not expected to extend beyond 72 hours. Extent of disability: animals are able to eat and drink unaided (typically offered fluid within 60 mins and food within 90 mins of recovery from anaesthesia), they are mobile but may exhibit limb weakness, aware of their surroundings and objects in their environment. Animals with bilateral lesions, in most cases, are more severely affected than those with unilateral lesions and we expect the majority of our lesions to be unilateral. In our experience all animals will make a full recovery with no lasting disability.

- Dural bleeds: The procedure to make craniotomies under chambers (~45% animals), or to extend a craniotomy (rare), is not intended to result in brain intervention, but sometimes if the dura is adhered to the cranium in the region of the craniotomy, or if there are superficial blood vessels in the region, there may be bleeding (<30% of cases). In rare cases there may be superficial damage to the dura and cortex that is visible but insufficient to lead to any neurological adverse effect. The bleeds are easily slowed and stopped.

- Neuromodulatory interventions: The neuromodulatory agents injected into the brain effect reversible and highly localised inactivation of target sites that it is temporary and brief (typically lasting 1-4 hrs, no longer than 24 hrs depending on agent and dose) but may last longer than the testing session. Because the effects of such drugs are transient and the area of effect small, we expect the effect of reversible lesions to be subtle and to have minimal effect on the animal's behaviour so to be largely indiscernible to observers in terms of home-cage behaviour.

- Implant cleaning: The animals may initially be stressed by the cleaning/maintenance but most animals tolerate it well as they are well used to being chaired and head-fixated and as cleaning is frequent they don't associate it with negative outcomes (and significant positive reinforcement / reward is provided). It normally takes ~ 10 minutes. If the animal has significant infection then there could be transient discomfort (periodically for a few seconds at a time, up to a typical duration of 10 minutes) associated with the cleaning of inflamed or sore areas.

- Dural scrape: There could be minor herniation (<10% occasions) if the dura is scraped very thin in a small area, wherein brain herniation could be observed; it is only minor herniation observed and animals do not show any discomfort. In ~50% occasions sometimes there is bleeding from granular tissue which usually stops as removed; it is only transient minor bleeding observed and animals do not show any discomfort.

- Head-cap related: It is rare for our animals to be fitted with plastic head-caps attached to the post to prevent wound picking, but if we do then it is estimated than in <20% of cases this may cause temporary distress evidenced by erratic behaviour including pacing/pulling (rare). This will be temporary as we can quickly remove the attachment.

Expected severity categories and the 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 ~70% Severe ~30%.

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

- Killed

A retrospective assessment of these predicted harms will be due by 11 April 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

Achieving these aims necessitates investigating how the activities of neurons relate to each other and to behaviour. Physiological neuronal recording (i.e. electrophysiology) via microelectrodes inserted directly in the brain is the only available technique to directly probe neuronal activity and function at a millisecond-by-millisecond level at the resolution of individual neurons, which is vital in order to determine the neuronal level mechanisms supporting behaviour. Non-invasive techniques used in humans for recording brain activity whilst people carry out behavioural tasks (such as fMRI) cannot measure individual neurone activity to the degree of resolution we require for these studies.

Further, we must record from multiple individual neurons in multiple brain areas at the same time in awake, behaving subjects, before and after intervening in specific brain regions. This combination of invasive techniques required necessitates using animals.

There are several reasons for this:

i)patients do not ever have electrodes implanted in the precise combination of network nodes we need to record from

ii) even those patients who do have electrodes implanted into some regions of their brains for clinical reasons are by definition not neurologically normal and we need to record neuronal activity in normally functioning brains

iii) we also need to gather data from a large sample of different neurons over extended periods of time to enable sufficient neurons to be analysed across sufficient repetitions of each trial-types (condition) to enable appropriate statistical tests to be conducted. This usually takes many months to a year. Patients with electrodes are rarely recorded from for more than a few days to a week so the detailed systematic sampling we need to do is impossible in patients.

iv) we also need to target interventions such as lesions or inactivations to specific limited areas that can only be induced with precision in experimental animals. For example, some patients acquire non- specific brain damage, known as lesions, that might include but are non-specific to an area, but this non-specificity of lesions will not suffice as it would be detrimental to any analysis and interpretation pertaining to a specific area. Sometimes we require reversible interventions that precisely (both the place and the time) target the same areas we record from. Patients do not have electrodes in all these requisite regions for the requisite time-frame (and it would not be ethical to stimulate their brains repeatedly in all the ways we'd need to do so over many weeks for non-clinical reasons).

v) we could not record the necessary neuronal activity both before and after interventions in patients because no healthy humans with completely normal neural activities ever have electrodes implanted. This means we could not compare of pre- and post-intervention behaviour with pre- and post- intervention neuronal recording which is necessary for us to achieve some objectives.

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

Alternative techniques or approaches would be to carry out experiments in humans using either invasive recording or non-invasive imaging techniques, and to use methods to interfere with the working of discrete areas of the human brain, or in-vitro cultures of primate brain cells, and computational/mathematical models.

The specific invasive techniques we have considered are recording from patients with deep-brain electrodes implanted for clinical reasons.

The specific non-invasive techniques we have considered are:

- Functional MRI, human magnetoencephalography (MEG), human Electrocorticogram (ECoG) through macroelectrodes placed on the cortex.

  The specific interference techniques we have considered are:

- Transcranial ultrasound stimulation (TUS) in humans.

  We have also considered the use of human brain organoids, in-vitro culture of brain slices and culture of primate brain cells.

We have also reviewed the current state of computer models and their suitability to replace any of our proposed studies.

Note we sometimes conduct our own parallel studies in humans to evaluate elements of our animal models in patients. Methods we have used to-date with humans include:

- testing neuropsychological patients with lesions on tasks like those we design and give to macaques
- testing humans using fMRI on similar tasks to those we design and give to macaques to assess whole brain activity (and to allow comparison with macaque fMRI).
- transcranial magnetic stimulation (TMS) in humans performing tasks similar to those we give to macaques
- recording local field potentials from rare patients with deep brain electrodes implanted in their brains for clinical reasons while they perform the same kinds of behavioural tasks we developed for our monkey
- behavioural studies comparing human and macaque performance on tasks
- mathematical and computational modelling

Whilst these human based studies provide some useful data they are complementary to our animal studies and cannot replace them.

Why were they not suitable?

Our aims are complemented by but cannot be achieved or replaced by a range of methodologies (see below for why those considered were not suitable). In short, the fundamental reason is we need to study neuronal level mechanisms that underlie cognition and that means we need to record neuronal level activity. Moreover, we need to do that from many electrodes in several areas simultaneously in order to study neuronal interactions. This can only be done in animals as it is invasive. There is no replacement technique with the spatial and temporal resolution required to get at neuronal level activities. We cannot use patients with electrodes implanted for clinical reasons as those patients have dysfunctional brin networks, do not have electrodes in all regions required, and we need to record for months to a year or more. Considering specific methodologies:

- Our aims cannot be achieved by human neuroimaging, e.g., fMRI (functional MRI). This is because fMRI cannot provide the level of detail we require about neuronal level mechanisms because it averages activities over a volume referred to as a voxel which is typically on the 1-4 cubic millimetre scale or larger (containing 10s to 100’s of thousands or more neurons). In addition, the temporal resolution (in seconds) of fMRI is also not sufficient as we need to measure neuronal activities operating between neurones that occurs at the millisecond level (whereas the temporal resolution of fMRI is at the seconds level). Moreover, fMRI is not a direct measure of neuronal activity, instead it measures localised changes in brain blood flow and blood oxygenation. The precise relationship between neuronal activity and the blood-oxygen-level- dependent (BOLD) signal response obtained with fMRI is controversial.

- Magnetoencephalography also cannot provide the level of resolution we require as the signals produced require concerted firing of around 50,000 similarly aligned neurons whose localisation is uncertain. The source localisation in MEG/EEG is 'ill-posed', meaning that the accuracy of the location is not certain.

- Some (e.g., epilepsy) patients have multiple ECoG (Electrocorticography electrode) arrays implanted over widespread areas of cortex but they do not record the speed or location of neuronal activity accurately enough. The technology only provides a temporal resolution of approximately 5 milliseconds and a spatial resolution of about 1 cm and cannot isolate the activity of individual neurons. This means that we would not be able to achieve our aims even if patients had arrays over all the regions we need to study. This is not the case as some of the regions we are interested in are subcortical or deep or inaccessible by ECoG too.

- Some patients have deep electrodes implanted in their brains but only in the presence of abnormal clinical conditions (and we need to study normal brain function).

- We cannot use human TMS (Transcranial magnetic stimulation) as an intervention method as it is not precise enough to target the precise circumscribed brain areas we study. Also, TMS is limited to targeting neurons dependent upon the local brain morphology of the gyral and sulcal patterns (and predominant orientation of neurons in cortex) with respect to magnetic fields induced by TMS (so it cannot target neurons in all brain areas equally).

- There are no current computer models of the complexity of multiple interacting cortical areas, their multiple layers, and myriad of connections. This means that we cannot use computer modelling to meet our aim of discovering new mechanisms of neuronal activities that underlie cognition.

- However, there are simplified conceptualized models of some interacting system components, or of behaviour, which we use in parallel to help explain our data. Sometimes these models generate new predictions which we can test. Sometimes reinforcement learning models, for example, can account for series of behavioural choices, and if they do that well, we can search for evidence of such neuronal encoding of those parameters which would suggest similar processes may be occurring in the brain.

- We have also considered in-vitro methodologies including human brain cell organoids. However these are entirely inappropriate and useless for our purposes; organoids do not have sensory inputs, they do not output complex cognition, and they do not possess the differentiated networked brain regions connected together in the same way as the primate brain that we know are necessary to underlie primate cognition.

We will continue to monitor progress in such technologies to see if future advances can provide opportunities for complementing or replacing any components of our studies.

A retrospective assessment of replacement will be due by 11 April 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

11 animals funded by three current awards/grants will be transferred from the current project to this licence

Funds are available for up to 6 additional animals by the current awards/grants.

The licence is for a plan of close collaborative work by two Principal Investigators and their associated teams of career development fellows and post-doctoral researchers that is envisioned to require up to 26 animals in total over the next 5 years. One major grant is already being prepared/submitted by both Principal Investigators for the next group of 3-4 NHPs. Several further awards/grants/fellowships will certainly be submitted over the course of the next years (as is our continued practice) for the remaining animals. Both Principal Investigators have a solid success rate in grant applications to-date, moreover both now have aspiring career development fellow applicants who have recently submitted applications and/or intend to make fellowships applications to work with us on these projects.

Hence amendments will be sought to increase the n of animals as each major award is obtained up to the envisioned 26 animals (this is the approach advised by the HOI and is why the n of animals at point of application is only 17). Although 26 total animals is envisioned, in rare cases some animals occasionally have to be replaced after entering the protocol (e.g. if diagnosed with untreatable illness unrelated to any protocol, or if determined to have brain abnormalities) and some contingency in total numbers is required under those circumstances.

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

It is important to first clarify that in the context of our within-subject designs our unit of replication is the neuron (more specifically it can be said to be the neuronal activity across the session) and not the animal. We do use power calculations (described elsewhere) to determine the number of trial-types to record per neurons, and/or the number of neurons to record per brain region, but power calculations are not relevant for numbers of animals per electrophysiology study. Rather, for a standard electrophysiology study data would be acquired from two macaques that performed the same task/s and from which we recorded from the same brain region/s we needed to analyse (occasionally a third animal may be used, but only if circumstances required). This is a long-standing international standard. This number of animals (i.e. typically 2, rarely 3) being necessary is a combination of: (i)

that required to show statistical reliability in the activity across neurons with respect to task 'conditions' and that these are not limited to neurons from a single animal, and (ii) our need to show that any new mechanisms we find evidence for (e.g., previously unknown patterns of task-related activity or synchrony or interactions within/between areas) are not specific to one animal.

In further explaining the above it useful to clarify that we typically seek experimental evidence for the 'existence' of such general mechanisms, our research does not aim to quantify the average level of such a mechanism in a population of animals (which would indeed require more animals). Hence the number of animals per 'study' is typically kept at 2 which is often sufficient and is also a 'Reduction' from using more animals than necessary. Only in cases where the behaviour or neuronal activities recorded across the 2 animals is inconsistent or ambiguous (e.g., due to rare noise of various kinds in occasional animals) is an additional 3rd electrophysiology animal used.

Steps taken to affirm the above include: retrospective reviews of decades of influential work by leading international experts; rigorous peer-review of our grants (where numbers of animals have to be justified); that our papers have had it deemed appropriate by the editors of the internationally respected journals and their expert reviewers. We have consulted statisticians in the past and this is a long-standing internationally agreed suitable n of animals per study for primate neurophysiology. If the consensus of thinking changes on the appropriate n of NHPs we will review the suitability of our numbers in that light and accordingly continue to apply the principle of Reduction.

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

Ways in which we will optimise the number of animals used include:

- ensuring that we use recording methodologies that have multiple recording channels so that maximum data is collected from each animal (e.g., recording from multiple areas in each animal is more efficient to recording from single areas per animal; similarly, when we study pairwise interactions between brain regions in a network involved in supporting a cognitive process, if we record from >3 regions we can analyse >2 pair-wise interaction per animal so fewer animals are required to study the interactions of interest).

- the use of only high quality, purpose bred animals, is also an optimising factor for the number of animals used.

- sharing of data in online repositories so that others can examine data without the need to perform further experiments of their own on additional animals.

- we harvest NHP tissue collected post-mortem for sharing with researchers.

A retrospective assessment of reduction will be due by 11 April 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

Which animal models and methods 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:

- **Adult rhesus macaque monkeys** are our only animal model that can be used to obtain the data we need to meet our aims. Other species do not have the brain networks of interest or do not exhibit the behaviour of interest. Specifically (1) Rodents are not suitable as we typically investigate cognitive functions mediated by, or in interaction with, cortical regions that rodents do not have analogues of in their brains. (2) We also investigate some complex cognitive tasks that require rapid and flexible decision-making and rodents cannot perform these tasks to a sufficiently high degree of competence and reliability. Macaques (which are Old World monkeys) can reverse rule competently in these tasks in only a few trials which is required for analyses. We cannot use marmosets either (which are New World monkeys) because they too cannot perform some of our flexible decision-making tasks to a sufficient level (e.g., they typically take a whole session's worth, or often more than one session's worth, of trials to reverse). (3) We use adult animals as we need to affix cranial implants and the stability is more robust if at the time of the implantations the cranium is not still likely to markedly grow as they transition to adulthood; adults also have larger heads so the devices may be suitably well spaced which helps stability and healing. We also use adults because it is not our aim to investigate development of cognition through juvenile stages; we are investigating developed adult cognition.

- Consequently, any data obtained using other species would not reliably achieve our aims. Unreliable investigations in an inappropriate model would not advance science and would require more follow-up investigations. This would result in wasted use of animals.

Methods:

- Our methods are multi-area, multi-electrode, electrophysiological recordings and interventions during complex cognitive task performance. By using refined training methods our methods operate in the context of an experimental paradigm wherein the animals voluntarily enter into the testing chair for reward. The animals perform the daily tasks for reward (with no punishment procedures). Due to refinements over the course of the last two licences we no longer typically need to require water restriction (<10% animals) so we know in general that macaques are not merely performing our tasks because they are thirsty, rather the tasks provide reward opportunities that are sufficiently motivating most of the time for most animals. The animals need to be restrained while testing (typically for a couple of hours), but do not endure pain when

being tested, and indeed are not unduly uncomfortable during testing as they can continually shift their body and limb position. Taking the above approach we minimise suffering, and distress; indeed the vast majority of days are of that kind of experience and are not deemed aversive or painful.

- We use predominantly positive reinforcement learning approaches in all our training stages. This includes right from the initial arrival and early husbandry training (e.g., clicker and/or target training to encourage animals to move around to cage zones as required, and to take food and drink from technicians). The chair training procedures and head-restraint training are again trained using predominantly positive reinforcement methods, as are the staged progression in cognitive tasks. We do not ever use punishment procedures. The dictionary definition of negative reinforcement is when something unpleasant or uncomfortable is removed or taken away in order to increase the likelihood of the desired behaviour; we do not use negative reinforcement according to this definition. The only reason we state above that we use predominantly positive reinforcement is because in additional to positive reinforcement we sometimes use mild negative procedures that are limited to: showing or raising up a net only if required (only proceeding to using a net in extremely rare cases, for example limiting space by inserting the net, but excluding actually catching the animal in the net); holding up a cage pull-back winder handle so it is visible only if required (or if that is insufficient proceeding to attach the handle to the winding pull-back mechanisms by which the cage insert may be slowly moved towards the animals to reduce volume of space and encourage the animal to move towards the cage exit). Even if these negative techniques are used they are only used temporarily, briefly, and they are immediately followed by positive reinforcement as a reward for success. We never use a pole and collar to force an animal to exit a cage and/or enter a chair (as many international laboratories do). Only if necessary, once an animal is in the chair, if it does not volunteer to do so itself for reward we might gently lift up the animals chin by lifting the collar (using a rod as opposed to directly manipulating the collar by hand to avoid potential bites to a person's hand) so to raise up the animal's head in the chair to enable neck-plate closure. Similarly, this collar-raising is only used if required and if used is followed immediately by reward. After these experiences animals quickly realise that raising their heads (for neck-plate closure) does not lead to an aversive situation as is it followed by positive reward, such that they then do it voluntarily typically within a day or just a few days. This use of a method beyond purely positive reinforcement avoids animals otherwise getting stuck in that phase and so avoids potential unnecessary repeated anxiety day after day by animals that might otherwise be stuck in that phase through unfounded fear of raising head. In short, predominantly positive reinforcement with rare/mild negative methods as described is an efficient, effective, and refined way to teach animals. Our lack of need to ever use fluid control to train animals to enter a chair and raise their heads in the chair further attests to this refined approach. We have ~25 years of experience in task-training. We also consult current good practice guidelines published by NC3Rs (Training laboratory-housed non-human primates, part 2:

  Resources for developing and implementing training programmes 2005; and An international Survey of Approaches to chair restraint of nonhuman primates 2017).

- On very few rare days (amidst all the standard training/testing days detailed above) they need to have devices surgically implanted to enable the neuronal recordings; on

these rare occasions they have general anaesthesia and receive pre-, peri, and post-operative analgesia, to minimise pain, suffering, and distress.

- To further minimise distress our animals are where possible housed in social groups and with environmental enrichment; animals typically live in pairs or larger groups even when they have implants as the implants do not change their normal behaviour.

- If any animals do experience significant adverse effects such as pain or suffering that cannot be ameliorated they reach a **humane endpoint** to avoid lasting harm and distress.

- **We largely avoid adverse effects of water restriction because most animals do not ever experience it.** This refinement arises from our use of refined training methods we introduced to electrophysiology (trial-by-trial high value smoothie reward; jackpot lunchbox at end of session) that are suitable to our tasks because they only typically require 100s as opposed to 1000s of trials per session. In the minority of animals that experience water control the majority of those only experience timed access control (not volume restriction) and so for several hours/day and over weekends they will have ad lib access to water. Also, if ever we need it, we only implement water control only for as long as necessary (i.e. temporary) and implement the lowest control necessary.

- To limit harms from surgeries we carry out all surgical procedures in the most refined way including best practice for sterile surgery. We have consulted human neurosurgeons about some of our approaches (e.g., methods for craniotomies). We have refined the postoperative care of animals with exteriorised implants by instigating regular inspections and cleaning and care as required. We have refined anaesthesia by using gaseous anaesthesia. We have refined surgical incisions by studying vasculature patters in the head in macaques to try to maintain good blood supply. We have shortened surgeries by about an hour by introducing a method of having pre-shaped legs on head-posts (by using an MRI scan to create a 3D model of the specific animal's skull so to shape the implant's legs to the skull model prior to the surgery). We have taken advice on best suturing techniques from veterinarians. We consult with other neurosurgeons about refined methods including approaches to brain regions. On account of all these refinements we have entirely avoided any of the most severe potential outcomes (e.g., brain haemorrhage and severe oedema and deep brain infection have never been encountered by the licence holder or his team in > 25 years of work). We further limit harms from surgeries by considering if we can combine required procedures into single surgeries to limit the number of surgeries to no more than required to meet the experimental aims balanced against animals' cumulative experience. We do occasionally need to make repairs to implants but our refinements with respect to improving implant stability (e.g., position of implants, combinations of screws and cement, refined procedures for skin margins, better implant-shaping-form-fitting procedures) have reduced the incident of repairs in recent animals compared to initial animals.

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

- We cannot use species that are less sentient because our aims are to investigate the neuronal mechanisms underlying higher cognitive function in primates, that are mediated by specific networks of primate brain areas that only exist in primates.

- We cannot use immature juvenile animals because our aims are not to investigate how cognition develops as the brain matures from juvenile stages into adulthood, rather our aims are to investigate brain mechanism in the mature adult brain.

- Investigations of brain mechanisms that underlie behaviour require animals to be carrying out the behaviour whilst measurements of neuronal activity are recorded. These behaviours cannot be performed by the animals whilst under anaesthesia,

- Similarly we cannot use organoids as they do not exhibit cognition and choice behaviour (nor contain the brain networks we investigate; nor have sensory inputs etc),

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

- recently we introduced a refined training procedure to minimise (and in majority of cases obviate) the need for fluid control for motivation in the kinds of complex tasks we use; we will continue to explore refined methods to train animals that focus on positive reinforcement including in early facility and lab acclimatisation and husbandry stages.

- we have refined our surgical approaches in many ways (optimising tools, and consumables, and surgical approaches, refining suturing methods, consulting with neurosurgeons) and will continue to do; most recently this has reduced the incidences of implant instabilities and infection for arrays and chamber respectively. We will continue these refine approaches and also continue to consult with experts to gain their expert knowledge (what worked well, what didn’t work so well with the latest iterations of these devices).

- in the future we are interested in wireless recording technologies which might, in combination with in-cage testing, open up some new avenues of research in unrestrained animals in their home-cages (this will likely complement as oppose to obviate traditional electrophysiology methods which we expect will remain necessary as we need to regulate and control and time behaviour in most studies which is not possible in home-cage investigations).

- we have just started to implement a home-cage testing system called Mymou which can recognise animals and provide a suitable task when they voluntarily engage; we will try to see if pre-training on some elements of some tasks using Mymou may reduce the number of restrained/chaired sessions required in some task learning stages.

- we have a range of options available to intervene/inactivate brain regions which include permanent and invasive surgical lesions, reversible and invasive muscimol injections, reversible and invasive electrical microstimulation, and reversible and non-invasive focal ultrasound neuromanipulation (FUN). Our facility has been using FUN

for years and is the most refined in terms of being both non-invasive and reversible, but the temporal and spatial resolution of all methods differs and the ability to target all areas effectively differs with or without other interfering devices/implants. We have not yet implemented muscimol injections as a way to reversibly inactivate a brain region for the duration of a testing/recording session but for some brain regions it may be the most appropriate technique taking all factors into account. If we introduce this new method we will first run a pilot study in one animal. It is a well-established technique in other laboratories and so we will use published parameters of stimulation known to be effective and safe. If we can use it to induce reliable inactivations that effect behaviour and neuronal activity then it may become even less likely we would decide to use surgical lesions as interventions as they are not reversible.

- the devices for recording we implant in this licence are likely to have higher channel counts and that either allow for more neurons in more brain areas to be recorded with the same number of implants (so getting more data per animal without increased cost), else equivalent numbers of channels can be recorded with fewer implants (e.g., the array pedestals used to house 128 channel connections and can now house 256).

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

Generally we follow:

- LASA guidelines to guide best surgical practice (e.g., Guiding Principles for Preparing for and Undertaking Aseptic Surgery).

- NC3R primate guidelines: NC3Rs (2017) Non-human primate accommodation, care and use; Training laboratory-housed non-human primates, part 2: Resources for developing and implementing training programmes 2005; An international Survey of Approaches to chair restraint of nonhuman primates 2017

- ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) are a checklist of information to include in publications describing animal research which we strive to meet.

- PREPARE guidelines (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) which is an aide memoire covering a range of topics for quality assurance falling in three main categories: study formulation, dialogue between scientist and facility, and quality control of study components.

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

- by myself and/or my team of researchers who work under this licence attending NC3R events and workshops (as speaker, presenter, or as attendee), including the dedicated NHP events organised by NC3R.

- by our attending welfare presentations at other national and international meetings (as speaker, presenter, or as attendee)

- by our attending NHP expert group meetings
- by communicating with national and international colleagues who are expert at similar techniques
- by consulting NC3R website and training resources (can be used as refresher training for staff)
- by running literature searches on Pubmed (which comprises more than 35 million citations for biomedical literature from MEDLINE, life science journals, and online books.)

A retrospective assessment of refinement will be due by 11 April 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

3. Genomics and population structure in wild British birds

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

birds, genomics, DNA sequencing, evolution, hybridisation

Animal types	Life stages
Birds (selected species)	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Uses endangered animals

Objectives and benefits

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

What's the aim of this project?

I will carry out genomic sampling of a small selection of bird species that can be found in the wild in the UK. This will allow me to identify relationships between population genomic structure/diversity and conservation status, at both national scale and local scale. Such analyses will identify populations of conservation interest, inform decisions about genetic management, and explore evolutionary and demographic histories.

A retrospective assessment of these aims will be due by 23 April 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 recent years, the ease of large-scale genomic study has increased. Despite this, there has been very limited research focussing on wild birds in the UK that makes use of whole-genome data. Due to the topography, geography and anthropogenic history of the country, the UK provides examples of species with differing population structure, conservation status and demographic history. Coupling fieldwork (using blood sampling) with computational analysis using high-powered computing systems, it should be possible to work with a small selection of bird species to:

Identify variation in population structure within and between the different islands of the UK, allowing us to identify subpopulations of particular conservation importance, and to explore the role of genetic differentiation (differences in the genomes among different populations) in population divergence (the distinctiveness of populations from one another) (which will aid our understanding of processes such as the formation of species, and interbreeding of different species). This will use four species which can be easily worked with, and which can be found across the U.K.

Explore fine-scale genomic variation/structure using a single focal species within a particular island group, including temporal variation and correlation of population genomic structure and parasitic/disease diversity.

Identify variation in genetic diversity between different populations of different species. This will include both newly expanding (e.g. introduced species) and declining species. Such work will pave the way for targeted conservation efforts and a stronger understanding of the biology of newly colonising birds, including those which have been experiencing range expansion due to climate change or reintroduction. This component will include a suite of 16 species (8 non-native, and 8 native species).

Working with this targeted selection of bird species will provide a wealth of data with which to explore the above questions. They are of interest both from an applied conservation perspective (e.g. identifying inbred populations, with limited diversity, that might be in peril - or studying newly colonising birds to gain a better understanding of population expansion and invasiveness) and in terms of basic research, exploring evolutionary biology and demographic histories. The species have also been chosen as they are easy to work with, ensuring that the project will be achievable.

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

The project will result in publications in high-impact academic journals, focussing on evolutionary biology and conservation genetics. The population genomics of almost all UK bird species is completely unknown, so there are significant opportunities for exciting published work. It is also likely that the aspects of the project focussing on declining or introduced species will inform conservation decisions. For example, we might identify populations which are suffering the genetic impacts of inbreeding and would require the introduction of unrelated birds to reduce the chance of extinction.

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

The study of birds of conservation interest (either declining or expanding populations) will benefit conservationists by informing conservation decisions and population management. This will allow us to incorporate information about genomic diversity and population structure into decision making in a way that has not been possible before. Working will a selection of tractable focal species will open the door to future work on a larger scale, which will use broad-scale inter-species genomic comparisons to inform conservation and evolutionary biology.

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

The work will often (depending on the species being sampled) be done in collaboration with other UK regulatory bodies, and sometimes other researchers carrying out e.g. behavioural studies of certain focal species. This will maximise the knowledge gained on each species as the genomics research is most valuable when coupled with a solid understanding of the biology of the species involved. Work will be disseminated at academic conferences and via peer-reviewed publications, and through presentations to the general public.

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.

I will use wild birds as these are the species I am interested in, given their population genetic structure. I will not sample freshly-hatched chicks as they are more delicate to handle than adults or juveniles (will not sample from any bird that is less than 50% of the average adult weight for that species). The species sampled will include both native and introduced species. As well as this, we will also sample from collections in aviaries for the naturalised focal species. This will allow me to generate genomes for comparisons with the free-living populations.

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

Birds will be caught (with permits from the British Trust for Ornithology) in nets and traps, before being measured, photographed and ringed. They will then have a small blood sample taken using a needle and collected using capillary action with a blood collection tube. The blood sampling procedure itself takes less than half a minute, and the birds will be handled, from capture to the point of release, for no more than twenty minutes. Up to 730 birds of 20 focal species will be blood sampled as part of this project.

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

No adverse impacts are expected, other than very brief low level pain. Birds will recover from having a small blood sample taken almost immediately (within five minutes, i.e. by the time the bird is released back into the wild). Rare adverse impacts include general stress response/shock (fluffed up feathers, closing eyes, panting) or haematoma (localised bleeding outside of the blood vessels). Neither of these are likely to have long-term impacts on the birds' ability to survive in the wild post-release, but any birds suffering from these adverse impacts will be held for observation for an additional 10 minutes to assess for further declines in 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)?

100% mild.

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

- Set free

A retrospective assessment of these predicted harms will be due by 23 April 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 requires DNA samples (blood) from birds. There is no way to get this other than to catch and take samples from the animals.

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

I considered using recent museum specimens (bird skins) rather than using live birds. I also considered using culled/hunted specimens.

Why were they not suitable?

For almost all species, there are not enough museum specimens to perform useful bioinformatic/genomic analyses. Also, I will be studying contemporary population status so current birds are required rather than museum specimens, which may have been collected some time ago. For culled/hunted specimens, I have trialled working with muscle tissue/feathers and DNA extraction is significantly less successful (especially given this project requires significant DNA quantities for whole- genome analyses). I would ideally need fresh blood which would need to be taken immediately after death of the birds. Hunters/cull operators are almost always unwilling to allow me to accompany them to carry out such blood sampling. It is therefore not a suitable alternative to catching and blood sampling live birds (and very few of the focal species are regularly culled).

A retrospective assessment of replacement will be due by 23 April 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

730 birds is the maximum number of blood sampling procedures which will be carried out as part of this project.

For objective 1 (national-scale population genomics): 25-50 individuals are needed to provide sufficient genomic variation to allow the common statistical analyses of population genomic structure over a region the size of the UK. For two of the four species, there is more complex known geographic variation in their distribution, or they are not studied commonly. This means that sampling cannot be as tailored to known areas of interest, requiring 40 individuals of each. The other two species are well-studied so sampling can be tailored to geographic regions of interest more easily, so 30 birds will be sufficient for these two species, minimising animal use to that which is strictly required.

For objective 2 (fine-scale population genomics): 70 individuals per year from one species, within a specific geographic area, will provide a large sample size allowing both population genomic structure analyses at the micro-scale, and also detection of presence/absence of

e.g. disease causing parasites. This will allow for both inter-year comparisons of population structure and also within-individual changes in infection status (recapture rate is currently 20- 40% in this study population, so this will ensure that there is a useable sample of data from multi-sampled birds within the dataset).

For objective 3 (genomics of increasing/decreasing populations): In order to generate accurate population diversity statistics, 7-10 individuals will be needed per species. For the eight non- native species (which are to be statistically compared not only with the other species, but as captive-wild within-species dyads, this means that a maximum of 20 individuals will be sampled (roughly half from wild populations and half from captivity). For the other eight (native) species, 10 birds maximum will be sampled, as these will be compared only among species.

The addition of further species and individuals may be suggested and discussed if additional funding is secured.

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

We will sample blood rather than feathers (which would not fall under ASPA and can be permitted by the British Trust for Ornithology) so that we can be certain to get genomic DNA from every bird. Trying to extract DNA from feathers would mean that not every individual would have its DNA successfully extracted - meaning that they would have been captured and have its feathers removed for no reason. Feathers also provide small quantities of DNA which are insufficient for whole-genome sequencing except low coverage or restricted representation (neither of these approaches is suitable for the analyses I propose).

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 (i.e. for the commoner focal species) I will liaise with British Trust for Ornithology bird ringers who would be trapping anyways, to keep overall bird disturbance to a minimum. Experimental design as such is not possible as this is not experimental but observational research, but the number of birds used for each objective is the minimum required to facilitate the suite of statistical analyses belonging to the relevant objective. I have been carrying out pilot work monitoring the single-species which is to be the focus of fine-scale population genomic structure work. This has allowed me to develop an understanding of expected recapture rate, and therefore to predict sensible sample sizes required. Finally, I have carried out pilot work catching and ringing in certain populations that will form part of this project, to optimise capture methods and liaise with landowners. This will allow me to quickly catch the required number of animals with minimal chance of by-catch etc.

A retrospective assessment of reduction will be due by 23 April 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

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

We will use wild birds and we will take blood samples. It is needed to use wild birds because the project is focussed on their genomic information. Blood sampling is the method to get (whole-genome, high coverage) genomic DNA samples which causes the least pain, suffering, distress or lasting harm.

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

My work focusses on British birds, which have the population genomic structure and between/within species variation that I am interested in. There would be no way to replicate this in laboratory animals or any less sentient animal. The procedure is simple blood sampling that will not require anaesthetic. Birds will be released back into the wild so terminal anaesthetic is not relevant.

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

I will couple the usual blood sampling procedure with British Trust for Ornithology guidelines for monitoring bird condition during handling - for example monitoring for signs of cold (fluffing the feathers up), stress (closing one or both eyes), heat (panting) or shock (going very still and unreactive). This will enhance my ability to react quickly to changes in condition and act accordingly (i.e. release immediately).

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

We will follow the guidelines outlined in the British Trust for Ornithology's Ringer's Manual when it comes to fieldwork procedures, catching and handling birds. This, as well as regular updates from other UK regulatory bodies (e.g. updates on the optimum ring size for tagging each bird species, updates on best practice for handling based on new information). All those working with birds are expected to be qualified British Trust for Ornithology bird ringers, in order to be deemed competent to work under this licence. Qualified BTO ringers will be deemed able to handle and catch birds, humanely dispatch them if required, and attach rings to them. They will not be deemed competent to take blood themselves unless they have undergone additional training.

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

advances effectively, during the project?

I will continue to read the literature, both relating to the 3Rs generally, and more specifically relating to working with wild birds. I will attend welfare meetings within the establishments to keep abreast of new initiatives. I will also regularly review the NC3R's website and interact with the Named Information Officer. I will keep up to date with updates in bird handling and welfare provided by the British Trust for Ornithology, and also relevant papers published in journals such as 'Ornithological Applications'.

A retrospective assessment of refinement will be due by 23 April 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

4. The Production of Laboratory Animal Bio- Products

Project duration

5 years 0 months

Project purpose

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

Key words

Vaccinations, Virology, Blood, Plasma

Animal types	Life stages
Mice	Adult
Guinea pigs	Adult
Ferrets	Adult
Rats	Adult
Beagles	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Uses cats, dogs or equidae

Objectives and benefits

Description 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 supply animal blood products, to generate data to support the development of effective and safe medicines to treat diseases in humans and animals where there is currently a clinical unmet need.

A retrospective assessment of these aims will be due by 27 April 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence? Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

Bio-products services providing animal blood products, will support the generation of scientific data to support the development, manufacture and testing the quality of new and approved medicines within the field of Human and Animal health.

The continuation of supply would further assist in scientific insight underpinned by this project license and will lead to a greater understanding of pathogens, contributing to research publications and advances in the prevention of human and animal diseases. The scientific data generated by using blood products taken from animals will also reduce the number of potential new medicines requiring further research in living animals by establishing ethically whether conducting experiments on living animals is necessary.

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

The supply of animal blood products will contribute to the development, manufacture and testing of the quality of new and approved medicines within the field of Human and Animal health.

The continuation of supply will further support the generation of data to enable understanding and predictions of drug concentrations to determine dose levels in pre-clinical phases allowing for more treatments to progress towards clinical trials and approval by the regulatory authorities.

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

Animal blood products supplied under this Project Licence will be used to validate methods to support GLP toxicity studies, by providing data to assess the stability of potential medicines in blood. This is to ensure the integrity of blood collection in GLP studies, and the scientific validity of data produced from the blood samples. This is in line with The International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) validation guidelines and to comply with the expectations of the Medicines and Healthcare products Regulatory Agency (MHRA) in performing validations to support GLP toxicity studies.

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

We carefully monitor and manage production levels of animals on a month-by-month basis looking at animals produced vs sales. Ex-breeding stock and animals not utilised for use in direct research will be considered to provide a pool biological material. As these products are required to allow for the continuation in contributing to the development, manufacture and testing of the quality of new and approved medicines within the field of Human and Animal health.

We will collaborate on a supplier level and gain insight into future demand and review ethical viability along with continued forecasting updates to allow for the continued supply at a minimal level.

Species and numbers of animals expected to be used

- Mice: 50
- Guinea pigs: 50
- Ferrets: 50
- Rats: 50
- Beagles: 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.

The animals used under this project licence are currently being bred to support scientific research. Only ex-breeding stock and animals not required in direct use for research purposes will be considered for use to supply blood products to the research community.

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

Under Protocol 1, ex-breeding stock and animals not required to be used for direct research purposes from species such as Mice, Rats, Guinea Pigs, Ferrets and Beagles will be reviewed and considered as potential donors. If ethically, there is a requirement to supply the research community, then in line with the protocol, animals will be placed under general anaesthesia and the required volumes of blood will be withdrawn. Once sampling has been completed, they will be humanely killed via a suitable scheduled 1 method without regaining consciousness.

Donor Beagle dogs for protocol 2 will be selected from a small group of ex-breeding stock or animals not required for direct use in research. These will be reviewed for suitability to be considered for blood sampling without anaesthesia. This group of beagle dogs will be housed at the establishment and used to supplying small quantities of fresh blood products to the research community.

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

Blood sampling under general anaesthesia is a non-recovery procedure conducted with the animals under a species-specific general anaesthetic regime that has been developed in consultation with the NVS. We ensure the agent(s) and route(s) used are the ones which will cause minimal discomfort and distress to the animal during the induction of anaesthesia.

For blood sampling without anaesthesia, the techniques used have been refined with reference to:

The guidance information on the general principles of blood sampling of the NC3Rs website

The EFPIA/ECVAM good practice guide to the administration of substances and removal of blood, and Wolfensohn S and Lloyd M (2003). Handbook of Laboratory Animal Management and Welfare.

We may use local anaesthetic (LA) and reward incentive on dogs to reduce any stress. Due to the possibility of side effects e.g., dermatitis from prolonged numbing of the skin, it was felt the LA would and should only be applied to dogs that require it.

We also ensure that no more than 10% of the total blood volume (TBV) is removed in any 24-hour period and no more than 15% TBV in any 28-day period. All dogs will remain under NVS care during the duration of the project license. There are no further impacts and/or adverse effected expected during the 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)?

Mice 100% general anaesthesia/non-recovery
Rats 100% general anaesthesia/non-recovery
Guinea Pigs 100% general anaesthesia/non-recovery
Ferrets 100% general anaesthesia/non-recovery
Beagles 86% general anaesthesia/non-recovery
Beagles 14% mild severity.

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

- Killed
- Kept alive
- Rehomed

A retrospective assessment of these predicted harms will be due by 27 April 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 a lack of appropriate synthetic material from existing alternative sources or instances where it is not possible to use cell culture techniques. Researcher protocols still require blood products, obtained from animals that are not required for direct use in research.

The scientific data generated by using blood products taken from animals will also reduce the number of potential new medicines requiring further research in living animals by establishing whether conducting experiments on living animals is ethically valid.

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

There are a number of early technologies in development which aim to utilise human cells to recreate the physiological functions without using animals, some of which have been made available on https://nc3rs.org.uk/3rs-resources/improving-human-relevance-cell-culture-using-animal-free-culture- media#animal-products-in-culture-media,

Why were they not suitable?

Although many technologies are moving quickly in the research sector favouring in-vitro approaches, it is not yet possible to fully offer synthetic material replacement for research animals and blood products, to support all protocols within research that contribute to the development of new medicines for humans and animals.

A retrospective assessment of replacement will be due by 27 April 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

The experience of supplying blood products under previous Production of Laboratory Animal Bio- Products project licences, has given insight into current and future demand. This, along with continued updates of researcher forecasting of further demand will form part of an ethical review of the donor colony levels. Which will be deemed appropriate to optimise and maintain the required number of donors, in order to reduce total numbers of animals used in research.

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

Previous knowledge and experience in the Production and supply of Laboratory Animal Bio-Products has enabled us to establish the minimum number of donors required to re-use for blood sampling without anaesthesia. This means that from this pool multiple samples can be obtained from a smaller number of donors thereby reducing the need to euthanise animals for the purpose of taking each sample. This current level allows us to meet current research sector demand whilst also allowing suitable rest periods for each donor prior to re sampling.

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 minimised by using proven collection techniques including taking blood under non-recovery general anaesthesia, to ensure that large volumes of samples can be obtained. The use of blood products, that are obtained from animals not required for direct use in research, will also reduce the total number of live animals required for research. Individual research requests will be co-ordinated in order to supply multiple samples from one animal, aiding in further reduction of the total number of animals used.

A retrospective assessment of reduction will be due by 27 April 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

Which animal models and methods 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 Ferrets, Mice, Guinea Pigs, Rats and approximately 86% of Beagle dogs will be sampled under non recovery general anaesthesia, which is deemed the least invasive method in causing suffering, distress, or lasting harm to the animals.

Approximately 14% of Beagle dogs will be used for live blood sampling and a suitable pool of donor individuals will be trained and assessed for suitability of this mild procedure.

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

Where possible, sampling will be performed under non recovery general anaesthesia.

However, some research protocols require low volumes of dog blood as fresh as possible to perform their regulated assays and currently no surrogate matrix is available. In cases like this, sampling via non-recovery anaesthesia is not deemed a viable option to fully utilize the donor dogs to the wider research community.

Therefore, blood sampling without anaesthesia is deemed the most appropriate method to supply these smaller volume demands. Donor beagle dogs used for the protocol will have been assessed for suitability. These beagle dogs are of an age to be able to receive suitable training allowing for an easy process and reducing associated stress.

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

Staff will be trained in suitable techniques for handling and restrain of donor beagle dogs during sampling. Staff required to perform sampling will have been trained and assessed for competency by a qualified PIL/NAWCO overseen by the NTCO for the techniques required to perform live sampling.

Donor dogs will also be assessed prior to being submitted and approved for sampling suitability. They will receive training to sit calmly on an examination table and acclimatized to manual restraint and the sound of the clippers beforehand. After blood sampling they will receive food treat reward making the process easier and reducing stress associated with the techniques when future live sampling is required.

Prior to each sampling request, a NACWO will check and ensure the blood volume to be collected is within the daily/ 28-day limits set in the project license and the donor has not been sampled in the last 7 days. Before each re-use, the NVS will perform a health check of the dog, to assess its suitability for re-use.

Additionally, once a month each blood donor dog is health checked by a NACWO (nails clipped, check for any visible abnormalities, check weight), followed by a monthly general health check by the NVS. Twice a year, a haematology and biochemistry review is carried out for each donor dog, to ensure these parameters remain within the required limitation.

What published best practice guidance will you follow to ensure experiments are

conducted in the most refined way?

Recommendations provided by the recent reports on the NC3Rs website such as Blood sampling will be sought. Any advances will be discussed with the Animal Welfare and Ethical Review Body (AWERB) for implementation and reviewed at recorded AWERB committee meetings to determine effectiveness.

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

Regular review of guidelines and recommendations provided by the recent reports on the NC3Rs website such as Blood sampling will be conducted and brought to the AWERB committee for discussion and actioned implementation.

A retrospective assessment of refinement will be due by 27 April 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

5. Pathophysiology and therapies for headaches and facial pain

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Headache, Migraine, Therapy, Pain, Neuroscience

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

The aim of the project is to investigate pathohysiological and molecular disease mechanisms implicated in different headache and facial pain conditions. It also aims to

identify novel treatments for these conditions and to understand treatments mechanism of action.

A retrospective assessment of these aims will be due by 24 April 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

Headaches and facial pain are painful and disabling. They are the commonest episodic neurological disorders and represent an enormous health burden, with considerable disabling, morbidity and social consequences. The clinical symptoms of primary headaches are more than just a head pain, as they are accompanied with sensory symptoms such as sensitivity to light and sounds, increase sensitivity to non-painful stimuli- like light touch, or even watering of the eye and running nose. There is little understanding of the mechanisms that trigger such neurological dysfunctions in headache and facial pain sufferers who are otherwise normal between attacks, and as a consequence, treatment is commonly unsatisfactory. Identifying disease mechanisms can aid the development of effective specific treatments. The ultimate objective is to translate pre-clinical observations to the clinical domain in order that we can better treat headache patients through specific pharmacotherapeutic applications.

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

New information on disease neurobiology, molecular and physiological mechanisms of different phases of headaches and facial pain. We are also expecting to create new information on the mechanism of action of established and new treatments, as well as of the potential therapeutic use of novel treatments.

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

Short-term Beneficiaries:

1. Scientific community by increasing knowledge on the subject. This will include knowledge on neurobiology and pathophysiology of disease, mechanism of action of treatments and identification of novel potential treatments.

Long-term Beneficiaries:

1. Headache health care providers by increasing their understanding in disease mechanisms and treatments efficacy

2. Patients by aiding their understanding on the disease and offering a wider range of treatment options

3. The pharmaceutical sector through collaborations on the development of novel, specific headache treatments.

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

1. Through collaborations with other scientists and the pharmaceutical industry

2. Through dissemination of new knowledge at scientific meetings and public engagement activities

3. Through publication of outcomes, including publications of unsuccessful approaches/potential treatments

Species and numbers of animals expected to be used

- Mice: 5000
- Rats: 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.

Adult rats and mice were chosen, due to their anatomical, physiological, and genetic similarity to humans. Advantages of rodents include their small size, ease of maintenance, short life cycle, and abundant genetic resources. The existing literature describes relevant methods particularly in rats, and availability of genetic alterations in mice. In the headache and facial pain field there are well described and validated animal models for translational research. We can also directly compare our data with previous work and minimize the need of repeating some experimental set ups. Historically, significant information on disease mechanisms and treatments outcome has been collected from these animals and successfully translated to the clinical domain.

In humans headaches and facial pain affect mainly the adult population and hence we choose to work with adult rodents for a translational medicine standpoint.

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

An animal may undergo injections or surgical procedures in order to induce a headache or facial pain phenotype. Following the animals may receive, injections of novel substances that could act as potential treatments for headaches and facial pain. The animals will be then tested with behavioural assays that assess their sensitivity to non-noxious or noxious stimuli or undergo non-recovery procedures that allow recordings of cellular or vascular responses. The number of procedures and each procedure individually will be kept at

minimum duration (for 30 days on the majority of non- recovery experiments and no more than 90 days on a minority of animals) depending on the specific objective of the project.

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

For both mice and rats adverse events are mild to moderate. A great majority of animals will undergo non-recovery procedures. Animals may experience abnormal behaviour either due to a genetic alteration (life-long) or by administration of drugs (hours to few days). Animals may experience weight loss (<10%) as a result of a surgical procedure or treatment administration, which will normally recover within a week. Animals may experience pain due to the nature of the project aiming to investigate the neuroscience of pain related to headache conditions (for 1 day for the majority of experiments, up to a maximum of 30 days on a minority 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)?

For both mice and rats adverse events are mild (~20% of rats; ~ 40% of mice) to moderate (~ 40% of rats; ~ 20% of mice). In a small minority of animals adverse events may be severe (~5% rats; ~ 5% mice). A great majority of animals (~ 50-60% of rats and mice) will undergo non-recovery procedures. Animals will be humanly terminated using appropriate methods if adverse events exceed expected severity.

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

- Killed

A retrospective assessment of these predicted harms will be due by 24 April 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

Primary headaches are more than just pain conditions as they are also accompanied by multiple abnormal sensory processing such as sensitivity to lights and sounds. The determination of neuronal changes associated with primary headaches not only requires the presence of neurons at a state of nociceptive condition, but also it requires intact brain pathways that influence each other.

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

The investigation of such pain pathways cannot be ethically conducted in humans as it involves major invasive approaches and removal of neural tissue for ex-vivo investigations. It will not be possible to develop non-animal alternatives for any part of the animal work described herein, which aims to dissect the anatomy and functionality of pain processing circuits involved in headache conditions. Some aspects of the pharmacology of headaches and facial pain, as for example, the presence of certain ion channels and expression of receptors, will be investigated in appropriate cell lines or in tissue taken from human skin or ganglia, however, how these may affect the biological sequences of events that occur in headaches cannot be fully predicted without the subsequent use of an animal model.

Additionally, computational analysis could be used where relevant to predict the response of future therapeutics using silicon cell models of trigeminal sensory neurons which are currently under development. However, prediction of safety and risks of novel therapeutics cannot be tested in silico models.

Why were they not suitable?

The alternatives cannot accurately replicate or model all the biologic and behavioral aspects of headaches and facial pain.

Cell lines or in tissue taken from human skin or ganglia cannot be used to predict the neurophysiological events that occur in headaches as they do not allow studies into neural pathway connectivity, pain processing and neurotransmitter influence on brain networks. Additionally, trigeminovascular activation that occurs in conditions like migraine and cluster headache, require intact vascular and neural coupling which cannot be achieved in vitro. Computational analysis and silico models will not be able to fully predict the efficacy of novel treatments and most importantly they will not be able to assess their safety.

Disease neurobiology, treatments efficacy and safety are important objectives of this project. Hence, there is no feasible alternative that would entirely replace the use of living animals and would allow these objectives to be met.

A retrospective assessment of replacement will be due by 24 April 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

1.From careful planning of the experimental protocols and focus on specific objectives

2. From previous experience of work done under a previous project with similar aims

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 of the results have been and will be further discussed as projects progress, with expert statisticians. The NC3R's Experimental Design Assistant was used during careful planning of experimental protocols. Additionally, recommendations based on the ARRIVE guidelines, such as the consensus of the IMI-Europain consortium on developing experimental design and reporting standards for improving the internal validity of pre- clinical studies in the field of pain, including minimization of methodologc bias (e.g. by utilizing randomization, blinding assessment) have been followed. Such recommendations will be also revisited during experimental planning to ensure optimum 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 proposed experimental designs and methods of analysis of the results have been and will be further discussed as projects progress, with statisticians within Establishment. Where relevant, the experiments will be designed in order to obtain maximum information without the need of repeating similar set ups. Technically, some of the main techniques we will utilize, allows us to perform multiple recordings in the same animal, which reduces the number of animals. For example, single cell recordings allow for multiple cell properties to be recorded in a single animal. Another example is the use of microiontophoretic application of drugs in pharmacological studies, which allows the study of multiple neurons in a single experiment, due to the minimum, diffusion of the drug in the neuronal vicinity. Where appropriate mathematical modelling of neuronal activity will be used, as well as other bench techniques that allow at least partial investigations of such neuronal activity or presence of molecules in neuronal tissue. An advantage of some anatomical work is that the tissue generated can be used to study several different molecules and neuronal markers. As most of the genetically altered animals (mice) that we plan to use, already exist, the minimum number of animals required for the project will be obtained from the relevant supplier or bred only for the purposes of the specific project. The requirements of each specific experiment (e.g. sample size) will be considered to maintain minimal experimental stock numbers, following appropriate breeding strategies (including best practices for archiving strains), as per NC3Rs colony management best practice. Good laboratory practice will be always followed.

A retrospective assessment of reduction will be due by 24 April 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

Which animal models and methods 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 the existing animal models of headaches. The majority of these models are induced in terminally anaesthetized animals. In animals where migraine model is induced with the use of a headache-inducing substance (e.g. nitroglycerine), these are not known to cause any additional harm other than the headache-like allodynia, as seen in humans. To induce an inflammatory orofacial pain model, the minimum dose of an inflammatory agent will be used. The majority of the experiments will utilize short acting inflammatory agents (e.g. recovery within hours). To induce a neuropathic facial pain model, only unilateral partial injury to a trigeminal nerve will be induced. Models of mild-traumatic brain injury will be used to induce post-traumatic headache. Using established experimental protocols with minimal side effects, the animals will be closely monitored during the whole period and spontaneous pain will be assessed without provoking unnecessary pain. In the above models, in general, behavioural and electrophysiological methods will be mostly used. Behavioral assays will include mostly von Frey testing, or orofacial pain assessment test in cold/hot conditions in free moving animals, which induce minimum, and only temporarily, discomfort. A good proportion of the animals used in this project will be terminally anesthetized for electrophysiological recordings. Under terminal general anaesthesia, which is sufficiently deep and stable to ensure that the animal is insentient throughout, the properties of neurons in different brain nuclei of interest will be assessed, and the actions of potential therapeutic agents on neuronal activity will be evaluated. Any complications that may induce additional harm to the animals will be discussed with the vet and animals will be terminated with an appropriate method. Good laboratory practice will be always followed.

In all animals (including terminally anaesthetised animals), dosing of agents 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. The dose of each agent will be chosen to avoid adverse systemic effects with veterinary consultation where appropriate, for novel agents or combinations thereof. As the majority of agents administered are produced to human clinical standards and their dosages have already been defined in animal models, adverse events are less likely to occur. In our experience, animals rarely die post i.v or i.p. injection (less than 1%) and generally recover well post anaesthesia.

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

Headaches are more common in adults and hence using adult animals is more fit for purpose. A good proportion of the animals used in this project will be terminally anesthetized for electrophysiological recordings (~50%). Headache models that allow translational experiments and pharmacological screening in species like drosophila or fish are not suitable due to the lack of anatomical and physiological fidelity to humans.

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

All animals will be closely monitored during the whole period and spontaneous pain will be assessed using the mouse/rat grimace scales and welfare assessment scoring sheets, without provoking unnecessary pain. Pain, outside the scope of the headache/facial pain model, will be minimized by offering appropriate post-operative care and post-operative pain management to increase animal welfare. Regular data revisions will be conducted to decide if earlier time points can be kept without reaching full duration of initial protocol. Training of animals and appropriate acclimatization will be offered for behavioral studies. All members of the lab working under this PPL will receive appropriate training for identify and minimize welfare costs (e.g. through learning modules on pain assessment by the Research Animal Training).

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

Welfare assessment NC3Rs library The grimace scale

Humane endpoints principles (e.g. Humane Methods of Killing (EU6.1)

Recognition and prevention of pain, suffering and distress in laboratory animals (EU5 module)

Other relevant published documents relevant to the work described in this PPL (e.g. LASA Guiding principles aseptic surgery; Refining procedures for the Administration of substances, https://doi.org/10.1258/0023677011911345

Andrews NA et al. (2015). Ensuring transparency and minimization of methodologic bias in preclinical pain research: PPRECISE considerations. Pain 17(4):901-9.

Knopp KL et al. (2015). Experimental design and reporting standards for improving the internal validity of pre-clinical studies in the field of pain: Consensus of the IMI-Europain consortium. Scandinavian Journal of Pain 7:58-70.)

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

Through information disseminated by our Establishment dedicated biological services and by attending NC3Rs meetings and through the NC3Rs monthly newsletter. We constantly seek new information on NC3Rs developments that could be applicable to this project through the literature, seminars and congresses covering 3Rs topics, especially on identifying techniques/models that cause reduce severity.

A retrospective assessment of refinement will be due by 24 April 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

6. Neuronal circuit mechanisms underlying cognitive memory judgements

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cognitive memory, Decision making, Object vision, Neuronal circuit, Causality

Animal types	Life stages
Marmosets	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Uses non-human primates

Objectives and benefits

Description of 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 reveal neuronal mechanisms by which animals detect objects or events and compare them with past experiences. In other words, the project aims to reveal how brain circuits allow us to discriminate familiarity from novelty.

A retrospective assessment of these aims will be due by 04 April 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence? Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 something from previous encountering and knowledge, or recognition, is critical for our life in many ways. For an example, loss of memory and the inability to recognise people, objects and events as familiar is a core symptom of dementia including Alzheimer's disease in which brain degeneration slowly destroys memory and cognition. In the UK, the number of people with dementia is estimated to exceed one million in 2025 (National Health Service - Dementia guide). On the other hand, our ability to read and write also requires recognition of letters and association with sounds, and its difficulty results in a learning disorder called dyslexia, which occurs in 10% of population despite no other cognitive disabilities including verbal communication (British Dyslexia Association). Therefore, understanding how our brain recognises objects is an essential step toward preventing, treating and improving various recognition-related symptoms which significant number of people are suffering from. However, a fundamental knowledge of the neural circuits that support our recognition is lacking despite that such knowledge is the basis of effective therapy and care. Whilst whole brain imaging studies in humans identifies the brain regions that show correlated activity with recognition, it is essential to gain information about the causal roles of the identified brain regions and how these regions interact to compare visual objects with memory, provide a sense of familiarity/novelty, and retrieve their associated meanings, which eventually induces a behavioural decision that is an essential component of human behaviour. To address this issue, detailed measurements and well-controlled manipulation of neuronal activity in behaving animals are critical.

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

This project will ultimately output the knowledge that determines the key brain regions and neural circuits that, if dysregulated, may underlie the different symptoms associated with disorders of fine vision, cognition and memory of objects. As a consequence, this knowledge will provide us with greater insight into the nature of clinically relevant symptoms which can be a seed of novel treatments to improve visual cognition and memory in disorders such as dementia and dyslexia which significant number of people are suffering from. Such a knowledge base would greatly improve human life.

Specifically, I aim to identify the neurons, and their connections and communication which causally guide the perceptual identification, and memory-based discrimination of visual objects. Such neuronal circuits would be potential targets of novel medications, behavioural therapy, and stimulation therapies. For example, by selectively stimulating the neuronal circuit that signals familiarity when a dementia patient sees a family member, it would be possible to guide the patient recognise that person as familiar. Such an application possibility would be implemented by developing a wearable device in which a small computer vision camera identifies a family member, and an implanted brain manipulator stimulates the neuronal circuit to generate a safe, familiar feeling to that person. Alternatively, by stimulating the neuronal circuit that signals the shape and identity of objects, such brain manipulation technologies would also help dyslexic people

discriminate letters and retrieve the sounds associated with the letters. Thus, the scientific output of this study will also have enormous socioeconomic impacts.

Equally importantly, I will gain a detailed understanding of the marmoset brain circuits and its operating principle. Marmoset is an emerging model animal to study neuronal circuit mechanisms of various cognitive functions and dysfunctions. The US, Europe, Japan, and China are making more and more investments on this small primate species because its small and smooth brain is highly suited for modern advanced technologies to investigate brain circuits underlying various cognitive functions.

Therefore, the new knowledge about the marmosets' behaviour and brain circuits produced in this project will serve as a highly valuable basis for many neuroscience researchers in both the present and future and further develop the value of this primate model species. The technical developments achieved in this project will be directly applicable for studying other higher cognitive functions that are specifically evolved in primates.

Publications will be an important part of the output for all information gained throughout the duration of the project. Because the marmoset is a rising but underdeveloped model species for neurophysiology, any results and developments will be highly valuable and published in well-read journals as important advancement.

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

The intended overall benefit of this project will be directed to the society as the intended advancement in the understanding of neurophysiological basis of visual cognition and memory will have far reaching social and economic implications.

The first, immediate beneficiaries of this project output will be the basic and clinical research communities because the researchers can directly use the findings and developments produced in this project to guide their own research directions. The journal publication with Open Access, which remains the most effective route to communicate research findings, allows the research findings to gain international impacts and prominence, relatively quickly.

The second beneficiaries would be the patients as the output of this project will help provide insight into the varied neural dysfunctions that can underlie the range of visual, cognitive and memory symptoms, guiding new treatment strategies as well as providing insight into the mechanisms by which current therapies have their efficacious actions. Those benefits will be provided to the patients through clinicians who directly advance healthcare protocols, and also through the healthcare industry which develops novel medications and devices to guide vision, cognition and memory.

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

I will communicate the output of this project as widely as possible to research communities. The outcome of this project will be published and disseminated to the scientific community in both high- impact scientific journals with Open Access and at domestic and international meetings, summer workshops, etc. My results will be targeted for publication in peer-reviewed high impact journals (e.g., Nature, Science, Cell, Nature Neuroscience, Neuron, Proceedings of the National Academy of Sciences, Journal of

Neuroscience, etc.). I have a strong track record in maximising the output of the own work, including both positive and negative results.

Manuscripts accepted for publication will be made open-access and archived in an institutional repository to ensure the widest possible accessibility and impact of my work, thus meeting the new HEFCE policy on peer-reviewed articles and conference proceedings. I will also publish conceptual papers and reviews which are often highly cited and increase the profile of my work and the field in general.

My results will be disseminated through presentations (symposium lectures, poster presentations) at international and domestic conferences and workshops. Particularly, since I am involved in the international network of researchers, I can very effectively communicate my results to the researchers who will make the most of the project output. In addition, the techniques to study neuronal circuits that I will develop through this project will be also directly beneficial for my collaborators in the same institute who works on the same animal models, and thus our collaboration will robustly advance the understanding of various cognitive functions.

I also communicate this research to non-scientific communities. The publication in journals with Open Access will only be the first step in a wider dissemination and communication strategy aiming to immediately increase our impact on the general public. Then, I will digest the scientific publications through Press Release as I did for my previous works which have been well discussed in media including Explica, Tech Explorist, Galileo, N+1, MedicalXpress, TechPlus, Le Scienze, New Scientist, The Scientist, Наука и Жизнь, Nauawpolsce, etc. I will also directly communicate with the society, as I previously gave lectures at a junior high school and a community centre. Thus, I will rely on publicisation by media groups, and also conversation with the non-scientific community.

Species and numbers of animals expected to be used

- Marmosets: 10
- Rats: 60

Predicted harms

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

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

In this project, I have chosen to work with adult common marmosets to study visual cognitive memory. I also work with adult rats for pilot experiments to validate and refine neurophysiological experimental techniques towards the future marmoset application.

The study of the neuronal circuit mechanisms underlying fine object vision and cognitive memory judgements requires a freely moving, behaving animal. It is not possible to investigate behavioural functions of the brain in a simplified experimental setting such as a tissue culture or in an artificial and biologically unrealistic computer simulation. Existing research techniques used in humans, such as whole-brain imaging, detect the brain activity only spatially less-finely and temporally less-precisely, and tell us only about the

correlation between brain activity and behaviour which does not address the crucial issue of the causal involvement of particular brain regions and neurons in specific psychological processes. While studies of human patients with neurological damage can provide causative information, they fail to provide neuronal or circuit specificity as the damage is not controlled nor specific to particular brain structures. Since there is abundant evidence that the normal functioning of much of the brain regions is comparable across species, not only structurally but also functionally, including the behaviour it supports, this facilitates the extrapolation of findings.

The marmoset has fovea (a small region with high visual acuity) in their retina which is crucial for human-like fine object vision but is missing in rodents. The marmoset brain, especially the cerebral cortex has an organisation far more similar to humans than that of rodents, the latter species most commonly used in brain studies. The visual, cognitive and memory functions under study are poorly developed in rodents and this is reflected in their poorly developed brains compared to humans. The brain regions known to be involved in complex memory-dependent behaviour in monkeys and humans, e.g., the prefrontal cortex (the most frontal part of the cerebral cortex) and the temporal association cortex (the side and bottom part of the cerebral cortex), are markedly reduced in rodents. Specifically, whilst the cortex makes up 80% of the brain in humans it makes up only 42% of the rat brain and the overall structural organisation of the rodent prefrontal and temporal association cortices is not comparable. Other mammalian species such as dogs have been much less studied in neurophysiology, and comparable knowledge is not available. Pigs have one stereotaxic brain atlas, but it details only about subcortical structures, and sheep have none. Thus, the anatomical and functional organisation of their cortex is much less understood. In addition, their brain (150 g in sheep, 180 g in pigs) is far larger than the monkey brain (100 g in macaques, 20 g in marmosets) and therefore they are less compatible with modern methodologies evolved in small rodents. Other vertebrates such as reptiles and birds have brain organisation that is not directly comparable with that of mammals. Cognitive memory capacities are very much linked with the complex decision makings that are the hallmark of most primates, hence why I have chosen the marmoset.

Rats are particularly suited for refining experimental procedures for marmosets, because rats weigh 300-400 g, and the length of the skull is 4-5 cm, which are very similar to those of marmosets. Mice are too small as they weigh only 20-30 g. This makes the rats highly beneficial as a model despite their insufficient similarity of the brain structure and functions to those of primates. Once the experimental procedures are successfully refined in rats, I will apply for amending this project licence to use those procedures in marmosets to further pursue the main objective of the study.

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

In this project, I will work with common marmoset to study visual cognitive behaviour with non-invasive brain imaging. I will also work with rats to validate and refine neurophysiological experimental techniques towards the future marmoset application.

Marmosets

Adult marmosets will be used in this study. Marmosets will be exposed to behavioural and experimental procedures with the cumulative severity which never extends beyond the moderate level. A typical study lasts between 12-24 months during which time a marmoset

may receive approx. 5 anaesthetics, for restraint purposes only, e.g., magnetic resonance imaging (MRI) and positron emission tomography (PET) which allow non-invasive visualisation of brain structure and activity.

Marmosets may receive short behavioural tests in the home cage that last no more than 20 mins, to determine their trait anxiety. The tests include measuring their responsivity to an unknown human that stands in front of their cage for 2 minutes and to a rubber snake placed in a box that sits on the floor of their home cage for 5 minutes. This test is important to allow animals to be directed into studies that they are most appropriate for.

Marmosets will be exposed to various behavioural procedures to aim to assess their psychological performance in tasks that measure a range of cognitive functions including perception, memory, and decision making. In most of these tests, visual stimuli are presented on the touchscreen and the monkey makes a voluntary response to the presented stimuli to receive a 'sweet' liquid reward through positive reinforcement. Testing in these tasks typically requires animals to be temporarily sequestered into a specialised testing apparatus. Behavioural tests typically occur daily, do not last more than 40 minutes per session, and only take place Monday to Friday. Typically, they have weekends off. When it is difficult for a monkey individual to maintain its stable test performance in intellectually demanding tasks across days, or a larger number of task trials is necessary for statistical analyses, additional motivation may also be provided by having the amount of sweet foods or the time of water access mildly restricted at home cage (the amount of water is not restricted when given) so the sweet liquid rewards in the tests are more valuable to them. The water access is restricted only gradually by analysing the behavioural performance of individual monkeys, and initially restricted only for a few hours immediately before testing. If needed, some animals may then gradually move on to longer periods of restriction to have the desired effect on their performance. This restriction only induces mild thirst, not dehydration, and to limit stress, the animals have two days of uninterrupted access to water every week and have a break from restriction for at least a week every six months.

For studying the behavioural effects of brain activity modulation, marmosets may receive injection of neuroactive drugs into the muscle or under the skin depending on types of drugs (typically 20-48 injections). Injection will be followed by behavioural tests, and injections of a drug and saline are interleaved to maximise the detectability of behavioural impacts.

Some marmosets may undergo brain imaging by MRI and PET (typically lasting 90 mins and 4 scans in total). For detecting brain activity by PET, an imaging agent may be administered by an intravenous route e.g. femoral or tail vein. Here, an animal receives anaesthesia for restraint purposes only. To compare the brain before and after a procedure by using the same marmoset as internal control, the scans will be performed both before and after the procedure with sufficient intervals (minimum 2 weeks).

Rats

Adult rats will be used in the study. Rats will be exposed to experimental procedures with the cumulative severity which never extends beyond the moderate level. Typical study will last up to 12 months. Rats will receive surgical implantation, and they may undergo electrophysiological experiments (to measure and manipulate electrical activity of neurons)

during behavioural tests or under terminal anaesthesia. Rats may also receive brain imaging by MRI or PET.

Rats will receive surgical procedures under general anaesthesia for implanting small MRI-compatible recording chambers and miniature micromanipulators (very small movable clamps) on the skull, and for inserting microelectrodes (very thin needles with tiny electrode contacts) to the brain regions of interest through a small hole in the skull and dura mater. In electrophysiological recording sessions, the rat is gently held by hand, and the microelectrode is advanced by driving the miniature micromanipulator. Then, a small, head-mounted electrophysiological amplifier (head-stage, approx. 20x35x20 mm3 / 15 g) will be connected to the microelectrode to record or stimulate the neuronal activity. The rats will freely explore in behavioural testing apparatus (open arena) or in their home cage, and they may collect scattered food flakes and receive neutral visual/auditory stimuli. Water and food are not restricted. Outside the recording sessions, the head-stage will be detached, while the microelectrodes, chambers and miniature micromanipulators will be kept implanted and covered by a cap to allow the animal to live normally.

Rats may undergo brain imaging by MRI or PET (typically lasting 90 mins and 4 scans in total) up to 5 times to help finely localise the brain regions of interest and their connectivity. The brain imaging may be performed before surgical procedures to plan implantation surgery, or after the surgery with the microelectrode inserted in the brain to finely determine the coordinates of electrophysiological recording. Here, an animal receives anaesthesia for restraint purposes only.

If the work with the rats is successful, I will be applying to amend the project licence to use those techniques in the marmosets.

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

In marmosets I will study visual cognitive behaviour with non-invasive brain imaging. In rats, I will validate and refine neurophysiological experimental techniques towards the future marmoset application.

Marmosets

In marmosets, the incidence of adverse events is expected to be low, with the majority of procedures not anticipated to produce adverse effects. This is a result of the many steps taken to ensure best practice and to mitigate adverse impacts at the facility. Everything will be done to limit the pain, suffering, distress, and lasting harm to the animals within our care at every opportunity and for every procedure. Nowhere in the project, it is expected that animals show clinical signs of ill health. They are checked routinely in case any such signs emerge.

In marmosets, behavioural testing is based on positive reinforcement with sweet liquid rewards, and the testing apparatus is highly habituated, so they do not produce adverse effects apart from transient mild anxiety. However, in some marmoset individuals who do not maintain stable task performance in intellectually demanding tasks across days, or when a larger number of task trials is necessary for statistical analysis, the access to water in the home cage may need to be restricted to add additional motivation. This water

restriction only limits the time that animals have access to water, so the amount of water is not restricted when given, but has the capacity to impact the animal's general well-being.

This is vigilantly watched for but rarely observed. Water restriction does not affect the weight of the animals, who often ignore the water when it is first returned to their cage, suggesting that they are not very thirsty.

Marmosets may experience transient discomfort when being handled and removed from their home cage. Discomfort associated with handling would only last a few minutes. However, they are habituated to this process over a period of time to ensure they are not stressed by the procedure and thus are not expected to experience much discomfort at all. They normally acclimate to this process quite quickly and are frequently rewarded with treats such as a small bit of marshmallow.

Animals receiving repeated injections could potentially experience soreness or bruising of the leg muscle (no incidence in the facility). After 12 injections, the animal will be examined by the NVS for signs of adverse effects. Most routes of peripheral drug administration do not result in adverse effects as a result of the injection per se. Injection sites will be alternated between legs to minimise the likelihood of this occurring and apply pressure after the injection to minimise bruising. If leg soreness or bruising is observed, injections in these animals will halt until the animal has recovered or been treated.

Given the cumulative nature of adverse effects, I do everything I can to limit the number of adverse effects experienced across their lifetime. This includes examining closely the transition between any procedures, especially if the animal has experienced any adverse effect or negative impact from a procedure.

Rats

In rats, most adverse effects expected relate to the initial acute recovery phase following surgery, whereby complications may arise from the procedure itself (e.g., localised facial swelling) or the prolonged use of anaesthesia (e.g., protracted recovery to normal behaviour). Such effects typically resolve within 2 hours but can extend to approximately 24 hours. With employment of best practice treatments (e.g., full analgesic regimen) and careful monitoring, the overall impact of surgery to the animal is limited as much as possible. The acute phase following a surgical procedure involves the animal being actively monitored very closely for any signs of deviation from the normal recovery process. Additionally, extra care is taken during the first week after surgery to observe any changes in normal behaviour or appearance. Long term implant sites are cleaned regularly throughout the life of the animal to prevent infection, and the cage furniture altered to minimise environmental hazards.

Surgery including implantation and craniotomy can cause a mild pain, but the pain will be alleviated by performing the procedure under anaesthesia. Insertion of microelectrodes itself can potentially produce transient mild discomfort but no pain as there is no pain sensor in the brain. Animals are watched very closely for adverse reactions i.e., tremoring, after all craniotomy, microelectrode insertion and electrophysiological recording and stimulation, and I have robust protocols to alleviate such reactions if they do occur.

In rats, no behavioural task training is required, and therefore food/water restriction will not occur. The head-mounted devices may initially cause slight inconvenience for behaviour,

but the animals will usually quickly adapt. The environmental enrichments of the home cage may also be adapted not to interfere with the implants and rats' 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)?

Marmosets

Mild 10%

Moderate 90%

Rats

Moderate 100%

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

- Killed
- Kept alive

A retrospective assessment of these predicted harms will be due by 04 April 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

In this project, I need common marmoset to study brain mechanisms of visual cognitive behaviour unique to primates. I will also need rats to validate and refine neurophysiological experimental techniques towards the future marmoset application.

Marmosets

The study of the neuronal circuit mechanisms underlying fine object vision and cognitive memory judgements requires a freely moving, behaving animal. Existing techniques used in humans, such as whole-brain imaging, detect the brain activity only spatially less-finely and temporally less-precisely, and tell us only about the correlation between brain activity and behaviour which does not address the crucial issue of the causal involvement of particular brain regions and neurons in specific psychological processes. While studies of patients with neurological damage can provide causative information, they fail to provide

neural circuit specificity. Thus, this research requires the use of animals engaged in specific behavioural and cognitive tasks.

There is abundant evidence that the normal functioning of many of these neural systems is comparable across species, thus allowing a certain amount of extrapolation of findings across species. Some non-animal techniques will complement the animal studies presented. The brains of all animals receiving brain manipulations will be analysed post-mortem to locate the positions of microprobes and/or lesions in the brain. This will inform future surgeries and refine surgical procedures.

It is essential to use an animal model that has fine object vision and cognitive memory functions translatable to/similar to those found in humans. These functions are poorly developed in the rat which is reflected in the organisation of their brain. The anatomical features of the brain known to be involved in complex cognitive behaviour in monkeys and humans, e.g., prefrontal and temporal association cortices, are markedly reduced in rodents. Specifically, whilst the cortex makes up 80% of the brain in humans it is only 42% of the rat brain and the overall structural organisation of the rodent cortex is not comparable. Other mammalian species such as dogs have been much less studied in neurophysiology, and comparable knowledge is not available. Pigs have one stereotaxic brain atlas, but it details only about subcortical structures, and sheep have none. Thus, the anatomical and functional organisation of their cortex is much less understood. In addition, their brain (150 g in sheep, 180 g in pigs) is far larger than the monkey brain (100 g in macaques, 20 g in marmosets) and therefore they are less compatible with modern methodologies evolved in small rodents. Other vertebrates such as reptiles and birds have brain organisation that is not directly comparable with that of mammals. The complex cognitive judgements are linked to the complex environments that primates live in including marmosets, which is the main species of choice for this work.

Rats

Because the reaction of the tissue and immune system to the procedures/implants as well as the performance of neurophysiological recording/stimulation system can be assessed only in live animals, it is essential to first validate and refine those experimental techniques in a simpler animal model, before applying them to marmosets. Rats are particularly suited for this purpose because rats weigh 300-400 g, and the length of the skull is 4-5 cm, which are very similar to those of marmosets. Mice weigh only 20-30 g, so mice are too small for the implants that can weigh about 10 g. Therefore, to study marmoset neuronal circuits in the future, the use of rats for technical refinement is essential.

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

Tissue cultures including brain organoids (three-dimensional cell culture model in developmental biology) and artificial computer simulations.

Why were they not suitable?

Tissue cultures (including brain organoids) are unable to contribute to a functional, behaving circuit, thus cannot perform cognitive tasks nor memorise things/concepts whilst artificial computer simulations are biologically unrealistic.

A retrospective assessment of replacement will be due by 04 April 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

In this project, I will work with marmoset to study cognitive behaviour with non-invasive brain imaging, and with rats to validate and refine neurophysiological experimental techniques towards the future marmoset application.

I am familiar with the PREPARE and ARRIVE guidelines (https://proecopa.no/prepare; https://arriveguidelines.org) and will ensure all our experiments are designed in adherence to these guidelines.

Marmosets

In deciding on group size, a number of factors are taken into account. My collaborators in the facility have extensive experience of publishing experiments on marmosets in high-impact journals with rigorous statistical peer review. I use the ‘appropriate number (n) of marmosets compatible with adequate statistical power for hypothesis testing, according to the "Reduction" principle from the 3Rs. In previous studies, the precise number of animals used in experimental groups has varied from study to study (from sample sizes of 3-8) depending upon prior knowledge about inter-individual variation in

(i) performance of animals on the particular task and (ii) brain activity, both of which affect the anticipated effect size. Smaller sample sizes (3-6) have been used in the more recent years owing to the refinements in the subject-study allocation, behavioural training and surgical interventions, which have led to enhanced effect sizes. When I embark on a new study, I start off with 2 lead animals in which I test out the hypothesis, and I look for large obvious effects e.g., behavioural changes and behaviour-related brain activity, which can be assessed in individual animals. Where possible, these lead animals will be incorporated into the main study if few, if any, changes have to be made in the experimental design before the rest of the animals enter the study. It should be stressed that the effect sizes are expected to be large because I have strong hypotheses based on previous findings and by having tight within-subject control over experimental variables. Based on recently published and unpublished studies of the collaborators and myself, I have planned for group sizes of between 5-8 in this behavioural study.

Rats

In recent publications about neurophysiological experiments in rodents, majority of studies use approximately 10 animals per experiment with nonparametric statistical tests (that is

more reliable but requires larger data size). In this project, rats are used to test several key technical points such as implantation of the recording chamber, semi-chronic implantation of the microelectrodes, recording performance of the microelectrodes, invasiveness of microelectrode, performance of the head-stage, etc. Accordingly, I estimate the necessary number of rats to be 10 for each test, 50 to 60 in total.

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

In this project, efforts are made to reduce the number of marmosets necessary for studying cognitive behaviour with non-invasive brain imaging. Rats will be used to sophisticate experimental techniques, which will reduce the number of marmosets required in the future studies.

Marmosets

i). Using a carefully controlled behavioural testing apparatus so that the particular cognitive abilities of interest can be studied in isolation which helps reduce variation in performance.

ii). Screening to ensure suitability of animal for particular study, thus also minimising variability.

iii). Re-using marmosets from another project held within the same facility when they suffered no significant adverse effects during or as a result of their previous use. Previous use will not prejudice the outcome of the study on which they are re-used, and after the completion of the previous procedure and before the intended re-use, the NVS (Named Veterinary Surgeon) has determined that they may be kept alive and that their health status and condition is compatible with proposed reuse in compliance with ASPA requirements. Re-use will not take place if an animal has received e.g., intervention surgery, but an animal is re-used if all they have received is e.g., non-invasive brain imaging, peripheral drug injections, or behavioural tests which have never exceeded the severity of the moderate level.

Rats

iv). Using MRI to target certain brain structures to ensure that the electrophysiological recording is effective in the majority of animals and reduce unnecessary repetition of the insertions of microelectrodes.

v). Using recording chambers and miniature micromanipulators to allow multiple tracks of recording in single animals.

Both species

vi). Using animals as their own internal controls (e.g., the conditions without manipulation, without drug, etc.) wherever possible to increase the power of statistical comparisons, minimise variability, and minimise the number of animals used.

vii). When separate controls are required, not necessarily matching the number of controls to the number of experimental animals but still ensuring they are sufficiently balanced to ensure statistical power.

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

Using pilot experiments in marmosets to increase the effect size in behavioural and imaging studies, and refine experimental procedures in rats towards future marmoset experiments.

When an animal is killed, organs and tissues other than brain may be shared with other research groups for various study purposes to help reduce the overall number of animals used.

A retrospective assessment of reduction will be due by 04 April 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

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

In this project, I will use common marmoset to study visual cognitive behaviour with non-invasive brain imaging. I will also use rats to validate and refine neurophysiological experimental techniques towards the future marmoset application.

Marmosets

Marmosets are a particularly valuable species for our work as their relatively small primate brain makes it possible to target cortical (surface) and subcortical (inside) structures and make regionally selective measurement and manipulation in the brain with relative ease, with minimal risk to the animal.

I use a wide array of methods in my research which have been, and continue to be, optimised to ensure least pain, suffering, distress, or lasting harm to the animals.

The behavioural tasks I use are designed to be able to detect neuronal activities most relevant with the cognitive memory judgment under study, maximising the ability to extrapolate findings from our marmoset studies to understand the underlying neuronal

circuit basis. To avoid unnecessary stress associated with behavioural testing, my animals are (i) trained to voluntarily get into a carry box in the home cage to go to the test apparatus by appetitive training, (ii) can move freely in the test apparatus,

(iii) trained in behavioural tasks by positive reinforcement, and (iv) are tested for less than 40 minutes per session in behavioural tasks.

When a marmoset individual does not maintain high task performance across days in intellectually demanding behavioural tasks, or when a larger number of task trials is necessary for statistical analysis, the access to water in the home cage may need to be restricted to add additional motivation. This water restriction only limits the time that animals have access to water, and the amount of water is not restricted when given. The restriction is introduced only gradually and only as needed through carefully analysing the task performance of individual monkeys. Initially, the water access is restricted only for a few hours immediately before testing. If needed as behavioural analysis suggests, some animals may then move on to longer periods of restriction, only having access to water for two hours at the end of the day to have the desired effect on their performance. This restriction only induces mild thirst, not dehydration, and to limit stress, the animals have two days of uninterrupted access to water every week and have a break from restriction for at least a week every six months.

Rats

Implantation of a recording chamber and use of a miniature micromanipulator attached to the chamber allow for insertion of microelectrodes according to the coordinate within the chamber, which increases the accuracy of targeting specific brain regions when combined with MRI than inserting the microelectrodes according to the stereotaxic coordinates (the coordinate relative to anatomical landmarks on the bone). MRI of the brain with the microelectrodes inserted will provide an accurate relationship between the chamber-based coordinate and the actual microelectrode position in the brain. This MRI-guided electrophysiological mapping of the brain increases the efficiency of localising the relevant brain regions and reduce the necessary number of microelectrode insertions in single animals, thereby reduce the total invasiveness of the experiment.

The use of semi-chronically implanted microelectrodes allows recording from as many neurons as possible in single insertions of the microelectrodes, thereby reducing the numbers of microelectrode insertions and the animals used and is much less invasive overall. Holding the rat for manipulating the microelectrodes for no more than 5-10 minutes allows for changing the depth of microelectrodes and recording from different neuronal populations in single insertions. Holding the animal avoids anaesthesia or the need to use of head fixation which offer no flexibility. All animals will be habituated to the holding procedure and thus it will cause only transient discomfort.

These are key techniques in the future marmoset application.

Both species

Terminal anaesthesia using a sedative followed by sodium pentobarbitone and trans-cardiac perfusion. Complete cessation of the heartbeat is confirmed via stethoscope prior to making incisions for the perfusion for absolute certainty the animal is no longer alive and does not experience any suffering or distress during the perfusion process.

Overall, my collaborators in the facility and I are geared towards optimal refinement, from the choice of animals to the methods, procedures, and skills. Additionally, I make sure that my group maintains high standards and training of staff in order to ensure all our refinements are actually implemented. I review all procedures and skills of the licenced researchers working in my group, under my supervision, regularly and discuss project licence-related matters at each of my weekly group meetings.

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

In this project, I will use common marmosets to study cognitive behaviour unique to primates, and rats to validate and refine neurophysiological experimental techniques towards the future marmoset application.

Marmosets

Marmosets are the least sentient organism with fine object vision and highly evolved prefrontal and temporal association cortices that interact with each other to control the higher-order cognitive memory behaviour.

Rats

Rats are the least sentient organisms with sufficiently large body and head that can be used to validate and refine neurophysiological techniques for future marmoset experiments. Mice are too small for this purpose.

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

In this project, I will study cognitive behaviour by non-invasive brain imaging in marmosets and refine neurophysiological experimental techniques in rats towards the future marmoset application. I have 17 years of experience in neurophysiological experiments in rodents and non-human primates.

Animals will be acclimatised to their home cage, experimental apparatus, and environment before starting experiments. For acclimatisation and pain management, palatable foods such as flavoured jelly, paste or milk shake liquid will be given. Animals' health score and body weight will be regularly recorded to detect and treat any potential harms/suffering as early as possible. Animals will be kept in a pair or group in home cage to maintain their social behaviour, unless temporary isolation is required for treatment purposes e.g., recovery periods.

Marmosets

I am closely collaborating with the groups within the facility which have been performing many of the described procedures for over 30 years and during this time the techniques have been refined either in house, or in consultation with outside experts in their particular field to minimise pain, suffering, distress or lasting harm. To avoid unnecessary stress associated with behavioural testing, marmosets are (i) trained to voluntarily get into a carry box in the home cage to go to the test apparatus by appetitive training, (ii) can move freely in the test apparatus, (iii) trained in behavioural tasks by positive reinforcement, and (iv) are tested for less than 40 minutes per session in behavioural tasks. Analgesic,

anaesthetic and antibiotic regimes have been developed in consultation with the NVS and are under continual review. I receive additional advice on anaesthesia from an experienced specialist veterinary anaesthetist with considerable expertise in primates.

A formalised weekly environmental enrichment programme with rotation of enrichment devices has been instituted in the colony, with new items regularly trialled and added to the rotation if successful. Items such as foraging boxes, highly palatable juice (frozen and liquid forms), swings and baskets, and textured bedding have all been utilised to enrich accommodation routinely, and/or as part of enhanced recovery after surgical procedures. Live foods (locusts and mealworms) have also been introduced to encourage more natural hunting and foraging behaviours. Animal staff and scientists interact with marmosets on a daily basis, continually monitoring the welfare and environment, ensuring that the NC3Rs guidelines on non-human primate accommodation and care are consistently met and exceeded wherever possible.

Rats

Rat experiments are performed in order to refine neurophysiological techniques towards the application to marmosets in the future.

The duration of surgeries is shortened where possible by technical refinements in order to minimise post-surgical complications when recovery is required. All surgeries, as well as microelectrode insertions, are performed aseptically. The home cage is enriched with various furniture and three- dimensional structure. I can gain advice from my collaborators and NVSs on best practice to reduce likelihood of post-operative complications and associated stress.

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

My research is constantly guided by and adheres to the Laboratory Animal Science Association (LASA), the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) and Animal Research: Reporting In Vivo Experiments (ARRIVE) Guidelines. Not only do I follow the LASA guiding principles of aseptic surgery (http://www.lasa.co.uk/wp- content/uploads/2017/04/ Aseptic-surgery final.pdf), but I will further these principles wherever possible as part of my constant refinement strategy, especially in the case of intra-jugular catheter implantation procedures. I will receive direct updates on best practice from the N3CRs through their mailing list, and the annual Primate Welfare meeting.

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

I have several lines of information that enable us to stay informed about advances in the 3Rs in order to implement them effectively. First, I have registered to the NC3Rs newsletter. Secondly, as all the project licence holders at my establishment, I receive tremendous support from the staff at the establishment, and I receive regular critical updates from the Named Information Officer to which I pay the utmost attention and that I share with all the members of my group. Third, I regularly hold project licence-related workshops with all the members of my group to discuss the changes in procedures. I also have an excellent working relationship with the animal care staff in our animal facility,

which facilitates the implementation of advances in the 3Rs. Finally, I am also part of an international network of marmoset users and rodent users and I regularly have meetings and exchange best practice.

A retrospective assessment of refinement will be due by 04 April 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

7. Developmental timing of symptom onset and therapeutic opportunity in genetic mitochondrial 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

Mitochondrial disease, mitochondria, paediatric disease, neurodegeneration, metabolic dysfunction

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

Mitochondria are a component of most mammalian cells and are responsible for energy production, regulation of cellular signalling, regulation of metabolic pathways, are involved

in immune responses, and are involved in cell survival and organism development. This project is focused on furthering our understanding of genetic mitochondrial diseases, a class of inherited disorders which include many different forms and lead to severe disease and early mortality, often in early childhood.

A retrospective assessment of these aims will be due by 07 May 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence? Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 mitochondrial diseases (GMDs) impact about 1 in 4,000 individuals and include devastating neonatal and paediatric, as well as adult onset, forms. GMDs are the most common cause of genetic metabolic disease and among the most common causes of genetic neurologic diseases. GMDs come in a great variety of unique forms, which has been a major obstacle in discovering how the diseases work and how to treat them. GMDs cause severe suffering in patients and exact significant economic, emotional, and mental health tolls on caregivers, as well as stressing healthcare systems. To date, no proven clinical treatment options exist for any mitochondrial diseases of any form.

Recent headlines in the UK surrounding so-called ‘three parent babies’ (resulting from cytoplasmic transfer of healthy mitochondria to an embryo) have raised public awareness in mitochondrial disease, but it is critical to note that this is simply an advanced method of conception. Cytoplasmic transfer does not diagnose or treat mitochondrial disease, and is only relevant to a very small subset of patients (those carrying a particular type of mutation, who have been diagnosed through DNA sequencing, are healthy enough to conceive and carry a child to term, and have chosen this reproductive option). As cytoplasmic transfer is not applicable for the vast majority of genes causing mitochondrial disease, and most cases are sporadic (no prior disease in parents so unpredicted), this advanced in vitro fertilization method cannot be thought of as a means by which to prevent mitochondrial disease.

In recent years, researchers have discovered a few therapies that can prevent disease in animal models. These include chronic mild hypoxia and treatment with drugs that block immune responses, both surprising discoveries which have the potential to lead to potent clinical therapeutic strategies. Unanswered questions about how they work, at what stages of disease they will be effective, and in what specific forms of GMDs they will work, must be answered before they can lead to clinically useful treatments.

The work outlined in this project will help further the development of our understanding of how GMDs cause symptoms and further efforts to develop strategies to treat GMDs. In addition to the needs of the GMD populations, this project addresses major basic science

questions regarding the role of mitochondria (which are an important functional component of nearly every human cell) in development, health, and disease. This project will enhance our understanding of the cell and molecular mechanisms involved in the role of mitochondria in both health and disease.

Mitochondrial dysfunction is known to play a role in a variety of neurodegenerative diseases, such as age-related and age-associated neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and Amyotrophic lateral sclerosis (ALS). The precise mechanisms by which mitochondrial dysfunction contributes is not well understood in any of these cases, but the links are clearly present – most notably in Parkinson's disease. Parkinson's disease can be caused both by mutations in genes coding for proteins involved in mitochondrial quality control (Pink1 and Parkin, both functioning in recycling of damaged mitochondria), as well as by exposure to toxins (such as the pesticide rotenone) which inhibit mitochondrial enzymes. We anticipate this project to contribute to breakthroughs in these diseases where mitochondria play. In summary, this research is important both as pre-clinical translational work and as basic research into the mechanisms mediating the relationship between mitochondrial function and health.

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

The studies detailed in this project will provide significant advances in our understanding of the basic biology of diseases arising from mitochondrial dysfunction. The information gathered will be disseminated to scientists and the general public via lectures and open access publication in peer- reviewed scientific journals. We anticipate that the results of the proposed project will also lead to the development of novel treatments for GMDs, as well as advancing basic knowledge of the relationship between mitochondrial function, development, and disease, which may lead to new treatments in other human diseases.

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

We anticipate that researchers, clinicians, mitochondrial disease patients, caregivers, and the general public will all benefit from the proposed research.

Researchers will benefit immediately upon publication of findings from the proposed experiments. Data will be available through publication in open access journals and, when relevant, upload to public repositories. We expect our findings will spur subsequent studies in this area and in other diseases where mitochondrial dysfunction plays a role.

Patients, caregivers, and clinicians will benefit in the years following these studies as new options for therapeutic intervention in genetic mitochondrial disease are developed and adopted based on our findings.

The general public will eventually benefit from improved patient care and reduced caregiver and healthcare strains as a result of research leading to treatment for GMDs. In addition, the advancement of our knowledge of the basic biology of mitochondria in human disease will likely contribute to advances in the understanding and treatment of other forms of disease where mitochondria play a role, such as in age-related neurodegenerative conditions including Alzheimer's and Parkinson's.

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

The information gathered will be disseminated to scientists and the general public via publication in peer-reviewed scientific journals and in scientific and public lectures. We will select open-access options for publication to allow for immediate public access without restriction. We will openly share our raw data. We actively collaborate with a number of researchers in our field and will continue to welcome new collaborations. Information regarding unsuccessful approaches will be freely shared with other researchers and published whenever possible.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

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

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

Choice of animal:

Many of the organ system interactions involved in these diseases do not exist in non-mammalian organisms, and many of the genes involved are not even present in lower organisms such as fish, flies, or nematodes (microscopic worms), while mitochondria do not exist in bacteria at all, as they are intracellular organelles ('mini-organs') inside of mammalian cells. There are specific tissues, tissue regions (small substructures within organs), and cell types involved. These specific factors do not exist in simpler organism such as fish, flies, nematodes, or other simper organisms.

Mitochondrial diseases arise from interactions between unique mammalian cell populations and body organ systems, such critical interactions between specific neurones in the brain and white blood cells of the immune system. The pathogenesis (process by which a disease develops) of mitochondrial disease involves an intricate (and still yet to be fully defined) interplay between neurones in the brain, macrophages (white blood cells) and other immune cells in the blood, stress factors released at certain times in development, and peripheral tissues such as liver and muscle. Many of these processes cannot be studied in isolation, and the overall disease cannot be studied without each of these factors present.

Choice of life stages:

Genetic mitochondrial diseases are complex diseases with multi-organ system involvement and post- natal onset which is specific to events occurring in mammalian development. The interaction with mammalian development is a component of disease. Embryonic animals are not diseased, and disease onset is specifically linked to postnatal events. These diseases are progressive and the onset and progression can only be studied in a living, developing, model.

Alternate models:

While aspects of mitochondrial function can be studied in isolation or in simpler models and we utilise simpler and non-mammalian models frequently, whenever it is possible, the diseases we study in this project cannot be studied in isolated cells, in organoids, at embryonic ages, or in non-mammalian models due to the reasons discussed. The disorders we see in humans which we term mitochondrial disease (what we recognise as clinical human diseases) do not exist in organisms other than mammals and cannot be studied in settings other than intact developing mammalian organisms.

Accordingly, mice are the best (least sentient) choice.

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

Animal experiences vary slightly specific protocol within the project. Common and protocol specific experiences are as detailed:

Some animals will be bred to produce experimental animals.

Some animals will have ultralight, non-reactive, ID tags placed in their ear.

Some animals will be given medicated food.

Some animals, assigned to chronic mild hypoxia (11% oxygen), will be housed in the mild hypoxia chamber.

In Ndufs4(KO) animals

Some animals will be allowed to develop neurologic disease symptoms. In these animals, in addition to routine welfare checks performed by the animal facility, disease onset and severity assessed by weight collection (low-stress handling), assessment of forelimb clasping, and documentation of visual signs of CNS (central nervous system) disease progression. In these animals:

Frequency of weighing will increase to daily when animals reach 17.5% loss from their individual maximum weight (humane endpoint criteria is 20% loss from individual maximum weight).

In some animals, blood glucose and lactate tests will be performed using tail nick blood collection and point-of-care glucometer/lactatometer, a maximum of three times per week on non-consecutive days.

Up to every 10 days of age animals will undergo assessment of performance on a rotarod assay.

In experiments using Tamoxifen mediated induction of gene targeting:

Animals will be injected with tamoxifen or vehicle (injection solution only) for five consecutive days at specific ages.

During the injection period and one day after the last injection animals will be weighed daily.

In Harlequin/Aifm1 mice:

Animals will be weighed 2 or more times per week.
In some animals, blood glucose and lactate tests will be performed using tail nick blood collection and point-of-care glucometer/lactatometer, a maximum of three times per week on non-consecutive days.

Up to once per month animals will undergo assessment of performance on a rotarod assay.

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

Mouse Models for Primary Genetic Mitochondrial Disease

The primary adverse effect in these studies is the disease arising from the genetic defect causing genetic mitochondrial disease. Animals presenting with mitochondrial disease experience ataxia, increased susceptibility to seizures, and weight loss. In both the Ndufs4(KO) and Aifm1 model these symptoms are progressive in nature, beginning with very mild symptoms at ~ postnatal day (P) 37 (P37) (Ndufs4) or ~4-5 months (Aifm1), and progressing until late-stage disease at median of ~P55 (Ndufs4) and 5.5-6 months (Aifm1); maximum age where humane endpoints are reached is ~P80 (Ndufs4) and ~6.5 months (Aifm1). In each case, these symptoms are not thought to cause pain (they do not in humans), and humane endpoints are designed to prevent disease symptoms from progressing to a severe stage. The phenotype of, and anticipated impacts/adverse effects in, the GABAergic (GAD2 specific) Ndufs4(KO) are also detailed below.

Disease Progression in the Ndufs4(KO)

To provide additional detail regarding symptoms in the Ndufs4(KO): animals show symptoms in a highly consistent order as follows: 1) Animals are born overtly healthy. Body weight is on average low compared to controls, but not low enough to be predictive. Ndufs4(KO) animals appear more likely to be the runt of the litter if a runt is present. 2) A transient but benign loss of the majority of fur occurs during the early postnatal period, around post-natal day 10. This coincides with a hair follicle cycle, and fur returns upon the next cycle. Penetrance (the likelihood that a clinical condition will occur when a particular genotype is present)is near, but not, 100%, and such hair loss is infrequently observed in normal or otherwise genetically altered mice, so alone this cannot be used to confirm genotyping. 3) Weaning generally occurs normally, but, as in normal weaning mice, transient weight loss can occur. More severe weight loss during weaning can occur in runts, and Ndufs4(KO) mice are more likely to be the runts of the litter, so while this is considered a normal part of breeding/weaning, it appears more frequently in the Ndufs4(KO) mice. All weaned are provided softened food to minimize weaning stress. 4) Ndufs4(KO) mice grow normally during the post-weaning period of rapid weight gain. Weight peaks around P37, where there is an inflection in weight gain in control mice. In Ndufs4(KO) animals weight peaks around this age, after which weight is slowly lost over the course of following weeks as symptoms progressively develop. 5) Mice subsequently

present with forelimb clasping upon being raised by the tail, ataxia (reduced speed of movement and altered gait), and circling behaviour (tendency to move in right or left leaning direction rather than straight), all signs of underlying neurologic degeneration in the brainstem and cerebellum. During this time animals are frequently assessed for weight and health status (as detailed above). Frequency will increase when disease is overt (all signs are present, weight loss reaches 10% from their individual maximum weight). 6) Mice are humanely killed upon reaching humane endpoint criteria – 20% loss of body weight from their individual maximum weight (measured on two consecutive days) or upon becoming unable to reach food normally. Our extensive experience with this model allows us to report that the majority of Ndufs4(KO) mice reach the humane endpoint criteria (the great majority the weight loss criteria), though a small percent (<10%) spontaneously perish, presumed to be due to SUDEP. Our humane endpoints are designed based on over a decade of experience with murine models of genetic mitochondrial disease to provide informative and reliable outcomes while avoiding any unnecessary distress.

Disease Progression in the Harlequin model

Aifm1 is an x-linked gene. Homozygous (the presence of two identical alleles) female carriers (hq/hq) and hemizygous (effectively homozygous because males have only one X chromosome) males (hq/Y) develop disease, while heterozygous females are normal and have no defects, including in breeding. We refer to the disease genotypes together as Harlequin mice. Harlequin mice are normal (apart from a very sparse hair coat early in life) until 4-5 months of age, when they begin to show mild ataxia.
Ataxia worsens slowly over the next 4-8 weeks without any other overt symptoms, until ~6.5 months. At this time they begin to show weight loss, which occurs over the next few weeks until they reach humane endpoint criteria (20% loss from their individual maximum weight). Few (<5%) Harlequin mice die before reaching this humane endpoint criteria defined by weight loss. The ataxia is thought to have a moderate impact on quality of life, and included in our score sheets for routine assessment of overall animal health and quality of life.

The Hq mutant of Aifm1 is a fully dominant/recessive allele. Diseased Harlequin female (hq/hq) and Harlequin male (Hq/Y) mice show an identical disease progression, while female carriers (hq/wt) have no detected phenotype.
Disease Progression in the GABAergic Neuron Specific Ndufs4(KO) (GAD2-Ndufs4(KO))

As detailed above, the GABAergic neurone specific Ndufs4(KO) model (GAD2-cre driven excision of Ndufs4 in a homozygous Ndufs4 floxed line) does not exhibit any signs of outward disease except SUDEP (as well as increased seizure incidence upon epileptogenic (seizure-inducing) stress, but we will not be inducing seizures in this project). Some mild metabolic symptoms may be present (altered glucose handling, etc), but these do not result in overt symptoms, and are not the focus of any work in this protocol. GABAergic neurone specific Ndufs4(KO) mice, both inducible and constitutive, experience the adverse effect of spontaneous mortality due to spontaneous (but thought to be painless) death which occurs in the absence of any predictive signs. While mortality is an endpoint to be avoided at all costs, in this model, death cannot be predicted, is thought to be painless, and SUDEP is an important cause of mortality in human patients that requires study.

Interventions

The interventions we are testing are well-studied, and we have significant experience with them. For the pharmacologic agents listed (rapamycin and pexidartinib), oral dosing via compounding in normal mouse food has been optimised to avoid more stressful treatment routes. In prior work, we found that weight loss can occur when the goal dose is provided with no ramp up, presumably at least partly due to changes in food palatability, but that a short dosage ramp-up period prevents any significant weight loss. All drug treatments include a ramp-up period, but some minor (<5%) weight loss can occur.

Chronic mild hypoxia at 11% is a well-established intervention strategy in the Ndufs4(KO), and should not be confused with hypoxic stress conditions. For points of reference, this level of hypoxia is roughly equivalent to living in La Rinconada in Peru, the highest elevation permanent settlement in the world, or the oxygen concentration that Sherpa's experience in the Himalayas at elevations below base camp. During the first 1-2 days of acclimation overall activity is reduced, but housing Ndufs4(KO) or control mice in hypoxia does not result in any significant alterations to behaviour after these first days

Health Monitoring and Assays for Overall Health Status

We utilise non-invasive and minimally stressful assays for health status in addition to routine standard health monitoring administered by the facility. These include the following, with adverse effects and duration noted:

Weight checks and visual observation of locomotor ability and behaviour. This will occur with the frequency noted above and detailed We will utilise low-stress handling practices designed to minimise stress.

Assessment of forelimb clasping by briefly raising mice by the tail (2-3 seconds) and observing for the presence of forelimb clasping – a well described early sign of neurologic disease. Forelimb clasping occurs when mice cross their front limbs and slightly curl their body inward (a 'sit up' motion), rather than reaching their two front limbs outward, as would be normal for a mouse raised by the tail. This is a quick and minimally stressful assay. Tail-prick for point of care blood glucose and lactate measurements. This is a minimally stressful method.

Rotarod. The rotarod is simply an elevated slowly rotating cylinder with a grooved rubber coating for grip and a padded area for if animals fall (we use Med Associates product ENV-571M). We utilise a customised protocol for assessing overall health status and locomotor function. Specifically, we use a constant low speed setting (6 rpm, equating to a slow walk for mice) and monitor 'latency to fall,' with a maximum assay time of 5 minutes. All control animals and all pre-disease onset animals reach the full 5-minute mark. Decline during disease onset and progression can be quantified using this method throughout the P40-60 age range (untreated Ndufs4(KO) animals), with animal performance progressively worsening throughout this period. Mice are given three of these 5-minute trials per test day, with a minimum of 15 min between each trial. Animals are placed in a normal cage (with all normal contents including tunnels, bedding, etc) with access to food and water between. This is a minimally stressful assay and causes no harm or adverse effects in control mice.

In subset of Ndufs4(KO) animals, seizures will occur in this assay. These are typically brief, and mice fully recover within 1-5 minutes. Seizures occur in ~30% of Ndufs4(KO) mice assayed at postnatal day 30, <25% of Ndufs4(KO) mice assayed at P40, and few mice at later dates (<10% - the duration of 'latency to fall' decreases as animal disease advances, and seizure risk in these mice is related to time on the rotarod). The susceptibility to seizures in a very mild exercise paradigm provides an important second endpoint in this assay. Critically for our severity categories below: the animals that show seizures at P30 are the ones at risk at P40, etc, so they represent one 30% group out of the total cohort (rather than an independent 30% set at each age).

Tissue collection by perfusion. This is a terminal procedure, and therefore not expected to have 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)?

Severe = 9.2%

Moderate = 22%

Mild = 68.8%

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

- Killed

A retrospective assessment of these predicted harms will be due by 07 May 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 clinically relevant symptoms of mitochondrial diseases are unique to mammals, and unique to intact organisms. The specific forms of disease we are studying in this project are specific to the mammalian brain, with key aspects of the diseases specific to the *developing* brain. In addition, the diseases involve multi-organ system interactions. Accordingly, the mammalian brain is necessary to study the unique temporal, spatial, and cell-specific aspects of the pathogenesis of these diseases. These cannot be studied in

simpler models which lack the cell types, cell to cell interactions, and even genes involved in genetic mitochondrial disease.

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

We routinely utilise the microscopic nematode (*C. elegans*), cell culture, and analytical models, such as human genome-wide association study data, in our laboratory for our work in this field of research. We also collaborate with colleagues in the development of organoid models for *specific* queries. All these alternatives remain at our disposal and are used whenever replacement is a viable option.

Why were they not suitable?

The pathogenesis of paediatric mitochondrial diseases involves a complex interaction between specific mammalian brain regions and cell types, the mammalian immune system, and mammalian development. Studying the necrotising brain lesions of Leigh syndrome or cerebellar ataxia of *Aifm1* deficient mitochondrial disease is not be possible outside of an intact mammalian system.

A retrospective assessment of replacement will be due by 07 May 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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 use published and preliminary data to estimate variance within groups, and to judge what effect sizes would be biologically significant. We use the key endpoints in individual studies which are limiting factors (based on variance, relevant effect sizes) to determine replicate numbers necessary. Our estimates include both male and female animals to ensure that our data are not sex biased or unable to detect significant sex differences that may be present. Our estimates are based on the known production rate of the genetic recessive animals. We have extensive experience with mouse models and the assays we will be employing.

Critically, this is the *maximum* number, many cohorts are optional, and we will take steps to end studies early if key scientific objectives are achieved early.

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

Preliminary experiments and prior data were used to determine the minimum number of animals needed for scientifically valuable data. We discussed our experimental strategies with statisticians and online statistics and experimental design references. Experiments are carefully designed so that control groups are common whenever possible, allowing us to reduce the number of groups needed and significantly reduce the number of animals needed.

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

In collaboration with the colony management team, we will use effective breeding strategies to reduce our breeding animal numbers and take every available opportunity to pool control groups and provide treatments in a manner designed to reduce the number of necessary controls. Pilot studies have already been completed for the experiments detailed here, allowing for optimised animal number estimates. We collect all tissues from animals used for tissue collection and store tissues and other biological samples not immediately used and are willing to share these tissues. This provides a potential avenue for reducing the need for additional mice in studies conceived at a later date by us or other groups. Experiments have been carefully designed so that control groups are common whenever possible, allowing us to reduce the number of groups needed and significantly reduce the number of animals needed.

A retrospective assessment of reduction will be due by 07 May 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

Which animal models and methods 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

The mouse models of Leigh syndrome, SUDEP, and mitochondrial cerebellar ataxia provide direct models of human mitochondrial disease, meaning that the respective genes cause the same diseases in mice and humans. These models are necessary to study

biological processes which only occur in mammalian brains. These models develop different symptoms of neurologic disease, necessary for us to study those diseases. Our extensive experience with these models allows us to minimise pain, suffering, and distress caused by the disease by using humane endpoint criteria based on weight cutoffs. This is in addition to the use of a score-sheet for regular health assessment. The use of weight loss criteria (20% from their individual maximum weight) rather than disease severity provides an early endpoint. The specific weight thresholds chosen are based on rate of disease progression and overall disease course in each model.

In the SUDEP model, no disease is present until SUDEP (sudden unexpected death in epilepsy) occurs, so an earlier threshold is not possible - there are no measures to base one on.

Methods

The methods in this project have been optimised to cause the least amount of distress and suffering possible in the experimental animals.

Drug treatments

Over many years we have optimised our drug treatments methodology to enable us to provide treatments compounded in normal food, rather than needing to treat animals by daily injections as in early work. This completely avoids the stress and potential harms associated with injections of any type, or with drug delivery via oral gavage.

In recent work, we refined our pexidartinib treatment in food to include dosing ramp up period, where the dosage is increased over the period of a few days. We found this fully prevents transient modest weight loss that was seen when animals were immediately given the full dose.

Assays for disease progression

Over years of research we have refined our assays to quantify the progression of disease in mouse models of mitochondrial disease and use a limited, minimally invasive and minimally stressful, set of assays which provide robust quantitative endpoints. Blood tests utilise the least stressful route (tail vein), and assays for neurologic function rely on non-invasive visual signs of neurologic state. The only available alternatives would require more invasive routes of blood collection or, for tracking brain disease, repeat MRI, which is a highly stressful procedure (in particular for the mouse models we use, as they do not tolerate anaesthesia).

The frequency of health assessment by rotarod has been designed to prevent stress from frequent testing while providing necessary experimental data.

Our rotarod assay has been optimised for use in diseased mice. We use a constant low speed of 6 rpm, rather that a speed ramp up which is the standard for most animal studies, and assess the ability to maintain rod walking for 5 minutes. If this is achieved, the trial ends. This paradigm is tailored for the animal models we use here, providing a mild exercise and balance test which all healthy mice can do without any issues and extremely minimal stress but which can detect the onset and progression of disease impacting

balance and muscle control. We believe this assay is less stressful than other tests for muscle strength or coordination (such as grip strength, balance beam, treadmill, etc), and assesses overall coordination and muscle strength in one test rather than multiple, minimising the overall stress each animal experiences.

We utilise a visual assay for forelimb clasping, and early sign of neurodegenerative disease. This involves a brief lifting of the mouse by the tail to observe the postural response. Low stress handling is used throughout apart from the brief lift. This assay is the most refined option available, providing a non-invasive, minimally stressful, method for tracking neurodegenerative disease onset. The only potential alternatives would involve serial anaesthesia (for MRI), which would be significantly more stressful, would require far more overall handling, would toxic in our anaesthesia sensitive model, and are less sensitive for early detection of disease (visual signs appear before MRI changes are visible).

Our disease progression monitoring is optimised for each model; neurologic disease does not present until post-natal ages in the models we use (around/after P37 in the Ndufs4 models, and around 4-6 months in the Aifm1 model), allowing us to limit handling and assessment at pre-disease onset ages.

ID Tags

For some animals, we will utilise non-reactive, ultralight, QR code ear tags. These are placed in the hole made in the ear during tissue collection for genotyping so that a separate procedure is not needed. These tags facilitate blinding of treatments and data collection, provide an added quality control measure to help ensure that all treatments and endpoints are properly assigned, and enable tissue cataloguing for further study. Each of these factors reduces mouse use for this and future studies (the tissue cataloguing will allow for this) and improves data quality.

Protocols Designated Severe

Protocols are classified as severe due to the occurrence of seizures in the respective models. In this project we do not employ studies designed to induce seizures, but seizures occur in mitochondrial disease. In the rotarod assay we anticipate that a portion of animals to show signs of seizures as a result of the mild exercise regimen. These are typically extremely brief with full recovery within minutes. Most are extremely mild (not status epilepticus), observed only as brief clonic movements. Where a seizure occurs, animals will be immediately returned to a warmed cage with wetted food for recovery and observed for 15 minutes to ensure behaviour is returning to normal. If animals are showing distress or failing to improve toward baseline by 15 minutes the veterinary staff will be consulted, and animals will be humanely killed or subjected to increased monitoring as advised by the veterinary staff.

In the model for SUDEP, seizures occur without warning and lead to sudden death. In the SUDEP mice, as in humans, this is thought to result from a sudden loss of brain cardiorespiratory control, typically during sleep and without any signs of distress. There are no alternative endpoints for SUDEP.

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

Many symptoms of mitochondrial diseases are unique to mammals, and the forms of disease we are studying in this project are specific to the post-natal mammalian brain. The mammalian brain is necessary to study these diseases. The pathogenesis of paediatric mitochondrial diseases involves a complex interaction between specific mammalian brain regions and cell types, the mammalian immune system, and mammalian development. Studying the necrotising brain lesions of Leigh syndrome or cerebellar ataxia of Aifm1 deficient mitochondrial disease cannot be done in non-animal mode or non- mammalian system. Less sentient species (for example fish, flies, and nematodes) lack genes, proteins, cell types, and organ structures involved in these diseases so cannot be used to model them. We are studying progressive disorders and the mechanisms involved in disease progression, so living animals are necessary.

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

Our monitoring strategy has been carefully refined based on our prior experience in mitochondrial disease models and maximises animal welfare over any other concern. It includes daily monitoring of animals when disease symptoms are overt and animals are declining. We will frequently consult with the veterinary staff and include any additional or more frequent monitoring as recommended. Our rotarod procedure and frequency of analysis have been optimised for the models used to allow for minimum testing and minimal stress. All these assays, as well as drug treatments and animal handling, will be subject to constant scrutiny. Additional non-invasive/non-stressful monitoring will be included whenever it is thought that the animals may benefit. We do not employ any surgeries or invasive techniques in this project.

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

Alongside the guidelines listed below, I will also adhere to local AWERB standards for research animals, and, where appropriate, support the development of new local standards for refinements discovered during the project licence.

Where these guidelines conflict, we will select the most up to date and refined option.

Code of Practice for Housing and Care of Animals Bred, Supplied or used for scientific purposes

LASA Guidelines

RSPCA Animals in Science guidelines

UFAW Guidelines and Publications

NC3R’s and Procedures with Care

I will consult with the Colony Manager, veterinary staff, and technical staff to review genetic health, breeding practices and overall colony health and management at regular

intervals. I will consult with animal facility technical staff and veterinary staff on a regular basis and seek out refinements through these interactions.

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

The local AWERB, NIO, NACWO, NTCO and Veterinary team regularly inform, and disseminate, improvements and recent studies involving reduction, replacement and refinement alongside external resources including (but not limited to); collaborators, peers, conferences and lab animal and animal welfare bodies.

During the 1, 3, and 5-year review of the project licence I will update implementation and consideration of the 3Rs that has occurred during the previous period, alongside a review of the linked training plan, score sheets etc. in collaboration with the NACWO, NTCO, NIO and Veterinary team with a particular focus on refinements.

In addition, I will regularly attend national and international conferences in my field, taking note of any advances in methodology that could be used to refine the work in this project, any advances in techniques that may lower the number of animals needed, and any new models which may be used to address any of the scientific questions raised in this project. Our laboratory is actively involved in developing additional models of disease, which may in some specific areas allow for further reduction in animal use.

A retrospective assessment of refinement will be due by 07 May 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

8. The Role of Inflammation in Efficacy and Safety Pharmacology

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

Pharmacology, Inflammation, Efficacy, Safety

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 required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of 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 efficacy and safety of new treatments for human inflammatory diseases, including respiratory diseases such as Asthma, COPD (Chronic Obstructive Pulmonary Disease), Pulmonary arterial hypertension (PAH) and Pulmonary fibrosis, treatments for pathogen (e.g. bacteria/fungus) induced lung inflammation and treatments targeting lung injury initiated by exposure to chemicals that may cause pulmonary (lung) inflammation.

No cosmetic products or chemicals that are exclusively intended to be used as ingredients in cosmetics will be tested.

A retrospective assessment of these aims will be due by 30 April 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

Although some of these diseases are controlled with existing drugs (such as Asthma), there are often side effects with these long term therapies. There are currently no cures for inflammatory diseases such as COPD, Pulmonary fibrosis and PAH, and these conditions often lead to premature deaths amongst those who suffer from them. This project will set out to find new drugs that have the potential
to both alleviate or cure such conditions, and in the case of asthma and other inflammatory diseases, find better drugs, that work better in patients with fewer side effects.

Governments require and the public expects that medicines are safe and/or well-characterised. Therefore, before humans are exposed to new substances, their safety and efficacy must be evaluated; this is a mandatory legal requirement. This safety and efficacy assessment requires the use of animals in studies to evaluate systemic exposure/efficacy/toxicity; currently, there are no scientifically, ethically or legally acceptable alternatives available that do not involve the use of animals.

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

The principal benefit of the project is the provision of data to facilitate sound decisions on safe/effective product development and appropriate regulatory decisions on clinical trial approval or marketing authorisation for new medicines to which humans or domestic animals will be exposed, thus contributing to their protection and safety.

The potential benefits of this project would include the discovery of new treatments for inflammatory diseases, and the confirmation of the safety of new treatments for inflammatory diseases prior to first administration in man.

Work under this Licence will also show which compounds are not suitable to move forward into patients due to them not being able to moderate the clinical condition examined, or they are not safe to go into humans, for example.

Work performed under this licence may identify more effective drugs for example to those already on market, with fewer side effects and that work better than existing drugs.

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

Patients will benefit from these studies as this work will contribute to the development of new drugs that help alleviate human inflammatory conditions. These new drugs may work better in the clinic, relieve or cure diseases and have better side effect profiles. We may, by our work, also contribute to better knowledge and understanding of these types of drugs, and that knowledge may be used to develop further new drugs.

One of the key benefits is the production of data that is required by regulatory authorities, to ensure medicines have their desired therapeutic effect in humans (e.g. reduce inflammation in the specific model). The drugs that will be tested are for a variety of conditions, in some cases where there is an unmet clinical need to treat such conditions.

In addition, the models on this project may be used to assess the safety or other in life properties of a new drug, and find a dose that causes no adverse effect. This is important when planning future trials in humans, to make sure any starting dose in a clinical trial is safe for the patients taking it.

Our customers will also benefit, as the data we generate will allow them to progress their new drugs into clinical trial, or otherwise if they are found to have adverse side effects.

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

The work will be shared with customers who will use it to determine their future strategy, or for submission in documents required by regulatory authorities. Whilst we have no direct control over what happens to the data after we have shared it, we trust from information given to us that it is used to support candidate selection or to support drugs progressing to clinical trials). Where appropriate, we collaborate with our customers to share data we have produced in the form of scientific publications that are in the public domain.

We are able to advise our customers on which studies are required in their development programme and on suitable study designs, based on our experience and on knowledge gained from previous feedback from customers and/or regulators, leading to focused and effective studies.

It is difficult to predict how the benefits of any work done on this project will be seen in the future due to confidentiality issues. However, this work will contribute to the efficacy and safety of pharmaceuticals that can be administered to humans, either by informing on safety and allowing to progress to clinical trials or preventing pharmaceuticals reaching the market due to safety issues), which in itself reduces the overall number of animals used, by preventing further testing.

Species and numbers of animals expected to be used

- Mice: 23500
- Rats: 29500
- Guinea pigs: 21000

Predicted harms

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

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

The project will use adult mice, rats and guinea pigs.

Mice and rats will be predominantly used in these studies. These species are used because they respond to inflammatory agents in a similar manner to humans and the data produced will help model inflammatory conditions that occur in humans (including inflammation cause by exposure to industrial chemicals), predict how well the potential medicines will work in humans, and predict the potential side effects of medicines in humans. In a limited number of studies, guinea pigs may be used, but they will only be used when the experiment required specifically requires these species (e.g. the guinea pig models the cough response in humans better than the mouse or the rat).

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

For the majority of experiments the induction, assessment and resulting inflammation will cause no or mild adverse effects such as slight weight loss. A small percentage of animals may show more significant adverse effects indicating moderate severity, e.g. more marked weight loss or reduced activity. To limit the animals discomfort, additional bedding, provision of moistened food within the floor of the cage, longer sipper tubes on water bottles will be provided. Specialist veterinary staff are always available to advise and assist in the welfare of the animal. Humane end-points are applied, under veterinary guidance as necessary, meaning that in the vast majority of experiments animals will be killed before they undergo anything approaching severe effects.

There are a small number of experiments where to be able to accurately recreate what happens in humans when they are exposed to chemical inducers of lung injury (e.g. Chlorine gas), it is necessary to expose animals to these same chemical inducers which may induce severe clinical signs in the animals. These animals will be closely monitored and appropriate humane endpoints applied. The severity of the adverse effects will always be kept to a minimum that is consistent with the scientific goals of the studies.

Occasionally we may perform surgery to help us achieve the aims of these studies. This may include cannulation of blood vessels to administer drugs or take blood samples. We may (rarely) implant small pumps that dispense drugs over a period of time, which prevents repeated injections. We also may need to cannulate the trachea to measure lung function, and implant microchips to take temperature.

These surgeries are only performed if its specifically required and the procedure means less overall suffering to the animals. Each animal will get pain relief and sometimes antibiotics under the supervision of a vet, and will get extra care after surgery much like a hospital patient would do after surgery.

Various clinical conditions are modelled in our experiments, and the animals may experience similar effects as humans do, eg. tightening of the chest in asthma (the condition modelled is usually mild), as they will be exposed to agents that cause inflammation (by injection, by inhalation or by dosing orally for example). This is under very controlled conditions, and the exposure to these agents is usually minimised to allow the development of the condition, without overly harming the animals.

The animals will also be dosed with putative drugs to help alleviate these inflammatory conditions by various means including injections and dosing via on oral catheter-these are techniques we are very proficient in and the minimum distress will be caused in these situations. On very rare occasions, other less standard routes including intrapleural dosing, maybe used. This will be in specific circumstances, and with full scientific justification given prior to the start of any study.

Animals may also be confined for periods to allow dosing, or to measure lung function for example, and may well be introduced to the chambers needed to do this, so they are prepared before experiments start.

Some surgery and a lot of the procedures performed in this project are done in animals who will not be allowed to recover from anaesthesia. This substantially reduces any potential suffering and distress animals may experience. Recovery Surgical procedures may result in some moderate effects like short term pain and weight loss, but the use of pain relief will be standard unless this is prevented by the type of study performed (not appropriate in studies testing some anti inflammatory drugs) as it will stop proper analysis of the results.

Rarely we may use mice that have been bred and altered to include either a human gene or have another genetic modification that, for example, models an inflammatory condition that is seen in humans. These mice can be used to test drugs against something very similar to the condition seen in humans, making it easier to see what a potential drug may do in the clinic in patients. This type of study is rare. The modifications seen in these animals will not be dangerous, but may be harmful to the animals, so like all animals on this project they will be very closely monitored under the care of experienced staff and vets.

At the end of all of our experiments all of our animals will be killed in a humane way. This is required by the law which covers the use of animals for the purpose of testing drugs for humans.

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

When dosing an animal by injection or taking blood, the degree of pain or discomfort an animal feels is similar to what a patient would feel having an injection done or blood taken by a doctor.

Animals undergoing surgery receive the same sort of care as a patient would in hospital. We discuss their pain relief and use of antibiotics with a vet before we start and administer drugs as necessary.

Inflammatory challenges inducing lung injury will induce mild to moderate adverse effects in animals depending on which protocol and which challenge is used.

Dosing with drugs may cause adverse effects in some studies, but this is rare as we often have a good idea of doses from other studies. Experience from the last licence shows that the majority (~95%) of animals display only mild severity with the remaining 5% displaying moderate severity. A lot of these moderate severities are due to surgical procedures. Lethality is not expected to occur, in any of the protocols in this licence.

We observe our animals at least twice a day, by trained staff familiar with the species used, and the people who do this know the signs when an animal is ill. If an animal is ill, we would check it more frequently, and consult vets and other senior animal care staff for advice and guidance in its care.

Most animals are expected to experience no, or only mild, adverse effects during the course of the study such as slight weight loss or piloerection (ruffled fur). A small percentage of animals may show more significant adverse effects, such as more marked weight loss, reduced activity or tremors. No animals would be expected to die or to suffer prolonged adverse effects as a result of the procedures, and due to defined control measures and application of early humane end points , under veterinary guidance as necessary, to prevent this; such end-points might include interventions to discontinue dosing, or to provide supportive treatments, or if necessary to humanely kill 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)?

On the last project, about 95% of animals were classified as having experienced mild severity, around 4% were classified as moderate. Around 1% of animals were classified as severe (this was mainly under a specific technique under a single protocol)

The moderate severities in the last project would have been largely due to a surgical procedure e.g. cannulation, or an implantation of a device was involved. It's impossible to predict the proportion of severities expected on a service licence like this, as this will be dependent on what study types we are asked to perform. However, a distribution between ‘mild’ and ‘moderate’ severities similar to those in the last project are anticipated.

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

- Killed

A retrospective assessment of these predicted harms will be due by 30 April 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

Inflammation is a complex process which is not fully understood. It involves differing body systems and processes, many of which are joined and therefore for the protocols listed in this Project, there is no adequate non animal model to replace the whole animal experimental model, as the complex inflammatory and cellular mechanisms under investigation cannot be adequately modelled in non- sentient laboratory preparations e.g. in test tubes (in vitro), for example.

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

In many cases the protocols listed in this Project will be used some way into the developmental life cycle of a test substance and in many cases for pharmaceuticals in vitro (tests in test tubes) tests will have been conducted earlier e.g. screening of potential test compounds for further development as part of the drug discovery process.

Experimental designs are constantly reviewed and alternative cell assays considered as technology improves, however due to the complex nature of the inflammation pathways there are no current alternatives but to use animals. Similarly, the regulators who decide whether potential new drugs are safe to be tested in man, will not accept tests solely using non animal methods

Why were they not suitable?

Although there are in vitro tests that can model inflammatory processes, and some tests showing how drugs get into our bodies, and how our body deals with them, and can identify undesirable effects, for example, there is no series of in vitro tests that brings all these complex events together, as in the whole human organism. It would be impossible to measure inflammation or inflammatory processes, for example, without using a whole animal, as its often an ongoing and long term process.

That is why we need to test new drugs in animals, as they have similar physiology and processes as humans, and that testing gives us a good idea what may happen if they are subsequently used in humans.

A retrospective assessment of replacement will be due by 30 April 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

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

The numbers of animals used in each study are in some cases specified in the regulatory guidelines; where not specified, numbers are based on established minimum regulatory expectation, or on scientific estimates of the minimum numbers required to meet study objectives.

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

Studies are designed to provide maximal data and statistical power (where appropriate) from the minimum number of animals considering that it is better to increase the number of animals used to achieve the objective than to use too few animals and risk not meeting the scientific objectives of the study.

For regulatory studies, guidelines require the number of groups and animals per group to be adequate to clearly demonstrate the presence or absence of an effect of the test substance; core study designs are based on international guidelines where these exist. Otherwise reference is made to standard study designs with input from the Department of Statistics, where appropriate, to identify the optimum number balancing the need to achieve study objectives while avoiding excessive animal use. These internal designs are reviewed and updated in line with changing external guidelines and internal refinements that either minimise numbers or reduce severity.

Whenever possible, common species of animals are used such that a large amount of control background data is available. This reduces the need for large control groups.

For less established experiments, a preliminary study may be conducted in which smaller numbers of animals may be used to generate data in order to ensure that the experiment operates as we would expect and to generate some data which may be used to get a better study design. From such pilot studies, the variability of the measurements are used by statisticians to determine the required number of animals per group required to identify whether the test substance actually has an effect in a main study.

In some protocols, animals that have previously undergone minor procedures (e.g. the use of eye drops for ophthalmoscopy) may be again subject to satisfactory veterinary examination and relevant re-use criteria set out in the regulations from the government, under supervision of a veterinary surgeon. This means that we reduce the total number of animals we use.

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

We will try to get as many outputs as we can from a single animal where possible, without adversely affecting its welfare. So if we need to take several different samples, for example, we will often do that in the same animal, rather than using separate ones, when possible. Histopathology is often performed in the same animals that have undergone lung function and subsequent washing of the lungs (BAL).

The experiments we perform are only permitted on the condition we use the least number of animals possible to get a meaningful result to assess safety or whether a drug has the desired effect. These numbers are sometimes based on a number set by a regulator, or based on our own experience. We regularly consult with statisticians when new study types are performed. They do a special calculation (power calculation) which takes into account the size of likely effect we will see to help determine the number of animals we use. Although we do use the least number of animals possible, its important to use enough animals to get a meaningful result, otherwise we would end up using more animals overall than we needed to.

Where we can we only use one control group per study (effectively dosed with the drug formulation without the drug in it) which acts as a baseline to compare any effect of the drug itself.

Studies are also carefully designed to combine the different aspects needed to evaluate the safety or whether a drug has its effect combined, and again this reduces the overall numbers of animals used.

Variables that may affect the study are kept constant wherever possible to make sure the experiments stay the same time after time. This actually means the data is more reliable and meaningful, and easier to make assumptions about.

A retrospective assessment of reduction will be due by 30 April 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 adult mice, rats and guinea pigs.

Wherever possible, lung function, experimental tissues and samples are collected under terminal anaesthesia or at post mortem to reduce the burden on the animal used in the protocols. In some circumstances other cells may be taken from the same set of animals to give the maximum amount of data for the fewest number of animals.

Highly trained staff use a rigid framework of welfare assessment to allow early detection of animals showing signs of discomfort or distress. We use pain relief as standard with anaesthesia and after procedures where relevant e.g. surgically implanted models. We sometimes, although rarely, use animals that have had their genetic material altered eg such that they are predisposed to developing a disease type or if a particular gene is important say in a specific component of the inflammatory process in which we have interest in.

Although we may use various species on this project, rats and mice are the standard species used as they are of the lowest sentience. We would only use other species where their physiology means that they would be the best animal to use modelling a specific disease type. For example, Guinea Pigs would be the best animal to use when assessing cough and constriction of the lungs because guinea pigs are the species of lowest sentience that has a robust cough reflex

This means we will use mice and rats unless other species will provide a better answer to the scientific questions we are asking due to their physiology being more suitable, or their reaction to a condition we are trying to induce being more like you would see in humans

Dosing and sampling procedures will be undertaken using a combination of dose volumes, routes and frequencies that of themselves will result in no more than low levels of discomfort and no lasting harm and will be the minimum consistent with the scientific objectives. Many of the procedures carried out produce only minor levels of discomfort, due to the nature of the procedure, and the skill of the person performing it. For example, an animal having a blood sample taken would feel the same level of discomfort as a patient in a doctors surgery having a blood sample taken.

Non-recovery surgical procedures will be performed in accordance with the principles set out in the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017) (LASA is the Laboratory Animal Science Association).

In some protocols, animals that have previously undergone minor procedures (e.g. the use eye drops for ophthalmoscopy) may be again subject to satisfactory veterinary examination and relevant re-use criteria set out in the regulations from the government. This means that we reduce the total number of animals we use.

Food and water withdrawal will be kept to a minimum. This is not routinely carried out and only occurs in very specific circumstances when it is an integral part of individual study requirement.

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

There is a scientific and regulatory requirement for safety/efficacy data in rodents (mice, rats and guinea pigs in this case), and they are considered the species of lowest sentience that give a valid comparison to human physiology.

Adult animals are used as we are modelling inflammatory diseases that are present mainly in adults.

Many of the processes we use to generate models of inflammation require days and weeks of administration to produce the inflammatory condition, therefore it is not practical to keep animals terminally anaesthetised (although some model outputs are generated under terminal anaesthesia).

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

Animal welfare is of utmost importance and Good Surgical Practice will be observed for any animal undergoing surgical procedures.

During dosing and restraint, animals are constantly and closely watched for signs of distress. If equipment is used to enable us to achieve the scientific aims of the study (e.g. confinement in a whole body plethysmography), then we would habituate animals to this equipment prior to dosing. Most animals habituate well to this equipment, but if they don't (rare) we remove them from the study.

If we have to repeatedly inject animals or withdraw blood using a needle and syringe, we would choose different sites to do this where possible to minimise local adverse effects. Where appropriate we place temporary cannulas in blood vessels to reduce the number of needle punctures necessary. If we can take blood samples when an animal is deeply unconscious then we do so. All personnel performing these procedures are trained to a high standard to minimise adverse effects.

All procedures are subject to ongoing assessment and technique improvement and we participate in cross-company working parties on best practice. Animals are regularly reviewed for general health and veterinary staff are on call at all times to assess any adverse events and provide supportive care and treatment as appropriate.

Refinements to improve the animals experience include but are not limited to group housing, environmental enrichment, including shelters for rodents, gnawing materials, extra bedding, human interaction, acclimatisation and training to procedures,

We have dedicated working groups on animal welfare for each species (in this case a rodent specific group) with a permanent brief to identify potential measures to improve animal welfare, and to trial such measures and make recommendations for adoption.

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

First report of the BVA/FRAME/RSPCA/UFAW joint working group on refinement, Laboratory Animals, 27, 1-22 (1993).
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How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

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

A retrospective assessment of refinement will be due by 30 April 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

9. Mechanisms of Cardiovascular Disease

Project duration

5 years 0 months

Project purpose

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

Key words

Cardiovascular disease, atherosclerosis, blood flow, inflammation, therapeutics

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 required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of 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 study mechanisms of how mechanical forces of blood flow regulate blood vessel diseases. An in-depth understanding of these mechanisms is likely to lead to the development of new therapeutics to treat diseases like atherosclerosis.

A retrospective assessment of these aims will be due by 16 May 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

Cardiovascular disease is one of the biggest killers in the UK and worldwide. Knowledge of molecular mechanisms that trigger and promote cardiovascular disease will lead to the development of novel therapeutics to ameliorate the burden of cardiovascular disease.

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

The project will advance our understanding of the genetic, molecular and cellular mechanisms of initiation and progression of cardiovascular diseases, with a particular emphasis on the role of mechanically-induced pathology. The results of the conducted studies will also enhance our understanding of therapeutic arteriogenesis. We anticipate that within the life of this license, we will identify 4-6 novel pathways associated with cardiovascular disease. Of the ones we identify, we anticipate that 3-4 will have a detailed analysis of the cellular and molecular mechanisms by which they regulate cardiovascular disease. For 1 or 2 of these pathways/genes we will further investigate the interplay between common co-morbidities and cardiovascular disease treatments. Once we have characterised these novel pathways, we will use our clinically-relevant models to examine the genes/pathways in relation to current therapies. Once characterised, we will make all new animals models developed under this licence available to other researchers . We anticipate that the finding from this work may have implications in non-cardiovascular diseases e.g genes which alter endothelial cell function may have an impact on other settings such as cancer or rheumatoid arthritis as the vasculature plays an important role in several pathologies. In addition, we will deposit our genomic and proteomic data to publicly accessible databases. Collectively, outcomes from these studies have the potential to identify new therapeutic targets for prevention of pathologic pathways and promotion/enhancement of protective pathways. In addition, the work conducted under this project will also result in high-impact publications, be presented at scientific conferences and filing of patents.

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

In the short/medium term, it will provide information on the mechanisms responsible for initiation and progression of cardiovascular disease (in both blood vessels and the heart) using mouse models of atherosclerosis, intima-media thickening and hypertrophy. These studies will determine how specific genes and pathways are related to disease initiation and progression.

This will benefit the wider scientific community and advance research in the field of vascular mechanotransduction [how the vascular system converts mechanical signals into electrical or biochemical signals] and heart disease.

Where appropriate, we will also collaborate with pharmaceutical companies to inform them of the novel pathways identified during this licence to establish if new or existing therapies are available to target these pathways in disease.

In the longer term, our research aims to identify novel approaches to diagnose and prevent (or reduce) cardiovascular disease. The identification of pathways causative for cardiovascular disease (described above) and their interactions with other conditions such as diabetes or pregnancy preeclampsia has the potential to identify patients at risk and/or inform efforts in how we manage patients. Work in this license may also help us identify people which may benefit from particular lifestyle changes. For instance, genes or pathways identified in this license may be associated with exercise intolerance and could therefore help us tailor lifestyle advice to these patients. This project will help advance our knowledge of cardiovascular disease by identifying novel pathways causative for the disease and therefore inform efforts for novel therapeutics development. We will publish and communicate our findings to the wider scientific community by publishing in open access journals and participating and presenting at National and International meetings

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

We will publish and communicate our findings to the wider scientific community by publishing in open access journals and participating and presenting at National and International meetings. Where appropriate, we will also collaborate with pharmaceutical companies to inform them of the novel pathways identified during this licence to establish if new or existing therapies are available to target these pathways in disease.

Species and numbers of animals expected to be used

- Mice: 28300 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 be using mice because of the ease of creating genetically modified models, that involve either knocking out our gene of interest, over-expressing it or editing it to express a mutant version of it.

Many of our protocols involve the creation of models of cardiovascular disease that involve dietary or surgical manipulation. These experiments can only be performed in an adult/mature animal and hence, we will not be able to use animals less sentient to achieve our experimental outcomes.

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

Genetically altered mice will be bred which is not expected to have any adverse effects. Some of our strains will have their genes inactivated to prevent any harms but will then need to be given a substance, typically Tamoxifen, to turn the genes on before we perform any further procedures.

In some cases, we will irradiate mice that are wild type, using a gamma source of irradiation. This will delete the bone marrow and then they will be given bone marrow from another strain of mouse to replace the depleted bone marrow. This process is similar to bone marrow transfusion in humans which is used to treat blood and immune systems diseases which affect the bone marrow.

Blood samples will be taken at regular intervals to show us the routine haematological and biochemical parameters as well as lipid profiles but no adverse effects are expected from this.

We will use imaging methods such as MRI and ECG throughout this project to allow us to monitor cardiac function as you would do in human patients. Blood pressure will also be monitored using an inflatable cuff on the tail. On occasions we will record blood pressure by surgically inserting a small telemetry device into the hind flank of the animal as this will give us the opportunity to take readings without the need to restrain the animals.

Animals will be given access to a running wheel in their home cage to evaluate the effect of exercise on cardiac function. This is voluntary running which the mice are happy to do.

When we have new mouse models, we will expose them to a small magnetic field to assess their body composition. This does not cause any harm to the animal.

These new models will also be maintained until they are considered ageing, typically up to 18 months of age.

The most refined way to induce clogging of the arteries (atherosclerosis) is to feed the animals a modified chow that is high in either fat or sugar. We will do this for periods of up to a year.

Some animals will undergo surgery to remove their spleen to exacerbate atherosclerosis as this causes more advanced plaque formation.

Animals will undergo one of three procedures to incur injury to the heart and surrounding blood vessels. The structure of the heart is damaged by a procedure called myocardial infarction where parts of heart muscle have reduced supply of nutrients and oxygen and hence get destroyed. Damage to blood flow is created by either Aortic banding, where a band is applied to the blood vessels, or a procedure know as Coronary artery ligation where the coronary artery is tied off. Animals that are subjected to these procedures can be kept alive between 7 days and 8 weeks depending on the scientific questions being asked.

Mice will be given substances to either modulate vascular health and disease or substances to label cells. These substances are not expected to cause any harms. We will

use the most refined route and if necessary, we will implant a small delivery device to deliver drugs over a longer period rather than give more injections.

We will be studying the effects of pregnancy on cardiac function (of both mother and pups). These females will undergo two pregnancies before being humanely killed.

At the end of the procedure all animals will be humanely killed.

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

The use of tamoxifen to switch genes on/off can cause a transient weight loss of up to 15% during the administration period which is typically 5 days.

Irradiation followed by the administration of bone marrow cells will cause a transient weight loss of up to 20% between days 7 and 10 post irradiation. Animals are expected to return to normal within 14 - 21 days.

Surgical implantation of osmotic minipump (for substance delivery) or telemetric devices (to monitor blood pressure, heart rate, ECG, etc. may result in transient pain and can also lead to wound dehiscence. All animals who undergo this procedure will be given pain relief, as would be done in a human subject.

Ageing an animal up to 24 months can lead to stresses on multiple systems and frailty.
Removal of the spleen can lead to transient pain and wound dehiscence.
Surgical interventions involving tying off blood vessels or putting bands around them are all classified as severe and associated with increased degree of pain (1 -2 weeks), reduced mobility and risk of spontaneous death.

Administration of certain substances to elevate lipid levels (e.g. cholesterol and/or triglycerides in the blood stream), such as LPS is associated with inflammation and may result in transient pain/discomfort, transient weight loss up to 15% and temporarily reduced appetite for the animal.

Administration of viral vectors as a carrier for therapeutic treatments may result in mild to moderate pain and increase in body temperature for a day or two, but this should resolve quickly. This response is similar to what the human experiences when given a vaccine.

Expected severity categories and the 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	Mice
Severe	10%

Moderate	25%
Mild	40%
Sub-threshold	25%

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

- Killed

A retrospective assessment of these predicted harms will be due by 16 May 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

Cardiovascular disease is a highly complex disease that involves a number of different cell types and processes including blood flow (shear stress), inflammation, and even different systems (vasculature, nervous system and the heart). Although there have been recent advances in computer modelling and in vitro cell-based systems, and access to patient samples is possible, these methods are still unable to fully model the complex environment of cardiovascular disease. Therefore the use of animals is unavoidable in order to fully model and answer important questions about cardiovascular disease, and there are no alternatives to using a whole mammal for studying heart disease.

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

Our laboratory has a long history of establishing and using cell based assays to test the role of genes implicated in cardiovascular disease, as well as the role of potential therapeutics for cardiovascular disease. We routinely perform in vitro experiments, e.g. transfecting cell lines with our gene of interest or knocking down with siRNA. We have also pioneered the use of in vitro haemodynamics models in order to model blood flow. These approaches have been useful in determining cellular consequences and probing mechanisms of action and exploring therapeutic potential.

Within our establishment we can access existing genomic data sets to enable us to refine our hypothesis before moving into in vivo work.

We also have access to blood and tissue samples from patients with cardiovascular disease and to umbilical cords, to isolate endothelial cells, from patients who have suffered pre-eclampsia

Why were they not suitable?

In vitro cell-based assays and staining of human tissue cannot fully address the effect of how our genes of interest regulate disease initiation and progression, or even regression following treatment with therapeutics.

A retrospective assessment of replacement will be due by 16 May 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

Using statistical analysis incorporating typical variations from our own earlier experimentation, we have calculated the minimum numbers of animals to be used whilst ensuring that the results are statistically significant. Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of 10-12 to achieve the quality of results we need. We have also referred to our annual return of procedures data to estimate the number of animals that we will need to use for breeding.

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

The majority of animals in this Project License will be used for breeding and their numbers will be managed closely to ensure that we minimise the numbers required for our phenotyping studies. During our weekly lab meetings, we review animal use and estimated upcoming needs very closely. Working as a team means that we ensure maximum use of all available tissue from each animal for different applications. For instance, if one lab member uses the lungs to isolate endothelial cells, another may use the aorta for immunohistochemistry and another may use the heart for their studies.

We will routinely use online tools such as the NC3Rs Experimental Design Assistant to help us design our experiments and keep animal numbers to the minimum.

We routinely use power calculations to guide our experimental design. For the majority of experiments, a significance level of 5% with 80% power will be used for statistical significance. Where possible, experiments will have a factorial design and good laboratory practice will be employed such as randomisation of treatments and blinded assessment of results. As an example: a standard study is a randomised block design consisting of 2 groups of animals subjected to hindlimb ischaemia: wildtype and genetically modified. Multiple comparisons are made using one-way ANOVA. Ischaemic hindlimb blood flow recovery is measured using a non-invasive Doppler imager with estimated variability of 15% SD. To detect a 30% difference at significance level of 0.01 and the power at 80% then 12 animals per group would be required.

A further consideration for reducing animal numbers (as an example, specifically for the hindlimb ischaemia studies which involve tying off the main artery supplying blood to the leg on one side) includes the induction of ischaemia on one side while leaving the other side as control (unoperated side) . This approach minimises variability and as a result the number of animals by using the same animal as its own control. Additionally, the use of non-invasive Doppler imaging for measuring blood flow recovery allows us to use the same animal at different time points.

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

Breeding colonies will be managed in line with the best practice guidelines. Particular attention will be paid to genetic stability and good breeding performance. Data from breeding animals are readily available from the in-house database and will be used to make decisions on future breeding animals and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

As already exemplified in the previous answer, we review animal use and estimated upcoming needs very closely. Working as a team means that we ensure maximum use of all available tissue from each animal for different applications. For instance, if one lab member uses the lungs to isolate endothelial cells, another may use the aorta for immunohistochemistry and another may use the heart for their studies. Pilot studies are an integral feature of all our research and any new intervention, when tried for the first time, will always be performed on a smaller subset of animals.

A retrospective assessment of reduction will be due by 16 May 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 animals that have altered cardiovascular function. These genetic models will be subjected to different cardiovascular health conditions - like clogging of arteries (atherosclerosis), heart attack (myocardial infarction), surgical procedures to create diseases such as thickening of the heart muscle (aortic banding), restricted blood flow (vascular injury and/or ligation).

We will use two surgical models that mimic the major causes of human heart failure; aortic banding and myocardial infarction. These are considered to be the gold standard in the field of cardiovascular research and findings are translational in nature. We will only use these models when we have strong indications from our in vitro studies on cells that the candidate gene/treatment will have an effect. For myocardial infarction, there is a proportion of initial mortality (similar to the clinical scenario in humans). The mortality can be reduced by inducing smaller infarcts, but animals in this situation do not develop significant contractile dysfunction and do not display the characteristics of left ventricular remodelling that are of clinical relevance. Specifically for myocardial infarction, typically up to 10% of animals may die of cardiac rupture at 3-7 days. This is highly dependent on strain and sex and we therefore use C57BL/6 and predominately females to reduce the incidence of death. Death from cardiac rupture is immediate, since the pericardium is removed during surgery leading to very rapid blood loss into the thorax. Typically animals are kept for acute, short term (up to 7 days) cell studies or longer term studies (4-8 weeks) to allow for gradual LV remodelling. During this time, animals are generally free of adverse effects, show normal levels of activity, and are housed in social groups. Occasionally, animals will suddenly develop deep abdominal breathing, which is the first sign of acute decompensated heart failure – suffering is minimised by using this as our humane end-point. It is not possible to predict the affected animals and the first sign of heart failure is often death. However, the majority of animals are killed humanely at the scientific endpoint without experiencing any adverse effects.

For aortic banding, surgery is via a trans-sternal thoracotomy, which is more refined than the alternative intercostal approach by avoiding lung deflation. Perisurgical mortality is typically less than 10% with such deaths usually immediate and under general anaesthesia. Adverse effects related to development of heart failure over time are minimised where possible by using echocardiography to identify mice for study before symptomatic heart failure. Drug delivery via slow release devices is a refinement over repeated drug administration via oral gavage/injection, thus allowing a more stable release profile and predictable pharmacodynamics and reducing fluctuations in drug impact on the physiology of the animal compared to bolus administration.

In each scenario, maximal attention will be paid to ensure the pain, suffering or distress to the animal are kept to the minimum possible. The disease models in this licence are established in our laboratory and we have published experience conducting them. We have refined protocols in collaboration with other groups within our establishment with whom we collaborate and who themselves use these techniques. For instance, it is now

standard practice that recovery surgery is performed earlier in the day to allow sufficiently frequent monitoring within normal working hours. Analgesia, heat support, access to water-softened chow, subcutaneous fluids and oxygen (as required) and application of liquid tears to the eyes are all routine practice. When surgical models are going to be used for the first time, research will be carried out before hand into best practice and to establish possible husbandry and welfare issues. Where practical possible a member of the lab will visit a laboratory where this technique is well established to gain first-hand knowledge of the techniques and also to enable consultation with veterinary surgeons and animal welfare officers as to appropriate post-surgical care and procedures.

For most surgical procedures on this licence, sham surgery will not routinely be carried out unless deemed necessary e.g. in the case of induced heart attack, sham surgeries are necessary to control for the confounding effects of the surgery on the disease process e.g. increased inflammatory response due to removal of the pericardium. We will try to use naive animals as controls or data from historic controls, but it may be necessary to give the control animals an innocuous substance such as saline so that the animals experience the same physical intervention as the experimental animal

Our surgical models of cardiovascular conditions are, as in humans, highly invasive (e.g. vascular ligation, induction of a heart attack and the application of a band to the aorta). These animals will have heightened levels of monitoring and provision of analgesia. For each experiment, the scientific end point will be the earliest time point at which we are likely to see a statistical difference between different groups, so that there is no undue suffering for the animals. However, experiments will be terminated earlier than planned if the humane end point is achieved.

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

Non-mammalian species can't be used as their cardiovascular systems are very different to that of mammals, and there are no relevant disease models in these species that can be of any research benefit to us. We also can't use embryos as some of our interventions involve surgical and/or dietary manipulation and these can't be done in the embryo. Nor can we use terminally anaesthetized animals as our surgical/dietary manipulation require several days/weeks for disease to initiate and progress.

Zebrafish have the remarkable ability to regenerate their hearts. However, they are not the optimal model for us to use as the primary focus of our research is mammalian cardiovascular systems. All the reagents (like siRNAs and customized antibodies) that we have been using extensively for decades only target mammalian genes and proteins.

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

Our refinements for this project have come from several collaborations between others performing similar surgeries within the establishment. Particularly, all instances of cardiac surgery are performed as early as possible in the day to allow for a full recovery and monitoring prior to the end of the facility day light hours. Our surgical models that mimic human heart failure (myocardial infarction and aortic banding) are defined as gold standard within cardiac research. There is a significant difference between strain and gender therefore we use a common inbred mouse strain (C57BL/6) and predominantly

female for the myocardial infarction procedure. The duration of these experiments can be between 7 days and 8 weeks thus keeping any suffering to a minimum. For our aortic banding model, we enter the chest cavity via an incision at the sternum (trans-sternal thoracotomy) rather than the intercostal route (between the ribs) as this avoids the lungs being deflated.

Mice undergoing surgical procedures, as in humans are given pain relief during and post general anaesthesia until they are fully recovered. We monitor these animals three times per day during the week and twice daily at weekends to try and identify any animal that may start to display signs of heart failure. Animals that show any signs of deep abdominal breathing are humanly killed as this is a very reliable sign of heart failure.

We use non-invasive imaging methods such as MRI, Electrocardiogram (ECG), Echocardiogram (ECHO) to make sure we can identify animals that may be at risk from heart failure.

When monitoring blood pressure, we use a non-invasive tail cuff, as opposed to invasive methods that require a general anaesthesia and the insertion of probes into a body cavity. The tail cuff is a smaller version of the arm cuff used in humans.

Substances that need to be administered are given by the most refined common route such as subcutaneous, intraperitoneal, and intravenous. If we need to give a drug over a longer period, we may implant a small delivery device under general anaesthesia to reduce the number of injections that the animal needs to receive.

Animals that are fed high fat or sugar diets, which are often softer in consistency, will be given wooden chew sticks to reduce the risk of the animals' teeth becoming overgrown. Animals on high fat diet are also undergo an increased frequency of cage cleaning to keep the level of fat in the cage to a minimum.

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

We will use the LASA guidance for the administration of substances and for advice on aseptic techniques during surgery.

The NC3R's will be consulted where we are looking for best practice in minor procedures and ensuring we follow best practice in terms of the welfare of the animals.

We will follow the ARRIVE and PPREPARE guidelines in ensuring our experiments are carried out in a manner that will allow reproducibility.

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

We will refer to the NC3Rs website www.nc3rs.org.uk frequently to stay informed. We also have termly animal welfare meetings, which are attended by the 3Rs information officer and they provide an update at each of these meetings. It is mandatory for all of our team to attend these meetings.

A retrospective assessment of refinement will be due by 16 May 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

10. Investigating immune dysregulation and inflammation 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

Acute liver injury, chronic liver injury, liver inflammation, immune dysregulation, immunotherapy

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

We aim to understand the underlying causes of immune dysregulation and inflammation in acute and chronic liver injury, and how these contribute to impaired antimicrobial host defence. This will enable us to identify and evaluate new targets for developing immune-modulatory therapies in liver disease.

A retrospective assessment of these aims will be due by 13 June 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

Liver disease is a global healthcare burden affecting over 840 million people worldwide and has a rising incidence. Irrespective of the underlying cause, persistent damage to the liver (e.g., alcohol, fat deposition) leads to inflammation, progressive formation of scar tissue (fibrosis), loss of liver function, and may ultimately result into scarring (cirrhosis). Treatment options are currently limited so there is a huge clinical need to develop new therapies.

The immune system plays a central role in the progression of liver diseases which are associated with impaired host antimicrobial responses; the latter further contribute to high risk of infections and increased mortality. Our immunological understanding of liver disorders is far from complete, and despite considerable pharmaceutical investments most clinical trials have shown modest impact on the disease trajectory.

We anticipate that the findings from this project will help us a) improve our knowledge of liver disease progression and its related immune dysregulation, and b) identify new targets for developing immune- directed therapeutic strategies that may ultimately enhance patient outcomes. Our findings may also be applicable to other important immune-mediated, liver and non-liver, inflammatory pathologies such as inflammatory bowel disease, infection, sepsis, and cancer.

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

This project will advance our knowledge on the underlying cellular and molecular mechanisms of liver inflammation and immune dyregulation. The information generated will be published in peer-reviewed scientific journals, presented at national and international conferences, and disseminated to the wider community through public engagement activities. It will further provide identification and testing of novel immune-modulatory therapies, thus can guide future studies and clinical trials. In addition, materials, protocols, and methods generated in this project will be shared with other researchers who are interested in studying liver disease pathogensis.

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

In the short-term, our team members and collaborators will benefit directly from the data generated as this will help to inform future experiments and research directions. Following data publication, other members of the wider scientific community (e.g., immunologists, clinician hepatologists) will benefit from the data being made freely available through open

access. In the medium-term, depending on the findings and whether new immune targets have been identified, pharmaceutical companies (globally and in the UK) might have interest in developing new candidate drugs targeting specific immune pathways or repurposing available drugs for treatment of liver diseases. In the long-term, depending on the results, specific drugs might be proposed and investigated in small- or large-scale clinical trials in patients with liver diseases.

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

The findings of this project will be disseminated to the wider scientific and clinical audience through peer-reviewed publications in open-access journals and data presentations at national and international conferences (e.g., immunology, hepatology). Through attendance at networking events we will have the opportunity to engage researchers from a wide variety of disciplines and disseminate our research in a less formal manner. We will aim to maximise the outputs of this project by seeking collaborations with other basic and clinician scientists to develop the work in the fastest and most effective way possible.

We will make all efforts to keep the public informed. We will engage with social (e.g., Twitter, Linkedin) media to disseminate our discoveries to a wider audience. The results from this research project will be published in peer-reviewed journals in open-access formats, will be made freely available and will be deposited in online databases. Aside from the above, we will engage with our institution’s public engagement and communications team to issue press releases and lay articles to ensure our work is shared with the public. We will directly interact with members of the public through events organised at various universities that are open for everyone.

Species and numbers of animals expected to be used

- Mice: 12000

Predicted harms

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

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

Mice have been chosen as the experimental animal for the studies detailed in this license as they have an immune system of comparable complexity to humans and represent the lowest vertebrate able to display liver fundamental features (cellular, subcellular, histological) and phenotypes with physiological similarity and genetic similarity. Their short intergenerational time, ease of breeding or availability of established genetic variants make them ideal models in which to perform genetic manipulations. Mice are an excellent model for understanding the immune responses to liver injury. Various models have been developed to study acute (e.g., paracetamol overdose) or chronic (e.g., dietary manipulations) liver injury and assess effective treatments and immunotherapies. A major advantage is that they provide a tightly controlled system (genetically, environmentally, temporally) that reliably yields well- defined stages of disease pathogenesis (e.g.,

hepatocellular/tissue damage, liver fibrosis) and enables relatively minor changes in biological pathways to be identified.

This project license will produce, maintain, and provide different genetically altered animals. Adult animals will be used in most cases. Under the breeding protocols, mice of different age will be used (life stages: embryo, neonate, juvenile, adult, pregnant) to allow generation of mouse strains with genetic alterations of particular interest (e.g., cell surface receptors) that modify the immune system. This will enable us to: a) facilitate in vivo immune cell tracking, b) study the role of specific cell types in liver inflammation and immune dysregulation, and c) understand whether these immune cell receptors promote or prevent specific liver disease pathologies. In addition, under the experimental protocols, both wild type and genetically altered mice will be used (life stages: adult, juvenile - up to 15 months old, before mice are considered aged). This will permit investigation of immune dysregulation across different liver injury types, to identify underlying mechanisms and evaluate potential immunotherapies in clinically relevant disease models.

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

This project will employ models of either acute or chronic liver injury to model different aspects of human liver disease. Wild type or genetically altered (GA) mice will be used and different experimental procedures/steps may be undertaken including induction of liver injury, blood sampling, immune system modulation, gut microbiome manipulation, infectious challenge, and imaging. Most of the suggested procedures in this license confer mild or moderate severity to animals, and the unexpected adverse effects are considered highly unlikely (expected incidence: <1%). We anticipate the cumulative effect of procedures (e.g., multiple injections of different substances, blood sampling, liver disease related adverse symptoms) to be moderate.

In some experiments, animals will be administered with toxin (paracetamol or carbon tetrachloride) to induce acute liver injury - typical administration route/frequency: single intraperitoneal injection; typical study duration: 1 week. In other cases, animals will be administered with toxin (carbon tetrachloride) to induce chronic liver injury resulting in fibrosis - typical administration route/frequency: twice weekly intraperitoneal injections; typical study duration: 6-8 weeks. In some animals, the induction of non- alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), conditions where fat accumulation causes liver inflammation and fibrosis, will be achieved through various dietary manipulations (e.g., western, high-fat diet) - typical study duration: 6-12 weeks.

Blood (usually taken via tail vein bleed) and urine samples may be collected from few conscious animals - typical frequency: once weekly. In specific experiments, animals may be administered with substances to modify gene expression (e.g., tamoxifen in GA mice with inducible genes - typical frequency: once, up to five times), modulate the immune system (e.g., monoclonal antibodies, small- molecule inhibitors targeting cell types of interest - typical frequency: once weekly) or treat liver inflammation (e.g., drugs - typical frequency: once weekly). Substances will be given via standard routes: orally (e.g., in the diet or water, oral gavage) or by injection (e.g., intraperitoneal, intravenous). On some occasions, manipulation of gut microbiome may be achieved through administration of different substances (e.g., antibiotics), live bacteria, bacterial cultures/solutions, or dietary supplements (e.g., prebiotics). These may be given orally by gavage, on one or several occasions, or integrated in food pellets or water - typical frequency: once weekly. In

specific experiments, animals may undergo an infectious challenge (e.g., bacteria or fungi) - typical administration route/frequency: single intravenous injection; typical study duration: up to 24 hours. All animals will be closely monitored and humanely killed when they reach defined endpoints. Our protocols and clinical monitoring criteria have been designed to keep animal suffering to a minimum.

Some animals may undergo non-invasive imaging (e.g., FMT, MRI, ultrasound) under recovery anaesthesia to immobilise them while images are being recorded - typical duration: 1 hour; typical frequency: once per lifetime. On few occasions, animals may undergo intravital microscopy imaging under terminal anaesthesia - typical duration: 1-2 hours. Animals will be humanely killed at the end of these experiments. In some instances, various tissues from animals may be collected for laboratory- based studies (ex vivo analyses) following terminal anaesthesia.

No individual animal will experience all the experimental procedures described above. All animals will be closely monitored and humanely killed when they reach defined endpoints. Few typical animal experience examples are provided below:

- Paracetamol induced acute liver injury model:

day 1: paracetamol administration (1x intraperitoneal injection)
day 2: substance administration e.g., therapeutic intervention (1x intraperitoneal injection)
day 4: bacterial challenge (1x intravenous injection)
day 5: terminal anaesthesia induction (1x intraperitoneal injection) - humane killing and tissue collection OR day 5: terminal anaesthesia induction (1x intraperitoneal injection) - maintenance of anaesthesia (e.g., agent inhalation) - intravital microscopy imaging under anaesthesia - humane killing and tissue collection

- Carbon tetrachloride induced liver fibrosis model:

week 1- 6: carbon tetrachloride administration (2x intraperitoneal injections weekly)
week 5: substance administration e.g., immune system modulation (2x intraperitoneal injections)
week 6: terminal anaesthesia induction (1x intraperitoneal injection) - humane killing and tissue collection OR week 6: infectious challenge (1x intravenous injection); after 24 hours terminal anaesthesia induction (1x intraperitoneal injection) - humane killing and tissue collection

- Diet-induced fatty liver disease model:

week 1-12: animal feeding on a western diet - animal weighing and monitoring - blood sampling
week 12: terminal anaesthesia induction (1x intraperitoneal injection) - humane killing and tissue collection

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

Some animals will be held and given an injection of a substance (e.g., saline, drug) into the abdominal area, some animals will be held and receive a solution/drug through oral gavage before being returned to normal feeding, and some animals may be given both. In other experiments, animals will be held and given an intravenous injection of a substance (e.g., saline, drug). These procedures may cause initial mild pain but animals do not show immediate adverse effects or sickness signs afterwards.

Possible adverse effects include a poor response to injections, however this rarely occurs. We expect the cumulative effect of these procedures to be moderate (e.g., injections, blood sampling).

Some mice may display signs of increased erection of hair, hunched posture, reduced mobility and experience loss of appetite and/or weight loss that can be associated with the administration of substances or the development of liver injury and inflammation. In some experiments, aimed to model the human liver disease more closely, mice will be administered high doses of toxin (e.g., paracetamol, carbon tetrachloride) with possibility of experiencing significant ill-effects (e.g., substantial liver tissue damage, high-grade inflammation, reduced level of consciousness, hypothermia) typically within the first 6-12 hours, and up to 24 hours; spontaneous death due to unpredictable severe effects of administered toxins such as paracetamol is rare (expected incidence: <2%). Animals fed with western or high-fat diets (typical duration: 6-12 weeks) may develop obesity and greasy coat which may lead to over-grooming or scatching, and as a result possible skin inflammation, ulceration. In some experiments, animals will also undergo an infectious challenge (e.g., bacteria) which can cause predictable systemic and/or organ disturbances with enhanced pro-inflammatory responses. Mice may therefore experience symptoms of generalised illness for brief periods (e.g., reduced activity and appetite, increased erection of hair, hunched posture, tachycardia, fever) typically within the first 6-12 hours. Thus, there is possibility for spontaneous unintended mortality due to response (to infection) variability in conjunction with toxin-induced liver injury (which is characterised by e.g., high-grade inflammation and profound immune dysfunction). Animals will be closely monitored for the duration of the protocols. All animals used in this license will be humanely killed by approved methods at the end of the experimental procedures or at any time if it becomes necessary to alleviate animal suffering.

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

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

- sub-threshold: 0%
- mild: 52%
- moderate: 45%
- severe: 3%

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

- Killed

A retrospective assessment of these predicted harms will be due by 13 June 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

Host's immune response to liver injury and inflammation is a complex, multi-staged process in which many different cell types (e.g., liver-recruited blood immune cells, liver-resident immune cells) may interact with each other. Subsequent immune dysfunction also involves the systemic vascular system and various extra-hepatic organs (e.g., spleen, lungs) which are often sites of secondary infections. This complexity can not be recapitulated using only cellular systems in the lab, thus necessitating experimental studies in a whole animal model. Moreover, human studies are limited by an inability to sample liver tissue at frequent time-points or to perform experimental manipulations for ethical reasons.

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

We have extensive experience in performing human immunological studies; where possible, our experiments will utilise human tissue samples (e.g., blood, liver biopsies) and in vitro migration models. We will also perform mouse in vitro experiments to investigate the cellular interactions mentioned in this project. However, some studies requiring systems level analysis (e.g., bacterial infections) are only possible in animal models. We are actively exploring the possibilities of novel methodologies and developing alternative in vitro assays (e.g., liver-on-a-chip technologies, liver organoid systems) to assess immune cell-specific functional aspects in an effort to replace our animal models.

Why were they not suitable?

Whilst much of our experiments will be performed using human samples, it's impossible to entirely replicate the immunological alterations or cell-to-cell interactions in the liver microenvironment. Some of our studies will require systems level analysis: a) cell trafficking from blood to liver during tissue injury, b) host response to infections, including bacterial dissemination/load to extra-hepatic organs (e.g., spleen, lungs), c) checkpoint inhibitor-related immune adverse events; such drugs may cause various side effects including liver or gut inflammation. Therefore, experimental animal work can't be totally replaced so that we can view the entire systemic effect of other organs on our in vivo models. Use of animals is necessary to confirm findings and ensure in vivo relevance.

A retrospective assessment of replacement will be due by 13 June 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

The numbers of mice for this project license have been estimated based on the:

anticipated numbers of experiments for the different liver injury protocols/models (e.g., evaluate the role of genes of interest, trial of new therapeutic agents),

numbers of different study groups required per experiment (e.g., study various time-points during liver injury, comparison of control versus therapeutic agent arms),

numbers of mice required per study group (derived from power calculations that were performed based on biological data variability observed in our previous animal studies); each experiment will consist of 10-16 mice to enable appropriate controls and experimental tests.

use of both male and female mice in every experiment; this will allow us to assess sex-dependent differences in immune-mediated regulation of liver inflammation.

Taking all the above parameters into consideration, the estimated “maximum number of mice” will be 4,400 for genetically altered animal breeding purposes and 7,600 for the experimental protocols, for the 5-year timeframe of this project license.

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

We carefully plan our experiments to minimize the numbers of animals we need to use while getting the most scientific information possible. Given our team's prior experience and considering data from experiments we conducted in the past, we ensure how these can be improved and animal numbers are reduced. We use tools like the NC3Rs Experimental Design Assistant to plan and organize our experiments and take advice from biostatisticians and other experts in our institute (e.g., consult with our institute’s Statistical Advice Service).

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 the reasonable steps to reduce the numbers of animals used in our project license. In addition to non-animal alternatives described in this application, we will minimize the number of animals using consistent experimental techniques and readouts, thus reducing variability. We will carefully plan our experimental timings to minimise the use of control animals. We will obtain as much information as possible per one animal; for instance, we will collect tissues and measure immunological and liver function in the same mice to reduce the animal numbers, thus preventing the need for repetition of procedures detailed in the licence.

The experimental mice will also have shared use of tissues within our group and with other researchers. Moreover, trials of new therapeutic agents will be commenced using small pilot groups using very few animals, to avoid unexpected welfare harms, toxicity or any side effects being experienced by large numbers of mice. Finally, many of the animals used will be from genetically altered mouse colonies which we will breed at expert facilities in ways that minimise over breeding, waste and ensuring that every single animal can be used in experiments.

A retrospective assessment of reduction will be due by 13 June 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

Which animal models and methods 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 have an immune system of comparable complexity to humans, and represent the lowest vertebrate able to display liver phenotypes with physiological similarity and genetic similarity. Their short intergenerational time, ease of breeding and availability of established genetic variants make them ideal animal models in which to perform genetic manipulations. Mice are an excellent model for understanding the immune response to liver injury. Various murine models have been developed to study liver injury and assess effective treatments. A major advantage is that they provide a tightly controlled system (genetically, environmentally, and temporally) that reliably yields well-defined stages of liver disease pathogenesis (e.g., hepatocellular damage, liver fibrosis) and enable relatively small changes in biological pathways to be identified. The liver disease models described in this project are well-established, published in peer-reviewed journals, and our team has extensive prior experience using them. We have carefully chosen well-characterised and reproducible models of acute (e.g., paracetamol overdose) or chronic

liver injury (e.g., diet-induced fatty liver disease). These models recapitulate many features of human liver disease immunobiology and represent the most valid mouse models in which immune-mediated tissue inflammation is known to contribute to the pathophysiology.

These models reflect the various liver disease phenotypes we observe in patient populations, in whom the hypotheses for this project have been derived.

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

We will use animals in the simplest and most humane models possible. We need to work with mammals, specifically mice, because they have anatomy, physiology, immune system and liver function that are similar to humans. Mice represent the lowest vertebrate able to display liver phenotypes with physiological similarity and genetic similarity with humans. We will work with juvenile/adult animals as our in vivo studies require a fully developed immune and gastrointestinal system, as we aim to study immune dysregulation in liver diseases and need to work with mice that are fully grown from a practical perspective.

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

We will use the most well-characterised and refined liver injury models to deliver as much scientific information and value as possible with minimum amount of animal suffering. For example, we have refined the paracetamol liver injury model; dosing titration studies have enabled us to distinguish between experiments that require induction of acute liver failure (substantial hepatocellular damage, high-grade inflammation) and those that can achieve our experimental objectives via induction of acute liver injury (mild hepatocellular damage, low-grade inflammation), thus reducing the number of animals that experience severe adverse effects. Similarly, we have optimized and use low doses of toxin (carbon tetrachloride) in the chronic liver fibrosis model. The liver injury protocols, and experimental procedures chosen will always considered to be the least severe ones that would produce satisfactory results and allow us to achieve our scientific aims.

We will always make efforts to make animals, undergoing a procedure or not, more comfortable. For instance, by providing them with a rich environment to live in and handling them in ways that don't cause stress. During experimental procedures, we will closely monitor animals for any sign of discomfort and distress using multiple criteria (e.g., weight, signs of pain, piloerection). Animals receiving injections will be closely monitored by trained staff; if any problems arise, we shall consult the named animal care and welfare officer (NACWO) or named veterinary surgeon (NVS) to offer pain- relief, treatment or proceed with humane killing. Refinement procedures will include:

Use of standardised monitoring forms established in conjunction with NVS and NACWO. We will perform frequent animal monitoring, especially during treatments; animal monitoring frequency will increase if any adverse effects occur; depending on the severity of harm, animals will be treated or humanely killed.

Extensive characterisation of any substances prior to injection in animals; having clear limits on animal injections will minimise the likelihood of adverse effects and suffering.

Strict adherence to humane endpoints, with pre-emptive humane killing of animals approaching endpoints.

Topical or general anaesthesia, pain-relief medication where possible, and adherence to best practice guidelines when performing procedures.

Use of short-acting recovery anaesthesia for non-invasive imaging.

Trials of new therapeutic agents will be commenced using small scale pilot group studies to avoid unexpected toxicity or side effects being experienced by large numbers of mice.

Throughout the lifetime of this project license we will continue exploring new ways to refine our experimental protocols and make animals more comfortable.

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

We will regularly review any published information relevant to this project license (e.g., guidance published covering the liver injury models proposed in this application) to identify the best and more refined ways to perform our work. We will follow the best practice guidance in performing animal experiments which will be designed, reported, and published following the PREPARE and ARRIVE guidelines. We will also make use of standardised monitoring regimen and proforma (using published information) and/or in conjunction with NVS/NACWO.

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

We will regularly review any published information relevant to this license (e.g., guidance published covering the various liver injury models) to identify the best, and more refined, ways to perform our experimental work. We will constantly review our protocols and experimental design to reduce, refine and replace the use of animals. We will keep informed about 3Rs best practice and any advances by reading scientific publications and discussing with colleagues within our own institution and elsewhere. This will include other scientists but also those directly involved in care and welfare of animals (our institution's animal facilities). In addition, frequent meetings and training events are held within our institution regarding the advances in the 3Rs and their implementation. All team members working under this licence will attend these meetings while frequent advice regarding animal well-being will be sought from the vets and the facilities' animal care staff.

A retrospective assessment of refinement will be due by 13 June 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

11. The Role of Pattern Recognition Receptors in Immunity and Homeostasis 2024

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Immunity, pattern recognition receptor, infection, autoimmunity, disease

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description 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 molecules found on our immune cells, called pathogen recognition receptors (PRRs), enable our immune system to combat disease causing organisms and how these PRRs are involved in the development of autoimmune diseases.

Our aim is to also understand how PRR-mediated immune responses to microbes influence the development of autoimmunity and vice versa.

A retrospective assessment of these aims will be due by 18 June 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 direct benefit of our research is the furthering of scientific knowledge of the underlying mechanisms of our immune system. These advances allow us to understand the disease process, and lay the foundation for new and better treatments for humans in the future. Our work has already led to substantial advances, as evidenced by our publications in scientific journals and the impact these have had on other scientists, measured by the number of times our work is cited by them. Our discoveries have also led directly to a greater understanding of what can cause disease in people, such as how alterations in PRR function cause predisposition to infections and autoimmunity, and have also led directly to the development of a novel therapy for a fungal infection.

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

In this research programme, we will generate statistically robust datasets, with appropriate controls, using methodological approaches that are accepted in the field, and that permit publication in leading open access journals. Additional outputs from this project will include data/information dissemination through conference presentations and seminars, cutting-edge new scientific insights that will support grant applications, student postgraduate degrees and outreach activities for the general public.

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

This project involves basic discovery bioscience that underpins human health by determining the mechanisms involved in the pathogenesis of infectious and non-infectious diseases (such as autoimmune disease). Thus the results from our studies will be of immediate and long-term benefit to academia, industry, clinical medicine, and the general public, both nationally and internationally. The training of post-doctoral researchers (PDRAs) and PhD students working on this project will impact the economic competitiveness of the UK through provision of highly skilled individuals, by training in state- of-the-art preclinical experimentation. We will also be producing monoclonal antibodies to novel antigens, by generating hybridoma cell lines from immunized animals. These hybridoma cell lines are immortal and represent the generation of an unlimited amount of key new scientific reagents, which may have clinical relevance (ie: for

diagnostics). These reagents will be made available to other scientists globally. The existence of these reagents will be shared through presentations at conferences and our publications.

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

We will share the new scientific information we generate via our publications in open access journals. Also, the data will be shared through direct discussions with other investigators and through presentations at national and international conferences. The data will also be shared through regular seminar invitations at other institutions, within the UK and around the world. Significant discoveries will be disseminated to the public, through our University Press offices and our engagement activities. All new antibodies we generate will be made freely available to the scientific community under appropriate agreements once their description has been published.

We will also ensure rapid translation of our findings to the clinic and industry. Exploitation of research discoveries will be supported by the University, which has established mechanisms to promote translational research and IP commercialisation.

Species and numbers of animals expected to be used

- Mice: 42800
- Rats: 20

Predicted harms

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

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

We study mice because the immune system, tissue organisation and development of all mammals are similar, allowing mice to be a model for humans and other animals. In some experiments we also need to study ageing mice, as these animals, like humans, start to develop conditions that can provide important insights into alterations of immune function. Mice are also used because scientists have created many genetically altered mouse lines that allow us to dissect in fine detail what happens during immune responses to infection and development of autoimmunity. Genetically altered mice, and many of the tools designed to work with mice, allow us to define in precise detail how particular cells and molecules of the immune system work together to develop immune responses. By manipulating these cells and molecules, we can identify the immune components that co-ordinate protective and non- protective responses. This provides key information that allows us to understand infectious and non- infectious diseases of humans, and enable the development of new therapies in the future. Where alternative methods are not possible, antibodies to human antigens will be generated in mice, whereas antibodies to mouse molecules will need be generated in rats.

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

We will use a variety of murine models to explore the role of PRRs in the immune system, so as to understand how they function in response to pathogens and/or alterations in the steady state (homeostasis) that can lead to autoimmunity. Indeed, many PRRs recognise both self (endogenous) and microbial (exogenous) ligands, and play diverse, and interrelated, roles in immunity, homeostasis and autoimmunity (PMID: 29581532). Our primary focus is on a family of PPRs (called the C-type lectins or CLRs), although we also study the roles of other PRRs (including other lectins and the Toll- like receptors, for example). Many of the receptors currently under study (Dectin-1, Dectin-2, MICL and MelLec for example) are at later stages of our discovery pipeline although we also have several novel receptors (e.g. CLR1 and CLR2) that we have just discovered and are still in the early stages of investigation.

In terms of how PRRs respond to infection, much of our work focuses on the role of these PRR in antifungal immunity, but we also explore their roles in immunity to other pathogens, such as mycobacteria, for example. Our discovery science normally begins in vitro with an observation about the ability of a PRR to recognise a specific pathogen. These in vitro experiments will require the use of primary cells isolated from WT and GA mice (protocol 1 and protocol 7), but we use transfected cell lines whenever possible. To understand the physiological functions of the receptor we then characterise its role in the immune response to the pathogen of interest during infection in mice, often testing multiple models [systemic (i.v., i.p) or particular sites of infection, such as the lung (i.t., i.n.), gut (gavage), onto or under the skin (s.c., i.d.), for example] (protocol 3). Where possible we utilize killed microbes (listed under protocol 2 as immune modulators) to elucidate specific functions of the PRRs in vivo, to minimize impacts on animal welfare. However, infections with live microbes are essential to be able to fully comprehend host-pathogen interactions and how PRRs contribute to these responses.

Consequently, we also need to use animal models (protocol 3) to understand how alterations in microbial virulence, adaptation, drug resistance and co-infections (for example the impact of virus infection on pulmonary fungal disease (PMID: 34788127) impact PRR-mediated immunity and subsequent host resistance to infection and disease. We may also need to induce autoimmune responses during infection, to enable us to dissect how the contribution of PRRs to antimicrobial immunity is altered during the development of autoimmune disease, and vice versa. Much of this work requires detailed comparison of various aspects of the immune response over time in WT, GA and in chimeric mice. For these in vivo experiments, we may need to alter the diet (to determine the impact of metabolism on immune responses), understand how immunity influences metabolism (tolerance to administration of insulin, glucose or pyruvate), use older animals (to determine the impact of ageing on immunity), fast the animals (to analyse blood chemistry), adoptively transfer in specific cells (to validate or explore specific functions like the development of immune memory - the adaptive response), collect blood samples (to monitor responses), administer substances to track cells or that enable us to control the expression of specific genes, or perform non-invasive imaging (to monitor the progression of an infection, for example). The cumulative experience of mice will typically be exposure to 2 procedures, with less than fifteen percent undergoing 4 or more of these procedures.

To better mimic human susceptibility, or to gain more detailed mechanistic understanding, we may need to use immune modulators, antibiotics, suppressants or stimulants (protocol 2 and used in protocol 3, 5 and 6) such as antibiotics, cytokine depleting antibodies, steroids or lipopolysaccharide (LPS), for example), and/or we may need to cross GA lines

to generate animals deficient in two or more immune components (protocol 1 and 7). The majority of the immune modulators will have little or mild adverse effects on the mice although a few, e.g. steroids and LPS, can have adverse effects that are dose dependent. Much of this work requires detailed comparison of various aspects of the immune response over time in WT, GA and in chimeric mice. For these in vivo experiments, we may need to alter the diet (to determine the impact of metabolism on immune responses),understand how immunity influences metabolism (tolerance to administration of insulin, glucose or pyruvate), use older animals (to determine the impact of ageing on immunity), fast the animals (to analyse blood chemistry), adoptively transfer in specific cells (to validate or explore specific functions like the development of immune memory - the adaptive response), collect blood samples (to monitor responses), administer substances to track cells or that enable us to control the expression of specific genes, or perform non- invasive imaging (to monitor the progression of an infection, for example). The cumulative experience of mice will typically be exposure to 3 procedures.

Understanding the role of PRRs in autoimmunity is much more difficult than infection, as the self (endogenous) ligands are often unknown and the functions and roles of the receptor can often only be established in vivo. Discoveries in this area often require experimentation using several mouse models of immune dysregulation and we make use of two main models arthritis (protocol 5) and asthma (protocol 6). Most of this work requires detailed comparison of various aspects of the immune response over time in WT, GA (protocol 1 and 7) and may need chimeric mice. In some cases, such as arthritis (protocol 5) for example, we may need to keep animals beyond the peak of disease to obtain data on the late stage or resolution of disease. For all these models we often need to use immune modulators, suppressants or stimulants to induce or regulate immune dysregulation (including protocol 2), such as proteins, microbial components, and other antigens, antibodies to collagen, steroids or lipopolysaccharide (LPS), for example. For these in vivo experiments, we may need to alter the diet (to determine the impact of metabolism on immune responses), understand how immunity influences metabolism (tolerance to administration of insulin, glucose or pyruvate), use older animals (to determine the impact of ageing on immunity), fast the animals (to analyse blood chemistry), adoptively transfer in specific cells (to validate or explore specific functions like the development of immune memory - the adaptive response), collect blood samples (to monitor responses), administer substances to track cells or that enable us to control the expression of specific genes, or perform non- invasive imaging (to monitor the progression of an infection, for example). The cumulative experience of mice will typically be exposure to 3 procedures.

As many of the molecules we are studying are new to science, we may need to generate monoclonal antibodies to these receptors or other immune or microbial components (protocol 4) to enable detailed characterisation of their expression and location. Monoclonal antibodies to human antigens will be generated in mice, whereas monoclonal antibodies to mouse molecules will need to be generated in rats. In some cases, these antibodies can be used diagnostically or used functionally, to inhibit or activate molecules in vitro or in vivo (and are considered immune modulators in this context) for example.

All experiments will end with animals (mice and rats) being killed humanely, sometimes under terminal anaesthesia.

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

The majority of animals under this PPL will experience no adverse effects or only mild adverse effects.

Although immune modulation can trigger systemic inflammation that can cause weight loss, piloerection, hunching and reduced movement, in most cases these effects should be mild or transient.

The generation of T and B-cell responses may induce transient inflammatory responses that cause some signs of moderate effects e.g. appear hunched with ruffled fur and gradual weight loss.

Our models of infection are classified as severe, since inoculation of mice with microbial pathogens will lead to infection. Only in a minority of experiments, will we need to allow the animals to get very ill, so we can functionally understand the contribution of the PRR to the development of disease, especially at the later time points that can more closely resemble human disease. Animals could experience weight loss, head tilting, ataxia, ruffled fur, reduced movement and very rarely breathing problems. In some cases we need to let the mice loose up to 30% of their body weight, as this enables us to gain important insights into mechanisms occurring at later stages of infection that cannot be gained earlier. However, we minimise suffering by implementing clinical monitoring with defined humane end points.

Additional measures taken to ameliorate the suffering of sick animals include increased monitoring and welfare assessment. Death is not an end point in these experiments.

Mice undergoing models of arthritis will show transient moderate effects from the induction immunomodulators that can cause weight loss, piloerection, hunching and reduced movement, in most cases these effects should be mild or transient. The mice will then present with swollen joints. The animals will develop arthritis (redness and swelling) in multiple joints that can reduce movement and persist for prolonged periods.

Our models of allergy are generally very well tolerated, though in rare cases can cause weight loss, head tilting and ataxia. Furthermore, in very rare instances (especially during choric models) mice may show breathing problems, i.e. laboured breathing, increased/decreased respiration. However, in all cases these will rarely reach moderate severity.

In all these experiments, animals will be carefully monitored and humanely killed before they exceed stated severity limits. Guidance will be sought from the NVS and NACWO should any animal display signs of abnormal behaviour or any unexpected change in physical appearance.

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

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

We expect 44% of the mice to experience mild severity, 16% to experience moderate severity and 5% to experience severe severity. 35% are sub-threshold. We expect >90% of the rats to experience mild severity.

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

- Killed

A retrospective assessment of these predicted harms will be due by 18 June 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

The mammalian immune system is highly complex, relying on the co-ordinated actions of multiple different cell types and molecules that collectively provide protection. As such, only in vivo experiments can provide insights on the mechanisms that underpin immunity. Unfortunately, in vitro systems are unable to reflect the cellular and molecular complexity of the immune system. Therefore, the use of mammals is essential for gaining a better understanding of the mechanisms underlying immunity that could ultimately be utilised for human benefit.

Mice will be used in these studies because their immune system closely resembles the human immune system therefore giving a better chance for translating potential therapies. Additionally, a wide array of wild type and genetically altered strains of mice are available that will allow us to better decipher the role of immune cells and molecules in immunity.

The mouse is the worldwide standard laboratory animal model and its immune system has been the most intensely investigated and for which there are the most information and reagents available, including genetically altered animals.

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

- other models such as insects, worms or fish.

- use of human or laboratory grown cells (including organ on a chip methodology). In fact, we do make use of non-animal models (such as human cells or laboratory grown cells) whenever possible and use in vitro assays to provide initial data that informs and complements our in vivo experiments.

- in silico modelling

- For the generation of monoclonal antibodies, we will use non-animal alternatives, such as phage display libraries from established providers, where possible. However, our previous experience has shown that such approaches to identify usable reagents can be limited, especially for closely related molecules such as the C-type lectins we are studying. We will always use antibodies from other sources (researchers or commercial companies) if they are already available. However, many of these reagents are not optimal in our hands (e.g: polyclonal and not sufficiently specific or they do not work in the assays we need them for such as Western blotting). Most of the the monoclonal antibodies we generate are to new molecules for which existing reagents do not exist.

Why were they not suitable?

- Rodent immune systems are much more complex than that seen in insects, worms, and fish. Hence, studying rudimentary immune systems will not lead to a significant increase in our understanding of the regulation of the immune system in health and disease.

- Experiments with cultured cells in tissue culture dishes cannot recreate the cell-cell and cell- matrix interactions that play such an important role in the regulation and functioning of immunity in vivo.

- For the production of monoclonal antibodies, our previous experience has shown that utilising non-animal alternatives to generate usable reagents can be limited, especially for closely related molecules such as the C-type lectins we are studying. In some cases existing reagents are not suitable for the scientific approaches we require (eg: use in Western Blotting), require the generation of new reagents.

A retrospective assessment of replacement will be due by 18 June 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

These numbers represent the theoretical maximum, and in practice will likely be less. Estimates are based on the historical numbers used to my previous PPLs, the numbers of

animals required for breeding (especially lines bred as heterozygotes), the required group sizes for experiments, experience on how many experiments are required to perform the studies over the course of this license, and on the number of researchers that will be working under this PPL.

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

We make use of online tools (such as http://www.biomath.info/power/prt.htm) to determine appropriate group sizes that will enable us to use the correct numbers of animals to ensure the most robust scientific results. We use highly inbred strains of mice of the same sex and age, in each experiment, to reduce variability in our results and cease breeding of mouse strains that we do not use. We reduce numbers by making use of multiple readouts from each experimental animal. We reduce variability by using aged matched animals, co-housing wild type and GA animals to normalise the microbiota. Where possible we use both males and female animals in experiments, but ensure we account for any sex-dependent outcomes in our results. In our experience, these approaches have historically helped to reduce our animal usage by at least 25%.

For the production of monoclonal antibodies, we normally utilise 3 animals per antigen to produce the specific reagent we need. All three animals are immunized, boosted and then tested prior to tissue harvest for monoclonal antibody production. We choose 3 animals so as to ensure we have sufficient cells for monoclonal production at the end of the protocol. In some cases, we need to try two or even three rounds of monoclonal production, and in different mouse backgrounds (eg: GA mice), to be able to generate the monoclonal antibody with the specific activities we need. We anticipate over 20 new antigens over the course of this project, for which we may need different monoclonal antibodies with different specificities (eg: against the intracellular or extracellular domain of the C-type lectin, for example). To reduce the need for further animals, we always fully test our new monoclonal antibodies for desired specificity and range of activities. Due to the structural similarity between most antigens we use, we are unable to challenge animals with multiple antigens either sequentially or simultaneously.

We cannot use large animals as we are not harvesting blood for antibody production, rather we need the immune tissues of immunized rodents to generate hybridomas for monoclonal antibody production. Hybridomas are immortal cell lines and will provide an indefinite supply of monoclonal antibody, thus we do not require repeated use of animals to produce antibodies from serum.

A significant proportion of our animal use is related to breeding programmes for genetically altered lines. We follow the advice of our animal facility staff to optimise breeding, and ensure that animal usage is carefully monitored so we do not overbreed.

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

We reduce the number of animals we use for experiments, by maximising the number of scientific measurements we make for each animal. For new experimental approaches we make use of small scale pilot studies to determine variation, which informs on numbers of animals required for experimentation. Experiments are then performed on a minimum of

two separate occasions to ensure reproducibility, following which data pooled from experiments are statistically analysed to reveal less pronounced effects without increasing overall animal use. We also carefully monitor the breeding of all our mouse lines, to minimise the production of excess animals.

A retrospective assessment of reduction will be due by 18 June 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

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

We will use mouse models to determine the mechanisms underlying immunity, and how these processes influence anti-microbial immunity and/or autoimmunity. Mice represent the most appropriate species for in vivo study of immunity, because of the extensive knowledge of their physiology as it relates to humans, the genetic and biological tools available and the ability to be easily bred and handled.

We will use rodents to generate monoclonal antibodies to antigens, for which there are no existing reagents or existing reagents that are not suitable for our experimental purposes. The generation of hybridomas expressing these monoclonal antibodies are immortal, and thus represent an indefinite supply of reagent. Where possible we will make use of non-animal alternatives, such as phage display libraries, and only use animals for production of monoclonal antibodies when such approaches are unsuitable (such as our previous experience where we were unable to identify a specific monoclonal from phage display libraries after multiple attempts). We plan to produce monoclonal antibodies to a novel family (around 20 receptors) of closely related human and mouse C-type lectins that we have identified. The antibodies will be used to determine the expression and functions of the receptors understudy, and will be of interest, and made available, to the broader scientific community and industry (we currently have monoclonal antibodies to over 5 C-type lectins available through commercial suppliers), thus having long term impact. We will use established approaches, involving either crude/purified antigen, nucleic acids (e.g RNA) or intact or dead cells in an appropriate adjuvant, as required, via an appropriate route.

While the majority of the mice entering these models will experience mild to moderate suffering, we employ stringent measures (e.g.: managed doses, clinical monitoring) to

reduce animals suffering as much as possible. We are constantly refining our models, based on previous experience, to ensure robust experimental results whilst minimising pain, suffering or distress.

For all our studies we will stay abreast of latest approaches to refine animal experimentation, through the NCR3R's website and discussion with our NACWOs and NVS's

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

The use of other animal species will not provide the key insights we need to understand human disease. We are studying immune processes that can take from hours to weeks to develop. Only experiments in adult animals would provide meaningful results, as the immune system is not fully developed in very young animals.

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

All procedures will be performed by trained and skilled personal licence holders, who will handle animals with care. Animals will be monitored for adverse effects using clinical scoring sheets previously developed in conjunction with the NVS and NACWO. These score sheets have proven to allow for objective measurements of clinical signs associated with adverse effects to determine when humane endpoints have been reached. Where we start a new line of experimentations, we first make use of a small number of animals (a pilot study) to learn about the impact of these new approaches on the animals and how best to minimise any suffering. In line with the establishment's policy, we will adopt the latest techniques in animal handling (e.g. tunnel handling) to significantly reduce the stress associated with procedures. Furthermore, where possible, the least invasive methods for dosing and sampling will be applied. Anaesthesia and analgesia protocols will be refined, through discussion with the NVS, to ensure they are the most appropriate for each type of procedure. Our group continuously attempts to refine our procedures, such as, for example, altering our experimental approaches to reduce harm, regularly reassessing our clinical monitoring sheets to refine experimental endpoints and minimise welfare costs, or modifying our breeding strategies to minimise impact and occurrence of adverse phenotypes (for example backcrossing of animals to reduce inbreeding, housing breeding colonies in house as opposed to buying animals from commercial breeding establishments for animal lines that are sensitive to transport stress).

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

We follow LASA guidelines, and consult the recommended https://www.nc3rs.org.uk/3rs-resources on a regular basis, including watching videos of best practice techniques. For specific models, we read papers from other groups doing similar experiments, as well as consulting directly with other researchers to discuss the most refined procedures. We are constantly scanning the scientific literature and talking to other scientists to make sure we are using the best possible approaches.

We will continue to use the NC3Rs and other online tools. This will include use, for example, of the Experimental Design Assistant to ensure we design experiments that will

allow us to achieve statistical significance whilst minimising the number of animals we need to use. We will also utilise the 3Rs self- assessment tool to ensure our researchers actively discuss 3Rs and how we can continually refine and improve our practice.

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

We hold frequent discussions with NACWOs, NIO and NTCO within our animal facility along with a team of dedicated veterinarians seeking to continually improve animal welfare and refine animal use. These are further facilitated by regular open meetings with NAWCOs, NIO, NTCO and all personal license holders participate to ensure we are following current best practices. We will also continually keep track of the latest advances in improving animal welfare via discussions with colleagues, attending national/international conferences and consulting published literature. We will continue to work closely with our local NC3Rs representative, and consult the NC3R website to ensure we stay informed about the advances in the 3Rs. The MRC CMM, in which we are based, also employs a dedicated senior manager to oversee all animal research, who will help ensure best practice and latest 3R's advances are incorporated into ongoing experiments.

A retrospective assessment of refinement will be due by 18 June 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

12. Neurobehavioural Mechanisms Underlying Mood, Anxiety and Stress-Related Mental Health Disorders

Project duration

5 years 0 months

Project purpose

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

Key words

Behavioural neuroscience, Mental health disorders, Memory, Brain, Individual differences

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description 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 neurobiological, neurochemical and molecular bases of psychological processes that vary across the population and can go awry in mental health disorders, particularly those associated with uncontrollable or inescapable stress.

A retrospective assessment of these aims will be due by 16 May 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

Mental health disorders are a major health burden, both in the UK and worldwide. The World Health Organisation estimates that worldwide, 301 million people live with anxiety, 280 million people live with depression, and 326 million people experience post-traumatic stress disorder. The cost of mental health disorders to the UK in terms of social, economic and health factors is extremely high (estimated in March 2022 as £117.9 billion per year).

Although treatments for mental health disorders exist, currently available treatments need substantial improvement, as they do not work for all patients. Treatment development for mental health disorders is a long-term goal for my laboratory and that of our collaborators, and we believe that treatment development will be facilitated by a better understanding of the psychological, neural and neurochemical mechanisms that underlie psychological processes that can become maladaptive or dysfunctional in mental health disorders. By understanding individual biological and environmental risk factors, it might be possible to identify those at greatest risk of developing mental health disorders, to give them access to preventative treatments (or at least access to treatments sooner). By characterising the individual psychological profiles associated with mental health disorders, it might also be possible to identify patient 'subtypes' would benefit more from specific treatments, allowing better treatment targeting to the individual.

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

The primary output from this project will be the generation of new information about how psychological, neurobiological and neurochemical processes that are relevant to mental health disorders go awry.

These will be communicated mostly through publication in scientific journals and presentation at scientific conferences, but we are also committed to communicating our findings more widely with the general public. Ultimately, the intention is that this research will lead to new treatments being developed for mood, anxiety and stress-related mental health disorders.

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

We expect our research to benefit the scientific community in the short to medium term, and in the longer term to benefit people living with mental health disorders through the development of new treatments.

In the short term, we expect to gain insight into potential drug treatments for the mental health disorders of interest (mood, anxiety and stress-related disorders). This information can be used by other researchers to test these drug treatments in small-scale and, in the longer term, large-scale studies in humans. We are also involved in the institution's animal tissue sharing initiative, so when animals are killed at the end of the experiments, we share this information with other researchers within our institution so that they can use tissues that we do not need to analyse for our research. This helps to reduce the institution's overall animal use.

We also expect, in the medium term, that our research will lead to the development of new behavioural treatments. The animal research allows these to be developed with an understanding of how these behavioural therapies can lead to changes in the brain, so that when they are translated to humans, we have a better understanding of how they work. We actively collaborate with researchers studying human participants and patient groups to ensure that our behavioural tasks work well and measure the same processes in humans and non-human animals.

In the longer term, understanding the risk factors that predict whether individuals are more vulnerable to developing mental health disorders may allow early interventions to be made for these people.
Furthermore, it may allow for the development of personalised treatments based upon the underlying differences in psychological and neurobiological functioning.

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

We are involved in collaborations with pharmaceutical companies, which will support the impact of our research, particularly with respect to treatment development.

I have a strong track record of publication and of speaking at scientific meetings, which will publicise the work. We are also fully committed to open science, and since 2017 we have made all supporting data for our publications freely available on the university repository, for other researchers to use as citable datasets. We also publish reliable null results, so that we contribute to a balanced interpretation of the scientific literature.

Species and numbers of animals expected to be used

- Rats: 1845

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 mostly be using adult rats. For some experiments, in which we are studying the impact of early life stress on behaviour in adulthood, we will use pregnant rats and their offspring so that we can control early life experience. As we are interested in adult behaviour, the majority of our behavioural tests will be conducted in adult animals. However, as one of our objectives is to predict which individuals are at risk of developing

mental health disorders, for some experiments we will take measurements (e.g. MRI images of the brain) in juvenile animals so that we can relate any differences in their adult behaviour to their previous brain development.

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

A maximum of 1845 rats will be used for this project. As the mental health disorders of interest (depression, anxiety, post-traumatic stress disorder) involve individuals being exposed to uncontrollable stressors, the majority of the animals used in our experiments (with the exception of a small number of controls, required to be naive for comparison) will undergo exposure to inescapable electric footshocks. Although trauma-inducing experiences in humans (e.g. road traffic accidents, sexual assault) are clearly different to the use of footshock in rats, these events share the fact that they are uncontrollable stressors, and the use of footshock in rats allows us to control the extent of the stress to a greater degree than using more naturalistic stressors (e.g. exposure to an aggressive male).

The typical response of rats to an electric footshock is to jump, to freeze (stay completely still other than the movements needed for breathing) for a few seconds, and then to resume normal behaviour. The number and magnitude of shocks that we use produces no physical lasting harm to the animal, but is sufficient for an aversive memory to form that subsequently affects behaviour on other sensitive behavioural tasks. We use the minimum number of shocks to achieve our scientific objectives. We typically use up to three footshocks to model adverse experiences that are within the ‘normal range’ of stressors experienced in adult life. We anticipate that ~73% of rats will experience this level of shock (and the dams required for early life stress manipulations, ~3% of the total animals, will not experience any shocks). However, to model the traumatic events that lead to post-traumatic stress disorder (PTSD), more shocks are required to model the sensitisation of the stress system found in this disorder. We have previously worked to refine this procedure, reducing the number and strength of shocks required, and allowing animals to be housed in groups rather than experiencing social isolation stress. We have published these findings in animal welfare journals. However, ~24% of animals will need to experience more than three shocks, with the maximum number of footshocks that will be delivered in a single session being 15. The maximum number of shocks in an animal's lifetime would be 20, which would allow us to assess the effect of prior trauma on subsequent learning about adverse events. While these aversive procedures produce changes in behaviour that can be measured on sensitive tests of cognition and affect, they are not sufficient to lead to changes in home cage behaviour or normal social interaction with cage mates.

Approximately 50% of rats will also undergo manipulations of early life stress, through the administration of drugs to the pregnant mother (e.g. to model stress during pregnancy) and/or periods of separation from the mother during early life (to model early life adversity). Early life stress is a prominent risk factor for developing mental health disorders in humans, and these experiments allow us to model this in rats. We perform these manipulations to test the impact of early life stress on the behaviour of the offspring as adults. Footshocks will only be delivered to adult animals; neonatal and juvenile animals are only used for early life manipulations in our experiments, with the same animals being tested as adults to model the interaction of early life stressors with uncontrollable stressful events experienced in adulthood.

Nearly half (~49%) of the animals will undergo experiments that only involve behavioural manipulations, or behavioural manipulations with injections (e.g. intraperitoneal or subcutaneous) or oral administration (e.g. in palatable food) of drugs to modulate the function of the nervous system so that we can assess the impact on behaviour. Often rats will receive different doses of drugs or different types of drugs, so that we can compare the effects of the drugs on the individual rats' behavioural performance. The drugs administered will depend on the specific experiment, but may include drugs proposed to act as novel antidepressants, drugs that alter motivational state or attention, or that affect learning.

Just over a quarter (~27%) of rats will undergo surgical procedures in addition to behavioural testing. These procedures are necessary to allow us to directly record the activity of the brain, to deliver drugs directly to specific brain regions, or to experimentally control the activity of specific types of brain cell (either increasing or decreasing activity).

Approximately 22% of rats will undergo brain imaging in addition to behavioural testing. The rats experience brain imaging under anaesthesia, and may undergo more than one scan, including some scans as juveniles. These scans allow us to measure brain changes between rats showing different types of behaviour, and the repeated scans allow us to track this across development and to determine whether differences in brain volume reported in human patients with the disorder of interest are a cause or consequence of stressful life experiences.

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

Most of the procedures will produce (at most) transient distress and no lasting harm. Extended or excessive distress would lead us to stop the experiment and either humanely kill the animal, or seek treatment under veterinary advice.

Some of the drugs that we give to the rats may cause short-term side effects (e.g. hyperactivity) but most of the time the subtle effects of these drugs can only be seen on our sensitive behavioural measures.

Early life stress produces extended (i.e. throughout the lifespan) effects on behaviour, but these are typically only measurable on our sensitive behavioural tasks and do not involve long-term changes in home cage behaviour. Modelling of trauma through exposure to massed inescapable footshocks (up to 15 in a single session) similarly produces changes in behaviour on sensitive behavioural tasks, but does not lead to changes in home cage behaviour or social interaction with cage mates.

Expected severity categories and the 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: 76%

Severe: 24%

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

- Killed

A retrospective assessment of these predicted harms will be due by 16 May 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

We need to use animals to achieve our research aims and objectives because:

We need to manipulate early life experiences and determine individual predispositions that make individuals more likely to develop mental health disorders

We need to understand the fundamental biological differences in the brains between individuals with and without mental health disorders

We aim to develop new treatments for individuals with mental health disorders.Our aims and objectives require long-term study of behaving individuals, where we have experimental control over their life experiences and can study both brain and behaviour.

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

Humans, cell culture, and computational models.

Why were they not suitable?

Human behavioural studies are useful for some of our objectives, and we aim to design experiments where the behaviours can be directly compared between humans and animals (i.e. 'translational' and 'backtranslational' research). However, we cannot fully understand the biological differences between those with and without mental health disorders in humans, as human studies are limited to correlational approaches (e.g. brain imaging) when we need to understand causal mechanisms.

Furthermore, it would not be ethical (or possible) to conduct studies in humans where individuals are deliberately put at risk of developing mental health disorders (e.g. early life stress studies).

Cell culture studies can be helpful for understanding fundamental biology, but they do not link readily to the behavioural measures in which we are most interested.

Computational models do not yet fully capture the behaviours that are relevant to our research questions, because the models are not sufficiently advanced. They do not capture individual variation or early life environmental differences. However, we are beginning to use computational models to give us better insight into the behaviour of each animal, and to increase the amount of data we produce per animal in each experiment.

A retrospective assessment of replacement will be due by 16 May 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

The numbers of animals have been estimated by referring to the numbers used on my previous Project Licence, the numbers required for our ongoing funded research, and projections based on future funding at similar levels. These experiments have been planned following power analyses, either using effect sizes from previously published literature or from pilot data in the lab.

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

I am well-trained in experimental design and statistics, and where additional expertise is necessary I have support from statisticians within my institution. Our statistical analyses are determined during the experimental design phase, and where possible we design experiments to allow us to measure within- subjects effects in addition to between-subject effects, to give us greater statistical power.

We refer to the PREPARE guidelines in designing experiments, with the design of each experiment checked by multiple researchers (including the Project Licence, PPL, Holder) and the Named Animal Care and Welfare Officer (NACWO), to confirm that the animal facility is capable of supporting the experiment. We report our experiments in accordance with the ARRIVE 2.0 guidelines, and make our data openly available to other researchers by publishing datasets on our institutional repository at the time of manuscript publication.

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

Where appropriate, we conduct pilot experiments to determine effect sizes, allowing us to perform power analyses where effect sizes are not available in the previous literature. We also design our experiments to collect the maximum number of behavioural measures from individual animals, including control measures to allow us to interpret our experimental effects in context. Most of our behavioural measures are collected automatically on computer, allowing us to interrogate rich behavioural datasets for the animals.

We often collect brains from the animals at the end of our experiments, to allow for further *post mortem* analyses that can be related to the individual behavioural data. We also participate in a tissue-sharing initiative run by our institution, to allow other researchers to access any unused tissues from our animals at the end of the experiments.

A retrospective assessment of reduction will be due by 16 May 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

Which animal models and methods 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 rats because they are the least sentient species that can model the behaviours, affect and cognitions relevant to the mental health disorders of interest. We do not attempt to model a mental health disorder 'in full' in the rats, but rather model specific psychological processes that have relevance to mental health disorders. This approach, called 'dimensional psychiatry', aims to understand the key processes that go awry in mental health disorders. These dysfunctional processes can be shared across different mental health disorders in humans, with different combinations of dysfunctional processes producing different mental health disorders. We take this approach to understanding mental health disorders, by studying these psychological processes in rats, often using behavioural tasks that can be used in both humans and rats (i.e. the tasks are both 'translational' and 'backtranslational').

As we are interested in mental health disorders where stressful environments impact the progression of the disorder, we have both scientific and ethical reasons to maintain high

standards of animal welfare and to reduce extraneous sources of stress that would add noise to our experiments. We are also committed, in our task development, to refining existing behavioural procedures and have previously published this work.

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

Our research relies mostly on sophisticated behavioural testing to assess the impact of uncontrollable stress on psychological processes such as responsivity to reward, cognitive flexibility and inhibitory learning. The behavioural tasks that we use are readily acquired by rats, but would be extremely challenging for mice. The majority of our experiments test complex behaviour in adult animals, and we only use neonatal and juvenile rats where this is necessary (e.g. in early life stress procedures). We could not conduct our behavioural experiments at a more immature life stage, because neonatal animals would not be capable of performing these tasks, which often take weeks to train.

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

The bulk of our experimental work relies upon sophisticated behavioural analysis, which typically involves daily contact (at least 5 days per week) with the animals. Any illness or pain would compromise the ongoing behaviour of the rats, giving us a sensitive measure of any welfare issues, often before any clinical observations could detect changes. We also seek to refine our behavioural procedures, and have published this previously in animal welfare journals (Lab Animal).

Where animals have undergone surgery, they are provided with pre-operative, peri-operative and post- operative pain relief. Post-operative pain relief is administered for several days (typically 3) following surgery, and if signs of pain are noted while rats are undergoing increased post-operative monitoring, this is continued for up to 5 days. In the very unlikely circumstance that a rat was still showing signs of pain after 5 days, the rat is humanely killed.

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

There are no specific best practice guidelines for the specific behavioural procedures used on this project (many of which have been designed or refined for use in my laboratory). We refer to more general best practice guidelines provided by the NC3Rs, LASA, FELASA and other learned societies (e.g. the LASA/BAP/BNA/ESSWAP Guiding Principles for Behavioural Laboratory Animal Science), including for surgical procedures and other procedures such as blood sampling and administration of substances.

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

I subscribe to the N3CRs newsletter and my institution's 3Rs mailing list. I also actively participate in 3Rs sessions at conferences.

Relevant guidelines (including a link to the Norecopa databases) are made available to all researchers working on this project through an online repository, and are uploaded by the PPL Holder as they become available.

A retrospective assessment of refinement will be due by 16 May 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

13. Evaluate current and new means of rodent control

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

Rodent, pest, control, rodenticide, humaneness

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description 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 protect the health and welfare of man and (non pest) animals by conducting research and testing to make available a range of effective rodent control products. In addition, the project aims to support the development of more humane rodent control solutions.

A retrospective assessment of these aims will be due by 14 May 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

Pest rodents (rats and mice) pose considerable risk to the health and welfare of man and (non pest) animals. Currently there is a drive to manage these pests more sustainably and humanely, while maintaining the efficiency of the solutions we provide. This means that many of the current techniques and products are under review, and some will be restricted and possibly banned in the next 5 years. It is necessary therefore to conduct research in order to preserve essential products and also to develop new chemical or physical solutions that mean effective pest control under all circumstances can still be delivered.

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

Maintenance of a range of effective rodenticide products based on anticoagulants
Information on efficacy of alternative rodenticides to anticoagulants (which pose less risk to non target wildlife) that may lead to a submission for a product registration in the UK and Europe

Information on the most humane rodent snap traps available to the establishment

New, more effective, more humane, physical devices for rat and mouse control, with multi kill capability.

Launch of a new 'twin kill' version of a CO2 trap

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

The anticoagulant products will be available globally to the establishment's businesses as required but will also be available to other PCOs in the UK.

The initial benefit in the lifetime of the licence will be insights for the establishment, but given a timescale of 5-7 years to achieve a UK / EU registration, there will be additional benefits in a reduction in risk to non target animals ranging from non commensal rodents to scavenging animals such as foxes, kites, and predators such as barn owls.
The establishment will benefit from being able to market traps proven to be more humane, but this will also lead to a reduction in the number of animals maimed or killed humanely by ineffective traps.

The establishment will benefit from being able to market these devices, but their use will mean more humane end points for controlling pest rodents than with snap traps or

traditional rodent baits, and customers will benefit from having a more sustainable solution than current techniques.

The CO2 trap, now with two killing chambers over just one, has increased efficacy, but this also means that the response time to the first capture is not as urgent, so allowing technicians flexibility over when to visit the site and service the unit - this means better route planning and therefore a lower carbon footprint for service vehicles.

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

The establishment's work has a global reach, so any new products and services will have a global impact and benefit for both commercial and residential customers of pest control services.

The establishment is involved across expert industry groups and trade bodies, therefore any new innovations will help provoke the industry, customers and stakeholders to respond and move towards a more humane, sustainable future.

In addition, work on key projects will be presented at key industry conferences (e.g. European Vertebrate Pest Management Conference) and used to inform various stakeholder groups (regulators, governments, industry working groups, etc).

Species and numbers of animals expected to be used

- Mice: 700
- 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 species chosen are the same species that the products or techniques being developed as part of this project are designed to be used against in any pest control activity in the field, i.e. rats and mice.

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

When testing rodenticides, animals will be typically offered a choice of a non toxic control bait and a test rodenticide. Usually this is for a period of 4 days, after which the rodenticide is withdrawn. Animals are monitored closely for up to 21 days for any signs of rodenticide poisoning. Over 5 years we expect to use 250 rats and 350 mice in the course of the project.

When testing physical controls devices (e.g. but not limited to snap traps) animals are observed in real time as they encounter and then trigger such devices and then the time to irreversible unconsciousness is recorded to determine if the trap is sufficiently humane.

Over 5 years we expect to use 250 rats and 250 mice in the course of this project.

Finally, mice will be tested against traps that release carbon dioxide as a rapid (and for pest control relatively humane) method of kill. We expect to use 100 mice during the course of the project for testing these devices.

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

For rodenticides the animals will die by internal haemorrhage (anticoagulants), depression of the function of the central nervous system (chloralose) or hypothermia and body weight loss (cholecalciferol). Minor symptoms may persist for a few days at most, animals will be euthanised if any (humane endpoint) major symptoms are observed.

For physical control devices, animals are expected to be struck on the neck or cranium with sufficient force to ensure a rapid kill (<180 seconds to Irreversible Unconsciousness). Any animal struck in such a way that death is unlikely to follow (e.g. struck on the tail, paw, lower body) is euthanised immediately.

For carbon dioxide, experience shows that rapid and humane death (within a minute) is the most likely outcome, although animals exposed to high concentrations of CO2 likely experience some pain due to the acidic effects of the gas on their airways.

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

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

All the products being developed or tested are lethal control products / devices, so death is the expected outcome (whether directly or by humane endpoint).

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

- Killed

A retrospective assessment of these predicted harms will be due by 14 May 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

Rodenticides and rodent control techniques need by their definition to be tested on the target rodent species themselves. The response of the whole animal needs to be studied, and the behavioural component of a rodent to a control measure is a crucial component that cannot be ignored if the final outcome is to be delivered: An understanding of the likely humaneness or efficacy of the rodent control product in a field setting.

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

There are no in vitro techniques that can replace the whole animal studies that are necessary to provide final confirmation of efficacy or humaneness.

Why were they not suitable?

Whilst there are no non-animal alternatives, there are huge opportunities for replacing animals at the earlier stages of product development - for example by using 'blank' formulations without the rodenticide present in order to minimise the number of animals challenged with the final rodenticide formulation. In addition, when it comes to registration dossiers, every opportunity is taken to 'read across' data from other products or to attempt to write 'waivers' in places where we believe data is not required. In addition, a great deal of non-procedural behavioural work can be conducted before final confirmation of efficacy, and mechanical testing options are available to screen for effective physical devices before a minimal number of live animals need to be used towards the end of the development process.

A retrospective assessment of replacement will be due by 14 May 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

For rodenticides, a review of currently known regulatory requirements and product development objectives has given a realistic estimate for previous licences.

For physical methods, a review of the establishment's Annual Operating Plan and range of potential product developments.

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

of animals being used in this project?

Regulatory tests of rodenticides are fairly rigid, and with a threshold of 80% mortality there is no opportunity to deviate from the usual 10 animals (5 of each sex) used in this protocol per product test.

For most tests of physical methods, numbers are too small to use statistics - animals are only used for validation at the end of a range of non procedural testing that has gone into product development as a strictly pass / fail test. Where official tests of humaneness are required, then up to 12 animals are required for testing (with a pass rate of 10/12 within humaneness norms).

See https://www.umweltbundesamt.de/en/topics/chemicals/biocides/non-chemical-alternatives-for-rodent- control for a more detailed testing protocol.

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 stages of product development, there are many options for Replacement, e.g. rodent carcasses can be used as part of testing lethal traps, and blank baits can be used to develop formulations to a satisfactory level before the rodenticide is added. This makes up the majority of the work carried out in this establishment, as opposed to Procedures which are the last resort.

Pilot studies of rodenticides (in order to perfect the formulation) may be required before full regulatory studies, and in this case the number of animals per treatment would be reduced to 6, rather than 10 - rodenticide testing by its very nature does not need to detect subtle effects by statistical means.

For physical control devices a lot of mechanical tests will precede any live testing, including tests on 'simulated rodents' and on rodent carcasses, and only when these are all satisfactory will live animals be used. In addition, for humaneness testing, if 3 animals fail the test then the testing can be aborted at that stage, limiting total animal usage, rather than just proceeding until all 12 rodents have been used.

A retrospective assessment of reduction will be due by 14 May 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

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

The species chosen (adult rats and mice) are the same species that the products or techniques are intended to be used against in any pest control activity in the field. Since for the most part the intended effects are required to be lethal, then it is these effects that must be confirmed during the course of this project.

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

Any such animals would not return results useful to predict the performance of rodent control products and devices. The natural behaviour of the pest species is essential in determining if a product will be effective, and standard tests for registrations of rodenticides require tests against adult rats and mice.

The use of anaesthetics is impractical for laboratory tests of rodenticidal products against free-feeding animals, and models or bodies of dead rodents would be more practical for testing physical traps.

Analgesics may alter an animal's response to any harmful effects of the rodenticide, for example by masking behavioural symptoms of poisoning from observers (and delaying humane end points) or by preventing a 'stop feed' effect normally caused by a certain dose of a rodenticide and thereby altering the palatability and efficacy outcomes of a trial. There could also be an unknown interaction between the mode of action of a rodenticide such as an anticoagulant and an analgesic. All these possible outcomes would render any findings highly dubious, and almost certainly invalid. However, if future studies build on the DEFRA Link desk study (WM0317) to demonstrate that a specific analgesic has potential to be incorporated into rodent bait without the above drawbacks, then this will be reviewed.

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

For rodenticides, establishing the 'palatability' of one bait sample against another is an important part of the Project, so baits are available in easily accessible hoppers so as to minimise any interaction between the bait format and the ease of access to it. This does however require the use of grid flooring over most of the floor in some trials – to allow 'spillage' to be collected and an accurate measure of consumption to be made (Johnson and Prescott, 2015). However, this period of any trial is usually short (4 days) and a solid 'refuge' is provided that is large enough for the rodent being tested to rest and sleep on. Outside of the test period a solid material will be provided over the grid floor for the rodents to use. Current refinement for rodenticide bioassays concentrates on monitoring rodents and euthanasing as soon as agreed end points are reached. These end points are those where the animal is clearly in distress and also highly likely to result in death before the next observation is made.

Monitoring frequency is increased when symptoms are first observed in order to increase the chances of reaching humane endpoints and therefore minimising suffering.

When the death of a pest rodent is the desired end product of the material being tested it is vital to ensure that the findings of any study are not compromised by imposing premature end points. End points have been established for the rodenticides in this project licence application, and these have been adopted in our internal procedures, according to a predetermined scoring chart (see examples supplied), with staff trained to ensure consistency in judgement. These end points are regularly reviewed with our Named Veterinary Surgeon and also through our internal Regulatory Team to ensure consistency with any changes in regulatory guidance. For example, currently, humane endpoints are identified and applied in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes and OECD [Organisation for Economic Co-operation and Development] . 2000 . Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation . (ENV/JM/MONO(2000)7).

The main refinement for lethal traps and control devices is to observe animals closely, and to euthanise any animals immediately it is obvious they will not become unconscious rapidly (for example if caught by a limb or tail or stuck on the wrong part of the body). Until the trap is activated, rodents are allowed to move freely around a pen or arena.

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

The Animals (Scientific Procedures) Act 1986 and subsequent amendments.

For rodenticides (including the CO2 trap), our protocols follow *Technical Notes for Guidance on Product Evaluation. Appendices to Chapter 7 Product Type 14 Efficacy Evaluation of Rodenticidal Biocidal Products* (https://echa.europa.eu/documents/10162/983772/bpd_guid_revised_appendix_chapter_7_pt14_2009 _en.pdf/6c05e11a-7a83-4769-9d1b-257a7458a031#:~:text=Rodenticides%20with%20a%20multi%2Ddose,(3%2D14%20days).

For Snap Traps, as already stated, we follow the methods in the Nochero voluntary code (https://www.umweltbundesamt.de/en/topics/chemicals/biocides/non-chemical-alternatives-for-rodent- control). This code can also be applied to the other lethal control devices that we plan to test.

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

I stay informed through regular updates from NC3Rs website, and learning about advances in general experimental practice through the contacts in other establishments. The work we do is very specialised, but through through our in house regulatory team and my contacts in industry I stay informed about any potential changes in regulatory requirements and testing method development.

Specifically, my membership of the industry groups RRAC (www.rrac.info) for rodenticide developments, and Nochero chemical-alternatives-for-rodent-control) for the development and improvement of trap testing methods.

I also use Research Gate to keep abreast of other developments, such as the latest research into humane euthanasia (Striving for humane deaths for laboratory mice: hypobaric hypoxia provides a potential alternative to carbon dioxide exposure, Clarkson et al 2023).

A retrospective assessment of refinement will be due by 14 May 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

14. Brain networks supporting memory and emotion

Project duration

5 years 0 months

Project purpose

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

Key words

memory, amnesia, anxiety, systems biology, psychiatry

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 required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description 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 different brain regions work together to support and integrate memory and emotion.

A retrospective assessment of these aims will be due by 21 May 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence? Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

Learning and memory are critical cognitive skills that are vital for every day existence and interaction with the world. More than that, our memories are a key component of our personalities, fundamentally making us who we are. Understanding how our brains enable us to learn and remember events, and how this is affected by emotional state, continues to be a crucial goal within neuroscience.

Furthermore, by fully understanding how the brain supports memory and emotional processing in “normal” systems we can far better understand how memory can be disrupted in numerous neurological and psychiatric disorders. Only by better understanding how these systems function in normal situations, and what happens when these systems break down, can we start to fully develop effective targets for treatment.

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

The project will advance our knowledge of the neural systems underlying memory and emotion and the neurochemical processes that support these functions in different brain regions. The data from the projects will be published in peer-reviewed journals and we will share data acquired from the project for others to use and analyse. The information gained from the project will be used to help identify possible targets to improve memory and anxiety in animals and potentially neurological disorders.

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

The neuroscience community will benefit from these outputs in helping to develop and test new models of memory formation and new models of neurological and psychiatric disorders.

The neuroscience community will also benefit from the shared data which can help with the development of new analytical pipelines and help reduce the number of experiments carried out.

In the longer term, patients will benefit as more detailed models of neural function will result in better options for treatments for a wide number of disorders that affect memory and emotion.

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

We will share our data so that it can be accessed and analysed by other groups.

We will publish all data in open access journals so that it can be easily accessed by others.

We will publish null effects as well as significant effects so that experiments are no repeated unnecessarily.

We will publish analytical pipelines and new methodological details to help with the improvement of experimental design and analyses.

Species and numbers of animals expected to be used

- Mice: 1700
- Rats: 1600

Predicted harms

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

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

We are using rats and mice because we have extensive knowledge of the brain anatomy for these species and the brain regions and connectivity for the areas under consideration are very similar across humans and rodents, increasing the translatability. Furthermore, rodents are particularly good at spatial learning and memory and again, this type of learning relies on the same systems and pathways as in humans. Rats and mice tolerate the intracranial recording devices well and the devices have been specifically developed for these species. There are also genetic modifications that have been developed in rats and mice that model the aspects of disease in humans and enable us to target specific cell populations. We will typically be using adults as the neural systems are more matured at this point. We will also look at more juvenile animals in some genetic models to potentially identify critical time windows within which neural populations become particularly vulnerable in the attempt to find the optimal points for neuroprotective interventions.

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

Animals will typically undergo some manipulation to affect neural activation in the brain and we will either look at the effects on behaviour and/or look at the effects on brain activity. This will involve surgeries where we either inject a substance into a specific brain region or implant a cannula into a brain region so that we can later infuse neuromodulators. Alternatively, animals will receive implants of miniscopes/optic fibres or electrodes that can be used to alter or record brain activity. Animals will typically either explore spatial environments that sometimes contain different objects or run up and down arms of a maze for a reward. We will modify brain activity either using systemic injections or infusions via in-dwelling cannulae or by activating cells using fibre optic wires. These experiments will typically last about 4 months.

We will also use genetically modified animals to help identify underlying processes in psychiatric disorders. These animals will undergo behavioural testing and we will also record brain activity. We will also alter brain activity in these animals using neuromodulators to determine how different receptor subtypes contribute to behaviour.

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

Occasionally animals can have a bad response to the anaesthetic and surgery procedures but typically animals recover well from the surgeries. The processes we are trying to examine involve animals' natural explorative behaviour and interaction with the environment. As such, the experiments needs to be designed so that the animals are behaving normally and are well. The infusions are typically short- lasting and only have effects on specific parts of cognition such as memory.

The genetically modified animals are also typically only minimally affected by the modification and behavioural impairments are only observed with quite specific testing - i.e., it does not cause gross impairments to 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 animals will typically be moderate severity given many of the animals will undergo surgery although recovery is expected to be without incident. This will be approximately 70% of the animals. For animals that do not undergo surgery the category is likely to be mild as they will either be a mild genotype or undergoing behaviour that is considered mild, potentially combined with a small number of systemic injections. Less than 5% of animals will be non-recovery, as they will undergo acute recordings/lesions/dye infusion and be perfused before coming round from surgery. This will typically be in cases where we are optimising co-ordinates and/or validating approaches before starting the full experiment. Less than 5% will be severe category when they have an adverse reaction to the surgical procedure.

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

- Killed

A retrospective assessment of these predicted harms will be due by 21 May 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

The goal is to understand how different brain regions work together to support learning, memory, and emotion. These interactions themselves are very complex, and there are many things we still do not understand about how the brain works, which means we needs to work with animal models that have reached a level of development that show similar behaviours to those seen in humans and have similar brain anatomy. It is also important to match brain activity and changes in activity to behaviour which is why we are not able to use alternatives such as cell cultures. While we carry out complementary experiments in humans where possible, we cannot achieve the same level of resolution in targeting/imaging brain regions. Furthermore, there are very few patients that have selective damage to the brain areas under investigation.

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

Humans - both patients and healthy volunteers.

Why were they not suitable?

We considered looking at the same systems in humans but it is not possible to specifically manipulate the brain areas under consideration with current techniques. Data from patient groups can be difficult to interpret because of lack of specificity of pathology and experiential differences.

A retrospective assessment of replacement will be due by 21 May 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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 determined numbers using the previous studies that have used similar approaches, both from our own labs and others. This enables us to estimate how many animals for each type of experiment would be needed to demonstrate an effect.

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

1.By refining behavioural tasks to improve performance/reduce variance in control animals so impairments can become apparent with fewer animals.

2.A number of approaches support a within-subject design, with repeated measures therefore reducing numbers required. e.g. in vivo imaging, electrophysiology, temporary lesions/neuromodulation.

3.Using techniques that enable the examination of multiple regions within the same animal instead of different animals for different brain regions. This includes in vivo recording from multiple brain regions using multichannel electrodes, the use of PET and/or immunohistochemical imaging which are appropriate for whole brain analyses.

4.By using anatomical tracers/antibodies with different wavelengths multiple markers can be assessed in the same animal.

5.The in vivo imaging studies naturally reduce the number of animals needed as extensive information can be acquired from even a single 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 use efficient breeding so that the minimum number of animals are being maintained at other times and co-ordinate studies with other researchers using the same animal lines so that all animals will be used or we can use animals that other groups are not using. The genetically modelled animals are all typically used by other groups in the unit which means there is much more efficiency in breeding the use of animals. Animals not exhibiting the desired genetic modifications are used as controls or in experiments designed for wild-type animals.

We also regularly use a resource at our establishment (Blackboard) where excess stock can be identified to be used by other groups as way of minimising numbers of unused animals.

For experiments where we are trying out new neuromodulators we will run small pilot studies first to identify optimum dose and optimum group size. We will also pilot new co-ordinates for implants on a small number of animals initially to ensure they are as accurate as possible for the main experiment, thus reducing the number of animals that need to be excluded due to incorrect placements.

We will also use machine learning for the analysis of data which results in us being able to obtain far more detailed experimental outcomes from each animal and much more nuanced data which reduces the number of animals needed.

A retrospective assessment of reduction will be due by 21 May 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

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

We will use rats and mice for the project. For the research to produce findings which can be applicable to the human brain it is necessary to use a model with the same anatomy, i.e., the same brain structures and connectivity, which is why rodents are required. Furthermore, these animals are able to perform a number of tasks that have similarities to those that are used with patients, in particular spatial memory tasks and object/location tasks, which increases the relevance of the findings.

We will use neural implants to monitor brain activity and neuromodulators to alter neural activity. The aim is to look at activity while animals are actively engaging in their environment and learning information. Gross changes in behaviour and sensory-motor impairments would make it very difficult to interpret results so the implants and neuromodulators are all designed to have the minimum impact on overall well-being.

All rats with implants are housed in larger double-decker cages with two floors and more head room. When rats need to be singly housed after surgery (e.g. due to implants) they are housed in the double- decker cages where they can see/smell rats in adjacent cages. These animals are also given supervised socialisation periods in an open arena. The animals are also given chewsticks in their cages as well as other enrichment e.g., hammocks.

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

For the research to produce findings which can be applicable to the human brain it is necessary to use a model with the same anatomy, i.e., the same brain structures and connectivity, which is why rodents are required. Furthermore, these animals are able to perform a number of tasks that have similarities to those that are used with patients with amnesia, in particular spatial memory tasks and object/location tasks, which increases the relevance of the findings.

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

After animals have undergone surgery we monitor them for 7 days and complete a post-surgery monitoring form during that time which helps identify issues in recovery and whether they need further analgesia or intervention. We have regular meetings with the vet to ensure our analgesia is appropriate for the experiments we carry out. For studies where animals need repeated injections we use the finest needles possible and the least invasive route possible for injection. All animals are regularly handled and often given rewards which make injections or infusions much easier and less stressful for the animals. We look to find alternatives to repeated systemic injections such as delivering drugs orally where

possible. We review and upgrade equipment where possible to ensure any implantable devices are as light as possible.

By using sophisticated viral vectors it is possible to selectively disconnect specific pathways rather than damaging the whole brain region under investigation. Likewise the use of neuromodulators that target specific receptors within a region rather than affecting the entire brain region means the overall impact on the animal is less. The use of temporary inactivation, rather than causing permanent damage, means that again, the brain region under investigation is only disrupted for short periods rather than permanently, i.e., the animals will not be rendered with a permanent memory impairment when using this methodology.

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

We will follow guidance laid out in the Prepare publication as well as guidance from the NC3Rs and Laboratory Animal Science Association.

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

The research group regularly attend webinars/symposia on improvements in 3Rs for example those put on by the NC3Rs. We also sign up to the NC3Rs newsletter. Furthermore, a senior member of the group is part of the Early Career Researchers 3R group at our establishment which provides an additional route to obtaining up-to-date information and also share our own improved methodologies.

There is an excellent knowledge-base within the University where we share best practice and improved techniques. At all points during the experiment we consider possible improvement to the design of the study and at the end of the experiment we critically evaluate what could have been done better or improved upon to ensure we are constantly learning and adapting, for example, improving behavioural tests to get more reliable data and reduce variance.

A retrospective assessment of refinement will be due by 21 May 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

15. Investigating novel combination therapies to activate anti-cancer immune responses and treat cancer

Project duration

5 years 0 months

Project purpose

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

Key words

Cancer, combination therapies, immune system, mouse models, cancer treatments

Animal types	Life stages
Mice	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description 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 develop new treatment options for patients with cancer by combining standard-of- care treatments, such as radiotherapy and chemotherapy, with novel, experimental drugs. In particular, this work will focus on using these new treatments to modify the activity of the immune system either to make it better able to attack tumours or less well able to cause damage to normal tissues. The research will be directed towards diseases that represent significant unmet needs in the clinic (eg melanoma, head and neck, thyroid, lung and breast cancer). The work will be based on a number of key themes that use: (i) gene and virus therapies as a means of killing cancer cells and alerting the

immune system to the disease's presence; (ii) drugs that make tumour cells more susceptible to being killed by radiation therapy in a way that also makes them more visible to the immune system; and (iii) gene therapy and drug treatments to reduce the risk of damage to normal tissues after radiotherapy.

A retrospective assessment of these aims will be due by 28 May 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 develop kinder, smarter treatment options for patients suffering from cancer.

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

We already have a very strong track record of taking new therapeutic approaches from laboratory studies in to clinical trials. For all of the approaches detailed in this application, we will use the data generated to inform the design and conduct of translational clinical trials that will be performed in the UK and internationally. We will publish the results from our studies in peer-reviewed scientific journals and present data in national and international conferences and meetings.

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

The goal of our studies is to improve outcomes (longer survival, less treatment related side effects) for patients with cancer.

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

We publish our results in peer-reviewed scientific journals. We collaborate with pharmaceutical companies and other research institutes as well as with teams in house to translate our scientific results into therapies to treat patients with cancer.

Species and numbers of animals expected to be used

- Mice: 30000

Predicted harms

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

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

We have chosen the least sentient animal species (mice) possible for all studies. For these type of cancer therapy experiments we need to use mammals in order to replicate tumour growth and immune system activation in humans. In the majority of experiments we will use adult mice. However in some cases there we will use juvenile mice for breast cancer studies to better reflect early onset of cancer in younger patients.

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

Most animals will receive an injection to grow a tumour. The majority of mice will receive treatment (or control) for cancer, this can include treatment through food or drink or through various types of injections for example into the blood stream or directly into the tumour. Some animals will undergo multiple treatments on the same day but care will be taken to design the experiments to use the minimum number of injections possible per day without compromising the results of the study. Some animals will undergo radiation treatments. Some animals will undergo surgery but anaesthesia and pre- and post-operative analgesia (pain relief) will be used to minimise suffering and distress. Some mice will be imaged to investigate cancer spread. The large majority of procedures are characterised as mild or moderate by the Home Office. A normal experiment lasts 4-5 weeks, with treatment ongoing for approximately 2 weeks time. All experimental mice will be monitored daily. At the end of an experiment, animals will be humanely killed and samples, such as tumour, lung, spleen and blood, will be taken for processing and detailed analysis to help improve future treatments for cancer patients.

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

The majority of the mice will develop tumours and will be treated with a variety of standard-of-care and experimental anti-cancer agents.

Some animals will experience discomfort from tumour growth, including laboured breathing if a tumour spreads to the lungs.

Mice will receive treatments similar to cancer patients through oral dosing or injections. Injections might cause pain but that is not expected to be more than mild, not sustained, discomfort. Different types of cancer treatment can result in weight loss. Some animals will undergo radiation treatments which might result in skin inflammation.

Expected severity categories and the 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 are characterised as mild or moderate by the Home Office. The majority of mice will experience moderate severities (~80%). Animal on breeding protocol will experience mild procedures (~20%). One of the protocols aim to test toxicity of new cancer

therapies, among those experiments we expect death due drug toxicity in a low number of mice, and is therefore characterised as severe.

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

- Killed

A retrospective assessment of these predicted harms will be due by 28 May 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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, detailed laboratory studies will be carried out on cancer cells growing in plastic dishes/flasks or as clusters of cells called 3D tumour spheroids. Such in vitro studies (studies of cells in dishes) will enable us to replace many experiments that would otherwise require animal testing. In addition, when it is possible to answer scientific questions directly from patients samples, we use this alternative instead of and as a complimentary approach to the animal work. However, no in vitro system is able to recapitulate (faithfully mimic) all of the effects of an intact (or partially defective) immune system. Therefore, it is unavoidable that some experiments will need to be performed in tumour-bearing animals to replicate human disease and response to treatment.

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

Cell cultures and 3D tumour spheroids in vitro (cell studies in dishes, not involving an animal). Human patient samples.

Why were they not suitable?

Human patients samples are not always accessible and are not treated in the same way as the exploratory research carried out in the laboratories where we aim to identify novel combination treatments to improve cancer therapies, therefore it is not possible to replace all animal experiments with human patient samples.

In vitro systems are not able to recapitulate all of the effects of an intact (or partially defective) immune system. Therefore, it is unavoidable that some experiments will need to be performed in tumour- bearing animals.

In vitro systems, human patient samples and in vivo mouse experiments all complement each other to best study cancer treatments.

A retrospective assessment of replacement will be due by 28 May 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

Animal numbers are based on the number of mice needed per group to reach significance in the experiment. Furthermore, animal numbers are based on treatments groups and control groups per experiment as well as number of experiments needed for the research and projects performed.

Experimental Design Assistant (EDA) from NC3R is a useful tool to help design experimental studies.

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

We will reduce the number of animals required for experiments by optimising experimental protocols through detailed preliminary in vitro studies. In addition, we will ensure excellence in the conduct of animal procedures by adherence to standard protocols and by the involvement of experienced animal technicians/consultant surgeons, to reduce the need for unnecessary repetition of studies. We have optimised group sizes to ensure that study objectives are met, without including unnecessarily large numbers 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 use each animal optimally by analysing as many parameters from each experiment as possible. This includes collecting and analysing multiple tissues at the end of the experiment. We perform pilot studies. We run efficient breeding programs.

For some experiments we will establish *ex vivo* tumour explants from tumours grown in mice. In this way, one tumour can be divided into several slices for further therapeutic drug testing *ex vivo*, leading to an overall reduction in number of mice used and an overall reduction of number of procedures performed *in vivo*.

A retrospective assessment of reduction will be due by 28 May 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

Which animal models and methods 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 this project.

Whenever possible, we will inject tumour cells to grow under the skin of mice for easy access and monitoring. However, the tumour microenvironment is best replicated in the real site for tumour growth, e.g head and neck, lung and breast sites) and, therefore, these models are used to study the complex interactions between tumour cells and immune cells. We almost exclusively use mouse strains which have a fully functioning immune system instead of genetically altered mouse strains that require more complex breedings.

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

Mice are the lowest species that are appropriate for drug development studies and for studies heavily based on the response and action of the immune system. Our studies involve analyses of complex interactions between immune cells, cancer cells and other cells within the tumour (e.g. blood vessel cells and structure support cells).

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

All animals will be looked after by trained, experienced personnel and scientists. Animals will be housed and experiments will be conducted in a designated research facility. Mice will be housed in cages with sterile bedding, food and water. Animals will develop tumours and will be treated with a variety of standard-of-care and experimental anti-cancer agents. Animal suffering will be minimised by robust and regular checks for tumour growth, as well as score sheets for animal well-being, tumour sizes, ulceration and behaviour. Maximum allowed tumour sizes will be strictly regulated by set guidelines to ensure that animals experience minimal discomfort and distress.

Some animals may undergo surgery, we will adhere to policies on the use of anaesthesia and pain relief to minimise stress and suffering.

Therapeutic drugs will be used according to published safe toxicities. If any, we only expect mild flu-like symptoms (e.g. fever, loss of appetite, tiredness and dehydration) from treatment with therapeutic viruses. In such cases, animals will be managed to help symptoms and, if necessary, animals will be humanely killed. Suffering will also be minimised by having experienced staff undertaking the procedures. Animals might lose weight but they will be monitored for weight loss at least twice weekly and mice losing weight will be supplemented with wet mash/diet gels. Mice will be humanely killed if weight loss exceeds 18%. If any mouse experience a sustained weight loss lower than 18%, advice will be sought from named animal care and welfare officer or named veterinary surgeon.

We perform pilot experiments to determine humane endpoints and thereby reduce the adverse effects that an animal may suffer. This information is useful not only for current studies but potentially also future and external studies.

Animals under the protocol for novel drug testing will be closely monitored for the occurrence of drug toxicity.

We will minimise the number of injections an animal receives every day to limit the suffering caused. It is anticipated that, in the majority of experiments, it will be necessary to inject animals for no longer than 3 weeks, using no more than 2 routes of injections / day and a low number of daily injections (typically 1-3 injections). However, some animals might receive longer-term treatments with increased risk of adverse effects, or might be part of triple combination studies which require more daily injections for therapy and anaesthesia (guided by administration regimens relevant to the clinical setting). We will design studies so that animals will receive as low a level of accumulated suffering as possible.

Wherever possible, we will avoid giving injectable anaesthesia on the same day as therapeutic agents via the intraperitoneal route (injection into the peritoneum / body cavity). Whenever possible we will use gaseous anaesthesia instead of injectable anaesthesia for imaging and radiotherapy treatments. If an animal needs to undergo injectable anaesthesia and be treated with a therapeutic agent using injections on the same day, whenever possible we will administer therapeutic agents injections whilst the animal is under anaesthesia to reduce accumulated suffering. We will minimise the number of injections given to an animal per day by, whenever possible, combining therapeutic agents in the same injection. Care will be taken to give the injections at different sites. Animals will be closely monitored and a switch of administration route will be effected if problems arise.

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

Animal Scientific Procedures Act, Code of Practice for the Housing and Care of Animals bred, supplied or used for scientific purposes, Guidance of the Operation of the Animals (Scientific Procedures) Act 1986, General Constraints, LASA, IAT, Experimental design assistant (NC3R), National Centre for the 3Rs, Home office links to all documentation regarding A(SP)A 1986 amended in 2012, animals in science procedures on ASPeL, FRAME, UAR, Arrive guidelines.

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

We constantly review our methods of working and keep up to date with new information and guidelines released including updates on the animal scientific procedure act and code of practices.

A retrospective assessment of refinement will be due by 28 May 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

16. Therapies & Biomarkers for Acquired Brain Injuries

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Traumatic Brain Injury, Acquired Brain Injury, Blast trauma, Neuroprotection, Neurodegeneration

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

Acquired brain injury (ABI) (*eg* due to a blow to the head, interruption of blood supply (ischemia), interruption of oxygen supply (hypoxia) or carbon monoxide poisoning) is a

major cause of death and disability throughout the world. In the UK alone approximately 1 million patients attend hospitals with a head injury, and the annual costs of their acute treatment is £1.4bn. The high economic and social costs of dealing with ABI combined with its high incidence mean that there is an increasing need to develop new treatments for ABI. Although the primary causes are different in each type of ABI, there are similarities in many of the mechanisms thought to play a role in delayed brain-cell death (*eg* excess of brain chemicals such as glutamate and glycine (excitotoxicity) and disturbance of the balance between reactive oxygen species and natural antioxidants (oxidative stress). The aim of this program of work is to develop novel treatments for ABI, to understand the pathophysiology underlying ABI, and to develop novel biomarkers for ABI.

A retrospective assessment of these aims will be due by 19 May 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

At present treatment options for ABI are limited. Therapeutic cooling is used in the treatment of neonatal asphyxia and ischemic brain injury following cardiac arrest; carbon monoxide toxicity is treated with oxygen therapy either at atmospheric pressure or elevated (hyperbaric) pressures; interventions for traumatic brain injury (TBI) focus on non-specific interventions to stabilize physiological parameters such as tissue oxygenation and intracranial pressure. The aim of this program of work is to understand the pathophysiology of ABI and to determine the effectiveness of novel treatments to prevent or minimise the brain damage resulting from these injuries. Greater understanding of mechanisms of injury may reveal novel therapeutic targets. We will also investigate whether novel biomarkers (*eg* electrical signals from brain (EEG), or chemicals detected in blood or cerebrospinal fluid (CSF) that surrounds brain and spinal cord) can be used to predict outcome, recovery after injury and response to treatment. The idea is that one or more of the biomarkers could provide a 'signature' that for example would identify someone with a (mild) brain injury that they were unaware of, or to determine whether someone has experienced a moderate or a severe injury. Such biomarkers could be of great use in both clinical diagnosis (*eg* of mild TBI or exposure to carbon monoxide) and in stratifying patients (*eg* mild, moderate, severe) such that they receive optimal treatments.

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

The outputs that are likely to be generated are:

- new information about the pathophysiology of ABI (*eg* which particular molecular pathways lead to development of brain injury), new information about the efficacy of

potential treatments for ABI, the identification of new treatments for ABI, the identification of novel biomarkers that could identify ABI (*eg* mild TBI or concussion) and that could be used to determine which patients would respond better to particular treatments.

- publications resulting from this new information.
- The ultimate aims are:
- to develop new treatments
- to develop new diagnostic tools (*eg* biomarkers)

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

Researchers in Acquired Brain Injury (ABI) and related fields Clinicians treating ABI patients

ABI patientsFamilies and friends of ABI patients NHS
Society as a whole Industry/Pharma

How will they benefit from this research:

Researchers in ABI and related fields will benefit from understanding of pathophysiologic mechanisms underlying ABI described in academic beneficiaries above. Timescale: during project duration and for 1-2 years after.

Clinicians treating ABI patients in general will benefit from a better understanding of the mechanisms of secondary injury development (secondary results in most of the morbidity & disability experienced by patients). Timescale: during project duration and for 1-2 years after.

Clinicians treating ABI patients will benefit from understanding mechanisms of action of potential novel neuroprotectantive treatments. Timescale: during project duration and for 1-2 years after.

ABI patients will benefit from improved treatments (*eg* drugs targeting ABI). Timescale: following completion of project 1-5 years after.

NHS will benefit in terms of improved diagnostics (*eg* novel biomarkers) and treatments (*eg* drugs targeting ABI). This could result in significant costs savings if novel neuroprotectants improve outcome or biomarkers facilitate patient stratification. Timescale: following completion of project 1-5 years after.

Family & friends of ABI patients will benefit in terms of reduced burden on family & carers that will enhance the quality of life of patients and their families. Timescale: following completion of project 1-5 years after.

Society as a whole will benefit in terms of reduced long-term care costs, less under-employment or unemployment of ABI patients. Timescale: following completion of project 1-5 years after.

New intellectual property (*eg* biomarkers, novel indications for specific treatments) may benefit both academia and commercial/pharmaceutical industry. Timescale: following completion of project 1-10 years after.

UK Pharma sector will benefit as research staff will develop skills (*eg in vivo/in vitro* pharmacology) relevant to this business sector. Timescale: following completion of project 1-5 years after.

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

We will publish our findings in peer reviewed journals, at scientific meetings and conferences, and online on the lab website. The journal publications are likely to occur towards the end of the project (24 - 60 months). However, as far as is possible without compromising the potential to publish the work in journals, we will aim to use conferences and poster presentation to disseminate findings and seek feedback and input to the ongoing work (12-24 months). If we develop novel methodology, we will aim to publish this so that others can make use of it. The translational nature of the research means that the conferences at which we would present at will have audiences of both researchers and clinicians. Examples of meeting presentations could include *eg* IBIA World Congress on Brain Injury; BrainPET Meeting; Society for Neuroscience Meeting; National Neurotrauma Society, British Neuroscience Association Meeting). We have ongoing collaborations both nationally and internationally that provide an opportunity to share the research findings with clinical & scientific audiences.

Species and numbers of animals expected to be used

- Mice: 4250
- Rats: 3750

Predicted harms

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

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

We will use rodent models of acquired brain injury. Rats and mice are the least sentient species in which we can determine effects of novel treatments on clinically relevant behavioural outcomes such as memory and locomotor function (*eg* balance and gait during walking). We will use both rats and mice in this program because each species has particular advantages: mouse models offer advantages in modelling certain aspects (*eg* locomotor function & gait) and are more convenient for studies on outcomes in aged animals, while rats offer the advantage that the effects on physiological parameters (*eg* blood pressure, heart rate, intracranial pressure) are more easily measured. In humans, ABIs can occur at all stages of life from infants to the elderly. We will use rats and mice

from juvenile and adult populations in order to understand how injury develops at different life stage and to determine whether treatments and biomarkers are effective following injury at different life stages.

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

Typically an animal will experience either an acquired brain injury (ABI) under anaesthesia or a sham procedure that does not involve brain injury. Some animals will receive novel treatments. Following ABI or sham procedure animals will recover for typically 24 hours after which they will undergo behavioural testing (*eg* locomotor or cognitive function). In almost all cases we require sham controls to establish baseline outcomes in *eg* behavioural tests or biomarker assays (these parameters can be affected by animal age/weight and local housing conditions/environment). Behavioural testing may be repeated at regular intervals over subsequent days and weeks in order to determine if there is functional impairment and recovery. A subset of these animals may have recording of electrical activity from the brain (EEG) or undergo magnetic resonance imaging (MRI) or 'brain scanning'. At the end of the period of behavioural testing animals will typically be euthanised and perfused to preserve brain tissue for histological analysis. The duration of the majority of experiments will be in the range of 1 week to 3 months. A subset of animals may be kept for longer periods of time after injury (*eg* up to 18 months) to determine the effects of novel treatments on translationally relevant long term outcomes.

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

The rodent models of ABI that we use are widely used in pre-clinical studies to understand pathophysiology of injury and to determine the efficacy of neuroprotectant drugs. We expect that animals will have some degree of locomotor and cognitive impairment after injury. Locomotor impairment is typically greatest at 24 hours after injury with recovery over subsequent days. There may be weight loss (typically <20% reduction) 1-3 days after injury with return to original weight around 5-7 days after injury. There may be persistent cognitive impairment (*eg* deficits in learning and memory). A minority of animals in some protocols (*eg* CCI) may experience severe impairment. All animals will be closely monitored and given appropriate pain relief to prevent and manage any resulting pain. Any animals in extreme distress will be humanely killed in order to minimise suffering.
The subset of animals kept for extended periods (*eg* 18 months) will be subject to additional monitoring. Animals will be monitored daily and have enhanced welfare monitoring at least every 2 weeks from the age of 15 months, increasing to weekly from 18 months of age. Monitoring frequency will be increased should any deleterious signs of ageing become apparent.

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

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

The proportion of expected severities over whole program are: Rats: non recovery (4%); mild (44%); moderate (49%); severe (3%)
Mice: non recovery (4%); mild (44%); moderate (49%); severe (3%)

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

- Killed

A retrospective assessment of these predicted harms will be due by 19 May 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

The events that follow TBI *in vivo* and the response to treatments are complex. Although some of these processes can be studied in *in vitro* models, that we will use where possible, there are no alternatives that can effectively model all these processes that do not use animals. In order to have relevance to clinical ABI and to allow progression of novel treatments or biomarkers to clinical use, the use of animals is unavoidable. We have already obtained a significant amount of information from our previous animal experiments, but there is much new information that remains to be determined (*eg* the optimum timing and duration of novel treatments in blunt TBI and whether these treatments are effective for other types of ABI such as sports concussion and blast TBI).

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

We have considered computational modelling, non regulated organisms (Drosophila) and *in vitro* models.

Why were they not suitable?

The study of secondary injury mechanisms and the efficacy of novel drug treatments is not amenable to computational approaches. We carried out preliminary investigations of injury models in Drosophila, but due to their very small size it is not possible to model impact trauma to the head alone. Trauma models that exist for Drosophila are whole body polytrauma. Some of the ABI injury processes can be studied *in vitro*. Using simple *in vitro* models of ABI we have identified novel neuroprotectant substances for different types of ABI. For example before moving to animal models we demonstrated that novel treatments reduced TBI development and investigated mechanisms of action *in vitro*. We will pursue further *in vitro* studies where possible, as part of the current program. One of the protocols is specifically to generate tissue for in *vitro* experiments. However, despite the usefulness of *in vitro* models, it is not possible to progress directly from *in vitro* models to humans. The response of a whole animal to ABI and to treatments is much more complex than can

be modelled *in vitro*. It is necessary to characterise the mechanisms of injury and response to treatment in mammalian species before clinical evaluation.

A retrospective assessment of replacement will be due by 19 May 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

The projected numbers above reflect the numbers necessary to achieve the scientific objectives of the program. Total numbers over the 5 year project are estimated based on an annual use of 850 mice and 750 rats over the 5 protocols. These numbers are based on our previous use in studies of similar animal brain injury models.

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

The proposed models, experimental design and statistical analysis have been discussed with a Statistician. For the quantitative experiments we will use power analyses to decide the sample sizes, if appropriate information on effect sizes exists. If this information does not exist, we will carry out pilot studies to estimate effect sizes. For the qualitative measures we will use the minimum amount of material required to provide an adequate description. We will make use of the NC3Rs experimental design assistant online tool in planning particular experiments.

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

We will carry out systematic review and meta-analysis of existing literature before specific experiments. We have already carried out systematic reviews and meta-analyses of the efficacy of novel treatments in animal studies of ABI. If the expected effect sizes are not known in advance, we will perform pilot studies in order to calculate appropriate sample sizes for the main study. Where possible we will use *in vitro* models in the initial screening of treatment efficacy, by for example establishing optimal dosing and treatment timing that will reduce the numbers of animals required in the *in vivo* studies. During the previous licence we developed a novel *in vitro* model of TBI that can be used for this purpose. One of the protocols in this licence is to produce tissue samples for these *in vitro*

models. We aim to maximise the benefit of each experiment by preserving as many tissue samples as practical for future research.

A retrospective assessment of reduction will be due by 19 May 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

Which animal models and methods 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 program of work will use rats and mice. Rat and mouse models have been widely used in pre- clinical ABI studies and are the most well characterised and reproducible models. There are a wide variety of behavioral tests available in rats and mice that allow quantification of deficits in multiple domains (*eg* locomotor, cognition, anxiety). The use of mice and rats will therefore maximise the amount of useful information we can obtain from the minimum number of animals. In order to increase the relevance of the results, the study designs will where appropriate, incorporate the principles of the STAIR (Stroke Treatment Academic Industry Roundtable), PREPARE and ARRIVE guidelines. In order to maximise the translational relevance of the work we will use well characterised outcomes (*eg* behavioural tests of learning and memory such as Barnes Maze, CatwalkXT gait analysis; objective histopathological outcomes such as lesion volume and neuronal loss). All animals will be closely monitored and given appropriate pain relief to prevent and manage any resulting pain.

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

We have considered the use of less sentient species (Drosophila) but the effect of trauma on their nervous system does not model human pathophysiology well (*eg* Drosophila brain has different mechanical properties and head injury alone without thoracic injury is not possible). Our aim is to understand the pathophysiology and effect of novel neuroprotective treatments in the medium to long term (for greatest clinical relevance). It is not possible to address these questions in terminally anaesthetised animals.

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

Our group has extensive experience with animal ABI models, particularly the severe protocol (controlled cortical impact) and we have published several papers with this model. We have had the opportunity to refine this protocol and the others. Most of the protocols that we use include physiological monitoring (*eg* temperature, heart rate, blood pressure, oxygen saturation). For the traumatic brain injury models the injury is performed under general anaesthesia with appropriate analgesia. Following recovery from brain injury animals are carefully monitored and additional analgesia is given if required. We will seek to maximise the amount of information we can obtain from behavioural tests that involve minimal stress to animals (*eg* analysis of video monitoring to extract additional parameters and/or multivariate analysis of data).

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

STAIR (Stroke Treatment Academic Industry Roundtable), NC3Rs PREPARE guidelines, CAMRADES (Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Stroke), LASA Good Practice Guidelines on Aseptic Surgery, Blood Sampling and Administration of Substances.

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

We will stay informed about advances in 3Rs via the NC3Rs website and publications, and *via* seminar series both within and outside of our institution. If we become aware of advances in 3Rs relevant to our experiments, we will first consult published material and we contact the developers and authors for additional information if this is required.

A retrospective assessment of refinement will be due by 19 May 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

17. Studies of the immune response to infection, tissue damage 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

Dendritic Cells, Cancer, Infection, Tissue Damage

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 required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

The fundamental objective is to understand how the immune system "decides" how to react to antigen challenge. Put simply, it is the study of how the immune system can be "switched on", directed to attack specific targets, and when necessary, to be "switched off".

A retrospective assessment of these aims will be due by 01 June 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

Immunity provided by T cells of the immune system (cell-mediated immunity) holds promise in treating cancer and many infections. Clinical success in the context of cancer or infection immunotherapy, therefore, depends on our body's ability to activate these T cells, which kill tumour and infected cells. Activating T cells is the principal function of another immune cell type called a dendritic cell (DC). The job of DCs is to show the T cells what they should and should not attack. We therefore study DCs and how they convey instructions to T cells. To do this, we also study how DCs develop and colonise tissues to act as local “sentinels”. This sentinel function refers to the unique ability of DCs to survey tissues for signs of infection, cellular damage or tumour progression, all of which might require induction of T cell responses. Therefore, we study how DCs integrate information from their tissue environment, for example receiving signals from damaged tissues or from cancer cells or from microbes or viruses. Although cancer, infection and tissue damage might seem like disparate phenomena, we believe they have commonalities that are unexplored and that reveal new facets of immune function. For example, we have evidence that sensing of extreme tissue damage (i.e., necrotic cell death) by DCs is used by the immune system to detect infection by viruses that kill cells. But it is also a means for the immune system to detect tumours, which often have a necrotic core. As such, decreasing the ability of DCs to sense the presence of dead cells leads to defects in the immune response to cell-killing viruses and to tumours. Conversely, augmenting dead cell sensing by DCs increases immune responses to several cancers and might be involved in fuelling autoimmunity. This is at heart a basic discovery research programme but has translational potential. It can lead to the design of better vaccines and immunotherapies for both infectious diseases and cancer, and to the development of immune "deactivation" strategies for autoimmune disease. As an example, based on results obtained under the current PPL, we have founded a spin-out company specifically aiming to harness the mechanisms of dead cell sensing by DCs as a means to improve cancer immunotherapy. We hope to start a clinical trial in 2025.

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

This project will lead to an improved understanding of the mechanisms by which the immune system reacts to various challenges, including infection, cancer and injury. It is envisaged that findings could lead to the design of better vaccines and immunotherapies for cancer and infectious disease. It can reveal processes that, when dysregulated, lead to the development of autoimmune conditions.

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

While our work is fundamental research, it has strong translational potential. This is due to the fact that the activity of the immune system impacts just about every human disease, from neurodegeneration to cardiovascular. The most likely short-term outputs of the present project will be in cancer immunotherapy and vaccine development. Indeed, discoveries under the current PPL have contributed to 4 filed patents in the vaccine and cancer immunotherapy fields, 3 of which have been licenced to companies developing novel cancer immunotherapy approaches. In addition, the work under the previous and current PPLs led to 7 clinical trials in immuno-oncology and the creation of a $53M spin-out company founded by the PPL holder and developing pre-clinical work in DC-based cancer immunotherapy. It is therefore expected that the present PPL will similarly lead to further development in these fields.

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

Dissemination of our results will be carried out at multiple levels to a diverse audience. The latter includes scientists, clinicians, health policy makers, patient groups and the public. Means include scientific publications (including pre-prints), seminars, presentations at conferences, press releases, radio and TV interviews, social media, podcasts and outreach activities (e.g., school visits, Pint of Science). Wherever collaborations allow us to improve or accelerate outputs, we will use them. We will also freely share reagents and publish negative results as far as possible, to minimise unnecessary repetition by colleagues. The Translation team in our institute will be involved early as promising targets are found to expedite bench-to-bedside development.

Species and numbers of animals expected to be used

- Mice: 70,000

Predicted harms

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

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

To understand the complex, multi-organ processes that characterise immune responses to infection, cancer and tissue damage, the use of in vivo models are indispensable, as no in vitro system is able to reproduce the complexity of the immune system and the immune response. Mice are the species of choice, given the unparalleled range of tools for analysis and manipulation. Many immunological therapies in use today were first discovered and studied in mice. As the physiology and pathology of the immune system is similar between humans and mice, mice are good predictors of mechanisms in action in human disease conditions. Data obtained in our mouse experiments are complementary to other studies in our lab using representative in vitro systems and human cells, as well as activities to compare our data to available patient data. As infections and cancer can affect humans at all life stages, from the very young to the very old, we may use mice covering the whole lifespan.

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

Typically, we will be comparing mock-treated (such as using a placebo) animals with those undergoing immune-stimulating treatments and challenges, such as infection, tumours or tissue damage and/or genetically wild-type to gene-modified mice. Apart from the protocol defining treatment, mice may undergo a limited number of other procedures.

For example, they may have been given immune cells from a genetically altered donor animal to assess their function within this new host, or they may have had a treatment to induce or activate a particular gene they are carrying prior to such a challenge.

In some cases, chemotherapy, other pharmacological interventions or radiotherapy may need to be used during the course of an infection or tumour progression to study their effects on the immune response.

Animals may also require an additional monitoring step(s) such as taking of a blood sample or measuring blood oxygen levels.

The infections and tissue damage models we study are generally chosen to be self-limiting and are usually resolved within three weeks, unless animals reach humane endpoints beforehand. Where pre- determined time points are chosen, we always aim to maintain the duration of experiments to the minimum required to address the scientific need and obtain meaningful scientific data. In some protocols, two infections/challenges will be applied in short succession or with longer periods between them, so as to better mimic the real-world human condition of sequential or overlapping infections, and to study the development and effectiveness of any immunological memory.

Whilst some of the protocols on this Licence have many optional steps, in reality, any single animal will only require a very limited number from those available.

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

The disease models we study do have the potential to cause animals to exhibit a wide range of symptoms; these may include weight loss, laboured breathing, diarrhoea, hunched position, lack of movement, all to varying degrees. For all models, we have detailed knowledge of the days of highest severity (depending on the pathogen, or the tumour position, the nature of any tissue damage, and the strain of animal) and we will monitor animals daily on these days, using a detailed clinical score sheet and/or body condition score/index where appropriate and/or weight loss as parameters. Once the critical days in any challenge are overcome, mice should recover rapidly and be undistinguishable from uninfected/unchallenged mice within a few days, as is familiar in the course of human influenza infection for example.

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

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

We will mostly utilise mild or or moderate protocols, depending on the scientific questions we are addressing. However, two protocols will require to be placed under the severe category in order to adequately address their scientific aims.

As a representative example, where influenza virus infection is to be modelled, we need to use a severe protocol due to the range of potential symptoms, including a rapid but transient weight loss of between 20-25%. However, only a few mice (<10%) will actually achieve this weight loss during the course of an infection.

As a general principle, the mildest possible protocol to model human disease will always be applied, for example, the lowest infectious dose possible will be used that is still able to generate the desired immune response whilst avoiding excessive pathology during infection.

For studying the response to and recovery from tissue damage, either infection-induced or in other damage models, we need to have certainty of the tissue damage occurring in the first place, which is more reliable and reproducible in severe protocols. We will weigh up in each individual experimental setting which is the earliest and mildest time point possible to assess crucial immune parameters.

Similarly, the vast majority of tumour models to be employed will involve superficial sub-cutaneous, non-metastasising tumour models, which cause relatively little impact on an animal’s' well-being before the termination of the experiment.

Due to the necessity to generate GM animals that are fully backcrossed to particular background strains (for which we employ "speed-congenic" genotyping strategies to optimise the process), as well as the need to assemble animals with the complex genotypes required to answer our very focused scientific questions, many animals will be used simply for breeding. Along with this purpose, the use of GM animals to provide material for ex-vivo studies and to act as donors for cell transfer experiments (both simple adoptive transfers and chimera generation), means a high proportion of animals are only ever subjected to a sub-threshold experience. The percentages recorded on our last Home Office return (for 2022) were: 68% Sub-threshold, 16% Mild, 16% Moderate and <0.01% Severe. We would expect a similar ratio to be maintained in our future work.

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

- Killed
- Used in other projects

A retrospective assessment of these predicted harms will be due by 01 June 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

The development and function of the immune system involves many different cell types interacting in a dynamic three-dimensional environment. For example, the progression of an infection within a whole organism's body involves changes in the expression of the antigens carried by the infectious agent that will evolve with both time and in their spatial distribution throughout the body. Similarly, cancer development and spread involves a plethora of interactions between cancer cells and their surrounding cells, governed by multiple signals originating from both their immediate neighbours and from distant tissues. These factors combined with the involvement of multiple host cell-types, as well as their expansion and migration around the body, means such research cannot be carried out in tissue culture alone and can only be addressed in the animal.

The mouse is one of the model organisms that most closely resemble humans. The human and mouse genomes are approximately the same size, and display an almost identical number of genes, the vast majority of which are functionally conserved. Further, mice have genes not represented in other animal model organisms (e.g. Caenorhabditis elegans i.e., nematode worm, and Drosophila melanogaster, i.e., fruit fly) such as those involved in the adaptive immunity. Mice can be genetically altered, there is extensive literature concerning the topics of our investigation, and our own studies can be enhanced by making comparisons with many complementary models developed by others in the field.

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

Wherever possible we will use in vitro studies to address specific scientific questions. Such as the use of dendritic cell lines when looking at the processing of antigens and their presentation to T-cells. As a principle we use cultured cell-lines wherever possible, and we are actively investigating the use of serum-free medium in all our tissue-culture models. However, there are no cell lines that reproduce the heterogeneity of DCs and, at present, there are no credible alternatives to the use of animals for studying the localisation of DCs in tissues. Further, studying the mammalian immune system usually requires the use of functional cells produced within an intact organism, and at best requires ex-vivo material to work with. The priming of T cells by DCs in lymph nodes requires spatiotemporal coordination with cells entering and exiting the tissue at specific times, which cannot be reproduced in organ-on-chip or organoid models. Current in silico models, including of DC movement and interaction with T cells, do not adequately explain or account for experimental observations although this is an area of active research. We collaborate with groups that have helped us model the distribution of DCs in tissues and, while this cannot be used as an alternative approach, it can complement our proposed studies thereby contributing to refinement through a reduction in animal use.

Why were they not suitable?

The complex regulation of the immune system involves the development of cells in one body compartment and their migration to other organs where, they may be "educated", undergo further maturation and differentiation, and become activated. All before eventually carrying out their effector functions in other peripheral locations. All these steps require

signals, some of which may be soluble mediators generated by cells in distal locations (including those generated by the microbiota of the gut), and/or cell-to-cell interactions, sometimes between multiple cell-types, to complete the process.

Currently this process cannot be fully reproduced by any in vitro system, including generating organoids and organ-chip methodology, nor by computer modelling. Therefore, in many cases, the intact immune system of a living animal is the only experimental model that can produce meaningful and potentially translatable results.

A retrospective assessment of replacement will be due by 01 June 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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 have calculated from our previous Project Licences and our experience in colony management the numbers of animals we will use for the various types of studies we will be undertaking. Our research group comprises around 16 PIL holders at any given time and this PPL application is meant to cover work by all of them, hence the high total number of mice. Many of those mice are used in breeding as in many cases we will be intercrossing strains and assembling progeny with complex genotypes for specific studies. We use our long experience in colony management to keep numbers to a minimum but it is worth mentioning that under our current PPL we presently carry upwards of 100 genetically-modified mouse strains

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

The use of mice will be minimised in several ways:

By minimising variability in results through utilising inbred strains and by housing them under identical conditions to limit variability.

By performing pilot studies using few mice when no information is available in the literature so that the number of mice utilised in experiments will be reduced to a minimal level. If some effects are worth investigating further we may perform larger cohort studies to determine if the observed difference is statistically significant. The size of the cohort will

depend on the observations made from the pilot studies and will be determined using power calculations.

By considering on-going statistical estimation of power requirements in each of the studies, using prior results in order to use the minimum number of animals while retaining sufficient numbers for statistical significance. We will always aim to use the minimum number of mice per group that will be informative.

By combining, where appropriate, different experimental groups with shared controls to reduce the number of mice utilized as controls. In experiments involving immunodeficient and/or transgenic/knockout mice, normal mice and/or wild type or heterozygous littermates will be used as control animals wherever possible.

By utilizing, where possible, tissues from different sites on one mouse for both treatment and control samples. e.g. by injecting two flanks of a mouse, one as a test and one as a control, when it is possible to use responses in draining lymph nodes as a readout, to minimise the number of control animals utilised. In this case, a paired comparison should also make the statistical analysis more accurate.

The proposed experimental designs and methods of analysis will be discussed with members of the laboratory, and those of our collaborators, and we will seek additional advice from the statisticians employed by our Institute.

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

The use of animals will be minimised by doing as much preliminary work as possible in culture models in vitro prior to engaging in studies in mice.

These studies will be carried out using normal mouse strains, natural mutant mice, and induced mutant strains (transgenic and knockout). These animals will be used to establish colonies of homozygous and/or heterozygous lines and will be bred and inter-crossed as necessary. We will avoid overbreeding, and lines under sporadic use will be maintained at low levels, and cryopreserved whenever practical, and/or maintained in collaboration with other licences to minimise redundant breeding.

A retrospective assessment of reduction will be due by 01 June 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

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

These models are broadly grouped as follows:

Cell transfer, depletion, and chimeric models for understanding the development and function of immune cells.

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

Inflammation and tissue injury models (intestinal inflammation, inflammation of lungs and other tissues) Infection models (Influenza A virus, other viruses, bacteria, fungi and helminths)

Tumour models (subcutaneous implantation of tumour cells, chemical or genetically-induced carcinogenesis)

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

In addition to these experiments, we will also use general breeding and maintenance methods for GA mice. The use of inbred and fully backcrossed mice in the field of immunology not only reduces intra- group experimental variability but also eliminates MHC incompatibility when cell transfers are carried out between various knockout, transgenic and wild-type strains. Without such a defined genetic background nearly all of these experiments would be impossible.

In addition, we use specific genetically-modified animals to understand the molecular events and steps involved in the immune activation process or as a way to direct immune responses against defined model antigens, thereby making analysis and quantitation of immunological effects easier. Whenever possible we will generate transgenic mice in which mutations are induced specifically and conditionally
e.g. using Cre-LoxP conditional alleles, where mice should not display a phenotype until the mutation in the candidate gene is induced.

During the development of new genetically modified lines it may be necessary to genotype or analyse the phenotype of mice. In the case of newly generated genetically modified

lines the use of a genotyping technique, such as Southern blotting, may require a 3 mm tail snip biopsy rather than an ear biopsy to provide sufficient DNA for analysis. In some cases, phenotyping by tail bleeding is required, for example to analyse strains expressing transgenic T-cell receptors. Strains expressing developmentally regulated markers and cell populations such as those carrying fluorescent markers of dendritic cells may require up to four repeated blood samples or ear-snips over a number of weeks to assess the colonisation of tissues by such cells. This characterisation may also require the use of imaging techniques.

Where the immune status of the animals might compromise health, they will be maintained in isolators or IVCs (individually ventilated cages) under barrier environment, to avoid infections. In our experiments we will set clear humane endpoints, for each and every experiment, and as part of good laboratory practice, we will write an experimental protocol which will include details of possible adverse effects. These experimental protocols will be provided to all the staff involved in the experiment.

In addition, when considering which route of administration of substances to employ, we will strive to use the least invasive route whilst maintaining direct control of dose. The choice of route to administer a substance or cells will be such as to achieve "best practice", i.e. to minimise or avoid adverse effects, reduce the number of animals used, and maximises 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. Although in the majority of 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 or cells may require administration through a non-standard route such as injection intratumourally or peritumourally.

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

Less sentient animal species do not provide the physiological similarity to humans and the wide availability of tools for analysis and manipulation. Using more immature rodents is not always an option as embryos and new-borns have only an immature if any immune system. We need a physiology in our in vivo models which is as close as possible to humans. Only mammalian organisms show the same degree of complexity as humans in their immune response, lung or gut structure, and other basic physiological parameters like metabolism.

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

We will continually review our experimental protocols to determine whether there are improvements that we can make in experimental design, techniques and animal husbandry to improve welfare and minimise adverse effects. This will involve both reviewing our own performance and also applying external knowledge / advances in best practice. As part of this, we will also maintain and share detailed information on the experimental procedures we use to benefit other groups. We will perform increased monitoring during all experiments to be able to detect any adverse outcomes and if we are performing a new procedure or an established procedure on a new strain, we will undertake a pilot study to establish optimal experimental conditions (that minimise suffering) and confirm the appropriate humane endpoints. We will work together with

animal care staff to ensure that animals are maintained in the most appropriate environment. We always aim to refine our procedures and husbandry techniques. For example, tunnel handling will be rolled out across the institute within the next few years, which causes less anxiety than traditional tail handling. We are guided by and seek advice from local NVS policy on non-invasive procedures, as well as improved pain management, to minimise harm.

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

Unless otherwise specified, the work in this project will be designed using the principle outlined in PREPARE guidelines for planning animal research and testing (2017). We will also follow LASA Guidelines on administration of substances. We will review guidance produced by NC3Rs expert working groups on specific disease models or more general guidance on common procedures, such as blood sampling (available via the NC3Rs website) and publications from the RSPCA Science Group regarding refinements to specific procedures and models. We will also use other sources, including the scientific literature (e.g. Nature Protocols / JOVE) and guidance available from companies such as Jackson laboratories, who have produced a series of manuals and guides for specific experimental models (see https://www.jax.org/jax-mice-and-services/customer-support/manuals-posters-and- guides/jmcrs-manuals-guides). We will also discuss with the NACWOs and technicians at the Biological Research Facility to determine whether they know of any recent developments in experimental protocols that could be used to further refine our experiments.

With regards to infection models, we take advice from the "Considerations for Infectious Disease Research Studies Using Animal " (Lesley A Colby, Lauriane E Quenee, Lois A Zitzow. Comp Med. 2017 Jun; 67(3): 222–231; PMID: 28662751), as well as from “Refining procedures for the Administration of substances” (https://doi.org/10.1258/0023677011911345) and from the “Guiding principles aseptic surgery” (https://www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf), and from the NCRI Guidelines for the Welfare and Use of Animals in Cancer Research (https://doi.org/10.1038/sj.bjc.6605642) in regard to cancer models.

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

We are regularly updated within the institute on advances in the 3Rs from NC3Rs (https://www.nc3rs.org.uk/nc3rs-newsletters) and NORECOPA, and we actively seek information on possible improvements in discussions with colleagues and collaborators at in-house meetings and external conferences. Whenever we are able to refine techniques without impacting the scientific validity of our work, we aim to implement advances, for example enriched environments for mice to reduce stress levels and avoid overgrooming. I can also contact the NC3Rs for specific advice as necessary. We are also aware that NC3R events and workshops are regularly posted on the NC3R’s website (events page). We will use this to identify any suitable opportunities to stay informed of 3Rs advances and new approaches.

A retrospective assessment of refinement will be due by 01 June 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

18. Animal models of brain tumours 2023

Project duration

5 years 0 months

Project purpose

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

Key words

Paediatric, glioma, preclinical, cancer, evolution

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 required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description 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 and maintain appropriate patient-derived animal models of paediatric brain tumours

To establish and characterise mouse models of paediatric brain tumours based on the manipulation of cancer genes.

To evaluate selected agents with in vitro anti-tumour activity for their tolerability, toxicity, and drug levels in animals

To evaluate selected therapies for efficacy in appropriate tumour models

A retrospective assessment of these aims will be due by 13 June 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

The primary aim of our work is to develop novel therapeutic agents for patients with paediatric-type diffuse high grade glioma (PDHGG). Current treatments for PDHGG are ineffective, and our goal is to generate that the data to support novel trials which improve patient survival.

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

We aim to increase confidence that agents entering clinical trial are optimised as far as possible preclinically, reducing the failure rate in the clinic. Therapies are designed to target the specific molecular alterations present in brain tumour subgroups, with fewer side-effects than current chemotherapy and radiotherapy regimens. Development of novel mouse models for brain tumour subgroups would represent a significant advancement in the field, as these are currently lacking. Such models may allow us unravel how these tumours arise in association with an intact immune system, and also allow for the testing of immune-directed therapies.

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

Paediatric diffuse high grade glioma (PDHGG) is a collection of high grade brain tumours of children and young adults with a median survival of 9-15 months, a figure that has remained unmoved for decades. Any advances in our understanding of disease biology, or the development of novel therapeutic options which may be tested in the clinic, have the potential to make a substantive difference to the life expectancy of children and young adults with these diseases, as well as their families. We may on occasion partner with other academic groups or Pharma to test novel therapies in our PDHGG models, which may otherwise be inaccessible to these groups. Such projects will be carried out under approved contractual arrangements and with assured publication such that any valuable data arising will be made available for the wider community.

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

Our findings are reported in academic publications and at national and international conferences. Validated models and assays are made available to the research community. Working closely with clinicians ensures our aims are focused on the unmet clinical needs

of childhood brain tumour patients, and will ultimately provide patient benefit. Therapeutics emerging from our research will be taken forward into clinical trials in cancer patients, utilising our leadership roles in International brain tumour working groups and clinical trial collaboratives.

Species and numbers of animals expected to be used

- Mice: Maximum 15,250

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.

Animal models are required to fully replicate the properties of tumour tissues growing within specific organs in cancer patients. Similarly, the effects of drugs need to be tested in vivo so that the effects of the natural microenvironment where the tumour resides, how the drugs access the tumour and how specific is the drug is to its target can be assessed. 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. As we study brain tumours which occur in children and young adults, we are using mice at the appropriate life stages which reflect this.

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

Mice will be housed in cages with sterile bedding, food, and water. Trained competent personal with experience of using animals in research will perform all procedures. The welfare of mice entering a study will be closely monitored throughout each procedure.

Mice may receive an injection of tumour cells into the brain to induce tumour formation, or possibly via a microinjection of genetic material into embryos still in the womb. Animals will be assessed for tumour initiation either by using non-invasive imaging techniques or by closely monitoring neurological symptoms. Tumour-bearing animals may be given either single or more usually multiple doses of therapeutic agents dependent upon the properties of the agent. Sometimes more than one therapeutic agent will be given (combination therapy) and sometimes therapeutic agents are given alongside other treatment methods eg radiotherapy or immune therapies, similar to what is experienced by human cancer patients.

Mice will receive the optimal drug dosing that has been assessed using (i) doses and schedules derived from the literature, (ii) previous studies carried out at our establishment or (iii) using dose tolerability studies for novel compounds, in either tumour-bearing or non-tumour-bearing animals, to determine the highest doses of drug that can be safely given to achieve their therapeutic effect.

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 or spread around the body. Others may be used to monitor biomarkers of cancer within the body.

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

The mice used in this project may exhibit clinical signs of cancer similar to those seen in humans such as weight loss, lethargy and pain. Where we are testing the effects of anti-cancer drugs, we need the mice to have established tumours and they will therefore inevitably show some or all of these clinical signs. Animals will be checked daily and weighed weekly . The body condition and behaviour of animals will also be assessed at these times. At all times humane endpoints will be established to ensure that the mice do not suffer any more than is absolutely necessary.

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

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

The expected severities are mild to moderate. Around 40-50% of animals will experience only mild effects with few side-effects and with tumour regressions due to therapy. Around 40-50% will have moderate effects with more loss of condition due to treatment (similar to that seen in humans) and having tumours of larger size, i.e. control animals who receive placebo treatment. A small proportion (less than 10%) may experience a severe severity. This is because with new test agents we need to make sure they are safe by conducting toxicity studies, and at times there are unforeseen effects from these new test agents.

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

- Killed

A retrospective assessment of these predicted harms will be due by 13 June 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 in three-dimensional space within the body. The surrounding tissue and normal cells contribute to malignancy and these conditions cannot be adequately modelled in vitro. The effects of drugs must be tested in vivo to determine that

adequate levels are achieved (and maintained) in tumour tissues, that adverse effects on normal tissues are minimised and that efficacy tracks with measurable endpoints – i.e. evidence that the compound reaches its molecular target and selectively inhibits it. This is particularly true of brain tumours, where the blood-brain barrier limits drug penetration into the central nervous system (CNS). The use of implantation of patient-derived cells into the brain has been shown to retain the complexity of human tumours and provide the possibility of studying truly personalised responses.

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

Extensive studies are first carried out in vitro to mimic as many of the basic cellular processes as possible. We use in vitro assays of tumour cell growth and movement, with cells grown either as single layers (2D) or as aggregates in suspenion (3D). In addition we measure many parameters in vitro associated with the drug itself including metabolism, drug-drug interactions, the ability of the drug to enter cells throughout the body, and toxicity against normal brain cells.

Why were they not suitable?

None of the aforementioned in vitro assays singly or collectively can adequately predict the full complexity of in vivo responses, including possible effects on sensitive normal tissues. Genetically engineered mouse models are necessary as there are no current alternatives for the study of cancer development and immuno-oncology treatment testing in an environment with an intact immune system.

A retrospective assessment of replacement will be due by 13 June 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

Statistical power calculations are carried out in advance to determine the minimum number of animals that can be used to provide a positive result according to previous experience. This is then used to guage expected animal numbers on the basis of estimates of future work necessary over the period of the licence.

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

The in vivo models are designed to provide maximum information from the minimum number of animals, compatible with statistical requirements. This is achieved by using well-characterised systems with predictable behaviour, obtaining multiple measurements of tumour development (e.g. tumour volume, degrees of cell death and intratumoral oxygen levels, amount of blood vessel penetration into tumour tissue) and analysing the tumours for the specifc effects of therapy on its intended target. A series of in vitro functional assays and well-established test cascades ensure that the minimum number of animals is used throughout the project. We use “microbleeds” (10-20µL blood samples) and highly sensitive analytical methods to assess circulating drug levels during therapy. This potentially reduces the number of animals in a standard study designed to measure drug levels from over 100 to six.

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 pilot dose-finding studies in small numbers of animals to help guide optimal further experiments. Non-invasive imaging measures of tumour burden such as MRI (magnetic resonance spectroscopy) are utilised which enables assessment of responses over time without harm to the animal.

A retrospective assessment of reduction will be due by 13 June 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

Which animal models and methods 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 may use either mice with a normal immune system, or those which have been engineered with an absent or compromised immune system to prevent rejection of implanted human cancer cells.

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

The use of mammals is required in order to most accurately recapitulate the privileged microenvironment of the human brain, and as such mice are the lowest species that are appropriate for in vivo drug development studies and are widely used for this purpose

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

Suffering is minimised by keeping tumour burdens within tolerable and acceptable limits and using non-invasive imaging. Compounds to be evaluated will have been previously assessed for potency, metabolic stability and specificity prior to evaluation in animals. Small pilot studies in small numbers of animals use the minimum dose predicted to be active based on cell-based assays and low dose
studies before more detailed studies are undertaken. The compounds are generally of low toxicity (e.g. agents targeted to molecules selectively overexpressed or mutated in human cancers). During surgical procedures aseptic techniques will be used to avoid and minimise the likelihood of wound infection, general anaesthesia coupled with pain relief will be administered to limit the transient pain/discomfort from surgical procedures.

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

All procedures are carried out in accordance with the United Kingdom National Cancer Research Institute guidelines for the welfare of animals in cancer research.

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

There are regular notices posted through establishment user groups. We keep up to date with literature advances in the modelling of paediatric brain tumours with a view to implement any novel technique which would assist our 3Rs goals. We are also signed up for the NC3R newsletter.

A retrospective assessment of refinement will be due by 13 June 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

19. Mouse models of paediatric cancer for pre- clinical studies

Project duration

5 years 0 months

Project purpose

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

Key words

Childhood Cancer, Refined therapy, Genetically Engineered Murine Models (GEMMs), Chemo- resistance, Patient derived xenografts (PDX)

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description 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 refined treatments for children with high-risk paediatric (children's) solid tumours - in particular - neuroblastoma (an abdominal tumour) and medulloblastoma (the most common malignant paediatric brain tumour).

A retrospective assessment of these aims will be due by 18 June 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

A significant proportion of children with these tumours are considered high risk, that is, despite intensive treatment they have a very poor prognosis and experience both resistance to therapy, and widespread development of secondary tumours. The events responsible for this are unknown. In addition, many of those children that do survive suffer a number of late effects that are severe or life limiting, such as long-term neurological and sensory impairments (e.g. hearing loss); endocrine deficits, including growth problems; and secondary tumours. There is a definite need to improve treatment options for these children.

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

Publishable data as to how tumours become resistant to therapy. Presentations of data at international meetings, collaborations with clinical, academic and pharmaceutical partners to further interrogate biological unknowns.

Genetically engineered mouse models that are representative of the human disease that can be used to test specific candidate drug treatments.

Publishable development of pre-clinical therapeutic strategies to treat resistant tumours, potentially leading to clinical trials in patients.

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

In the short term we will generate novel in vivo models that better mimic the genetic landscape of human disease and gain more insight into the mechanisms behind metastasis and treatment resistance in these tumours. We will use these to pre-clinically test standard treatments and compounds obtained through our collaborations within our organisation and with external researchers or organisations. This data will also be of use to the wider scientific community, pharma companies and the clinical profession.

Our medium-term aims are that by characterisation and analysis of newly developed models and further development of our existing models we will be able to target the relevant mechanisms and suggest new therapeutic approaches in order to carry out pre-clinical trials with monitoring of markers to measure response. This will allow us to strengthen our relationship with industry as well as the academic and clinical community.

In the long term we will produce data leading to clinical trials as previously achieved under previous project licences. We hope to develop therapeutics that have greater specificity

and hopefully fewer side effects this will be of huge use to industry, the clinical community but ultimately to patients.

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

We aim to disseminate our findings through publication in peer reviewed journals and by presenting data at relevant conferences.

Species and numbers of animals expected to be used

- Mice: 50000

Predicted harms

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

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

We will use genetically engineered mice that are pre-disposed to develop tumours post-weaning, these represent the high-risk disease seen in children. These are the lowest sentient species that are appropriate as they faithfully recapitulate the complex physiology which leads to the development of this type of disease and have comparable metabolism and immune responses.

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

We will breed mice that are genetically engineered to develop tumours. These may be neuroblastoma tumours which arise in the abdominal cavity or medulloblastomas which arise in the brain. We will inject some mice with tumour cells either from patients or mice and observe these for tumour development. Mice will be assessed for tumour formation using the most sensitive techniques to detect tumours as early as possible. These mice may undergo non-invasive imaging to follow the tumour progression. These mice may be given multiple drug treatments over a number of weeks that mimic the type of approach used to treat these tumours in patients. The tumours will initially shrink in response to treatment, but when the tumours return they will be euthanised and the tumours taken for studies as to why they have relapsed with the aim of understanding what happens in patients. In some mice we may obtain tissue and blood for assessing the effectiveness of treatments.

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

Neuroblastoma tumours normally arise in the abdominal cavity and occasionally in the thoracic cavity, the primary adverse effect is weight loss, if the animals lose 18% body weight that doesn't respond to diet supplements they are humanely killed. The primary symptoms of medulloblastoma, a tumour in the back of the brain, are either circling behaviour or domed heads, when they show these signs they are humanely killed, where possible we use non-invasive imaging to detect tumours before the onset of these

symptoms. Using our existing models, we have a great deal of knowledge as to the expected symptoms and time of onset and thus are able to prevent progression of tumours to the stage that they cause ill health. With our new models we will monitor the health of the animals carefully and where possible use non-invasive imaging techniques prior to onset of symptoms. We will test methods of treatments such as surgery and drugs. This could cause pain and weight loss. Pain relief and diet supplement will be given and if they don't respond they will be humanely killed.

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

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

For our genetically engineered models we expect 30 - 40 % of animals to develop a tumour. We expect 60-70% to be non-tumour bearing and hence will have a sub-threshold severity. The majority of tumour bearing animals will be placed on a pre-clinical trial and may be given either a vehicle control - whereby the tumour will continue to grow or given the experimental compound, where the outcome depends upon the efficacy and the type of trial undertaken. The expected severity for most of these will be moderate (25-30%) or mild (5-10%). For our implanted tumour models we expect 50 - 70 % to develop tumours, depending upon the cells/tumour type implanted, these may be used in pre-clinical trials or may be used for tumour passage, these would have a moderate severity. A small proportion of animals used on our dose limit protocol may experience severe suffering, we expect these to be rare as we will be using substances similar to those that we have administered before. However previous data may not take in to account different strains of mice or an escalation of drug concentration which may give rise to adverse reactions that have not been previously experienced or anticipated. In this protocol novel combinations of drug may be administered, this may give rise to adverse reactions due to drug/ drug interactions which we had not anticipated.

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

- Killed

A retrospective assessment of these predicted harms will be due by 18 June 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

The development of tumours depends upon a number of interactions between the cancer cells themselves and the local tissue environment. Tumours can be classified as organs as they comprise a multi-tissue organisation and are continually responding to different environments and stimuli. They are comprised of cellular, structural, and molecular components collectively known as the tumour microenvironment (TME), which hosts a mixture of cancerous, and noncancerous cells which migrate from the bloodstream and neighbouring tissues.

There are many studies aiming to differentiate the relative contributions of these structural, molecular, and microenvironmental processes in disease progression in neuroblastoma. There are also many attempts to model neuroblastoma and medulloblastoma in vitro, using cells cultured to grow as 3- dimentional structures. These 3-dimentional cancer models offer many advantages over 2-dimentional cultures, and they more closely reflect the in vivo tissue organisation. Unfortunately, they are limited in the ability to fully simulate in vivo tissue conditions at the organ level as they lack other tissue properties such as a blood supply and other cell-cell interactions. They represent short-term or static conditions, in contrast to the continually progressing in vivo system.

There have been models of both neuroblastoma and medulloblastoma developed in zebrafish, which have some similarities to their human counterparts. There is, however, a difference between zebrafish physiological temperature (28/29°C) and human body temperature (37°C) which is likely to affect many cellular processes which may be required to make the compounds “active” and are thus fundamental for a therapeutic response. For therapeutic studies there is also the complication of administration via water which may not be suitable, and the absence of general guidelines to calculate mammalian equivalent doses from zebrafish doses.

There is also a model of neuroblastoma using fertilised chick eggs, which has been used for the investigation of tumour differentiation, proliferation, invasion, and migration. This model has the disadvantage of a lack of immune system and that drug metabolism in birds is different from humans.

Medulloblastoma has the additional complication that it occurs in the brain, and as thus any therapeutic compound has to be able to penetrate the blood brain barrier - this is difficult to model in other systems.

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

Currently our workflow involves selecting compounds that have either shown to be effective in another type of cancer or have been developed to target an aberration seen in these paediatric tumours. We assess them for their effectiveness against their target in 2-D cell culture systems before proceeding to in-vivo trials. We have also considered developing 3-D models from patient derived tumour samples in order to triage our compounds before they are tested in mice. However, the availability of material is not consistent, biopsy samples are small and the priority for these is obviously for diagnostic testing not research. Resected tumours are often unsuitable material for culture as they are usually taken after the patient has had chemotherapy and therefore there is a lot of dead tissue and hence it makes it hard to establish cultures.

We are also trying to develop models where we implant tumour samples from patients into mice (patient derived xenografts.) This could lead to a reduction of mice needed for trials by providing reproducible reliable cohorts of animals harbouring identical tumours, avoiding the need to breed animals some of which are non-tumour bearing. However, this work is hampered by the availability and suitability of the source material.

Why were they not suitable?

Although 2D and 3D cell culture models are useful for the initial screening of anticancer compounds and for investigating the biology and characteristics of neuroblastoma and medulloblastoma, they do not completely replicate the complexity of cancer as they do not have the involvement of other features, such as the immune system, which are important in therapeutic testing.

A retrospective assessment of replacement will be due by 18 June 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

Prior knowledge of our genetically engineered murine models allows us to predict how many mice to breed to obtain numbers for our experiments. We will optimise our breeding strategies using the expertise available from collaborators at a national facility and will base calculations of numbers on these strategies. Using information from previous experiments we can determine how many mice we need to use to give 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 a workflow whereby we test the validity and relevance of each new model we develop and therefore make decisions as to how to proceed with minimal number of animals. This depends on the particular model. For those models that are more predictable in their time of tumour onset and with a higher proportion of animals developing tumours, we can vastly decrease the size of animal cohorts used in either biological or preclinical studies. For evaluation of interventions including drugs/biotherapeutics we determine a minimally effective biological dose in pilot experiments and then test in optimised cohorts. We will refer to tools such as the NC3R's Experimental Design Assistant and local statistical expertise to assist. Experiments will be designed to use the minimum number of

mice whilst providing statistically and biologically significant results. We use the most sensitive and least invasive detection and imaging techniques that are available to us to ensure that we select only those animals that form tumours, very early, and then streamlining the duration of their treatment. In our experiments we use both sexes to optimise the use of mice of the desired genotype.

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

When breeding our genetically engineered mouse models we will design breeding programmes that are optimised to deliver the maximum number of tumours by breeding the fewest numbers of mice for each colony, and as above, we optimise cohort size using biological and imaging endpoints. We use protocols that have been optimised to standardise the way experiments are run and data collected in order to reduce variability and sample size. We measure the response by collecting samples that we know indicate whether or not a particular treatment is effective.

A retrospective assessment of reduction will be due by 18 June 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

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

We will be using genetically engineered mouse models of neuroblastoma and medulloblastoma. We will use models that faithfully represent the disease as it presents in patients. We have previously demonstrated that we are able to produce and utilise our models to advance clinical trials. We are advancing our previous research as there is increasing data as to the importance of the immune system in cancer development and in response to therapy. We are able to interrogate the immune response in tumours using recently developed technology that allows us to examine the presence of several different cell types all in one section of tumour and to look at interactions of cell types. This technology also allows us to make important comparisons between the mouse models and human cancers, which may inform us how to proceed. Depending upon the type of study we will define the humane endpoint based on our prior experience but with the exception of our dose limit protocol, these are all within the moderate severity rating and intervention occurs at the earliest point possible that will give meaningful results.

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

There are not currently any other organismal models of cancer with the appropriate metabolism and immune system in which to under take this type of research. Zebrafish have a lower physiological temperature making them unsuitable for therapeutic intervention trials. We use models that are very representative of the clinical disease. The location of tumours is relevant to the treatment plans, for example a major consideration as to treatments for our brain tumour models is whether the drugs are able to cross the blood brain barrier, thus testing in a model that addresses this issue is crucial. We need to assess the effects of drug treatment on living animals.

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

Using information from our current models as to potential symptoms we will closely monitor all animals that are expected to develop tumours. Any new drug regimens will be discussed with Named Persons, i.e. the vet and animal care and welfare officers, in advance. We will use refined techniques such as clinical score sheets, regular observations, combining drugs to reduce the number of injections and use of minimally effective biological dose with analysis of how drugs are distributed in the body and tumour, to minimise harms. Imaging and biomarker based tumour detection will also be employed to minimise uncontrolled growth of tumours and symptoms. We will use analgesia and anaesthesia as recommended to minimise any pain from surgery.

When animals start to show any of the specific symptoms relating to pain from the tumours they carry or the procedure they are undergoing, they will be given analgesia.

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

We will follow The ARRIVE guidelines 2.0 and the NC3R experimental design assistant. For surgical procedures we follow the LASA guidelines on aseptic procedures (LASA 2017 - Guiding Principles for Preparing for and Undertaking Aseptic Surgery).

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

Through our collaborative network we are exposed to data from other model organisms and we will follow developments in alternative technologies, especially at conferences and in publications. We receive regular updates from our animal care teams and Animal Welfare and Ethical Review Board; we will share refinement and animal care knowledge, specifically of cancer models, with all involved in this project.

Staff at the facility we are using adhere to the highest welfare standards and are proactively committed to implementing the 3Rs for the benefit of mouse health and welfare. Staff regularly attend animal care meetings and keep up-to-date with the current guidelines.

A retrospective assessment of refinement will be due by 18 June 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

20. Studies of malaria host-parasite interactions

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Malaria, Plasmodium, Gene function, therapeutics

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

Our research aims to improve our understanding of malaria parasite infection within the host, and to identify and investigate the function of malaria parasite proteins and regulatory pathways that play important roles during the parasite’s lifecycle and so are potential therapeutic targets.

A retrospective assessment of these aims will be due by 19 June 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 one of the most serious challenges to global human health (causing over half a million deaths per annum, mostly children, under the age of 5) and new approaches to the development of anti-malaria therapies are urgently needed. To address this challenge we need a greater understanding of malaria parasite biology and infectivity, and we need to identify, validate and prioritise urgently-needed new targets for anti-malaria therapeutic development.

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

Malaria parasites cause some of the most serious disease in humans globally, despite decades of research into their immunology, cell and molecular biology, and few new treatments or control measures are in the pipeline. This project is therefore aimed at increasing our understanding of parasite biology, infectivity and the host response to infection. We will develop new technologies to investigate these processes, and we will validate potential anti-malarial drug and vaccine targets, and prioritise new targets for anti-malaria therapeutic development. The generation of genetically modified mouse malaria parasite lines, will open the way to directly analyse and image host-parasites interactions. These parasite lines will be important for our own research and will be made available immediately to the scientific community to benefit further studies on malaria.

New knowledge and information from our research will be shared in open-access publications and published datasets, regardless of the outcome, and will be posted to pre-print servers as soon as manuscripts are ready for submission. Outcomes will be also be maximised through the building of collaborations and presentations at local, national and international conferences.

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

Malaria parasites have high medical importance and the development of new control strategies requires a better understanding of their biology. This project will continue to build the foundational knowledge about parasite biology that will form the basis for the development of control/treatment strategies. The scientific community benefits from our expertise in molecular approaches to investigate the malaria parasite, and our development of model systems to investigate host-parasite interactions, attested by our publication record and >20 requests/year we receive from colleagues in the international scientific community for advice on the development and use of mouse malaria model systems, and to receive our genetically modified mouse malaria parasite lines. Up-to-date

information on our transgenic Plasmodium lines and on the range on genetically-distinct rodent malaria parasite lines that are being curated locally is immediately made available on our dedicated European Malaria Reagent Repository website (http://www.malariaresearch.eu). We will also provide information and protocols of improved Plasmodium genetic modification technologies through this website and through publications. We therefore expect that our work will continue to have a significant impact on basic research throughout the project.

Building upon the knowledge and resources we have generated, further studies will pursue an understanding of how parasite gene function affects disease progression *in vivo* using molecular, biochemical and imaging approaches. These studies will additionally use a panel of genetically- modified parasite lines that will be developed within the first three years of the project, leading to impact and publications from year one. During our studies we also aim to characterise parasite genes that are essential for parasite infection and transmission, and to identify and test compounds that block their function (from year two), with the further long-term goal of developing these potential therapeutics to the point where they enter the malaria drug pipeline.

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

We will disseminate information about our research and parasite lines in open-access publications and at national and international meetings, and through regular updates on a designated website. We will also work in association with academic collaborators and outreach advisors to identify new opportunities to collaborate with industry and deliver translational benefits .

Species and numbers of animals expected to be used

- Mice: 3800

Predicted harms

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

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

Infections with malaria parasites (Plasmodium) in adult mice share many characteristics with human malaria infections and transmission, so mouse malaria models offer relevant and amenable model systems for our research. These models have proven immensely useful for studies of the biology of malaria, particularly aspects of the regulation of parasite development in the complex environment of the host that require in vivo work. Advances in our understanding of host physiological and immune defence against malaria have been achieved thanks to the uniquely well-characterized biology of strains of lab mice, Furthermore, the availability of genetically-distinct and transgenic mouse malaria parasites has greatly enhanced our ability to identify and evaluate proteins involved in host-parasite interactions and to assign function to parasite proteins, facilitating the prioritisation of potential drug and vaccine targets for further detailed characterization.

For example, genetically modified mouse malaria parasites have been used to demonstrate that conserved Plasmodium surface proteins play an essential function during parasite transmission into the mammalian host, thus validating them as vaccine candidates, and facilitating the development of transgenic attenuated live parasite vaccine lines. Fluorescent mouse malaria parasite lines have proved invaluable in whole-body and tissue imaging studies aimed at investigating aspects of parasite biology that are associated with parasite virulence and the generation of immune responses that provide protection or sensitivity to infection and severe disease.

In our studies therefore we will use adult mice, as these provide a relevant model of *in vivo* malaria. Where host-parasite interactions are being investigated, we will use specific inbred strains of mice which have well-characterised physiological and immune responses to malaria infection. To generate parasite material for studies of parasite genetics and biology we will maximise output by using outbred mice.

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

Mice used in this project will typically undergo infection with malaria parasites, either by injection or exposure to infected mosquito bite (under anaesthetic), and will then be monitored at least twice a day throughout the course of infection. To monitor infection and collect data, small volumes of blood may be withdrawn by venepuncture /venesection or tail snip (removal of the tip of the tail, and/or the scab formed) daily, mice may be weighed and their faeces may be collected.

Where large volumes of blood are required for parasite purification, ex vivo and biochemical analyses, exsanguination by, e.g. cardiac puncture under terminal anaesthesia may be performed. Such techniques will be completed by an appropriate schedule 1 method. Where histological readout of tissue pathology/parasite accumulation are required, mice may be killed by perfusion with fixative under general anaesthetic without recovery. In all other circumstances, mice will be killed by a schedule 1 method.

Experiments will range from short infections of 3 days to chronic infection of up to 3 months and in the majority of cases, infected mice will experience subclinical, mild or moderate symptoms and make a full recovery. All mice will be euthanized at the end of experiments.

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

The expected adverse effects will result from malaria infection as we need to use in vivo models of malaria to achieve the objectives of this project. Half of the infected mice will experience sub-clinical or mild symptoms. Those mice that do display clinical signs may show the following possible moderate clinical symptoms at days 5-9 of infection (depending on the infection regime); weight loss of up to 10%, limited piloerection, transient hunched position, subdued but responsive behaviour (with normal behavioural patterns and interactions with cage-mates), paling of feet and ears due to malarial anaemia. However, these symptoms are modest and are unlikely to persist for more than 24-48-hours.

In a small minority of cases, mice may experience severe clinical symptoms, including hypothermia, anaemia, reduced activity & responsiveness. However, severe disease is usually transient, and almost all mice make a rapid and full recovery. Importantly, all infected mice are closely monitored (at least twice daily when exhibiting symptoms of infection) and mice not expected to recover are immediately euthanised. In this way, no individual will experience > 48-hours severe disease. We work closely with veterinary staff (consulted whenever unexpected adverse effects are observed), and the endpoint for all mice is a humane method of 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)?

Mice.
Mild; 50% (short-term infection, generating parasite material for analysis, culture and preservation)

Moderate; 46% (acute and chronic infection with parasites that have attenuated virulence; generating parasite material for analysis)

Severe; 4% (acute and chronic infection with parasites that are virulent or have unknown virulence; generating parasite material for analysis)

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

- Killed

A retrospective assessment of these predicted harms will be due by 19 June 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 interaction between malaria parasites and their mammalian hosts, including humans, is highly complex and is dependent on an appropriate immune response and the structural integrity of the vascular system and tissues in which the parasite accumulates. Therefore, to fully understand the role of parasite genes mediating interactions that contribute to parasite virulence and immunity to malaria, they must be studied in the context of the complete malaria parasite life cycle; in the liver following transmission and in the

bloodstream during proliferation. These studies cannot be pursued in detail in humans for ethical reasons and there is currently no in vitro system that can recreate these complex interactions between mosquito, parasite and host. Therefore, the objectives of this project can only be achieved through animal research.

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

The human malaria parasite, *Plasmodium falciparum,* can be cultured in human blood *in vitro*. Although, it cannot reproduce the complex in vivo environment, where appropriate, aspects of the project are carried out using this system.

Why were they not suitable?

In vitro culture systems cannot recreate the complex environment in which malaria parasites interact with host tissues and the evoked immune host. Therefore, to fully understand the role of parasite genes mediating interactions that contribute to parasite virulence and immunity to malaria throughout the parasite lifecycle, animal models must be used.

A retrospective assessment of replacement will be due by 19 June 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

Animal numbers are estimated based on our current usage, and the anticipated number of researchers who will carry out experiments. We calculate the minimum numbers of mice that can be used to achieve our objectives by drawing upon our extensive experience in the design and analysis of molecular malaria experiments and by analyzing all available relevant data sets (including our own previous and pilot studies) which can provide indications of expected effect sizes and observed levels of variation between experiments. We then use advanced statistical and modelling methods to calculate the size of experimental groups and to maximize the quality of the data obtained from each mouse used.

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

We work closely, and share resources and methodologies, with other malaria researchers to reduce overall mouse numbers used and to ensure that each mouse is used to answer as many research questions as possible. Where appropriate, we will carry out pilot studies before designing larger cohort experiments. All experimental design will be carried out 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 use statistical analysis of datasets and computer modelling methods to maximize the quality of the data obtained from each mouse used. Wherever possible, we coordinate experiments amongst the group and with collaborators to maximise the amount of data and tissue obtained and minimise mouse usage.

A retrospective assessment of reduction will be due by 19 June 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

Which animal models and methods 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 research involves malaria parasites that infect rodents in nature, and our experiments require monitoring parasites and hosts during infections and examining host and parasite development and genetics. Adult mice are infected either by mosquito bite (under anaesthetic) or by injecting parasites, and our procedures for infection and sampling are generally expected to cause no more than transient discomfort and mice.

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

Malaria infections in young adult mice reproduce many of the important features of malaria in humans and are therefore the most appropriate choice of model system for studying aspects of host-parasite interactions, parasite biology and parasite gene function that may have relevance in the design of anti- malaria therapies in humans. Malaria parasites only infect terrestrial vertebrates so model systems do not exist for species that are less sentient.

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

I have worked to improve and refine mouse models of malaria throughout my career and, in particular, have pioneered new molecular techniques that have allowed us to move away from using mouse models of malaria that give rise to severe disease, to using a malaria model that more often causes mild or moderate disease symptoms and more closely reflects the complexity of disease and symptoms of human malaria. These refinements have allowed us to obtain more relevant data using fewer animals at a lower degree of severity.

We will continue to revise experimental design and monitoring to further reduce the number of mice used and to minimise suffering, whilst still achieving our project aims.

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/), and will follow local guidance for blood sampling.

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). The University communicates advances and development opportunities regularly. During the current PPL we have implemented procedures to communicate more closely with animal care technicians to inform on the monitoring of animals and how to make interventions more effective. We will continue to foster these lines of communication. In addition, we collaborate with malaria research groups locally and nationally and continue to discuss best practice with them.

A retrospective assessment of refinement will be due by 19 June 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

21. Understanding the mechanisms regulating fibrosis in the lung

Project duration

5 years 0 months

Project purpose

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

Key words

Fibrosis, repair, TGFβ, integrins, signalling

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 required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description 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 ways of treating of Idiopathic Pulmonary Fibrosis (IPF), a chronic lung disease in which the lungs develop abnormal levels of scarring and become stiffer so that breathing becomes increasingly difficult. We hope to identify exactly which substances in the body are involved in these processes and how they promote such stiffening and scarring.

A retrospective assessment of these aims will be due by 19 June 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

IPF is an incurable lung disease which has a prognosis worse than most cancers, including lung cancer. The incidence of the disease appears to be rising but currently around 6000 people are diagnosed every year in the UK. Newly diagnosed patients will receive the news that their likely survival is 3-5 years unless they are eligible for lung transplantation, and that their symptoms of shortness of breath, chronic cough and tiredness will become worse over time, ultimately requiring an oxygen mask to perform simple tasks such as getting dressed. Although there are two drugs available on prescription for treatment of IPF, they are only able to slow progression of the disease, they can neither halt or reverse the scarring in the lungs once it has developed. For many patients, these drugs are either ineffective, or produce unacceptable levels of side effects including diarrhoea, nausea, vomiting, loss of appetite, abdominal pain and sensitivity to sunlight which are significant enough for them to discontinue treatment after a short period of time. In those able to tolerate the drugs, the current treatments can prolong life by a few years although there is no evidence to suggest that they provide a meaningful improvement in the quality of life. Research is urgently needed to improve the speed of diagnosis and to develop treatments that, if started early enough in the disease course, could halt progression or even reverse the lung scarring and provide a cure for this devastating disease.

The lung damage that occurs in patients with IPF is a result of complex processes that occur when wound repair goes wrong in the lung. One of the key substances released by the body during injury and which promotes repair of damaged tissues is Transforming Growth Factor beta (TGFβ). This substance also appears to play a central role in causing the abnormal scarring seen in patients with IPF. What is currently unclear is how the natural repair process goes wrong in some individuals leading to a build-up of scar tissue in the lungs. Understanding how TGFβ is involved in the scarring process is central to developing new therapies for the treatment of fibrotic lung diseases. Recent studies have identified a number of genetic mutations which appear to increase the risk of developing lung scarring and some of these mutations may affect key processes including how much TGFβ is found in the lungs. This project will investigate how TGFβ works, how its levels in the lung are controlled and how, when these levels are not properly controlled, this leads to the development of chronic fibrotic lung diseases. These studies will increase our fundamental understanding of how lung injury, inflammation and fibrosis occur. This will improve our knowledge of wound repair principles in the lung and allow us to identify potential areas for drug development. It is hoped that these investigations will ultimately lead to the development of desperately needed therapies to prevent fibrosis, repair the lung and treat fibrotic lung diseases.

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

In the short term, we hope to advance knowledge of the basic biological mechanisms which regulate the normal and abnormal responses of the lungs during repair. We may improve the chances of developing methods for classifying patients based on the underlying cause of their lung disease. We will share this information through high impact publications and presentations at national and international scientific conferences.

In the medium term, we will work with academic and pharmaceutical industry collaborators to develop and patent new drugs or to re-purpose existing drugs which act on targets that we identify in our studies. We will evaluate and report their effectiveness through publication and presentation of the research. We will aim to generate at least one new drug candidate suitable for progression into clinical trials to treat currently incurable fibrotic lung diseases.

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

In the short term the beneficiaries would be the research team and collaborators both within our institution and across the wider lung fibrosis research community. It would be expected that these data will result in an increase in scientific knowledge about the underlying reasons for the onset and progression of disease in IPF patients and result high impact publications and grant funding.

In the medium term, collaborators in the drug discovery or pharmaceutical fields may benefit from our research.

In the long term it is hoped that novel therapeutics would provide benefits to patients with chronic progressive lung diseases and ultimately provide a cure for conditions that are otherwise fatal.

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

We will maximise outputs and share our findings widely by publishing in open-access journals, by presenting data at national and international scientific meetings and by utilising our institutional website resources and social media eg Twitter feeds to amplify and share the results. The fibrotic lung disease research community is highly collaborative, and our research group has a large network of collaborators with data, protocols and samples often being shared with other researchers ahead of publication to speed up the progress in finding a cure for lung fibrosis. We also have close links with Action for Pulmonary Fibrosis UK, a patient support charity which also funds research, and we regularly share our latest research findings with their members and amplify them through their extensive network of partner organisations. We will continue to actively pursue this collaborative approach to research wherever possible. We are also committed to sharing all data from our animal and other studies including reporting the outcome of negative studies

Species and numbers of animals expected to be used

- Mice: 7800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 the lung injury and fibrosis studies, and these may include studies in which mice are aged. Whilst some of the key processes which regulate the develop of fibrosis might be common across different fibrotic diseases and occur in people of all ages, idiopathic pulmonary fibrosis is typically considered a disease associated with aging. As a result, there may be specific substances in the body or specific 'pathways' which are involved in the development of lung fibrosis which only become active in later life, requiring us to study these in aged animals. Mice are the lowest vertebrate group in which lung injury and fibrosis models are well understood and are therefore the most appropriate models to use in these studies. The models selected for our studies will be chosen based on the disease processes being studied and the endpoints required. Special types of animals (wild type) with predictable responses to lung injury will be used to reduce the number of animals required. Studies using genetically altered (GA) mice may be crucial to improve our understanding of lung fibrosis if there are no substances available which mimic the effects we wish to study. These genetically altered mice will be specifically bred for any proposed studies and depending on experimental requirements, may have different genetic make-ups from the normal, wild type mice.

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

Studies to identify the best dose levels for novel drugs will be conducted in healthy uninjured mice where possible to minimise the number and impact of the procedures being performed. These studies will typically involve a single administration of a drug by the most appropriate route followed by taking very small samples of blood across a 24 hour period and collection of body tissues when the animal is humanely killed at the end of the study. For some studies, techniques may be used to take images of the animals using a CT scanner to study drug distribution in the lungs and/or to see how effective the drug is the drug once it is in the lungs. This is a non-invasive technique and does not hurt the animal.

In some studies animals will be exposed to a chemical drug called bleomycin which causes injury to the lungs and triggers the development of the abnormal repair process associated with the development of IPF. We will monitor these changes in the lung over time, typically for up to 14 or 28 days. By adjusting the amount of the bleomycin used, the level of lung injury and the symptoms experienced by the animal can be varied and we will always use the lowest degree of injury, and the shortest timeframe to answer our scientific questions. In many of our experiments, animals with injured lungs will be administered a drug on one occasion and be monitored for up to 24 hours to measure the short-term effectiveness of the drug. In later studies, we may aim to study the effects of that drug over a longer period, typically up to 10-14 days to see if they might be beneficial in either preventing development of lung scarring, or in slowing down the development of the disease.

Aged mice may also be used in these studies if process driving the abnormal repair response we are studying appears to only happen in older animals. Mice may be aged up to 18 months and then given bleomycin to cause a lung injury to investigate how fibrosis develops in aged individuals. Alternatively, some of these studies, particularly those in genetically altered animals, may involve monitoring of mice for the signs and symptoms of lung fibrosis as they age naturally for up to 18 months even in the absence of a chemical lung injury. This may allow us to understand whether a specific genetic mutation in the lungs, can trigger the spontaneous development of fibrosis and scarring. The monitoring methods used for these long-term studies may include the use of non-invasive CT scanning of the lungs every few months with tissue and blood samples being collected at the end of the study.

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

Most of the animal models require a lung injury to be brought about which then leads to the development of fibrosis as part of wound repair process therefore animals will be carefully monitored to ensure their well-being throughout the course of these studies.

Our extensive previous experience with these lung injury experiments using normal and genetically modified mice shows that they are associated with altered patterns of breathing, including increased rate and depth of breathing and are always accompanied by brief weight loss which peaks 10-14 days after giving the specific chemical to cause the lung injury. Mice may have a hunched posture and fur standing up on end when experiencing weight loss, although this typically resolves over time. The symptoms resulting from damage to the lungs may last throughout the study.

In aging studies, mice may spontaneously develop lung diseases including emphysema or fibrosis, resulting in the development of symptoms similar to those exhibited by animals following lung injury.

A small number of animals may undergo surgical procedures for implantation of controlled release drug pumps and this procedure will result in brief discomfort around the surgical site which will be alleviated by administration of pain relief.
Other groups of animals may undergo non-invasive imaging after being given a radioactive substance which can be tracked, but this process is well tolerated and apart from the stress of being restrained prior to receiving the injection of radiotracers and the effect of prolonged anaesthesia (up to 1 hour) they are not expected to show significant adverse effects.

Animals may also experience mild, brief discomfort where repeat blood sampling is required, for that reason blood and tissue samples will be collected post-mortem where possible.

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

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

Mice - 60% moderate, 30% mild, 10% severe

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

- Killed

A retrospective assessment of these predicted harms will be due by 19 June 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

The processes which regulate the development of lung scarring following injury are complex and appear to involve interactions between the cells within the lung and the cells which circulate in the blood. Although where possible we have replaced screening or "blue sky" experiments in animals with those performed in tissue culture and test tubes and using healthy and diseased human tissues, it not always possible to mimic all of these potentially important interactions using these systems due to the lack of blood supply and interplay between multiple different organ systems. As a result, addressing some of our key scientific questions will require live animal experiments.

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

We routinely use different types of cells isolated from human tissue samples, tissue samples themselves and simplified 3D living models of the lung created using isolated human cells in our studies. We will continue to use these as appropriate to address fundamental biological questions. We are also exploring the potential of organ-on-chip lung systems to address some of our key questions.

Why were they not suitable?

These non-animals model systems are not yet capable of fully replicating a living multi-system organism with lungs which are constantly moving during the act of breathing and interact closely with the circulatory and immune systems. Although they are being rapidly improved, in their current form, they do not reliably model the complexities of the tissue microenvironment in the lung, or the interactions between the 40 different cell types found there. We will continue to use the models where possible and will also work to further develop and improve their complexity for modelling human systems. We will also use them as a screening tool to ensure that animal experiments are performed only where there is the possibility of generating meaningful data that has potential to change the way we treat patients who suffer from lung injury and fibrosis.

A retrospective assessment of replacement will be due by 19 June 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

Many of the procedures and protocols described in this project have been developed and used extensively under previous PPLs held by my research team since 2009. I have developed and refined these models over many years and generated scientifically robust datasets which have been published in peer reviewed journals.

The total number of animals estimated on this licence is based on my previous experience using our established protocols, the effect sizes we would expect to see based on previous studies and data from our annual HO returns over this period.
When planning to establish a new model for the first time, I have had personal communication with the researchers responsible for the published literature and have estimated the animal numbers required to establish, refine and utilise this model for our studies based on their experience with the model and the effect sizes they have seen consistently.

The overall estimate includes sufficient animals for pilot and repeat studies where required, includes control animals eg those which might receive a placebo in place of a drug whose effects are being assessed. They also include a significant number of animals required within the breeding programme. We will aim to use breeding methods which maximise the use of as many littermates as possible when creating genetically altered animals for use in this project and will utilise experimental designs which allow data to be collected from animals of both sexes.

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

All experiments using animals will test a specific theory or have a defined objective and be conducted in a manner that ensures that high quality, reproducible and useful data is extracted from the minimum number of experiments and allows publication according to the ARRIVE guidelines. We have utilised both the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines, the NC3Rs experiment design assistant and historical data to inform both our choice of model

and the study design, aiming to ensure that the minimum number of animals are used to achieve our scientific objectives. Advice on the proposed experimental designs and planned statistical analysis has also been sought from a dedicated statistician within our research team to ensure the projects include an appropriate number of animals for results to be scientifically useful.
These consultations will continue as the projects develop and experimental designs are refined.

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 mice known to give consistent lung injury using our established protocols to reduce variability and therefore minimise the number of animals required for our studies. The majority of the wild type (normal) animals using in these studies will be obtained from commercial breeders. We will generate new genetically altered mouse strains only if they do not already exist and cannot be obtained from another research group. Where breeding of mice is required for our studies, we will use the most refined breeding methods in order maximise the number of mice of the required genetic background being generated for our study and minimise the creation of littermates which would be unsuitable for our experiments.

The lung injury models developed by our research team have been refined over many years to produce the most reliable data. Where possible we will use non-invasive measures of injury and fibrosis, such as CT scanning and blood microsampling to obtain as much experimental data as possible from a single animal.

We will use pilot studies to see how effective new drugs might be, using methods with the lowest severity and in the shortest timeframe possible. Only drugs with scientific evidence to suggest they may be effective in treating disease will be used in lung injury models which may have a higher severity of impact.

We have an extensive network of collaborators, both within our own institution and across the UK and will endeavour to share tissues and/or biological samples with other licence holders to maximise the data generated from the use of any one animal. We will also use these connections to source samples eg tissue for laboratory analysis from previous animals studies if it allows us to address scientific questions without repeating a study in-hous

A retrospective assessment of reduction will be due by 19 June 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 clear that to date, no animal model fully replicates all the features of lung fibrosis seen in patients with IPF. Therefore the aim of model selection is to develop or utilise the model which mimics as many of the molecular, biological and structural changes observed in IPF patients as possible whilst minimising the level of pain, suffering, distress, or lasting harm experience by the animals involved.

The pattern of fibrosis seen in patients with Idiopathic Pulmonary Fibrosis (IPF) is characterised by the development of parenchymal fibrosis, scarring which is found specifically around the lung airsacs in response to damage to the cells lining these airsacs. Any model to be utilised for our research would therefore need to demonstrate this characteristic feature to some extent.

In considering the choice of model for the study of the biological processes which result in the development IPF, we evaluated a number of different models each of which has been shown to elicit a fibrotic response in the lungs. These included a transgenic mouse model in which the key molecule which promotes fibrosis, TGF beta is produced chronically within specific cells in the lungs (Clara cells) in response to drug treatment (tamoxifen). Although this model was associated with mild lung injury and was well-tolerated, the resulting fibrosis was confined mainly to the airways and the resulting disease pattern more closely resembled asthma than IPF.

We also considered the elastase and cigarette-smoke models of lung injury, both of which have been shown to develop some degree of early-stage fibrosis post-injury both in the airways and lung parecnchyma following a significant inflammatory injury. Although these models may be considered less severe than the bleomycin model, the major feature of both is that the fibrotic repair response occurs after the development of inflammation and emphysema, a process which results in destruction and enlargement of the airsacs occurs in the lungs. Significant inflammatory white blood cell recruitment and enlargement of the alveolar airsacs is not a typical feature of patients with IPF, indeed these two models more closely resemble the disease pattens associated with Chronic Obstructive Pulmonary

Disease (COPD). The enlargement of the airsacs in response to injury makes the lungs floppy, and as a result these models are not suitable for studying the mechanical signals responsible for development of lung scarring and stiffening of the lungs in IPF, a key feature of our research plan. In addition, the presistent presence of a high number of white blood cells, not typically found in the lungs of IPF patients in the absence of an underlying infection, may interfere with our ability to extrapolate our findings to the human disease condition.

The development of fibrosis is a complex process and there may be some shared pathological mechanisms involved in a range of lung diseases including asthma, COPD and IPF. However, given the body of published evidence and our experience with the use

and refinement of the bleomycin model, we believe that bleomycin models replicate more of the key features and biological processes involved in development of human parenchymal fibrosis and IPF, and that bleomycin-induced models are the most relevant model in which to study the disease-causing pathways and identify and evaluate new ways to treat the disease. We will typically use one of three different versions of the bleomycin-induced lung injury and fibrosis models for our research studies and will always aim to conduct studies in the model with the lowest severity possible to address the scientific objectives. Each of these models are based on delivery of bleomycin directly into the lungs which results in lung injury. As a result of the injury, lung cells being to divide rapidly to repair the injury, filling the airspaces and stiffening the lungs with the development of parenchymal fibrosis which ultimately makes breathing more difficult.

In the most widely published bleomycin model, a single high dose of bleomycin is given causing a predictable response which can be divided into 2 phases. The first phase (the acute injury phase) lasts up to 10 days and is followed by a fibrotic repair phase in which fibrosis is fully established 3-4 weeks after the injury. This lung injury model is associated with brief but significant weight loss which peaks after 7-10 days, and this is followed by development of breathing symptoms which get worse as the fibrosis progresses. We have used this model extensively and our previous data has shown that the degree of weight loss is proportional both to the amount of lung injury caused and to the level of fibrosis in the lungs. In order to better understand the ways in which fibrosis develops after a lung injury and to test new ways to treat the resulting lung scarring, it is necessary to perform studies in a disease model in which animals develop pronounced lung fibrosis. We have found that a significant degree of fibrosis is detected only in the lungs of animals which experience weight lose greater than 10% of their body weight within the first 10 days of the study. Due to the high dose of bleomycin used, the degree of lung injury can sometimes be variable with this model and unlike human fibrosis, if left for up to 3 months, the lungs of animals treated with this high dose approach seem able to resolve or repair the scarring itself. As a result these high dose studies are limited to a maximum duration of 28 days.

In order to continue to refine out methods to improve the modelling of human fibrotic lung disease in mice, to reduce variability and also lower both the amount of lung injury and reduce the symptoms experience by the animals, we will also establish and test a new lung fibrosis model based on recently published work. Mice will receive a low dose of bleomycin into their lungs on 3 occasions, as this is reported to promote a repair and fibrosis response which better mimics the features of human fibrotic lung disease and the lung does not spontaneously undergo repair if left for up to a year post lung injury. In our studies, his repeated low dose bleomycin procedure typically lasts 56 days. Although the overall severity is unlikely to be reduced compared the single high dose bleomycin protocol, the degree of lung injury, weight loss and breathing issues after each bleomycin challenge are reported to be lower in addition to the model showing more features of human fibrotic lung disease. Itcan therefore be considered to be an improved and more refined lung injury model.

Where possible, and where potential drug targets are present in healthy lungs, we will use healthy uninjured animals to investigate the way newly designed drugs behave, minimising the impact on animals in the study. However, for some drug studies it may be necessary for animals to be subject to lung injury. We have therefore developed a single low dose bleomycin method which allows us to investigate many key pathways involved in fibrosis over a shortened 7 or 14 day time frame. This model typically results in only 5-10% body

weight loss and is associated with minor breathing issues. Coupled with more sensitive methods such as non-invasive imaging or analysis of substances of interest in lung cells or blood, this model allows us to perform many of our studies investigating the effectiveness of new anti-fibrotic drugs thereby reducing our use of the high dose bleomycin model by 30-50% .

We will continue to refine our models based on the newest scientific evidence and will select the model most appropriate to provide the data required to address each biological question but with the lowest risk of causing pain, suffering, distress and lasting harm.

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

Mice are the lowest vertebrate group in which lung injury and fibrosis models are understood and are therefore the most appropriate models to use in these studies. As fibrotic lung disease is primarily an adult disease with an increased risk of developing in ageing populations, immature life stages would not be appropriate for our research studies.

Injury to the lining of the lung is one of the key features of many fibrotic lung diseases and as these cells are highly plastic and undergo significant changes during lung development it is important to use adult or aged mice in the studies. To understand how fibrosis develops and to develop new methods for preventing or reversing this process, it is important to perform studies over a period of days or weeks in animals with lungs which retain the ability to breath, extract oxygen and nutrients from a constant blood supply, and which are protected by a working adult immune system. For this reason, it will not be possible to utilise anaesthetised animals for many of our studies due to the progressive nature of the disease and the need for an initial lung injury to be followed by a period of lung repair resulting in fibrosis.

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

We have refined our laboratory procedures to increase the quality of the data we obtain, and this has allowed us to develop animal models with different degrees of lung injury for use in our studies. As a result, in many of our studies we have been able to shorten the duration of experiments, reduce the severity of the symptoms and minimise the suffering that might be experienced by the animals whilst still obtaining meaningful data. These model and endpoint refinements will continue.

As a result of our extensive previous studies, we have established a detailed health assessment and scoring system which closely monitors weight loss, body condition scoring, breathing and behavioural changes in the animals on a daily basis and ensure that any potential suffering of mice on procedure is minimised. We also have a rigorous training programme to ensure that all studies are conducted by researchers who are both competent and highly trained in undertaking the procedures but also in assessing and managing the health of the animals involved in our experiments. All our studies have detailed protocols with clear humane endpoints which have been developed in consultation veterinary and animal facility staff and these endpoints will remain under constant review.

Under our detailed health assessment procedures, the frequency of monitoring and weighing is increased in animals which are more likely to deteriorate eg after dosing with new drugs. Uncommonly some animals do fail to recover completely, or show signs of pain, distress or ill health during the studies which were not expected. Any animal showing signs of pain, distress or of significant ill health will be humanely killed unless a programme of enhanced monitoring, potentially with supportive therapy, is appropriate and can be put in place until the animal fully recovers. This detailed monitoring may involve health assessments being made for up to 8 hours after symptoms are identified, and these checks will be performed outside of normal working hours where necessary to 247inimize the risk that animals may suffer from being retained in a study overnight.

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

We will use guidance issued by the NC3Rs and LASA, the guidelines published by PREPARE and will also ensure that we design studies and generate data which comply with the ARRIVE guidelines.

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

We will regularly check for information on the NC3Rs website, and attend the Regional NC3Rs symposia to ensure we are aware of advances in the field specifically related to the 3Rs. As an NC3Rs grant holder, the PPL holder will also be in regular contact with the NC3Rs Policy and Outreach team and with the network of other NC3Rs funded researchers to share information and best practice relating to non-animal alternatives for our in vivo models. Finally, we will attend national and international conferences to ensure that we aware both of refinements to our current in vivo models which may be suitable for adoption into our programme of work, or of any new, cutting edge in vitro models with increased complexity which may be suitable alternatives to our current in vivo models.

A retrospective assessment of refinement will be due by 19 June 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

22. Identifying the molecular mechanisms of appendage and organ regeneration in zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Regenerative biology, Regenerative medicine, Appendage regeneration, Heart regeneration

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

The aim of this work is to identify the molecular mechanisms allowing zebrafish to regenerate their caudal fins and hearts following injury.

A retrospective assessment of these aims will be due by 21 June 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

Regenerative medicine is a rapidly evolving field of medicine, which aims to identify and ultimately implement novel therapies that will promote the repair and regrowth of damaged tissues and organs in humans, as popularised by the medical potential of stem cells. Rather than studying stem cells per se, or how they might be used clinically, my lab studies organisms that naturally have high regenerative capacity, such as fish, in order to tease out the mechanisms that bestow them with such high regenerative capacity, such as regrowing fins or regenerating their hearts following injury. The goal of this project is to identify the molecular mechanisms that zebrafish employ, enabling them to regenerate their caudal fins and hearts following injury.

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

The primary output of this project will be a greater understanding of the cellular and molecular mechanisms of appendage and organ regeneration in zebrafish. During the work, we will generate and/or characterise a number of genetic mutant strains in zebrafish, which we will make available to the scientific community. To facilitate the distribution of the genetic mutant strains that we generate as part of this work to the scientific community, we will deposit them in the European Zebrafish Resource Centre. We aim to publish all our findings in high quality, peer reviewed and open access journals.

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

Short-term benefit - the identification some of the key mechanisms that zebrafish employ to promote regeneration of complex tissues, such as appendages and organs.

Mid-term benefit - the development and testing of new drugs that promote regeneration in zebrafish.

Long-term benefit - the development and testing of new drugs and/or therapies that will promote a greater regenerative response in human patients, following injury.

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

This work is part of an International Training Network, primarily funded by the European Union. This network is multidisciplinary and includes internationally leading labs across 10 European countries. As such, this work is part of a large consortium of labs, and we will be meeting and discussing our findings on a regular basis. This collaborative consortium will be a primary avenue for us to disseminate our findings, but also in maintaining abreast of the current progress of the other consortium members.

Apart from these mechanisms, we will also attend international conferences on regenerative biology / medicine in order to disseminate our findings to the scientific community and to also learn the latest advances in the field. We will aim to publish our

findings, whether negative or positive, in high impact, open access journals, thus ensuring maximum impact of our work in the field.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 26500

Predicted harms

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

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

Zebrafish have extraordinary regenerative capacities, which allow them to fully regenerate their fins after amputation, and they can regenerate many organs, including the heart, following injury. They retain this high regenerative capacity throughout life. As such, we can investigate some of their regenerative capacity at the larval, pre-feeding and thus pre-protected stages. Indeed, we plan to perform many of our experiments at these early larval stages. However, it is essential that we also carry out some of our appendage and organ regeneration studies in adult animals, because only adults have a mature immune system and greater physiological complexity. Thus, our work aims to investigate the cellular and molecular mechanisms that permit zebrafish to regenerate complex tissues and organs throughout life.

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

The main intervention that will be performed on the animals used in this project will be genetic intervention / alteration / modification. This will involve either the generation or maintenance of genetically altered (GA) zebrafish lines. Small biopsies or skin swabs may be taken from some of the GA fish in order to isolate genomic DNA for genotyping. In addition, given that the primary aim of the work covered by this licence is to investigate the genetic, molecular and cellular mechanisms responsible for zebrafish's high regenerative capacity in the adult, there are two additional interventions that some of the adult zebrafish will be subjected to. One is caudal fin amputation and the other heart injury. These two injury models are used to activate the regenerative response in the adult zebrafish. However, given their very high healing and regenerative capacities, the adult fish can fully regenerate their caudal fins within 3-4 weeks and to fully regenerate their hearts within 2 months.

Finally, some of the adult fish that have had their fins or hearts injured may be subjected to chemical/drug treatments, either via immersion or via injection. The amounts of the chemicals used and their frequency of delivery will be carefully adjusted so to minimise any adverse or toxic effects to the fish, while ensuring maximal efficacy of the chemicals/drugs used.

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

We do not expect that any of the genetic alterations / modifications generated or maintained under this licence will cause lasting harm, therefore this is listed as mild interventions. Indeed, our hypothesis is that mutations in genes we are investigating encoding will be able to heal and regenerate better than wild-type animals. In addition, obtaining eggs and sperm from adult fish will also not cause lasting harm to the fish, and there are also listed as mild interventions. The two injury models induce a full and complete regenerative response, but these take from 3 weeks to 2 months to complete. Therefore, the caudal fin injury model is listed as Moderate given that full recovery of the fins takes up to 3-4 weeks. And, while the adult heart injury model also culminates with full functional regeneration of the heart, this takes up to 2 months, therefore the heart injury model is listed as Severe.

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

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

We expect a significant proportion of the GA animals (maintenance and breeding of GA zebrafish) to experience sub-threshold severity, as the genetic modifications are not expected to cause any harm to the animals. For the subset of the GA animals that need to be anaesthetised in order to obtain samples for genotyping and those anaesthetised for the purpose of obtaining eggs and sperm will be returned as having experienced mild severity. We expect that the majority of adult fish subjected to the injury models (caudal fin amputation and heart cryoinjury) will experience moderate severity, with a minor number of the latter experiencing severe symptoms, such as excessive and persistent bleeding following surgery.

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

- Killed
- Kept alive

A retrospective assessment of these predicted harms will be due by 21 June 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 objective of this project is to uncover the mechanisms that allow adult zebrafish to regenerate complex tissues, such as appendages and organs, following injury. It is

currently not possible to fully emulate the complexity of multi-tissue environments present in appendages and organs, including circulation, in vitro over the time required for these complex tissues and organs to regenerate fully in vitro. It is thus necessary to perform much of our work in vivo. While we can and will perform some of our preliminary studies in pre-feeding stage larvae, the bulk of the work aimed at understanding the mechanisms employed by the adult animals to repair and regenerate appendages and organs will necessitate using the adult stages for our analyses. For this project, we have focused our work on adult zebrafish, not only because of its remarkable capacities to heal and regenerate complex tissues and organs, but also, because it is an aquatic vertebrate with lower neurophysiological sentience.

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

While we can perform experiments using cell culture systems, as for example to test whether chemical inhibitors have the expected activity in cell culture assays before using them on zebrafish larvae or adults, these non-animal alternative strategies cannot be relied on to determine fully the genetic, cellular and molecular mechanisms of regeneration of complex tissues, such as appendages and organs, as such complex structures cannot be reproduced using cell culture. Instead, the primary way in which we replace the use of adult animals in some of our work is to use pre-protected larval stages.

Why were they not suitable?

We use cell culture and pre-protected stages for preliminary studies but given that the primary aim of this project is to investigate the mechanisms employed in adult zebrafish to repair and regenerate their appendages (fins) and organs (hearts), the alternative non-animal or pre-protected stage experiments cannot be relied on exclusively to achieve the overall aim of the project.

A retrospective assessment of replacement will be due by 21 June 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your 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?

The number of animals has been estimated based on previous licences covering our work. We have based our assumption that we will use a similar number of animals as in the previous 5 years.

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

In all our studies we aim at reducing animal numbers to a minimum by using the NC3R's Experimental Design Assistant (https://www.nc3rs.org.uk/experimental-design-assistant-eda) web application and the PREPARE guidelines (https://norecopa.no/PREPARE). We will also consult with our in-house statistical service for advice during this project. More specifically, we have applied to all our experiments very stringent methods to obtain statistically meaningful results with the minimum number of animals possible.

Data analysis will be conducted according to a pre-specified statistical analysis plan drawn up in conjunction with establishment-based statisticians. Important experimental results will be designed with biological replicates and repeated and validated via an alternative follow-up experiment to minimise the likelihood of spurious non-replicable results.

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

Most of the animals estimated to be used in the project will be breeding and maintenance of GA zebrafish. Thus the primary means by which we aim to reduce the numbers will be through improved husbandry and efficient breeding approaches.

We will also endeavour to freeze down our GA strains when they are not immediately needed and, in this way, reduce the number of animals needed simply to maintain the strains. We also will aim to reduce the numbers of animals used for the regeneration experiments through refinement of our regeneration assays.

A retrospective assessment of reduction will be due by 21 June 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

Which animal models and methods 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 larvae and adults are particularly well suited to this project because they have remarkable capacities to heal wounds quickly, without leaving scars. In addition, they can fully regenerate complex tissues and organs within weeks following injury. This makes

zebrafish particularly useful for investigating the genetic, cellular and molecular mechanisms of tissue repair and regeneration of complex tissues, such as appendages, and organs, such as the heart. While performing injuries in zebrafish will cause discomfort, the fish fully recover and given their remarkable regenerative capacities, they fully recover from these injuries.

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

Zebrafish is a low-complexity vertebrate model with low neurophysiological sentience. While there are some invertebrates that also exhibit remarkable regenerative capacities, such as planarians, hydra and some crustaceans, these invertebrates lack the complex tissue composition and anatomy representative of vertebrate appendages and organs, which are the subject of study in this project.

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

Most of the protocols in this project, including the husbandry and injury models, are well established for zebrafish both in the literature and in our laboratory, and this includes refinements that minimise the welfare costs of the animals. All procedures that may result in transient distress are carried out under anaesthesia, which also helps immobilise the animals during imaging sessions or when gametes are stripped from the animals. Nevertheless, we are constantly looking to further refine all our procedures to minimise any potential harms. As for example, we recently refined our caudal fin regeneration experiments such that we no longer assay regeneration in groups of animals, but rather, in individual animals over time. This has allowed us to obtain much higher quality data on fewer animals, as the animal-to-animal variability is now removed. We will continue to aim to refine our experimental regimes like this so that we can further reduce the number of animals needed to obtain high quality appendage and organ regeneration data. We will also continue to monitor the published literature for further refinements in these injury models in zebrafish, and we will actively investigate the use of analgesics, such as lidocaine, during the injury models, in terms of the efficacy and also in terms of whether their use impacts negatively on the healing and/or regeneration responses that we are investigating.

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

There are many useful resources on the NC3Rs website (e.g. NC3Rs experimental design assistant). We are constantly reviewing and improving zebrafish husbandry techniques following the advise from other labs and from advised from The Zebrafish Information Network (ZFIN).

The new PREPARE Guidelines will also be strictly adhered to (https://norecopa.no/PREPARE.)

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

We have previously submitted several applications for funding from the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs), and we have previously held an NC3Rs grant. We also maintain strong links with local and national advisors. NC3Rs regularly holds online workshops and institutional events aimed to improve experimental procedures with a focus on the 3Rs. We meet regularly, review and refine our protocols following discussions between our researchers, Named Animal Care & Welfare Officers (NACWOs) and NVS.

A retrospective assessment of refinement will be due by 21 June 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?