



Home Office

# **Animals (Scientific Procedures) Act 1986**

Non-technical summaries for project  
licences granted January - March 2024  
that require a retrospective assessment



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# 1. Glial Cells in development and adulthood

## Project duration

5 years 0 months

## Project purpose

- Basic research
- Translational or applied research with 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

Myelin, Glial cells, ageing, Regeneration, plasticity

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

The brain and spinal cord, which make up the central nervous system (CNS), is made up of mainly 1) nerve cells (neurons) which conduct electrical impulses and 2) glial cells which include many different diverse cell types with imperative roles that among other things, support neuronal function. We are interested in how glial cells in the CNS contribute to maintaining overall CNS health/repair when we are young, how these mechanisms change (and mainly deteriorate) as we age, and how we can identify targets of intervention to promote the health and regenerative capacity of the CNS with ageing.



## **A retrospective assessment of these aims will be due by 05 July 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?**

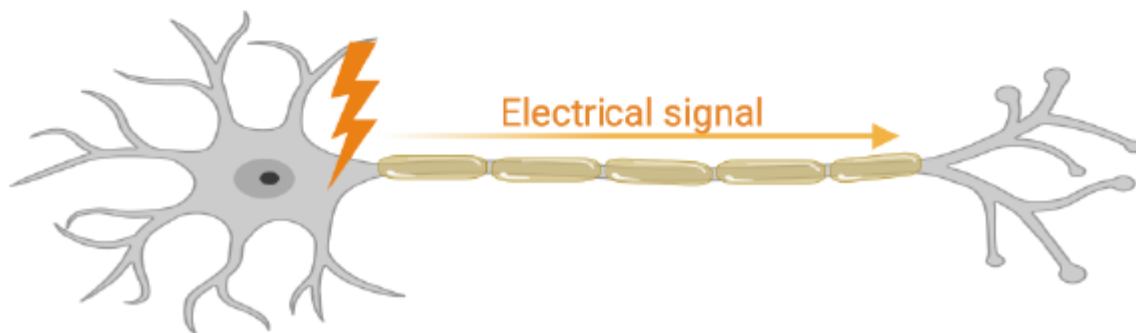
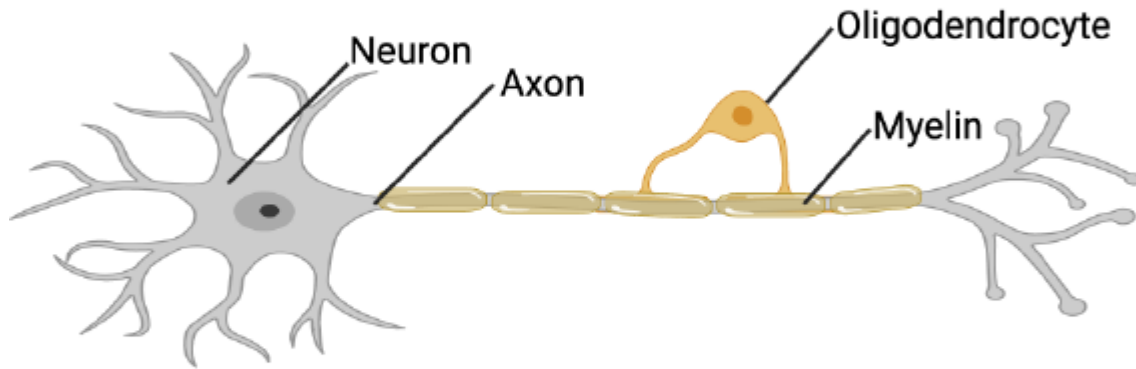
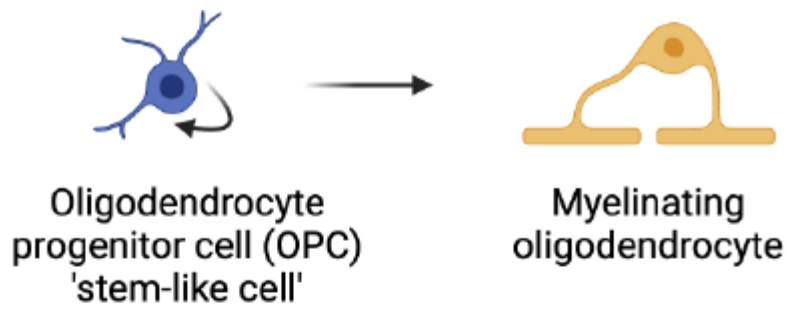
Research into the ageing brain is important for several reasons. Firstly, as the global population ages, the incidence of age-related neurological disorders such as Alzheimer's disease, Parkinson's disease, and other forms of dementia is increasing.

Understanding the function of glial cells in the ageing brain can help us develop treatments for these conditions. Furthermore, studying glia in the ageing brain can provide insights into the process of ageing itself, which is still not fully understood. This could lead to interventions that slow down ageing or improve health in old age.

Here we use rodent models to look closer at glial cell biology. Glial cells provide support and protection for neurons. They are responsible for tasks such as providing nutrients to neurons, repairing the nervous system following injury or damage, maintaining the balance of ions in the extracellular environment, and modulating signals transmitting across neurons. There are several types of glial cells, including astrocytes, oligodendrocytes, and microglia, each with their own specific functions.

Our main glial cells of interest in the lab are those in the oligodendrocyte lineage which includes the oligodendrocyte precursor cell (OPC) and the oligodendrocyte, both of which have important roles in supporting neuronal function. Neurons conduct electrical signals along their axons (long process extending from the neuronal cell body). Axons are wrapped in layers of fatty membrane called myelin. A good analogy for myelin is that you can think of it like the insulation along an electrical wire which helps to protect the wires underneath and helps to speed up the current in the wire.

Myelin not only allows nerve cells to communicate rapidly but also maintains the health of nerve cells. Myelin in the CNS is produced by oligodendrocytes, which are generated by their progenitor cells (OPCs) mainly during early development after birth but also throughout life. In some areas of the brain, the distribution of myelin adapts to the functional conditions, for example, new myelin can be produced by newly formed oligodendrocyte from OPCs during learning a new skill (and we call this adaptive myelin). Damage of myelin in diseases and injury can also be restored by newly formed oligodendrocytes (and we call this regenerative myelin). If loss of myelin is not restored, the axons will fail to function properly and therefore the neurons are prone to dying. Oligodendrocyte lineage cells and myelin are illustrated in the diagram below.



Both adaptive and regenerative oligodendrocyte and myelin generation decline with age, contributing to most age-related neurodegenerative disorders. One of these diseases is multiple sclerosis, in which the destruction of oligodendrocytes and the failed regeneration of myelin leads to loss of axons and progressive and irreversible functional deficit, for which there is no treatment in the clinic. How changes in myelin contributes to age related decline and neurodegenerative disorders is understudied, often because of the sheer cost of ageing rodents and the perceived complications specific to testing outcome measures in aged animals. With that being said, that underpins the reasons this work is so imperative to conduct in an ageing rodent population making the work proposed here even more clinically relevant and valuable to the scientific field moving forward.

Understanding the key cellular process from OPCs to oligodendrocyte is crucial to identifying the cause of age-associated decline. The successful process is not only determined by the machinery inside the myelin forming cells, but also driven by numerous factors created by all other cells in the tissue environment. By identifying these intrinsic and extrinsic regulators and their changes in ageing and disease conditions using animal models, we will be able to gain new insights for developing new therapeutic strategies for the unmet clinical needs for multiple sclerosis, which will likely also benefit the treatment of a wider spectrum of neurodegenerative diseases.



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

The outputs of the project would be a deeper understanding of the biology of myelin in the central nervous system. We expect to reveal novel factors which play indispensable roles in myelin maintenance and regeneration, and those contributing to age-associated functional decline. Periodical achievement will be primarily in the form of publications in peer reviewed journals, presentations in scientific meetings, and filed intellectual properties. The new mechanisms of action identified from this project may offer targets for interventions for clinical translation.

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

The project allows us to conduct research programmes towards our goals. In short-term, the findings from individual experiments answering specific questions will culminate in advancement in understanding the biology of myelin in the central nervous system including its normal functions and regeneration following damage. It will not only address the predefined objectives of the current project, but likely help get new directions of research and create new projects. The publication of interim achievement can benefit researchers in the science community which will contribute to the progress in the subject field. Ultimately, we expect the genes, regulatory pathways and novel targets of intervention identified in the current project will facilitate clinical translation in age-related CNS disorders.

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

We will ensure that the work under this project will be conducted properly and efficiently. The results will be shared in various platforms such as publications on high impact peer reviewed journals, international conferences and other forms of talks and presentations. These include not only the research findings, but also the development of experimental approaches and technical advances. A large proportion of our experiments will be conducted in collaboration between researchers within the group, with other groups within the institute or from other institutes worldwide, through exchanges in experimental design, supplementary technical expertise and material sharing to maximise research output. We will ensure the unsuccessful approaches and negative data are also disseminated in scientific papers and other presentations provided they are validated and with sound experimental design and appropriately executed.

### **Species and numbers of animals expected to be used**

- Mice: 11600
- 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 will use rodents in this project ranging from embryos through the entire adulthood. Although we focus on myelin health and regeneration, which are relevant to adult and



ageing, understanding how myelin emerges at early life, is extremely helpful to inform how damaged myelin is restored, because the process in making myelin forming cells from their parent cells is identical.

Myelin, the main model system we focus on, is a feature of vertebrates. Although myelin is present in lower vertebrates such as fish, and zebrafish have been very valuable as a model to learn many fundamental processes of myelin in development, it is considerable different from mammalian regenerative systems. In addition, they are less ideal for studying ageing, and not as versatile as mammals in testing interventions potentially translatable for human use. We therefore require the use of rodents to study regeneration of myelin such that it can be potentially relatable to myelin regeneration seen in humans. Myelin in rodents has close resemblance to human in both structure and composition, which has been widely used to study myelin development, its changes through ageing, and regeneration after injury and disease. For this reason, a considerable variety of valuable rodent models have been established for myelin research. Importantly, much of our focus will be on experiments in the adult as well as the aged system, the latter is currently understudied. It is crucial that we look at changes in myelin and myelin regeneration in aged animals which best models aged- associated decline in humans. We will characterise how age effects myelin in normal conditions, and then determine how age alters the capability of regeneration of damaged myelin in injury and disease conditions, and importantly, try to identify treatments that can slow, stop or even reverse the decline in myelin health and regenerative capacity.

We will use both rats and mice in the project. Rats offer larger volume of tissue allowing better yield of material for subsequent analysis; Larger lesions can be created in rat tissue which are more relevant to human pathologies; rat tissue has better compatibility in assays involving mouse monoclonal antibodies. Mice are the most used species in biomedical research which offer unrivalled capability of genetic manipulation which are irreplaceable in our study.

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

We will breed genetically altered (GA) mouse lines in the project including enhancing or eliminating the function (gain- or loss-of function) of genes which are expected to affect glial cell functions or labelling of specific cell types for as a experimental tool for analysis. The GA mice may be further tested throughout their life for changes in functions of brain or spinal cord as a result of such a genetic manipulation. The wild type or GA animals may undergo additional procedures such as administration of substances, to enable gene manipulation, cell labelling or testing drug candidate compounds. The substances may be delivered by adding to food and drinking water, or administered by injections through various routes. A proportion of the animals will be induced for a focal myelin injury, by injection of a small volume of toxins into brain or spinal cord following surgical exposure, or wider area of myelin injury with a systemic toxin. The injury (lesion) models may be combined with delivery of substances, or transplantation of cells into tissue or into blood circulation. Alternatively, myelin injury may be induced by feeding a toxin selectively damaging myelinating cells. Typically, injury models are created, and allowed to recover for 2-4 weeks from lesion induction, occasionally, a shorter (24 hours) or longer observation (3-6 months) after lesioning may be undertaken. A surgical procedure in this project normally takes 20 to 45 minutes. Some animals will be tested for one or more behaviours designed for measuring performance of the nervous system, including learning, memory, movement and muscle strength, and sensory functions, these are typically performed on genetically manipulated animals.





Since effects of age is one of our main research questions of this project, many experiments involving these procedures may be carried out on aged animals with a maximum of 24 months.

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

In this project, the animals will be maintained under standard conditions with controlled temperature, humidity and 12-hour light/dark cycles.

We expect the following to be among the impacts/adverse effects associated with the proposed protocols:

- Generally, food is given ad libitum with the exception where food needs to be removed in the context of every-other-day fasting protocols or in preparation for a behavioural outcome measure or training where food-based motivation is required.
- Generally, animals are grouped housed in individually ventilated cages with the exception where 1) male breeders are temporarily separated from breeding set-up to avoid injury from fighting; 2) when we need to measure activity (activity cages, running wheels etc). In any circumstance, lone housing will be minimised.
- Animals undergoing genetic manipulations from early life may display detectable signs as result of disruption of a gene expressed in glial cells, such as slow or delayed growth (smaller pups), mild difficulties in movement control which do not affect feeding and drinking. We expect these signs are usually temporary and compensated at around 4 weeks of age.
- Abnormal signs may occur in some animals, which may not be specifically related to the altered genes, such as malocclusion, the overgrowth of misaligned front teeth in rodents which affects normal eating, which can be managed by early identification and tooth trimming.
- A significant proportion of animals will be maintained to and used at old age which will exhibit functional decline and higher incidence of signs associated with ageing, such as tumours, rectal and vaginal prolapse, reduced vigour and activity.

The animals undergoing behaviour tests (many of which will be adult or aged rodents) will be acclimatized to handling and behavioural rooms and testing environment in most cases, with the exception where initial learning or initial responses to a test is recorded. The animals for behavioural tests may undergo a short-term fasting which is no more than 20 hours at a time and followed by substantial recover time.

We will occasionally need mice with reduced immune function for testing grafted cells across species, e.g. testing human cells in mice, so that the transplanted cells can survive the host rejection. These animals will be housed in a more rigorously clean environment and closely monitored for general conditions as they are prone to infection.

- Some animals will be used for creating lesioning models via surgical procedures, which usually take 30-60 minutes under anaesthesia. This is done by surgically exposing the surface of the brain or spinal cord, and injection of a solution of toxin into the tissue to induce a small area of tissue damage. The animals will experience pain following surgery within the first 72 hours, which can be managed well by providing pain relief before and after surgeries. The injury models used in this project do not usually lead to detectable abnormal signs though very occasionally, the rat brain lesions may cause significant loss in movement control and balance, the affected animals will be killed





humanely. Lesions in the spinal cord may lead to transient paralysis of one rear limb in some animals, which usually does not hamper the movement and access of food and water, and the animals recover completely within 72 hours. Animals undergoing surgery or other procedures require anaesthesia, we will predominantly use inhalational general anaesthetics (i.e., isoflurane) which is of high safety range and rapid recovery (within 10-15 minutes). Although all types of anaesthesia pose risk to animals such as respiratory suppression, our records show that the adverse effects in our settings are extremely rare. There will be a transient loss of body weight directly due to surgical trauma, usually around 8-10%, but may reach 15-20% in aged and obese animals.

- Some animals in this project may receive administration of substances, alone or in addition to the treatment described above, which may cause certain adverse effects depending on the nature of the substances and the routes of administration. Side effects may arise from local irritation, or as a result of reduced food and water intake, leading to loss of body weight and sign of dehydration. The animals will be closely monitored for their general conditions.
- Finally, some animals may be subjected to long-term treatment which deviates from standard maintenance conditions such as diet and nutritional manipulation, change in circadian rhythm, training, and behavioural tests. Mild stress is expected to be experienced by the animals at the early stage of the experiments but normalised after a short period of time in 7 - 10 days. These animals are classed as mild unless other procedures are combined.

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

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

The majority of animals used in the project will experience mild or lower grade of adverse effects, a considerable proportion will experience moderate severity, and very small proportion may show substantial adverse effects which fall in severe category.

The estimated proportion of each category by animal type is as below:

<b>Mouse</b>	
Mild or lower	70%
Moderate	25-30%
Severe	5%
<b>Rat</b>	
Mild or lower	80%
Moderate	15-18%
Severe	2-5%

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

- Killed
- Used in other projects
- Kept alive

**A retrospective assessment of these predicted harms will be due by 05 July 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?**

Myelin, the primary subject and model system in the present project, functions only when it forms a unique structure wrapping the nerve fibres in the central nervous system (CNS) including brain and spinal cord. Its generation, maintenance and repair following injury and ageing are complex biological processes involving interaction of many cell types in three dimensions and also affected by factors of whole body (e.g. from blood). At present, there is no non-animal substitute that can accurately replicate the structure and function of the central nervous system. Although non-protected animal alternatives (non-vertebrates) may be useful for studying fundamental cellular processes, they do not exhibit the same level of complexity in the CNS, i.e. their nerve fibres do not have myelin sheaths. For this reason, vertebrates are the only option from which we can gather enough information to address the objectives of the project.

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

There are no non-animal alternatives for this project since we are studying biological processes not only involving physical interactions between molecules but a system capable of maintaining equilibrium through precise self-regulation. However, there are some techniques which will be considered as alternative to using live animals which include: cultured cells isolated from animals or immortalised cell lines, especially human induced pluripotent stem cells (cells capable of making any cell type in culture); synthetic materials to mimic tissue environment for cells to grow, or simulate particular structure, e.g., axons. Although these techniques can only serve a partial surrogate of work using animals, we will conscientiously explore the opportunity to develop and maximise the use of these techniques wherever possible. In addition, human tissue from patients via biopsy and autopsy is a valuable material for learning pathophysiology and pathology in disease conditions.

**Why were they not suitable?**

The techniques such as cultured cells or incorporating synthetic materials in the experiments have their unique values in addressing specific questions as a simplistic model system, which are often difficult to obtain from using live animals. However, these approaches cannot accurately reproduce the biological processes which are invariably contributed to by multiple cell types and changes of systemic environment such as circulation. In addition, promising interventions for the health benefit of humans are required to be demonstrated to be both effective and safe before potential clinical translation.

Moreover, although human samples are directly relevant to the questions we pursue in the



project, but they often only represent a snapshot of a particular stage (usually late stage) of disease and are often unable to provide information of a time course of disease development, and normally feature very high variability.

### **A retrospective assessment of replacement will be due by 05 July 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 estimated the numbers based on the statistics from previous projects spanning more than 20 years. The numbers are adjusted according to evolution of the research questions and technical advance such as genetic manipulation, as well as the current member of researchers in the group, and the planned experiments can be carried out around the objectives set out in the projects.

In order to achieve the objectives in the project, we design experiments to determine the roles of candidate factors (genes) in the maintenance and regeneration of myelin, then to test various conditions and possible interventions to alter the activities of these genes to enhance myelin health and regeneration, especially in the context of ageing.

In experimental cohorts for testing these interventions, the control and test (treated) groups should be randomly chosen from age, sex, genetic background matching animals, so that the controls only differ by omitting the treatment. Use of sham operated control will be minimised, for lesioning models that we will use, contralateral part of the lesioned tissue will be ideal to be used as uninjured tissue control because it is on the same animal. The number in each group (group size) is determined by two factors, how big an effect is of biological relevance and how variable the measurements are between animals. If the extent to which animals respond to a treatment is small, or the variability is great, then more repeat of the experiments on different animals, i.e, larger numbers of animals are required to obtain robust results, and vice versa. The information about the effects and variabilities can be obtained from a previous experiment with similar setting, or a small pilot experiment. Thus, for each type of experiments, we can estimate the minimal numbers of animals required using statistical methods. Too small or too large number will both lead to waste of animals. Minimising variability and maximising the response of treatment are key measures for minimising the use of animal numbers. We will consult biostatistician for advice to ensure our experimental designs are sound and robust.

A number of other factors have also been considered:

We often need to obtain optimal dosage for testing a drug candidate, since it is quite



common that low dose and high dose may cause opposing effects. Breeding and maintaining of genetically altered mice will result in surplus animals with intermediate type of animals which will not be used in experiments; there is a small proportion of animals to be used at advanced age will be lost due to their developing age-associated ill-health signs such as tumours which are no longer suitable to be used in experiments.

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

To optimise the number of animals used in this project, we will maximise the use of tools such as Experimental Design Assistant (EDA) to define the number of animals per experiment that is required to detect statistically significant outcomes in each experimental setting. We will use established online tools and consult biostatisticians to ensure sufficient statistic power without using an excessive number of animals in our experiment.

Experimental groups will be randomly selected to ensure that they are balanced for age, sex, and strain. The number of animals in each group will be determined by a statistical principle based on existing data from similar studies or early phase so called 'pilot' experiments. In addition to randomisation, proper controls and sample sizes, we will avoid the introduction of bias that may otherwise influence interpretation of results by “a blind” approach, so that the researchers are hidden from the information and identities of the animals (e.g., control or treated) during the experiments until all the data have been obtained. Where possible, repeated non-invasive tests (e.g. imaging, behavioural tests) will be performed on the same animal to maximise the efficiency and robustness of data acquisition, reducing overall animal usage whilst simultaneously ensuring that there is no increased harm to animals used. We will continue to use cell and tissue culture in our studies to obtain preliminary data before using animal models. This ensures only the most promising experiments are progressed to be performed on animal models.

The PREPARE (<https://norecopa.no/prepare>) guidelines contains rich source of information not only offers guidelines on robust experimental design, but also provides a thorough workflow to ensure a smooth running and high-quality execution of animal experiments.

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

We will strive to achieve consistent, reproducible, non-biased data acquisition to minimise the variation in studies. We will ensure researchers carrying out the work to have adequate training to produce data of the highest quality possible to minimise the wastage. We will continue to adopt and develop modern tools for genetical manipulation, such as CRISPR (a latest technique in molecular biology which enables inserting or deleting DNA sequences in live cells – i.e. gene editing) and viral vectors (modified live, non-disease causing viruses which help transfer genetic material into cells) - mediated gene manipulation, so that we can use more wild type animals for experiments to enhance or disrupt gene expression, therefore reducing the requirement of creating genetically altered animals and the surplus from breeding.

We will maximise use of published and other publicly available datasets, e.g., sequencing data, aiding for selecting new candidate genes and pathways. Where possible, we will use surplus animals from other projects, and offer our own surplus animals to other projects. We will also ensure materials such as tissues not needed for the particular experiments are made available to other researchers. We will also share control tissues where appropriate, between projects. These measures will facilitate optimising the use of animals



and minimising unnecessary repetition of the same experiments using animals.

### **A retrospective assessment of reduction will be due by 05 July 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, we will use models which manifest deviation in functions of myelin in a small area of white matter in the central nervous system (CNS). The models were chosen because of the minimal functional loss that they induce. The animals will experience 1) genetic manipulation which potentially leads to functional changes in a particular type of glial cells; 2) acute focal myelin injury in defined locations in the CNS; 3) Local and systemic administration of biological agents which may affect the regeneration process.

The animal models proposed were chosen because of the minor functional deficit that they induce in general. The toxin-induced rodent demyelination models have been demonstrated by many laboratories extremely valuable in addressing specific questions in regenerative biology and preclinical research. Among the models available, we will always choose the one which is least harmful to the animals, gives the clearest results and is the most consistent.

Amongst the above 3 broad aspects, surgical procedures required for creation of injury model can cause relatively more serious harm such as pain for a short period, usually 3-5 days in the animals. Injury at certain locations may cause temporary reduction of loss of functions, such as voluntary movement of a limb. Handling, training animals required in functional (behavioural) test, administration of substances will cause mild stress to the animals, and brief pain in the latter depending on the routes, long-term, repeated administration may be associated with local discomfort, inflammation, reduction in food and fluid intake.

Manipulation of genes involving CNS can potentially lead to halted development or abnormal functions in the adulthood, though the abnormalities are usually mild if the changes only take place in specific cell types or induced at adult stage. The harmful experiences are more likely exacerbated when aged animals are used in the project. Except for the likelihood of additional signs related to ageing, aged animals recover more slowly following surgical procedures.

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





Emergence of myelin is a significant milestone in animal evolution. Myelin is only present in vertebrates. In mammals, myelin formation, or myelination occurs only at postnatal stage, and the extent of myelination correlates closely with the development of advanced functions of the central nervous system, such as movement and cognitive skills. In addition to developmental stage, myelination also plays an essential role in acquisition of new skills throughout adulthood. Therefore, the models capable of being assessed for these relevant functions are essential for this project.

Moreover, regeneration of myelin is perhaps only pertinent to fully developed stage. However, animals at more immature stage will still be used to study regulations of the key cellular process, which is recapitulated during the injury. Samples from developmental stage provides clearer, more consistent readout compared to injury model. As for less sentient species, such as zebrafish, a valuable model which has helped elucidate many fundamental mechanisms of CNS myelination, not only does it require specific expertise, but there are substantial differences in the complexity comparing with the mammals. For these reasons, we choose rodents as our model animals which are the most suitable for our main objectives.

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

The animal models proposed were chosen because of the minimal functional loss that they induce. The toxin-induced rodent demyelination models have been demonstrated by many laboratories as extremely valuable in addressing specific questions in regenerative biology and preclinical research. Among the models available, we will always choose the one which is least harmful to the animals, gives the clearest results and is the most consistent.

In conducting animal work, we will always ensure most appropriate ways for handling and housing, to minimise the impact of single housing of animals (which is at times scientifically necessary) and other sources of stress. We will take appropriate measures (pain relief and anaesthesia) to minimise pain in the animals under procedures. We will limit the numbers of procedures on individual animals to prevent cumulative and unnecessary harm. Whenever it is feasible and scientifically appropriate, we will choose the least harmful models for our tests.

Non-specific developmental defects or other abnormalities may occur in genetically altered mouse lines, such as retarded growth, or 'runt', and malocclusion, overgrowth of misaligned front teeth. We will always ensure proper selection of breeding animals to avoid the presence of such traits in the background based on their recorded health status in the 'family tree', so that the occurrence of the defects may hopefully be eliminated or minimised. The impact of abnormalities (e.g., obesity) will be mitigated by providing special housing conditions (e.g., soft bedding and easy access of food) for comfort, claw clipping to limit skin damage, and additional monitoring for breathing when anaesthesia is in use.

Before testing a new compound, which has not previously been used in which not previously been used in animals, we will perform small scale 'pilot' experiments with low numbers of animals to determine effective dosage and toxicity to minimise potential harm on full scale studies.

We will continue to improve the method of detecting the impact of adverse effects of procedures on animals, using a consistent, objectively measurable way to record pain, stress, and functional deficits e.g., utilising 'scoring' systems where appropriate. For new procedures potentially leading to obvious harms we will always start with small scale pilot



tests first and create a suitable monitoring programme to minimise suffering.

We will ensure adequate training of all researchers who carry out animal work and continue to develop effective measures to reduce pain and discomfort for animals under procedures, such as incorporating pain relief agents into food supplements after surgeries as an alternative to giving by injection, and work with animal technicians to perfect relevant monitoring system.

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

There are a number of guidelines available online that covers every aspect of working with laboratory animals. The LASA Guidelines (<https://www.lasa.co.uk/>) are an important source of references for legislation, theoretical and practical guidance. Likewise, the Handbook of Laboratory Animal Management and Welfare by Sarah Wolfensohn and Maggie Lloyd (Blackwell Science) serves the same purposes. The NC3R website provides a library of 3R resources covering ethical, technical, and regulatory advances. For instance, to ensure the robustness of our animal research, we will consult the NC3R's Experimental Design Assistant (EDA) tool in our experimental design and statistical consideration. Similarly, the PREPARE Guidelines, in the form of a two pages checklist, is also a valuable tool for assisting designing animal experiments.

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

We will regularly monitor publications on advances concerning the 3Rs. These include advances in experimental design, novel or improved experimental models and new techniques in data acquisition. We will regularly visit the NC3R website and follow its Newsletters to receive latest updates on 3R development and advances in methodology. There is a wealth of resources on 3R guidelines from government and various institutions and authorities such as the Animal Scientific Procedure Act (ASPA), Laboratory Animal Science Association (LASA), Understanding Animal Research (UAR), Institute of Animal Technology (IAT), the Animal in Science Regulation Unit (ASRU), and online resources such as <https://norecopa.no>. Finally, we will assess the outcome of our experiments in a timely manner to identify issues and imperfections in our models and procedures, then we will seek advice from members of Animal Welfare and Ethics Review Board (AWERB) and experienced users where possible, to maximise implementation of 3R's principles in future practice.

**A retrospective assessment of refinement will be due by 05 July 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. Function and dysfunction of the basal ganglia and their partner brain circuits

### Project duration

5 years 0 months

### Project purpose

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

### Key words

Brain, Behaviour, Nerve Cell, Health, Disease

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?**

To provide new knowledge about the operations of the basal ganglia, a group of brain structures, and their partner brain circuits in health and disease.

**A retrospective assessment of these aims will be due by 05 August 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 basal ganglia are a group of brain structures that play vitally important roles in many of our daily behaviours, including purposeful movement. When nerve cells in the basal ganglia and their partner brain circuits do not work properly, like in Parkinson's disease, behaviour is greatly affected. The diverse functional roles played by the basal ganglia and their partners in controlling behaviour likely stem from the diversity of the nerve cell types within each structure. The properties of most cell types are not yet fully understood, and it remains difficult to confidently ascribe particular functions to them. This is one of the key reasons why we still cannot be sure about how, when and why the basal ganglia and their partners do what they do. However, a mature understanding of these issues is essential for the rational design of new and better treatments that might slow, stop or otherwise lessen brain dysfunction and impaired behaviour in human disease.

It is clear that we need to fill major gaps in our knowledge about the operations of the basal ganglia and their partner brain circuits, including how their different cell types make their specialised contributions to complex behaviours. The overall aim of this work using animals is to meet this need by; (1) mapping the molecular building blocks and connections of these nerve cells; (2) defining the electrical and chemical signalling of these nerve cells; (3) clarifying how these nerve cells influence behaviour; and (4) establishing how the properties of these nerve cells are altered in models of brain disease, especially Parkinson's. The new information to be gained will be important because it can then be used by us and other researchers to strategically interact with the brain in efforts to correct nerve cell dysfunction and improve behaviour in disease.

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

The main outputs stemming from the new knowledge gained in this project will include:

- Scientific publications containing original data that better define the molecular properties and connections of basal ganglia nerve cells and their partners, the ways these nerve cells organise their electrical activities and chemical signalling in time and space, and how these relate to and support specific behaviours.
- Scientific publications containing original data that clarify how the properties and operations of basal ganglia nerve cells and their partners are disturbed in brain diseases, such as Parkinson's, as well as fresh rationale and proof-of-principle for novel therapeutic interventions in these diseases.
- Datasets on the properties and operations of basal ganglia nerve cells and their partners that can be used by others.
- New technological developments and novel tools (e.g. computer code) that can be used by others.

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

- In the short- to medium-term, i.e. within the timeframe of this licence, the outputs from this work will benefit:
- The sizeable community of researchers working on the function/dysfunction in the basal



ganglia and their partner brain circuits, including those investigating the causes and consequences of brain diseases.

- A wider community of researchers working in diverse fields (e.g. biology, psychology, computational science, engineering, neurology, neurosurgery).
- Stakeholders with an interest in research using animals, including research funders, policy makers and the public.

In the longer term, the outputs from this work are anticipated to benefit:

Clinicians and industry (e.g. pharmaceuticals, medical devices) needing new insights into when, where and how to interact with the diseased brain (e.g. through administration of drugs or the use of devices) to most effectively combat brain dysfunction and disease symptoms.

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

We will disseminate the new knowledge, technical developments and tools we generate in a timely manner, using a variety of means, to diverse audiences:

- We will publish our results on 'preprint' servers and in peer-reviewed journals. Our papers will be published in an Open Access format, and will also be deposited in full on a freely-accessible public database.
- We will communicate our results (published and unpublished) through presentations at conferences as well as at specialist user groups (e.g. collaborators, clinicians, industry, research funders, policy makers).
- We will share our data and computer code via publicly-available online repositories.
- We will share our new knowledge through public engagement activities, including those designed for schools and patient/carers groups.

### **Species and numbers of animals expected to be used**

- Mice: 16000

### **Predicted harms**

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

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

We will use mice with and without genetic alterations in our work because:

- The fundamental properties of nerve cells in the mouse basal ganglia are similar to those of the human basal ganglia, such that the outputs to be gained from our use of mice should be applicable to humans in many respects.
- Mice are the most widely-used mammals in neuroscience research that have sophisticated motor and cognitive behaviours, so we can build on a good knowledge base of past research that has provided insights into related human behaviours.
- The experimental models and methods to be used have been specifically designed for, and successfully used in, mice.
- We can harness the power of genetics (and, more specifically, of genetic alteration) to give us the selective access to certain nerve cell types that we will need to achieve our scientific objectives.



We will mostly use adult mice because our work is focused on understanding the operations of the basal ganglia and their partner brain circuits in adults. However, will also use aged mice because we aim to establish how the properties of nerve cells are altered in models of brain disease over time, and we know that age is a big risk factor in some human brain diseases like Parkinson's. In rare cases, we will also use juvenile mice because their smaller, more transparent brains make it easier to study the physiological connections between nerve cells in brain slices.

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

- Some of the animals bred will have their genes altered, for example, to give selective access to certain cell types or copy the genetic changes that are important for some human brain diseases.
- Some animals will be aged up to 28 months to model the progression of disease over several pertinent life stages.
- Some animals will be administered substances by injections to the body (away from the brain) or orally. Some substances will mark or temporarily alter the functions of nerve cells so we can study cell properties. Other substances will damage cells so that we can model disease processes.
- Some animals will be anaesthetised and have surgery to inject substances into their brains. Some substances will mark nerve cells so that we can trace their connections or monitor and manipulate their activity. Other substances will damage cells so that we can model disease processes.
- Some animals will be anaesthetised and have surgery to place or implant devices in their brains, so that we can monitor and manipulate animals' nerve cells and behaviour.
- Some animals will have their heads temporarily restrained in place while they are awake, so that we can monitor and manipulate animals' nerve cells and behaviour.
- Some animals will be trained to perform simple behavioural tasks, such as moving on time to get a reward.
- The motivation of some animals to perform certain behaviours will be changed by controlling their access to food or fluid intake.
- Some animals will have their blood sampled to better understand the effects of experimental interventions.
- Animals will often have a combination of two or more of the above procedures.
- Typically, experiments will take place over a period of between three weeks and six months.
- Animals will be killed by a humane method and, typically, tissues taken for analysis after death.

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

We do not expect any of the genetic alterations to impact greatly on the animals' wellbeing.

Old animals can become unwell, just like old people can become unwell. Age-associated conditions in old mice include weight loss, reduced organ function, skin abnormalities, abscesses/tumours, eye disease, dental disease, joint disease, and mortality. Many of these conditions can be treated as advised by a vet.

Animals that have had surgery will experience some pain and discomfort, and may



temporarily lose a modest amount of weight (e.g. up to 15% of their starting weight) for a few days, but are expected to recover quickly and will be given painkillers and post-operative care, just like people recovering in hospital.

Some animals administered substances may temporarily lose a modest amount of weight (e.g. up to 15% of their starting weight) for a few days, which may be alleviated by giving access to nutritional supplements.

Some animals receiving experimental interventions designed to model disease processes will show behavioural signs related to the specific nerve cells affected, e.g. slower movements in models of Parkinson's. These behavioural changes are not expected to affect the animals' ability to perform other behaviours normally (e.g. eating, drinking, grooming).

Animals on controlled access to food or fluids will be hungry or thirsty for short periods (a few hours), and will typically lose weight but with weight stabilising at a lower level (e.g. at 90% of their starting weight) that does not greatly impact on their wellbeing.

When awake animals have their heads restrained, they might initially experience frustration or stress for short periods (over a few minutes), but we do not expect this to impact greatly on their wellbeing.

Animals will experience mild and transient discomfort from blood sampling.

The final procedures will be undertaken under terminal anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience brief discomfort, but no pain.

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

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

**Mice:** 61.0% Subthreshold, 4.0% Mild, 34.5% Moderate, 0.5% Severe.

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

- Killed

#### **A retrospective assessment of these predicted harms will be due by 05 August 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 use of animals is essential to achieve our scientific objectives because it provides unmatched opportunities to define the molecular, structural and physiological properties of specific sets of nerve cells at sufficiently high resolution and in the context of the whole brain. This knowledge is in turn critical for understanding how different types of nerve cell work together to control complex behaviours in health and disease. Animals can be used to accurately model specific features or symptoms of human brain diseases.

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

Several non-animal alternatives have been considered, including clinical investigations, the use of computational/mathematical models, and the use of cultured nerve cells.

We anticipate that computational modelling will continue to offer some opportunities for replacing a small part of the proposed animal use during the course of this programme of work. Indeed, we have shared (and will continue to share) our data obtained using animals with computational neuroscientists who can use our discoveries to help us test key hypotheses with computer simulations.

## **Why were they not suitable?**

It is not feasible to study the human brain, either living or dead, and obtain sufficiently high-resolution definitions of the specialised properties of different types of nerve cell in the basal ganglia and their partner brain circuits.

Although computational models have provided important insights into basal ganglia function and dysfunction, the models currently available in our field are not yet sufficient (in detail or physiological relevance) to address all of our scientific objectives.

We cannot use organoids, organotypic or other types of cell cultures because these preparations will not allow us to observe the full range of nerve cell activity that has been reported in living organisms, presumably because the necessary connectivity between nerve cells is only maintained in the whole brain. Moreover, cell-culture preparations have the potential to give confusing results because not only are the cultured nerve cells 'immature' (in several important respects), but also abnormal connections may develop between nerve cells grown in cultures.

In conclusion, alternative approaches, such as clinical investigations or *in silico/in organo/in vitro* studies are not suitable for addressing our scientific objectives here. This programme of work using animals will nevertheless generate valuable data sets that can in turn be used to inform future studies employing non-animal alternatives.

## **A retrospective assessment of replacement will be due by 05 August 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?**

Estimates are based on our experiences of the models, methods and experimental design needed to address our scientific objectives and that have been acquired by delivering on related research projects. Typically, we will need to use groups of 6-15 animals for each experiment. We have used our annual return of procedures data to estimate the number of animals that we will need to use for the breeding of genetically-altered mice.

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

The use of experimental designs that minimise and control sources of variability by, for example:

(1) taking into account the sex, age, weight, genetic status and circadian phase of animals; (2) using Standard Operating Procedures; and (3) ensuring that animals comprising an experimental group receive near identical procedures.

The use of experimental designs that minimise and control subjective bias by incorporating randomisation, 'blinding', 'blocking' and computerised data analysis.

The use of experimental designs that allow for statistically powerful within-subject comparisons (e.g. before, during and after an intervention).

The use of experimental designs that incorporate carefully-chosen control groups that allow for powerful 'statistical pairing' and help with interpreting the effects of experimental interventions.

The use of methods that are optimised and based upon repeated measurements to enable data averaging and assessment of statistical outliers, as well as maximise the amount of data obtained from each animal.

We plan to conduct and record our experiments in a manner suitable for publishing our results according to the ARRIVE guidelines (<https://arriveguidelines.org/>).

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

We will use a variety of measures to help optimise the number of animals we plan to use:

- Breeding colonies of genetically-altered mice will be managed in line with 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.





- We will use both male and female animals in our experiments.
- Before starting on full-scale studies, we will carry out extensive literature searches to avoid unnecessary duplication of work.
- Before starting on full-scale studies, we will use pilot studies as necessary to test or finesse an experimental tool, method or hypothesis. Pilot studies will be carried out using small groups of animals; our prior experience is that as few as 2-5 animals in each pilot study are sufficient to reach a robust decision point, i.e. 'go or no go'.
- We will share the data generated by our research using animals with computational neuroscientists, who can then use our discoveries to test key hypotheses in computer models. The computer models can generate fresh rationale and new testable predictions that can in turn guide the optimal use of animals in our future experiments.
- We will bank and share postmortem brain tissue for use by researchers in our lab and elsewhere.
- We use perfusion-fixed brain tissue for most of our anatomical experiments. Typically, the work of an individual researcher in the group is focused on one or two discrete parts of the basal ganglia and their partner brain circuits. However, we will encourage our researchers to collect tissue from as much of each brain as possible, thereby generating a valuable materials resource for other researchers working on other parts of these circuits or entirely different brain regions.
- We will use methods, tools and technologies that maximise the data obtained from a single animal, thus reducing the number of animals needed. For example, we will use special devices that can monitor the activity of hundreds of nerve cells from a single animal.

### **A retrospective assessment of reduction will be due by 05 August 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 mice to study the basal ganglia and their partner brain circuits in health and disease because the core features of these brain circuits are similar in all mammals. The use of genetically altered mice represents a refined way of giving the selective access to certain cell types that we will need to achieve our scientific objectives. We do not expect any of the genetic alterations to impact greatly on the animals' wellbeing.

We will use aged mice because only natural ageing captures the full range and appropriate timing of biological processes that may affect the age-dependent emergence



of traits in models of brain disease. We will not age the mice beyond a certain time (28 months).

To help understand what goes wrong in brain diseases like Parkinson's, we will investigate the influence of abnormal clumps of proteins called Lewy bodies, we will inject animals with proteins that cause the formation of Lewy bodies. To mimic other aspects of disease, for example, the loss of a chemical messenger called dopamine that underlies many of the symptoms of Parkinson's, we will use established genetic models or toxins that cause these dopamine-producing nerve cells to malfunction or die. We will carefully choose models that allow for the selective targeting of certain nerve cells; selective targeting of e.g. small groups of cells can minimise the potential for more widespread effects on the animals' behaviour and wellbeing.

To map the molecular building blocks and structural connections between nerve cells, we will inject substances that mark cells into the brains of live animals and then study brain tissue after humanely killing the animals. The basal ganglia lie deep in the brain and so, typically, we will need to make a small hole in the skull to give direct access to these brain structures in live animals. Injections of substances into the brain is a refined method of accurately targeting selective cell types for experiments; selective targeting of e.g. small groups of cells can also minimise the potential for more widespread effects of substances on the animals' wellbeing.

Animals are expected to recover quickly from surgery.

To understand the electrical and chemical signalling of nerve cells, we will study cells in living brain slices from animals that have been humanely killed. We also will surgically implant small, light-weight devices (e.g. fine electrodes and optical fibres) to monitor and manipulate the activity of nerve cells in the brains of animals that are under general anaesthesia as well as in animals that are conscious. The implantation of devices within the brain is a refined method of accurately targeting selective cell types for experiments and can minimize the potential for more widespread effects of manipulations on the animals' behaviour and wellbeing.

Animals are expected to recover quickly from surgery, and thereafter, the devices should not in themselves impact greatly on the animals' wellbeing. The choice of invasive monitoring/manipulation technique will be made according to the resolution and type of data that are required to meet our scientific objectives. Non-invasive devices do not give the resolution needed. The parameters of manipulations applied to the brain will be carefully chosen, aiming for minimal interventions that are sufficient to evoke a measurable physiological response without impacting greatly on the animals' wellbeing.

To understand how the basal ganglia and partner brain circuits control behaviour, we will monitor and manipulate nerve cells in the brains of animals that are freely moving or that have their heads restrained. We will monitor animal behaviour in tasks and environments that are not expected to impact greatly on the animals' wellbeing. Restraining the animal's head is a refined method for the monitoring and manipulation of nerve cells in experiments where good physical stability of the head is essential to gather data. The duration of head restraint will be limited, typically for an hour or two in a day and for 100 hours over the lifetime of the animal, so that it is not expected to impact greatly on the animals' wellbeing.

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

Our scientific objectives are firmly focused around understanding the operations of the



basal ganglia in the adult brain. We cannot use less sentient (non-mammalian or non-protected) species, such as fish or fruit flies or worms, because they do not have well-developed basal ganglia in their 'brains'. We cannot use very young mammals or embryos because their brains are still developing and the properties of their nerve cells are different in many respects to those of nerve cells in the adult basal ganglia. Relatedly, very young animals and embryos do not exhibit the complex independent behaviours that we need to study. Although we can and will address parts of our scientific objectives by studying adult animals that have been terminally anaesthetised, we must also study adult animals that are free of anaesthesia to determine directly how the activity of basal ganglia nerve cells compares to and controls complex behaviours.

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

The weights, body conditions and behaviours of animals will be carefully assessed to minimise the impact of potential adverse effects on their wellbeing; refinement measures and controls specific to each procedure will be used, as will humane endpoints.

Ageing animals will be carefully monitored by staff trained to work with them. Group sizes in ageing experiments will be increased to accommodate any loss of animals and to avoid single housing. Animals will be assessed for age-associated conditions. If these are observed, animals will be treated accordingly; those developing severe effects will be humanely killed.

Animals undergoing surgical procedures will receive peri-operative analgesia, which will be continued after surgery for as long as required to alleviate any pain.

We will use feeding regimes that lessen weight loss after surgery or other procedures.

Surgical procedures involving the head and/or brain will be performed under precise three-dimensional control ('stereotaxy') to minimise tissue trauma and facilitate accurate targeting. Animals will be given sufficient time to fully recover between surgical procedures.

Typically, the substances to be administered will have known safety, tolerability and efficacy from the literature.

Animals will be gradually habituated to head restraint (before experiments start) to minimise any frustration or stress.

When motivating the behaviour of animals by controlling their access to food or fluids, we will weigh the animals daily with a view to animals reaching a target weight (e.g. 90% of their starting weight) that should not greatly impact on their wellbeing.

Animals below target weight will be given additional or free access to food and fluids as appropriate.

We will optimise experimental parameters to give reproducible effects with the minimum intervention.

We will take advantage of refinements in tools and technologies, for example, using the smallest and lightest-weight devices for implantation.

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



### **conducted in the most refined way?**

We will follow best practice guidance from:

The National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs; <https://nc3rs.org.uk>)

The Laboratory Animal Science Association (LASA; <https://www.lasa.co.uk>) e.g. guidance on aseptic surgery and the administration of substances.

The PREPARE Guidelines for planning experiments (<https://norecopa.no/prepare>).

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

We will continue to attend the local (Departmental) animal welfare meetings, which are held several times per year and provide effective forums for sharing advances in the 3Rs. These meetings are attended by vets and NACWOs who provide us with updates on best practice and advice on how to implement it. We will also continue to attend the Annual 3Rs Symposia ('Research Days') and 3Rs specialist workshops that are regularly hosted at the primary establishment. We are also emailed the establishment's 3Rs Newsletter, which provides timely updates on 3Rs events, developments and resources. Aside for these local initiatives, we will stay informed by following the websites of the NC3Rs (<https://nc3rs.org.uk/>) and the RSPCA Science Group (<https://science.rspca.org.uk/>).

### **A retrospective assessment of refinement will be due by 05 August 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. The role of iron in health and disease

#### Project duration

5 years 0 months

#### Project purpose

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

#### Key words

Iron, deficiency, physiology

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	adult, pregnant, embryo, 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 understand how perturbations in iron levels in the body drive disease and how they can be corrected to prevent or treat disease.

#### A retrospective assessment of these aims will be due by 09 August 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?**

Iron deficiency is the most common nutritional disorder in the world. It is particularly common in individuals with underlying conditions, such as heart disease, lung disease or kidney disease, with prevalence reaching 80%. It is also very common in women of reproductive age, because of excessive blood loss associated with menstruation. Its presence is associated with worsening of existing disease. For instance, the presence of iron deficiency in patients with chronic heart failure is associated with 60% increase in risk of death over the subsequent 3 years. The presence of iron deficiency during pregnancy increases the risk of the mother developing heart failure, and her babies to subsequently have cardiovascular disease. The presence of iron deficiency in patients with lung disease seems to be associated with worse decline in ventilation and respiration. Furthermore, patients who have both iron deficiency and anaemia appear to worse off than those who have anaemia alone, indicating that iron deficiency worsens the effects of anaemia on the body. Currently, we do not fully understand how iron deficiency affects the physiology of various organs. Work undertaken previously suggests that iron deficiency impairs the ability of organs to respond to stress and to generate energy.

Continuation of this work is necessary in order to further understand the detrimental effects of iron deficiency on the physiology of organs, and to determine if existing iron supplementation strategies can prevent those detrimental effects.

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

#### **New information to address pertinent questions in the field:**

The impact of local iron deficiency on the functioning of different tissues and their responses to stress are not known. This question is important because iron deficiency is endemic in many patient populations as well as during pregnancy and childhood. Under current practice, iron supplementation is triggered by haemoglobin levels dropping below an arbitrary anaemia threshold. However, anaemia is a relatively late manifestation of iron deficiency. This work will be the first to describe the impact of non-anaemic iron deficiency on the body, and to define a set of accessible circulating iron marker(s) that best predict whether iron levels are sufficient to support the functioning of vital organs. In doing so, our discoveries could underpin a shift away from haemoglobin-centric approaches to an approach that anticipates the iron needs of all the organs of the body.

Additionally, the advent of novel intravenous iron replacement therapies that can replenish iron stores quickly is still hampered by the haemoglobin-centric view of iron. The findings of our study will give a first assessment of whether intravenous iron can replenish iron levels in all the organs that depend on it for normal function.

#### **Publications & International Communications.**

In terms of scientific meetings, we will focus on the meetings of professional bodies that are concerned with promoting research into the cardiovascular system, physiology and





haematology. Findings will be disseminated at the meetings of these societies and any relevant satellite meetings.

In terms of disseminating results that are ready for publication, we will target journals with a broad medical and scientific interest as well as more specialist cardiovascular journals. We will make all findings available open access upon publication.

Evidence to trigger change of practice in the clinical management of iron deficiency. Findings, disseminated through publications and presentations will put us in a strong position to engage with public health bodies, and with pharmaceutical companies with an interest in iron.

The work to be carried out under this project goes hand-in-hand with clinical studies in our laboratory, to ensure our discoveries in animals reflect human pathology and are informative of better diagnostic and therapeutic approaches that can be deployed in the clinical setting. Our team has existing links with clinicians, public health bodies and pharma. We will ensure that our discoveries generate the impact needed to translate their benefits into patient care.

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

**Basic scientists interested in iron homeostasis-** The discovery of the mechanisms of iron control at the turn of the century resulted in a significant expansion of the iron research field. By uncovering the first evidence of local iron control in the heart, and exploring its function further in other tissues, our work is producing a shift in our understanding of iron homeostasis, and in particular of how individual systems/tissues control their iron levels. This shift will stimulate further research into the role of these mechanisms in local iron control in other tissues where it is known to be expressed e.g. brain. Our experimental strategy, i.e., the use of tissue-specific models of disrupted iron homeostasis, will further provide a blueprint for those studies. Benefits already being delivered through collaborations that have resulted in publications.

**Clinical scientists-** A number of clinical trials have recently shown that iron supplementation benefits patients with chronic heart failure and pulmonary arterial hypertension, even in the absence of anaemia. Until recently, the mechanisms underlying those benefits remained unclear. Our work is showing that increasing iron supply to individual tissues improves metabolism and regeneration, providing a mechanism for the benefits seen in patients supplemented with iron. This will help strengthen the case for further clinical trials of iron supplementation in a greater proportion of patients (i.e., those with iron deficiency but not anaemic) and in a wider range of pathologies. Benefits delivered in the short to medium term.

**Public health bodies-** Currently, there are international and UK guidelines on the management of “Iron deficiency anaemia”, but no specific guidelines on iron deficiency itself. This means that, in the clinical setting, patients presenting with non-anaemic iron deficiency are not supplemented with iron. By contributing to our understanding of the link between iron deficiency and disease, our work is providing a rationale for more specific guidelines relating to iron deficiency itself. Benefits delivered in the short to medium-term.

**Clinicians-** Currently, clinical literature around the effects of iron deficiency and the benefits of iron supplementation is contradictory. This is particularly the case in the settings of acute heart and kidney disease, where there are concerns that use of iron





supplements may exacerbate acute injury by increasing the generation of reactive oxygen species. More specific guidelines on the treatment of iron deficiency itself, together with better understanding of how iron deficiency affects specific tissues in certain disease settings will fulfil an unmet clinical need and achieve an evidence-based shift in clinical practice. Benefits delivered in the medium to long term.

**Pharmaceutical companies-** In the past decade, there has been a surge in interest in targeting iron homeostatic pathways, because it is known to be altered in inflammation of chronic disease and to account for reduced iron absorption and anaemia in that setting. Our work on the role of this pathway in local iron control is forcing a re-think of how it should be targeted to treat anaemia, because of a concern that such strategies could undermine local iron homeostasis, and hence iron supply to tissues such as the heart, the muscle and the kidney. Benefits delivered in the short to medium term.

**Patients-** Ultimately, by enhancing understanding of iron in the disease setting, contributing to a change in public health policy, clinical practice and influencing the direction of pharmaceutical research, our work has the potential to both improve outcomes for patients with cardiac, pulmonary, renal and musculoskeletal diseases, and to prevent such pathologies through better management of body iron stores in healthy individuals and particularly in pregnant women. Benefits delivered in the medium to long term.

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

We will make use of existing collaborations with different epidemiological and clinical trial units to ensure basic discoveries in animals reflect and benefit human conditions.

We will make use of our exiting positions on scientific advisory boards.

We will make use of existing collaborative networks to deliver the benefits of discoveries to the clinic. Indeed, we already work with clinicians who are interested in developing better ways to diagnose and treat iron deficiency in patients.

We will share our mouse models with the international iron research community in order to further promote research into the clinical consequences of iron deficiency.

### **Species and numbers of animals expected to be used.**

- Mice: 6000

## **Predicted harms**

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

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

### **CHOICE OF MICE**

Mice are amenable to a wide range of genetic manipulations that will enable targeted disruption of iron homeostasis needed for mechanistic insights.

Mice regulate their iron levels using similar pathways to those operating in humans.



Their physiological responses to iron deficiency in various organs closely mimic those in humans.

For example, iron deficiency in the heart leads to heart failure both in mice and in humans through similar mechanisms involving a defect in energy production within cardiac cells.

Their responses to iron supplementation drugs (such as iron tablets and iron injections) appear to be similar to those in humans.

## **CHOICE OF LIFE STAGES**

We use adult animals because we are modelling disease conditions that normally occur in human adults.

In some instances, we will use all life stages up to adult, because we are aiming to model the effects of maternal iron deficiency on the progression of pregnancy, as well as on development of offspring, both in the womb and after birth.

## **USE OF TRANSGENIC ANIMALS**

So, we can delete certain iron regulatory genes to help us investigate the importance of iron regulation in certain organs.

So we can image changes in tissue iron by using a genetic change that help produce a light signal in the presence of iron, which can be detected non-invasively (without the need to kill the animal and extract its organs for direct quantitation of iron).

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

Genetically altered mice will be bred by natural methods. The genetic mutations are not expected to cause any welfare concerns that are beyond mild and transient. Some of the genes affected may be turned off at this stage.

Animals will receive substances that will activate the gene of interest in a specific organ so that iron levels in that organ are increased or decreased.

Some animals will be monitored to allow investigation of how the iron changes in the organ affect the organs function. Animals will undergo MRI at multiple time points of a period of months to trace disease progression and how different organs are affected by too much or too little iron. This is performed under general anaesthesia which lasts no more than 1 hour.

To test how muscle iron deficiency affects exercise some animals will be exposed to treadmill exercise, which the mice tolerate well just like humans.

In patients, the combination of iron deficiency and anaemia produce distinct effects from either iron deficiency or anaemia alone, indicating that there is an interaction between their effects. To investigate the nature of this interaction, some animals will have anaemia induced by either being fed a low iron diet for a period of up to 24 weeks or by taking a larger than normal blood sample (phlebotomy) on two occasions. On other occasions they will be housed under hypoxic conditions (low oxygen levels as experienced by humans at altitude). The oxygen levels will be altered so that they decrease from 21% to 8% over period of days. The animals tolerate this very well. These procedures are known as



physiological stresses.

Another physiological stress that is of interest is pregnancy. We will aim to establish if females who have iron-deficient hearts are less able to increase heart rate and heart output, as is expected in normal pregnancy.

To allow us to study the effects of iron levels in specific organs we will induce pathological stress to the relevant organ. In this project it will involve the animals being given a heart attack by surgically ligating one of the blood vessels that leads to the heart or the induction of chronic kidney disease by surgically ligating a blood vessel to the kidney. Surgery is performed under general anaesthesia, with strict aseptic techniques being followed. Pain relief is given during and after these surgeries.

To investigate whether iron supplementation therapies can prevent or reverse the detrimental effects caused by organ-specific iron deficiency, animals may receive a form of iron supplementation either in their food or by an injection into the blood stream.

Animals will undergo blood sampling at time points throughout the experiments to allow us to monitor iron levels.

Tissues and organs will be harvested post-mortem to allow for further biochemical analysis.

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

Weight loss up to 15%- This is the most frequent adverse effect, and will be the result of either the diet, or the physiological or pathological stress. Weight loss typically occurs in the first few days after the procedure, after which weight typically stabilises.

Changes to breathing pattern and heart rate- This will be experienced by some animals that have physiological stress such as hypoxia or cardiovascular stress such as myocardial infarction.

Following hypoxia exposure, changes in breathing and heart rate can happen quickly within hours, but resolve almost immediately after the end of the hypoxia exposure. For cardiovascular stress, changes in breathing and heart rate typically happen in the later stages of heart failure (about 6 weeks after heart attack) and last for up to 4 weeks.

Pain- Pain is expected because of the surgical procedures however this is controlled by the administration of pain relief both during and after surgery until the animal displays no clinical signs of pain, which is typically 24 hours post-surgery.

Unexpected death- In rare cases, some animals might die during or in the immediate aftermath of surgery, as a result of the size of the injury. Additionally, some animals might die unexpectedly from the rupture of an aneurysm in the abdomen, but this is not preceded by any clinical signs.

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

### **What are the expected severities and the proportion of animals in each category (per**



### animal type)?

Severities	Mice
Severe	1%
Moderate	10%
Mild	67%
Sub-threshold	23%

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

- Killed

### A retrospective assessment of these predicted harms will be due by 09 August 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 have considered carefully the use of alternative strategies to using animal models of disease and have been unable to find non-sentient alternatives to answer the important medical, biochemical and physiological questions posed by these studies. To obtain relevant measurements of cardiac, lung, skeletal muscle function, or to investigate the progression of pregnancy, it is necessary to use intact animal models, because these organs act in concert with each other and with the circulation to produce function.

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

We have considered the use of in-vitro approaches such as cultured cells to answer some of the questions. Where possible, we have indeed incorporated the use of cells to investigate some of the specific regulatory mechanisms. For instance, we will use cultured cardiac cells (purchased commercially) to investigate which iron formulations can be taken up directly.

### Why were they not suitable?

In most cases, cultured cells do not allow us to address the questions of physiological function and disease. This is because the physiological function and disease progression of the organs we are interested in necessitates complex interactions between different cell types and with the blood circulation. These interactions cannot be replicated under cell culture conditions. Furthermore, assessing the progression of disease necessitates longitudinal monitoring of the animal over time. Cells do not replicate disease and cannot be typically maintained in culture for long enough to model chronic disease.

### A retrospective assessment of replacement will be due by 09 August 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 in experimental protocols were estimated on the basis of three factors. The first factor is the level of spread expected in the experimental measures. For example, we know that typically, animals who have been given a heart attack show a reduction in the proportion of blood pumped by the left chamber of the heart with every beat. That reduction ranges between 20 and 40%. Because of this spread, we need at least 10 animals in the heart attack group and 10 animals in the sham group to be able to say, with confidence, that the ejection fractions are different between the two groups. Where experimental measures show a smaller spread (for example haemoglobin), we use smaller number of animals. We know the size of the spread in different experimental measures on the basis of previous experience. The second factor is the number of experimental groups we plan to compare, for example heart attack with normal diet, with or without iron supplementation, vs heart attack on an iron-deficient diet, with or without iron supplementation. The third factor is whether the experimental measures can be obtained non-invasively or not. For instance, measures of heart function can be obtained, by MRI, in the same animal over time, whereas bone marrow smears cannot.

The numbers estimated for the breeding programmes reflect the numbers required for our experiments plus additional numbers to reflect the rare cases where the genotype of the animal is not compatible with the experiment. We have also used our previous experience to help with this estimation.

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

We will utilise highly tested and validated readout techniques which will remove some of the artefactual variation from our studies. For instance, we have established that quantitation of tissue iron by ICP-MS is more accurate and reproducible than the use of the old fashioned ferrozine assay. Therefore, we have now adopted ICP-MS as a standard method in the lab, meaning that less animals are required to detect differences in tissue iron quantities.

We have favoured non-invasive techniques over invasive ones, so that repeated measurements from the same animal can be obtained longitudinally, increasing thereby the statistical power and reducing the number of animals needed to achieve it. Examples of non-invasive techniques that we use include MRI, echocardiography.



By regular exchange of information within the group and with other research groups, we can ensure that tissues are shared whenever possible, or banked for future use.

Animal research data submitted for publication have to adhere to ARRIVE guidelines ([www.nc3rs.org.uk/arrive-guidelines](http://www.nc3rs.org.uk/arrive-guidelines)). We ensure all our experiments are designed to meet these guidelines in advance, to avoid the need for repeating experiments.

**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.

Our breeding strategies are designed to minimise the number of surplus animals of the undesired genotype. We typically ensure that we pair animals so that all of the animals in the litter contain one of the desired genotypes, including the control genotype.

Before incorporating any new procedures or substances into our protocols, we conduct pilot experiments such as dose-finding experiments and safety experiments to ensure that the procedures we use and the doses we administer generate reliable and reproducible effects, and in a safe manner.

For our endpoint measures which require tissue extraction, we will be economical with the tissues extracted, so that they can be used for obtaining the maximum amount of data.

This involves splitting the tissues into multiple batches and using sensitive techniques that only require a small quantity of tissue.

We will continue to review our existing methods to determine if they have been superseded by newer methods that are less invasive and require less animals.

**A retrospective assessment of reduction will be due by 09 August 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 have an investigative strategy that involves an incremental increase in the level of harm or stress that the animal might experience. So we will first investigate the phenotype of transgenic animals under baseline conditions (without any further challenge). If no change is seen in baseline conditions, we then apply a physiological stress. If no response is seen to physiological stress, we apply a pathological stress.

### Physiological stress

Our research is focused on understanding the role of iron in the ability of our bodies to respond to physiological stress. Therefore, we have carefully chosen the types of physiological stresses to replicate those typically seen in humans. For example, we will make the animals iron deficient by provision of an iron-deficient diet for a number of weeks. We have previously refined this procedure by identifying minimum concentration of iron in the diet, and the length of diet administration, needed to produce iron deficiency with or without anaemia. This protocol is well tolerated by animals.

The other approach for producing iron deficiency anaemia is to remove blood, similar to blood donation in humans. We have a refined protocol for doing this, for instance, we have calculated the minimum amount of blood to be removed to produce a well-tolerated drop in haemoglobin levels, and we will also mitigate against any undesired effects of a reduction in blood volume by injecting an equivalent volume of saline to maintain the animal in a healthy physiological state. We will measure haemoglobin levels, and only remove the amount of blood needed to achieve the haemoglobin cut-off needed (80g/L). To minimise the amount of blood removed, some animals may be provided an iron-deficient diet for some weeks prior to bleeding. This should deplete their iron stores, meaning that less blood needs to be removed for the haemoglobin cut-off to be achieved.

The third type of physiological stress is hypoxia (low oxygen), which is used to replicate the clinical conditions typically found in patients with suboptimal lung function. Again, our protocol has been refined to allow incremental decrease in oxygen levels over a number of days, so the animal can acclimatise. This protocol is also well tolerated by animals.

Another form of physiological stress is pregnancy. As in humans, pregnancy places physiological stress on the cardiovascular system of the mother. Nonetheless, pregnancy is well tolerated, even when the mother is iron deficient. Our protocol for studying the effects of iron deficiency on pregnancy has been refined to identify the critical timepoints at which we need to assess the pregnant mother. Thus we have been able to reduce the number of scans and blood sampling steps to a strict minimum. This protocol is also well tolerated.

### Pathological stress

Our research is focused on understanding the role of iron in the context of human disease, and so it is necessary to recreate the clinical conditions seen in humans, such as heart failure and chronic kidney disease. In each disease model, we have opted for a protocol that best replicates human disease but without generating any further unnecessary pain or distress to the animal. For instance, we know the heart attack protocol is well tolerated by the animals because they are provided with sufficient analgesia to manage pain in the post-surgery period.

We have carefully considered the choice of severity limits, to account for the minority of animals that might manifest the disease pathology more overtly than usual.

We have carefully considered the length of each protocol to ensure that we allow sufficient





time to capture the natural progression of disease (and of its reversal or prevention upon treatment), but without maintaining the animals for any longer than necessary. For example, we know that heart failure develops progressively within 6-10 weeks of heart attack, and so typically only maintain animals for 10 weeks post heart attack. We also know that, post pregnancy, the maternal heart returns to normal function within 6 weeks, and so again, we only maintain females for 6 weeks postpartum.

For new disease models, we first set up a small pilot study to chart the timeline of disease progression. This helps us establish the appropriate humane endpoints and identify the critical timepoints at which data should be collected, and the experiment terminated.

We ensure animals are maintained in the best physiological state, by monitoring their weight and general level of activity and state of alertness. Following a given procedure, we additionally monitor for specific signs of pain and ill health that are likely to have resulted from that procedure. For example, we look for signs of blood in urine, following surgical intervention on the kidney.

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

Non-mammalian animals are limited in their use because they do not have the same need for iron and do not respond to iron therapies in a way that would help us understand what happens in humans. Additionally, the techniques for assessment of cardiac, lung, skeletal muscle function have been developed in rodents, this precludes the use of fish or frogs, in which the range of investigations is limited by the small size of the tissue of interest and the lack of adapted techniques.

In most experiments, we have to use adult animals because we seek to model disease conditions that normally occur in human adults.

In some experiments, we use all life stages up to adult, because we are aiming to model the effects of maternal iron deficiency on the progression of pregnancy, as well as on development of offspring, both in the womb and after birth.

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

Animal suffering will be minimised to that which is unavoidable in the conduct of scientifically valuable research. We will adopt a number of steps to minimise pain and suffering to the animals we use:

When setting up new protocols, we will initially include a range of doses and observation timepoints. This approach maximises the likelihood of observing the desired phenotype, and provides information as to the optimal dose and timepoint. This information can serve to refine the protocol further, in order to keep pain and suffering to a minimum and only that which is necessary for the scientific purposes of the experiment. Frequent monitoring will help identify precisely the appropriate humane endpoints

Peri-operative analgesia will be administered and continued after surgery for as long as required to alleviate pain.

Where suitable we will use non-invasive techniques (ECG, MRI, plethysmography), slow release devices, such as osmotic minipumps, to reduce the need for repeated



administration of substances and telemetry devices that allow blood pressure to be monitored over a long period of time.

Animals will be monitored closely after surgery using score sheets, to help early identification of animals that aren't recovering at the expected speed. This means supportive measures and additional analgesia can be provided at an earlier stage.

We will continue to observe strict guidelines of animal husbandry. All of the procedures will be carried out by competent and fully trained researchers using the necessary aseptic techniques (to minimise risk of infection). In addition, all our experiments are designed to avoid single housing of animals.

We will continue to have open and effective communication with the veterinary surgeon and animal welfare officer. For example, we inform them if we plan to carry out any new techniques, so that they can advise on best practice, help us anticipate any caveats and troubleshoot any unexpected adverse effects afterwards.

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

We continue to access online resources such as [www.nc3rs.org.uk](http://www.nc3rs.org.uk) <https://norecopa.no>  
<https://www.lasa.co.uk>

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

Prior to implementing any new protocols, contact the named information officer to help identify researchers who have the relevant expertise. We will seek advice and feedback from those with expertise in the relevant technique.

Attend regular 3R meetings both internal and external to the establishment, to remain informed on best practice in animal experimentation.

Review our techniques regularly in light of updated guidelines published on relevant websites such as [www.nc3rs.org.uk](http://www.nc3rs.org.uk) and <https://science.rspca.org.uk>

**A retrospective assessment of refinement will be due by 09 August 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. Cell therapies for neurological disease

### Project duration

5 years 0 months

### Project purpose

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

### Key words

Neurological disease, Stem cells, Therapy, Alzheimer's, Multiple sclerosis

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged
Rats	juvenile, adult, pregnant, neonate, 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 stem cell therapies for the treatment of neurological disease.

### **A retrospective assessment of these aims will be due by 22 August 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?**

There are over 2 million people living with progressive neurological disease in the UK. These conditions, which include multiple sclerosis, Alzheimer's disease, and hereditary ataxia, are a cause of significant suffering and disability to those affected. The damage and loss of nerve cells associated with these conditions, result in a wide range of symptoms and functional limitations that pose a daily challenge to both patients and those caring for them. These conditions thereby impose a considerable social and economic burden on society. In the NHS alone, expenditure on neurological conditions is approximately £4.4 billion per year.

Despite increased understandings of the underlying causes of neurological diseases, as yet there are no effective curative treatments. Whilst current treatments may slow the progression of the associated nerve cell damage, they all fail to bring about the effective repair required to restore normal function. Consequently, the symptoms of neurological diseases nearly always worsen with time and ultimately are either fatal or result in a shortened life expectancy.

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

The data from this project will determine if stem cell therapies can be used to promote nerve cell survival and repair and thereby restore neurological function. If successful, the study will provide the proof-of-concept and safety data needed to help secure funding streams to support the progression of this work towards clinical trials in human patients. The study findings will be disseminated through publications in high-quality scientific journals and presentations at national and international scientific conferences.

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

In the short term, it is expected that the knowledge gained through these studies will be of direct benefit to scientists working to find cures for degenerative conditions by improving insight into the processes involved in nerve cell repair. Specifically, this project will help advance knowledge of the molecular and cellular processes that occur in response to nerve cell injury. It will also give insights into the protective and regenerative mechanisms of adult stem cell populations and how these processes may be manipulated to improve nerve cell repair and achieve functional recovery. In the medium-term, the work is expected to contribute to the advancement of stem cell-based therapies towards first in human clinical trials for a range of neurological diseases. Within an estimated 10-15 years of project completion, the work is expected to benefit patients and health care providers (such as the NHS) through the development of restorative treatments for neurological diseases.

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

The benefits of the work will be maximised through the dissemination of the data generated via publications in scientific journals, presentations at national and international conferences and publication on our laboratory website. If our studies are successful, we will also engage with pharmaceutical and/or biomedical companies to enable the



development of the regenerative therapies resulting from this project.

### **Species and numbers of animals expected to be used**

- Mice: 1500
- Rats: 100

### **Predicted harms**

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

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

In order for the findings of the studies to be relevant to the clinical need, it is necessary to use an animal that is anatomically and physiologically similar to humans and for which models of neurological disease are established. Rats and mice are the least sentient of the species that meet these criteria. In addition, rats and mice are available as inbred lines, thereby minimising the risk of donor cell rejection, a step essential to the treatment under investigation. Since the study requires the brains of the animals to be fully mature, the outlined studies will be conducted using aging rats and mice.

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

Animals will be bred using conventional methods to create the genetically modified animals required for experimental studies. These animals will either be used as cell donors or cell recipients.

Cell donor animals will carry genetic modifications that either 1) enable specific cell types to be identified, using florescent tags and or 2) enhance/alter the function of specific cell types. These genetic modifications are not expected to result in any adverse effects on the wellbeing of the animals.

Cell recipient animals will either carry genetic modifications or natural mutations that predispose to the development of neurological degeneration or will be normal animals and have neurological disease induced.

Recipient animals will have their bone marrow depleted by exposure to a non-lethal dose of radiation or by giving chemotherapeutic drugs, prior to being given donor cells by intravenous injection. In some cases, animals may also be given agent by injection to enhance cell migration, survival, and proliferation. A few weeks after receiving donor cells, a small blood sample will be taken to confirm engraftment.

A small proportion of recipient animals will undergo a surgical procedure, under general anaesthesia, to implant a cranial window and head fixation post to enable the cerebellum to be imaged while the animal performs simple tasks using a microscope. Post-surgery, the animals will be given pain killers, which will be maintained until the animal is showing no signs of pain. Thereafter the animal will be habituated to head restraint before imaging is undertaken. Head restraint and imaging in habituated animals is not expected to result in the animals experiencing any suffering or distress.

Animals predisposed to the development of neurological disease are not expected to



develop signs until they are quite old (6-12 months) and are not expected to show more than mild signs characterised by poor balance and coordination. The neurological function of these animals will be assessed at time points throughout the study period (up to 18 months of age) using non-aversive tasks to assess balance and coordination such as climbing ladders or balancing on a slowly rotating rod.

Around 20% of animals will have a model of neurological disease induced that mimics Multiple Sclerosis. The model is induced by giving agents by injection (two intraperitoneal and one subcutaneous). The neurological disease induced in these studies peaks at around two weeks, before waning. Animals will be assessed at time points throughout the study period (1-2 months) using non-aversive tasks that test balance and coordination.

At the end of the study period, all animals will be killed to enable tissues to be harvested for analysis. In some instances, a terminal procedure will be conducted under general anaesthesia, prior to killing, to assess aspects of neurological function including nerve conductivity.

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

The majority of animals used in the outlined work will be bred with genetic modifications that do not result in any harm. The animals will be bred using conventional methods and killed humanely to enable their tissues to be harvested for use either in tissue culture assays or as donor cells.

Some animals will be bred with genetic modifications or natural mutations that predispose to the development of neurological disease. These animals live normal lives but in adulthood (from 6-12 months onwards) develop signs indicative of poor balance and coordination however, the disease state is quite mild and is not usually detectable by observation alone and is not thought to impact on the wellbeing of the animals.

Throughout the study, the animals balance and coordination will be assessed, for approximately 6 months after developing signs of neurological disease (up to 18 months of age), using non-aversive tasks, such as ladder climbing and balancing on a slowly rotating rod. These tests are not expected to cause distress and most animals engage in them readily.

Animals undergoing radiation treatment or chemotherapy to deplete the cells within their bone marrow will experience a period of sub-optimum health during which they are vulnerable to infection. To minimise this the dose given will be the lowest required, and following treatment the animals will be housed under conditions that minimise the risk of infection. These animals will be given donor cells, to restore their immune function, within 24 hours of treatment by intravenous injection.

Approximately 1 month later, a small blood sample will be taken to check that the donor cells have engrafted. The injection and blood sampling procedures, outlined above, are expected to cause no more than mild transient pain and distress.

Animals undergoing a surgical procedure to implant a cranial window and head fixation post are expected to experience some level of pain upon recovery however, as far as possible, this will be prevented by giving pain killers, which will be maintained until the animal is showing no signs of pain. Thereafter the animal will be habituated to head restraint, to minimize potential stress, before imaging is undertaken.





Up to 20% of animals will be used in an induced model of Multiple Sclerosis. The majority of these will be normal but some may have genetic modification to label specific cell types however, these modifications are not expected to cause any harmful effects. The model is induced by the injection of agents (2 x intraperitoneal and 1 x subcutaneous). Disease induction is not expected to cause more than mild transient pain. The model induces varying degrees of limb paresis and weight loss but is not associated with pain. The disease state commences at around 10 days post induction, peaks at days 14-16 and then wanes. Where clinical recovery is not complete, the disease progression may be associated with the development of an irreversible loss of tail tone or hind limb weakness due to suboptimal nerve tissue repair. These effects have the potential to cause distress, due to reduced in mobility. To minimise this mice are group housed, and provided with high-energy supplement foods at cage floor level and an easily accessible sources of water. Animals used in these studies will have the severity of the disease state assessed daily and will be killed if the disease progresses to a point where the animal is restricted in its ability to eat or drink. Animals used in the induced model may be retained for up to two months.

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

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

#### **Mice:**

>50% mild  
<30% moderate  
<20% Severe

**Rats:** 100% 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 22 August 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?**

It is not possible to assess the effectiveness of treatment for neurological diseases without the use of animals since it is only in the intact brain that neurons are organised in a



manner that enables their functionality to be assessed. Whilst extensive experimental work, conducted using both cell culture models and human tissue specimens, underpins the proof-of-concept of our scientific approach, it is only through the use of animals that the actual effectiveness of a treatment, in terms of functional change, can be determined.

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

The outlined work is supported by the findings of extensive experimental work, using cell culture models and the analysis of human brain tissue samples. These studies will continue in parallel with the outlined work but do not provide viable options for assessing the effectiveness of treatments aimed at restoring functionality to neurons within the brain.

### **Why were they not suitable?**

Neurons grown in cell culture remain unorganised and do not form communicating units suitable for modelling brain function. It is therefore not possible to assess the effectiveness of treatments aimed at restoring functionality using cultured neurons.

### **A retrospective assessment of replacement will be due by 22 August 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 estimate of the number of animals required to reach the scientific objectives is based on usage under the closely related studies that underpin the outlined work and an assessment of the experimental design and approach to data analysis undertaken with the assistance of a bio- statisticians and the use of the online tools 'Experimental Design Assistant' (EDA) provided by the NC3Rs.

The animal breeding strategy and colony management were designed with the assistance of my institutes genetically altered mouse breeding unit, taking into consideration factors including strain, genotype, age, and sex.

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

All group sizes are calculated on the basis of either published data, (as yet) unpublished data from closely related studies, or pilot studies. The experimental designs and methods of analysis of the results were designed with the assistance of bio-statisticians and using the online tools 'Experimental Design Assistant' (EDA) provided by the NC3Rs.



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

All studies are designed with the assistance of a qualified statistician who will regularly review the group size calculations and update them accordingly. This ensures that animal numbers are minimised, and that the most appropriate analysis is carried out to enable the correct interpretation of the data generated.

All research staff are trained to a high standard and closely supervised to ensure that procedures are carried out with minimal animal suffering and performed in a reproducible manner that minimises variation.

Tissue samples obtained from a single animal are analysed using several different methods to address multiple objectives, thereby minimising the number of animals required for the project.

All tissues samples are stored in an appropriate manner that preserves them for use in future projects and by other research groups.

The breeding strategy used for transgenic mice has been designed to minimise wastage.

Where feasible, surplus tissues from the animals used in these studies will be made available to other researchers through my institute's internal tissue sharing network.

**A retrospective assessment of reduction will be due by 22 August 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 and rats will be used for these studies because they are the least sentient of the species that share a close similarity to humans in their genetic makeup, neuroanatomy, and physiology. In addition, the normal behavioural repertoire of rodents facilitates the assessment of neurological function which is critical to the study and cannot be done using models based on less sentient species. Mice are also the most refined mammalian species in the context of genome manipulation and transgenic modelling.

The animal models selected for the study have been chosen because they all reliably



replicate human disease well, thereby minimising animal usage whilst increasing the likelihood of translating our approaches from rodents to humans. All of the models used have been validated in previous studies, including my own, and are the least severe models to investigate our approaches to discover new therapies for patients with neurological disease. To further reduce the potential for animal suffering in these models, refinements to housing and husbandry, such as acclimation periods, grouped housing, welfare scoring, easy access to both food and water, and use of high-energy supplement foods will be implemented.

Several treatment approaches necessitate the depletion of the animal's bone marrow to enable the engraftment of donor cells. In animals undergoing this procedure, treatment doses, donor cell infusions and post-transplant care have all be refined by our group. The treatment given leaves the animals temporarily vulnerable to infection consequently, these animals will be given antibiotics and housed under conditions that minimise the risk of infection.

Non-invasive brain imaging will be used to reduce the number of animals needed to achieve our objectives. This approach enabling data to be captured at several time points, thereby greatly increasing the data yield from each animal. All surgical procedures will be performed under strict aseptic conditions, following the induction and maintenance of general anaesthesia. Post-surgery, the animals will be given pain killers, which will be maintained until the animal is showing no signs of pain. To ensure stress levels are minimized in animals undergoing imaging, they will be fully acclimatized and habituated to head restraint before imaging is undertaken.

High animal welfare standards are consistently maintained within our animal facilities. All work is conducted by trained and experienced staff. Close monitoring will be in place for all animals under our studies and in the event that an animal show unexpected signs of poor health or distress, veterinary advice will be sought and followed.

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

The outlined studies aim to evaluate novel cell therapies for neurological diseases of humans, with the primary aim of generating the data needed to progress effective therapies into a medical setting for the treatment of patients. In order for the results of the study to be meaningful, it is essential to use an animal with a Central Nervous System that is anatomically and physiologically similar to humans. It is therefore not possible to conduct these studies in less sentient animals as they cannot meet the anatomical and physiological criteria required.

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

The procedures required for the outlined work have been extensively refined by my research group, under previous studies, to minimise the welfare impact on the animals. These include refinements to housing and husbandry for animal models of human neurological disease, such as improved welfare scoring, easy access to both food and water, and use of high-energy supplement foods; and refinements to prevent potential adverse effects, such as skin irritation, using barrier or topical creams.

Prior to the start of the study, the animals will be habituated to human contact by regular handling. The behavioural tests used are non-aversive and do not cause any harm. During the period when animals are susceptible to infection, following the depletion of bone



marrow cells, they will be maintained behind barriers to minimise the risk of infection. Animals used in the Multiple Sclerosis model, which caused varying degrees of limb paresis before waning, will be closely monitored and killed immediately if paresis develops to a point where it impairs their ability to access food or water. To ensure stress levels are minimized in animals undergoing brain imaging, they will be fully acclimatized and habituated to head restraint before imaging is undertaken. At the end of the study, all animals are killed humanely under terminal anaesthesia.

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

All procedures will be conducted in accordance with LASA and NC3Rs Guidelines on best practice for handling, husbandry, and in vivo techniques.

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

I receive regular 3Rs newsletters from our local NC3Rs representative and participate at my institutional 3Rs events. In addition, I monitor advances in best practice by regularly attending conferences in the field, liaising with national and international collaborators, and through weekly journal clubs appraising recent publications. I also maintain live collaborations with engineers and technology developers in the field, optimising opportunities to pilot and/or implement the most efficient and minimally invasive methods of neurophysiological data collection.

**A retrospective assessment of refinement will be due by 22 August 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. Development and validation of New Approach Methodologies (NAMs) for Endocrine Disrupting Chemicals (EDCs)

### Project duration

5 years 0 months

### Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man or animals

### Key words

Endocrine disruption, Xenoandrogens, Thyroid disruptors, Metabolic disruption, Adverse Outcome Pathways

Animal types	Life stages
Zebra fish ( <i>Danio rerio</i> )	embryo, neonate, juvenile, adult
Medaka ( <i>Oryzias latipes</i> )	embryo, neonate, juvenile, adult
Three-spined stickleback ( <i>Gasterosteus aculeatus</i> )	embryo, neonate, juvenile, 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?

Endocrine Disrupting Chemicals (EDCs) interfere with the hormone systems of animals and current regulation requires a high number of vertebrate tests to assess their hazards.





This project licence aims to develop and validate New Approach Methodologies (NAMs), a recognised international priority to modernise the hazard assessment of EDCs.

### **A retrospective assessment of these aims will be due by 6 September 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 issue of endocrine disruption in the environment, known for almost three decades now, is still of very high concern from a policy perspective and generates an ever-increasing need for vertebrate testing. It is widely recognised that the number of chemicals that need to be assessed for hazard and risk in the environment is too high for empirical testing, hence new, high throughput, reliable and preferably animal-free methods for assessing their hazard are urgently needed. In addition, the European Chemicals Agency (ECHA) recognised three areas of regulatory challenge in terms of chemical toxicity, namely neurotoxicity, immunotoxicity and endocrine disruption. For the latter, ECHA suggested that "NAMs for both EATS (estrogen, androgen, thyroid and steroidogenic) and non-EATS modalities (e.g. the Retinoid system pathway) need to be improved and Adverse Outcome Pathways (AOPs) should be developed to facilitate the assessment and interpretation of observed endocrine activity and adverse effects (e.g. metabolic disorders)". A prerequisite for NAM incorporation into regulatory decisions is that they should at least guarantee a similar protection level for humans and the environment that is in place currently. Under REACH (Registration, Evaluation and Authorisation of CHemicals) and CLP (Classification, Labelling and Packaging) regulations, it is only for hazard identification and classification of skin sensitisers that NAMs are sufficiently developed to substitute *in vivo* methods.

The proposed work is fully aligned with ECHA's recommendations (ECHA, 2023), aiming to characterise important endocrine pathways to develop and validate NAMs and AOPs that are useful to regulators. NAMs include a wide variety of methods, from frameworks where the knowledge is organised, such as AOPs, to non-animal (or non-protected life stage) testing and *in-silico* methods, including computational approaches. This licence aims to generate new endpoints and check the validity of existing proposed endpoints, for hazard assessment of EDCs, mainly in non-protected fish life stages (embryonic stages of fish, prior to first feeding).

**Importantly, this licence seeks to validate these early endpoints by demonstrating their presence is prognostic of adverse outcomes that are of regulatory concern.**

Ultimately, the outputs of the proposed work aim to replace or partially replace the need for using protected sentient vertebrates for regulatory testing of chemicals.

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



This project licence is seeking to obtain new or enhanced information on the adverse outcomes associated with EDCs, whilst minimising the future use of protected vertebrates to obtain such knowledge. The work will build confidence in a range of new, emerging or proposed endpoints of endocrine disruption in non-protected early life stages of fish (embryonic forms prior to first feeding). In addition to the (partial) replacement benefits for assessing harm from EDCs in the environment, the work may have refinement implications for human health. For example, one of the current methods used for such assessment, the Hershberger assay, requires the use of young rats that are surgically castrated before being tested.

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

The proposed work is of international relevance as management of chemicals in the environment is a global issue. We aim to better protect wildlife and halt the biodiversity loss experienced in aquatic environments, including marine, by acquiring new mechanistic knowledge on how chemicals affect the endocrine system. This new knowledge will not only allow better management of environmental chemicals benefiting society by protecting natural capital, but it will also help governments shape their policy on the risk management of EDCs. Importantly, the work aims to evaluate the validity of endpoints in non-protected fish life stages and characterise their relationship with apical endpoints that are currently of regulatory relevance but require vertebrate testing. Hence, the benefits of this work are directly relevant to (partial) replacement of animal testing, a strong societal drive in the UK and internationally.

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

The establishment is in a very good position to undertake relevant assessment work due to long term experience and a state of the art aquarium. The research team has representation at OECD Validation Management Group for the Environment (VMG-Eco), which oversees the development and validation of test guidelines for the assessment of chemical hazards. The team has been working in this area for over 25 years and has participated in the conception, development and/or validation of many OECD test Guidelines and guidance documents: the fish endocrine screen (TGs 229 and 230); the androgenised female stickleback screen (AFSS, GD148); the fish sexual development test (TG234); the fish embryo toxicity test (TG236); the two molluscan TGs on reproductive toxicity (TG242 and TG243); the most recent review of the fish acute toxicity test (TG203); the RADAR assay (Rapid Androgen Disruption Activity Reporter; TG251); the REACTIV assay (Rapid Estrogen ACTivity In Vivo assay, draft TG pending acceptance in April 2024).

In addition, we are contributing to PARC (Partnership for the Assessment of Risks from Chemicals), a 7-year partnership under Horizon Europe, which has total funding of €400 million, 50% from the European Union (in our case, UKRI) and 50% from Member States. PARC brings together chemical risk assessors and managers with scientists and stakeholders to accelerate method development and the production of necessary data and knowledge, responding to the needs of end-users.

The outputs of the proposed work will feed directly into test guidelines and guidance documents: AOPs with the endorsement of OECD (see AOP WIKI; <https://aopwiki.org/>) and IATAs (Integrated Approaches to Testing and Assessment).

Unsuccessful approaches will directly inform TG development at OECD and PARC, whilst all data will be published in the peer review literature.



## Species and numbers of animals expected to be used

- Zebra fish (*Danio rerio*): 2500
- Other fish: No answer provided
- Medaka (*Oryzias latipes*): 5000

## Predicted harms

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

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

We are requesting to use three fish species (all common models for regulatory ecotoxicology) to underpin the aims of the project: new endpoints identified in non-protected life stages must be validated with adverse endpoints in protected stages as used for regulatory decisions. Although the focus will be the development and validation of endpoints in embryonic, non-protected life stages, a few targeted experiments will follow these early endpoints into later, protected life stages to ensure they are not transient and linked with harm that is of regulatory concern, such as lack of reproduction, growth or development. The endocrine system is subject to homeostatic responses, hence early markers of disruption do not always translate into adverse outcomes, primarily because life has evolved multiple pathways to bring back homeostasis when the system is perturbed.

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

The fish will be exposed to the chemicals of interest in an environmentally relevant way, which includes waterborne and dietary exposures. The former is a very common route of exposure which we have implemented for over 20 years in our establishment. The latter is of great environmental importance especially for chemicals of low solubility in water but is used less frequently in regulatory testing.

We have recently developed a robust system for dietary fish exposure, under a previous, short-term licence based on the same principles as those used by the fish bioaccumulation test (TG305).

Endocrine Disrupting Chemicals (EDCs) are not typically toxic, i.e. they do not induce visible signs of toxicity; rather they alter the signalling pathways of the endocrine system, affecting key processes for population sustainability, such as growth, development and reproduction. So, we do not expect routine animal suffering, as often anticipated in chemicals that present general toxicity (i.e. narcotics or irritants).

Exposures can be as short as few days or as long as a life cycle. In most cases we will follow the design of existing TGs for the assessment of EDCs as the aim is not only to seek new endpoints in fish embryos, but also to enhance existing test guidelines with additional endpoints. Most animals will be terminated before they reach sentient protected life stages, but some will need to be maintained for longer to link the early potentially diagnostic endpoints with a later adverse outcome of regulatory relevance.

In general, the only stressor we intend to apply is exposure to a single chemical or a binary mixture of chemicals. In some cases, however, when investigating metabolic disruption, we may need to additionally deprive fish of essential food micronutrients (e.g. vitamins) or



alter the photoperiod and temperature cues of natural endocrine signalling. It should be emphasised that the endocrine system, which is the subject system of the proposed work, receives cues from the environment (light, temperature and food) and utilises them in a synchronised way to orchestrate development, growth and reproduction. It is therefore important to understand the interplay of these factors along chemical exposures as they may affect the outcome.

Photoperiod and temperature changes will be applied well within the tolerance of each species (i.e. not outside the normal physiological range), whilst feed deprived from micronutrients will only take place in a few, selected cases and only when we have strong suspicions that adversity may be a combined effect of poor food quality and chemical endocrine disruption.

In addition, fish may be confined in a restricted water volume for a limited period to obtain non-invasive samples of hormone levels. Hormone levels are a key determinant of endocrine system functionality.

Fish may be confined at specific life stages including juvenile, peripubertal, pubertal and sexually mature animals. The team has pioneered the development and validation of non-invasive techniques to obtain information on several sex steroids and cortisol as a stress marker under previous PPLs, and have produced more than 50 relevant peer review publications. The fish may be returned in their tanks following confinement or euthanised. Our multiple observations in applying this method suggests that fish become accustomed to it very quickly and show little evidence of stress after confinement sessions. For example, male fish have displayed nest building activity within 15 minutes of confinement following habituation involving two past trials. Finally, behavioural assessments on feeding, swimming and reproduction may be conducted, involving potentially and principally transfer of fish into different tanks. These transfers are viewed as a mild procedure, similarly to common husbandry practices.

At the end of the exposure period protected animals will be terminated either using a Schedule 1 Method (S1M), or a humane non-schedule 1 method (non-S1M). The non-S1M method most likely to be used is immersion in liquid nitrogen, which is the only way we can obtain tissue gene expression data unaffected by an anaesthetic. All fish used in the proposed work are small teleosts, in which loss of consciousness (and death) are judged as instantaneous from freezing in liquid nitrogen.

Infrequently, we may need to use another non-SM1 method, namely exsanguination (terminal bleeding) as this allows the collection of blood, which is required in some of the regulatory TGs. In this case, killing is under deep anaesthesia.

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

We don't expect the fish to experience pain or any other discomfort except in cases where developmental exposures result in organ or systemic failures to perform basic functions e.g., inability to swim and feed normally, an expected outcome of swim bladder impairment following exposure to thyroid disruptors (AOP WIKI #155-159). The way most EDCs cause adversity is by altering important functions such as growth and reproduction without resulting in mortality, irritation or visible discomfort. Changes in behaviour are expected and are part of the series of endpoints investigated, hence they will be analysed both as humane intervention points (see below) and as supporting evidence of the affected pathway. It is expected however, that the study of androgenic xenobiotics, one of the main



elements of the proposed work, will affect aggression levels that will need to be managed. Another element of expected adversity is weight loss, anticipated in investigations of the thyroid axis and of metabolic disruptions. On such occasions, the actual adverse effects will be managed by defined humane intervention points implemented by intensive monitoring which involves both direct visual checks and software analysis of videos from both in-tank underwater and external (glass aquaria) camera systems. Weight checks, a common husbandry procedure, albeit intrusive, will only be applied in cases where weight gain or loss are diagnostic endpoints for the chemical hazard assessment.

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

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

For all fish species seeking authorisation in this licence we expect the following severities:  
5% Non recovery - this percentage includes control and baseline (stock) animals killed via S1M or exsanguination (non-S1M)  
25% Subthreshold - this percentage includes control and baseline (stock) animals killed via immersion in liquid nitrogen (non-S1M)  
50% Mild - most of the exposed fish, and baseline (stock) samples subject to weight checks, skin swabs and/or confinement for non-invasive hormone measurement prior to humane killing.  
15% Moderate - those animals that display clinical and/or behavioural signs that merit humane intervention.  
5% Severe-those animals that are found moribund or dead in the first day check as the period of suffering may have been more than 10 hours (overnight).

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

- Killed

#### **A retrospective assessment of these predicted harms will be due by 6 September 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 protected animals to validate the early endpoints identified for non-protected stages. This validation data will bridge the current gap between traditional regulatory endpoints and NAMs, ultimately waiving the need for further regulatory animal wastage. The use of animals is inevitable as they currently underpin all regulatory requirements, especially for EDCs. Collecting data to support NAM validity and confirming the relevance of early endpoints to the apical endpoints familiar to regulatory decision





making, is the only viable way of generating confidence for the uptake of NAMs in regulation.

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

We are working towards the development of non-animal alternatives via this work, as currently there are high animal data requirements for assessing hazards from EDCs. Specifically we will:

Generate a series of Adverse Outcome Pathways (AOPs), with full OECD endorsement, to guide future decisions in chemical read-across. AOPs are chemically blind, which means that they can capture any chemical with the same biological target, allowing chemical read-across which will waive the need for further empirical testing. The chemicals used for this purpose under the proposed licence, will be listed as prototypic stressors in the OECD AOP wiki.

Generate a series of IATAs (Integrated Approaches to Testing and Assessment), another important framework that amongst other benefits helps to identify conservation of toxicity targets across species, allowing cross species extrapolations, and reducing duplication of testing in different countries.

Validate fish embryo endpoints, contributing directly to the development of NAMs that allow both the protection of the environment and reduction of the number of animals used. The exception here is the medaka, as under current UK HO guidance medaka embryos are protected as soon as they hatch. This means that the UK will not be able to demonstrate replacement via the application of at least two NAMs that have become part of the OECD TG programme (TG251, the RADAR assay) or expected to be as early as in April 2024 (the REACTIV assay); both require transgenic medaka embryos to be maintained for 24-72h after hatching.

### **Why were they not suitable?**

These methods are under development and require validation to be suitable for regulatory decisions; we wish to contribute to this via the proposed work. NAMs need to demonstrate that they are fit for regulatory purposes rather than represent transient perturbations of a gene or metabolite. We strongly believe that most, if not all, of the non-animal or non-protected animal stages present great opportunities for (partial) replacement, and we strive to generate the validation data needed for their regulatory acceptance. In this perspective we are in an ideal position as institutionally we are directly connected to UK government needs for evidence and can prioritise work to meet requirements.

### **A retrospective assessment of replacement will be due by 6 September 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 propose to use two highly used species for chemical hazard assessment (medaka and zebrafish) and an endemic UK species (the three-spined stickleback), which is also a commonly used fish in OECD TGs. The validation of markers for endocrine disruption in the stickleback will add significant weight to the AOPs and provide the regulator with confidence that the markers of disruption are universal and not just relevant to laboratory fish strains.

The estimated numbers of fish are based on 1) an expected number of tests and 2) the number of fish per test. The number of tests is based upon the level of funding achieved over a 5-year period and our capacity to perform experiments over this time. Both tank and staff availability have been considered, including staff fatigue as monitoring will be intensive over long periods of experimentation.

The numbers of fish per test were calculated using requirements for existing relevant OECD TGs which already take into account the power needed for regulatory endpoints. These include the Fish Early Life Toxicity Test (FELT, TG210), the Fish Sexual Development Test (FSDT, TG234), the Fish Short Term Reproduction Assay (FSTRA, TG229) and the fish endocrine screen (TG230). Additional fish have been added as they will be sampled throughout the experimental duration, to provide means of tightening up AOPs (i.e. looking for relationships between molecular initiating events and key events). Most sampling will involve non-sentient embryonic stages, which are not counted; however, the higher medaka numbers reflect that under UK HO guidance all medaka post-hatch stages are protected, so account for additional animals in this species in the RADAR (TG251) and REACTIV (draft OECD TG) assays.

The point of protection for the three-spined stickleback has been calculated carefully for different temperatures during development over our long-term experience with this model. Embryonic development in fish is temperature-dependent, hence common use of degree-days. Protection for fish starts when capable of independent feeding, and for three-spined stickleback occurs later than 4dph (ca 11dpf) when raised at 17°C. We have documented evidence (can provide videos to justify this suggestion) that exogenous feeding (ingestion of offered feed) doesn't take place prior to 5dph as we have repeatedly offered suitable prey (live *Artemia nauplii*) that was not ingested. Hence, the choice of 4dph (11dpf) at 17°C as the point of becoming capable of independent feeding is conservative and protective. For zebrafish, we will follow current HO guidance which states 5dpf.

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

Sampling of animals over the duration of the exposures has been calculated to account for biomarker or assay variability as well as natural variability so that no animal goes to waste because the power for analysis was too low. In general, we use 6 animals per time point for gene expression work. We also plan to use a novel technique that provides concomitant information on gene expression in all tissues - even in small specimens of fish embryos and early life stages. This novel platform operates on histological slides so all tissues are visible, both for histopathology and for gene network analysis associated with



the pathology. The application of this novel technique alone dramatically reduces the number of fish used as all tissues can be simultaneously assessed in a single animal, adding statistical power to the gene network analysis.

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

In addition to the experimental design and the pioneering techniques we propose to use, we will further optimise the number of animals used for endocrine disruption method validation by:

Careful consideration of chemicals. As the work we plan to do involves the development of AOPs which are chemically blind (i.e. they need to stand up on their own as adverse outcomes, regardless of which chemical initiated the perturbation) the chemicals used in our experiments will either be listed as such under the AOP wiki or selected using a thorough review of the existing literature and examining the chemical structure in terms of ability to bind to proteins. This is because many of our targets are proteins (enzymes or receptors) that, although generally conserved across different animals, they do often present structural differences that hinder or promote interactions with chemicals.

Focus on endpoints for non-protected life stages of fish. All of our animals are bred in-house so that large numbers of eggs/embryos can be collected and used for screening selected chemicals including environmental EDCs before the main experimental procedures start.

In addition, pilot data and observations collected from stock fish will be used to better define sampling points which need to be concomitant with major physiological changes like hatching, initiation of feeding, metamorphosis and sexual maturation.

**A retrospective assessment of reduction will be due by 6 September 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 propose to use three different fish species to provide the necessary confidence for the resulting NAMs to be used at regulation. Two of the species are highly used for regulatory testing of EDCs (zebrafish and medaka), whilst the third (three-spined stickleback) is environmentally relevant and represents a reproductive strategy, which is shared with all



fish that live in temperate waters. This contrasts with the core regulatory species that, although convenient model species, once they reach sexual maturity they spawn until they die which is not representative of the annual reproductive cycle of most fish species. This is of key importance for the development of AOPs as we expect differences in feedback loops between the brain and the gonads, stemming from the different needs inherent to the reproductive strategy.

We are extremely well positioned to undertake research with these species due to substantial past experience. However, we plan to establish our first medaka colony (both wild type and transgenic animals that will be used for the RADAR and REACTIV assays) only after receiving formal training.

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

In many ways, the work will focus on the lowest possible level of sentience as fish are considered lower vertebrates and within this category, embryonic or very early life stage animals are presumed to have even lower capacity to experience suffering. The whole research project aims to validate endpoints in non-sentient life stages of fish (embryonic forms, prior to the initiation of independent feeding). To do this, a number of fish need to be assessed at later, protected stages so the harmful endpoints currently used in these tests are linked to the early markers.

We are very familiar with the physiology of the species we are proposing to use. The exception is the medaka, a species with which we have limited experience (under 3 years). For medaka, we plan to receive robust training before we initiate the in-house colony.

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

The procedures employed (dietary and/or aqueous exposures to chemicals with endocrine activity) are generally mild in nature and so are the anticipated harms. Toxicity of EDCs is subtle, not acute, and often linked with failure to reproduce, grow or behave in a natural manner rather than induction of pain or suffering. Nevertheless, the following steps are in place to ensure refinement:

Daily observations (a minimum of two but if signs of toxicity are evident this will increase to a minimum of four) and video analysis of fish appearance, behaviour and clinical signs via a comprehensive list as developed by the applicant's team for TG203 (see OECD TG203, 2019, Annex).

Application of moribundity *in lieu* of mortality as an end-point of chronic toxicity. This is defined by the presence and severity of clinical signs. This can be considered as a humane intervention point (when suffering ends) to avoid confusion with endpoint (here meaning the response variable to the treatment that is measured and analysed).

Termination of treatments where mortality/moribundity exceeds 40% (acceptable mortality in the regulatory tests employed ranges between 25-30% as they are juvenile fish) of the initial population at any point during exposures.

Application of dietary exposure for chemicals of low solubility in water to avoid the use of high solvent levels; this option presents a refinement as high levels of solvents in waterborne exposure are associated with welfare issues.

Application of photoperiods and temperatures that are within natural seasonal variation for



each fish species.

Provision of suitable environments, including enrichment (e.g. spawning substrates).

Active management of aggression levels by physical isolation of fish where observed. This is expected to be more intense in male fish as the levels of internal androgens are much higher than in female fish.

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

The applicant's team aspires to apply fully the 3Rs principles in their scientific use of animals. Some key literature that is relevant to the programme and is guiding our experimental approaches is listed below:

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

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

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

This body of work aims to improve current test methods for regulating EDCs, and develop the next steps to meet future regulatory needs and minimise animal use. Engagement with the regulator and up to date good practice on animal welfare, chemical toxicity and regulatory needs will be maintained over the project through close links with: the NC3Rs; Defra (policy leads); Environment Agency (the UK regulator); the OECD VMG-Eco; the OECD Extended Advisory Group for Molecular Screening and Toxicogenomics (EAGMST); the Society for the Advancement of Adverse Outcome Pathways (SAAOP);





SKIG, the advisory body of international experts that ensures the AOP wiki is functional and uses standardised information and nomenclature (ontology); SETAC Endocrine Disruptors and Animal alternatives special groups; the International Consortium to Advance Cross-Species Extrapolation in Regulation (ICACSER) which is providing shared resources/modern tools for the 21st century toxicology needs (<https://www.setac.org/page/scixspecies>). In this context a successful outcome would both refine the testing required and reduce the number of fish used by predicting chronic toxicity outcomes via early molecular initiating events.

### **A retrospective assessment of refinement will be due by 6 September 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. Neural basis for adaptive auditory processing and plasticity

### Project duration

5 years 0 months

### Project purpose

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

### Key words

Brain, Behaviour, Hearing, Auditory perception, Deafness

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

This project aims to advance our understanding of the brain circuits responsible for the adaptive auditory processing that enables mammals to perceive and learn about their sensory environments and how this is affected by hearing loss.

**A retrospective assessment of these aims will be due by 6 September 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?**

Our ability to hear and respond to sounds in the world around us often seems effortless. The long-held view is that this remarkable feat is based on the successive activity of millions of neurons arranged into a hierarchy of brain areas between the ear and the neocortex. However, in reality, messages are sent both up and down the auditory pathway to incorporate information from our other senses, motor activity and past experience. We will examine how these bidirectional connections contribute to active listening, enabling auditory neurons to adapt to the sensory and behavioural settings in which sounds are heard.

This research will provide fundamental insights into how the brain processes and adapts to constantly- changing listening conditions and therefore into real-life hearing. By addressing the impact of both sensory and behavioural context across different levels of auditory processing, it has the potential to fundamentally change our view of how sensory systems operate. Furthermore, investigating the adaptability of these circuits will improve our understanding of the neural and behavioural consequences of age-related hearing loss, which affects half the population and is a major risk factor for dementia. We expect the outcomes of this project to inform future treatment strategies for hearing impairments and tinnitus.

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

The primary outputs of this research will be increased scientific knowledge through peer-reviewed journal publications, presentations at scientific conferences, and the generation of extensive datasets and computer code that we will make publicly available for use in future research.

We hope to advance our knowledge in the following two main areas:

First, this project will deepen our understanding of the neural circuits and computational principles that underlie auditory perception, particularly in noisy or reverberant environments. Specifically, we will show how ascending and descending pathways in the auditory system interact to support active listening, enabling neurons to adapt to the sensory and behavioural settings in which sounds are heard.

Second, we aim to show how these circuits respond to hearing loss in adulthood. We will characterise the brain plasticity that provides the basis for training-dependent recovery in the accuracy of sound localisation following conductive hearing loss in one ear, as well as the physiological changes that accompany noise-induced tinnitus and determine whether they can be reversed.

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



Our research findings will primarily benefit the scientific community by providing fundamental insights into the neural basis for auditory perception in challenging listening conditions and how this is affected by hearing loss. This project will therefore help to guide the development of further scientific research by many others. Furthermore, our findings will be of broad interest to the general public. They will advance our understanding of how the brain processes natural sounds – including speech and music – and compensates for the presence of background noise and reverberation, which represents a particular challenge for people with hearing impairments. Our research will also directly address how the brain changes as a result of hearing loss, the commonest form of sensory disability, and the origins of tinnitus, a potentially debilitating condition in which phantom sounds are perceived in the absence of any acoustical input.

Through our close links with ENT clinicians and hearing charities, we are well placed for our research to benefit the future diagnosis and rehabilitation of patients with hearing loss and tinnitus, e.g., through the development of targeted training strategies that are based on the remarkable capacity of the brain to adapt to altered sensory inputs.

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

We will present our findings at national and international conferences and submit our manuscripts to pre-print servers, in addition to publishing them in fully open access journals, to ensure that they have the widest possible reach. We will endeavour to include the results from unsuccessful experiments and will select journals that welcome this. As a recent example, we included apparently conflicting results obtained using two different inactivation methods in a pre-print review paper. We will also make our datasets and computer code publicly available, so that others can utilise them.

Although we have a large research group encompassing a wide range of experimental and theoretical expertise, we will continue to collaborate actively with other researchers, in particular those conducting theoretical research or cognitive studies in human participants.

### **Species and numbers of animals expected to be used**

- Mice: 5500
- Ferrets: 150

### **Predicted harms**

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

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

We will use mice and ferrets in this project. We have obtained a wealth of data in both species, with each offering specific advantages for different aspects of our research. Experiments will typically be conducted in (predominantly young) adult animals to avoid any confounding effects of ageing.

Mice offer unique opportunities for investigating the neural-circuit basis of behaviour due to the availability of transgenic strains and the consequent ease with which proteins for monitoring and controlling neural activity or for tracing connections can be targeted to particular cell types. Behavioural methods are well established and used worldwide for



measuring sensory performance in head-fixed mice as they navigate through virtual environments. It is therefore possible to achieve the stability required for monitoring the activity of the same neurons, dendrites or axon terminals over days or weeks in a behaving animal. Furthermore, mice are widely used for research on auditory processing and learning, and offer direct access to both the auditory cortex and midbrain for imaging and optical stimulation of neural activity.

Ferrets will also be used because of their particular suitability for hearing research and longer-term studies of how the brain changes as a result of hearing loss. Unlike mice, ferrets have good low-frequency sensitivity and their hearing range includes the full range of frequencies audible to humans. Ferrets can be readily trained in auditory behavioural tasks and their sound localisation abilities are comparable to those of humans. This species has been used extensively for investigating task-dependent changes in auditory response properties and most of the evidence for how the brain compensates with training for partial hearing loss has been obtained in ferrets. We have also recently shown that they provide an excellent model for noise-induced tinnitus, in which behavioural and neural assessments of this condition can be made over a period of several months. This is important because in humans, tinnitus tends to fluctuate in its level over time. It is also possible to conduct behavioural experiments in ferrets whilst monitoring or manipulating neural activity without having to use head restraint.

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

### **Mice**

Genetically altered mice will be bred using natural mating methods that will not cause any harms. In addition, the mutations our mice carry are not expected to exhibit phenotypes that will cause the animal to deviate from its normal wellbeing.

Mice will undergo a surgery under general anaesthesia in which we will attach a light-weight device that allows us to hold the head in a fixed position during behavioural training and testing, brain imaging and recording, and/or manipulations of neural activity.

Depending on the scientific question, we may additionally perform one or more of the following:

implant a transparent glass window in the skull to allow imaging of the underlying brain  
make a small opening in the skull that allows administration of various substances or insertion of fine microelectrodes.

These additional procedures may be carried out in the same surgery as head-plate implantation or in separate surgeries with full recovery between each procedure.

After 5-7 days of recovery (depending on the procedure), mice will be habituated to consuming their daily water at a controlled time rather than from a water bottle in their home cage. They will also be habituated to human handling as well as head fixation in experimental setups.

Mice will then be trained daily in head-fixed behavioural tasks that use entirely non-noxious auditory or other sensory stimuli. Mice may respond to or classify objects by licking one or two ports to receive a small water reward. Behavioural sessions typically last less than 1 hour, during which mice perform hundreds of trials. Our simplest sound detection tasks are learned in 1-2 weeks, whereas our most complex behaviours may require up to 2-3 months of training.

After the mice reach a high level of performance on the task, we will record/image and/or manipulate brain activity as trained mice perform the task. In studies of learning, brain



activity will be monitored or manipulated as mice transition from novice to expert stages of performance.

The daily water amount for each mouse will be calculated based on weight, which will either be received in full during task performance or topped up after testing. Body weight will be monitored daily while their access to water is being regulated, with water amounts being adjusted as necessary to keep the animal's weight within a target percentage of their starting weight. The duration of each continuous period of water regulation will depend on the experiment. Typically, data collection will be complete after 3-6 weeks, and the maximum period of time over which an individual mouse will have a chronic implant is 6 months.

Some mice will only undergo behavioural training and testing, whereas if behavioural data (and therefore water regulation) are not required, recordings may be carried out under either terminal or recovery general anaesthesia (with the latter limited to 6 recording sessions, each of a maximum of 3 hours duration, and separated by a minimum interval of 24 hours).

In a minority of mice, after the collection of baseline data, one of the following sensory manipulations may be included:

- reversible plugging of one ear with a standard foam earplug inserted into the ear canal, plus ear impression material in the inner part of the auricle (to help secure the earplug in place and make it more visible), for up to 4 weeks on a maximum of 4 separate occasions.
- exposing one ear to loud noise or mechanical damage to the cochlea to induce a sensorineural hearing loss in that ear. This will be carried out under general anaesthesia and in the case of noise over-exposure, the other ear will be protected with an earplug to reduce the sound level on that side to safe levels.
- whisker trimming as a control for the effects of whisker stimulation and in order to investigate the effects of crossmodal plasticity on auditory processing.
- with behavioural and/or physiological measurements carried out as before.

## **Ferrets**

The majority of ferrets will undergo behavioural training and testing using water regulation to motivate them to perform a sensory detection, discrimination or localisation task using entirely non-noxious auditory or other sensory stimuli. This involves training freely-moving animals to approach and lick one of several water spouts in order to receive a reward comprising a specific volume of water. They will typically be tested twice a day, with each period of controlled water access usually lasting for 5 days before the animals are provided with free access to water for a minimum of 2 days. The volume of water consumed will be measured and topped up at the end of each day's testing to ensure that their expected daily consumption is always met. The animals will be weighed daily when their access to water is being regulated to ensure that their body weight remains within the permitted range.

These behavioural tests will be carried out over several weeks to months, depending on the experiment, at the end of which the animals may be used in a terminal recording procedure or undergo a recovery surgery under general anaesthesia for performing a craniotomy and implantation of a pedestal to house microelectrode recording arrays, a transparent glass window for imaging neural activity, or a small device for shining light on the brain in order to alter neural activity. In some cases, this will require the intracranial injection of substances (e.g., viral vectors to express particular genes in specific neurons),



which may be done at the same time or as part of a separate surgery. Young adult female ferrets will typically be used for these studies, because the thinner skull and much less pronounced temporal muscles mean that surgery duration is shorter and the implantation procedure much simpler than in the larger males. Furthermore, using female ferrets will ensure that animals can be group housed. Once they have fully recovered, behavioural testing may then resume in the tethered but otherwise unrestrained animals whilst recording or manipulating neural activity at the same time.

Individual sessions will typically last 30-60 minutes. The maximum period of time over which an individual ferret will have a chronic implant will be 24 months, with the overall duration of the behavioural protocol limited to 36 months. Alternatively, if no further behavioural data are needed, up to 6 recording sessions under general anaesthesia may be carried out, each limited to a maximum of 6 hours in duration and separated by a minimum of 48 hours.

In a minority of cases, after the collection of baseline data, the animals' hearing will be modified transiently by plugging one ear for up to 4 weeks on a maximum of 4 separate occasions, or permanently by exposing one ear to loud noise or by mechanical damage to the cochlea on one side, with behavioural and/or physiological measurements carried out as before.

After the experiments are completed, all animals will be humanely killed and the brain recovered post-mortem for further analyses.

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

Transient pain after a surgery is expected and therefore controlled for with pain relieving medications.

Controlled water access during our behavioural paradigms typically causes mice to lose 10-20% of their body weight and ferrets to lose up to 15%. Some degree of weight loss normally lasts throughout the period of water regulation, which, depending on the experiment, ranges from 1 week to 4 months (spread out over a 6-month period) in mice and up to 18 months (spread out over a 36-month period) in ferrets.

The sensory manipulations to be used (earplugging or cochlear ablation on one side and whisker trimming) are expected to cause transient pain/discomfort only and to have no lasting effects on the animals' behaviour, other than the intended scientific goal of altering auditory or somatosensory inputs.

### **Expected severity categories and the 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: 83% Subthreshold, 9% Mild, 7.5% Moderate, 0.5% Severe

Ferrets: 23.5% Subthreshold, 73.5% Moderate, 3% Severe

### **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 6 September 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?**

This project is concerned with the neural basis of auditory perception and the behavioural and physiological consequences of adult-onset hearing loss. Specifically, our aim is to investigate how information about the auditory world provided by the ears is processed in the brain in ways that allow neurons to adapt to changes in their inputs, including those resulting from hearing loss, and to integrate sensory and motor signals. These questions can only be studied in the intact brain by direct measurement of the response properties of individual nerve cells and manipulation of neural circuits in order to establish causal relationships between brain activity and behaviour. Our measurements therefore need to be highly detailed, down to a millisecond temporal resolution and a spatial resolution of a few micrometres. This can only be achieved through microelectrodes implanted in the brain or through imaging of exposed neural tissue with advanced microscopy techniques. Moreover, only animal studies provide the necessary control over acoustic experience (by occluding each ear independently or inducing partial sensorineural hearing loss by exposure to loud noise) and the opportunity to manipulate the activity of specific neural circuits using optogenetic, pharmacological or lesioning methods. Progress in this area can therefore only be achieved through animal experimentation.

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

We have considered *in vitro* preparations, computer simulations, and non-invasive human studies.

### **Why were they not suitable?**

This project is concerned with the neural basis of auditory perception and the behavioural and physiological consequences of adult-onset hearing loss. *In vitro* approaches (isolated tissue, cell cultures, organoids) are therefore of limited value because there are no behavioural measures. We are also primarily interested in how the interplay of information transmitted along the ascending and descending pathways of the auditory system underlies adaptive, context-dependent auditory processing, which can only be studied *in vivo*.

Too little is currently known about the relevant anatomy, physiology, and cellular properties of the brain circuitry in which we are interested to replace *in vivo* animal use by computational modelling.



Nevertheless, deep artificial neural networks trained to perform specific tasks can reproduce some aspects of sensory system organization. Our group is at the forefront in using these approaches to model the auditory system, which has reduced our use of animals and led to refinements in experimental design.

Non-invasive human studies do form an important part of our work, particularly in our behavioural research, but functional imaging approaches currently available for studying activity within the human brain lack the necessary spatial or temporal resolution to achieve the objectives of this project.

### **A retrospective assessment of replacement will be due by 6 September 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 is based on sample size calculations we performed for typical experiments in preparing the applications for the awards that fund this research. These numbers are highly consistent with our published past studies and with the numbers published by other groups performing similar experiments. They represent the estimated number of experimental animals necessary to generate statistically significant results, allowing for the fact that a small proportion of experiments may not work.

We have also used our previous Home Office returns to estimate the numbers required in our breeding programmes.

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

We have consulted the NC3R's Experimental Design Assistant as a tool for planning our experiments and make extensive use of computational modelling for generating specific predictions that can be tested using lower animal numbers than would be the case if we did not have access to these computational methods. Through the scientific literature and conferences, we regularly seek the most appropriate genetically engineered mouse lines and labelling methods that are best suited for our experimental questions. We have embraced the recent advances in large-scale recording techniques, such as highly-parallel silicon probes and two-photon calcium imaging microscopy, which enable data to be collected from hundreds of neurons at the same time in a single animal. We were the first group to combine more than one Neuropixels probe, which are currently the state-of-the-art recording electrodes, in chronically implanted ferrets and to adopt anterograde trans-



synaptic labelling methods in the auditory system. These approaches have been extremely effective in reducing animal numbers. Moreover, our within-subject design in which behavioural and physiological methods are combined in the same animal for investigating the effects of hearing loss has largely avoided the need for separate experimental and control groups and further reduced 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?**

Mouse 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 assist in maintaining a suitable colony size to ensure that only those animals needed for experiments are produced.

We routinely use advanced statistical methods (e.g., generalised linear models) to analyse our data to determine when a sufficient number of animals have been used to achieve significance in our results, while our regular use of normative modelling approaches (most recently for investigating how auditory neurons might adapt to the distorting effects of room echoes on the sounds reaching the ears) has led to specific hypotheses that can then be tested in smaller numbers of animals than if we did not have the benefit of these predictions.

**A retrospective assessment of reduction will be due by 6 September 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 both wild-type and genetically engineered **mice**, which enable the targeting of proteins to report or manipulate activity in specific cell types. The genetic alterations we use are harmless to the animals and provide the same quality of life as experienced by wild-type mice. These genetically altered animals also reduce or eliminate the need for additional surgical procedures, such as injections of viruses to deliver genetic modifications. Mice are particularly suitable for *in vivo* 2-photon imaging during behaviour, which involves using a special microscope positioned above the animal to measure emitted light from active nerve cells, thereby avoiding the need for more invasive approaches.



**Ferrets** will also be used both because of the wealth of data already available on auditory processing and plasticity in this species, and because of their particular suitability for behavioural studies of hearing. Their hearing abilities also closely resemble those of humans. In particular, ferrets have good low-frequency hearing, and therefore provide a better model than mice for studies of pitch perception and sound localisation, which are a major focus of our research. Because of their size, ferrets can readily be used for measuring the activity of nerve cells, which will generally be carried out in freely-moving animals using lightweight implants that are easily supported by the animal. Their longer life span will also allow us to study the behavioural and neural consequences of hearing loss without the potentially confounding effects of natural age-related hearing loss.

Animals will be group housed to provide social interaction and stimulation. Any surgical procedures always involve deep anaesthesia as well as analgesics during post-operative recovery.

Animals will be motivated to perform tasks by regulating their access to water during behavioural testing. This provides an effective way of training the animals and is readily tolerated by both ferrets and mice. Measurements of body weight provide a robust measure of health before any serious signs of dehydration are observed (e.g., hunched posture or piloerection), allowing us to provide supplementary water as necessary to prevent any adverse effects.

Beyond the initial implantation surgeries, the methods are relatively non-invasive. Cellular imaging and optogenetics only require shining light on the brain. Modern microelectrodes, when necessary, are very small (only 1 or 2 cell bodies thick), minimising the possibility that they may cause damage in the brain.

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

Certain parts of this project will be carried out in animals that have been terminally anaesthetised. In order to investigate aspects of hearing that depend on sensitivity to low frequency sounds, such as pitch perception and sensitivity to interaural time differences, the principal cue used by humans to localise sounds, we have to use species with a comparable ability to humans to hear these frequencies. Rodents are not suitable because they have poor low-frequency hearing and lack sensitivity to interaural time differences. By contrast, the hearing range of ferrets entirely overlaps that in humans. Ferrets are also more dependent on their hearing than rodents, have a more similar cortical organisation to that of humans, are very easily trained to carry out auditory tasks, and their longer life span enables longitudinal studies of the effects of hearing loss on auditory processing and perception to be carried out. For aspects of this project that do not have these requirements, we will instead use rodents. The mouse has been chosen because this is one of the lowest-order mammalian species that engages in complex sensory behaviour and learning and the major organisational features of its brain are shared by humans and other mammalian species. Animals less sentient than mice, such as invertebrates, lack a comparable brain organisation and do not exhibit the adaptive sensory behaviour and learning that are a characteristic feature of mammals and the topic of this project.

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

We closely monitor the health of our animals on a daily basis and regularly engage with the NVS and animal care staff to ensure that welfare costs are minimised. For example, veterinary staff often attend our recovery surgeries in ferrets. These discussions have led



to the provision of additional measures for any animals with specific needs, as well as more general refinements, such as using more appropriate bedding material for animals recovering from anaesthesia, keeping periods of single housing as short as possible, and ensuring that sufficient intervals are included between steps on a protocol for animals to fully recover. We will follow the latest guidelines and advice as set out at Animal Welfare and Ethical Review Body meetings or in publications from the NC3RS (including the recent paper published by the working group that looked at optimal approaches for head-fixation and controlled fluid intake for motivating animals to perform sensory tasks).

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

Our experiments in mice that involve head-fixation and controlled fluid intake for motivating the animals to perform sensory tasks will follow the recently published findings from an NC3Rs international working group convened to consider best practice in this area. I was a member of this working group and am one of the authors of the resulting publication.

In addition to using existing and any new standard operating procedures, we will follow any equivalent best practice guidelines that relate to other aspects of this project. This includes Laboratory Animal Science Association guidance on substance volumes and administration routes and the Animal Research: Reporting of *In Vivo* Experiments recommendations for the reporting of research involving animals in publications and other outputs from this research.

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

We continually look out for improvements and innovations in our experimental protocols and technology to reduce and refine animal numbers. For example, we have been involved in the testing of Neuropixels silicon probes since they were first developed, and have been promised early access to the next generation of these probes, in which the number of recording sites will be increased by an order of magnitude. We also regularly reassess the design and construction of our cranial implants to ensure that they are as light and small as possible, and secured to the skull in the most refined way.

We presented an earlier ferret implant design at an NC3Rs meeting on cranial implants, and frequently attend technical workshops on recording and imaging techniques at scientific conferences.

We will also continue to engage with institutional and national 3Rs efforts (e.g., through my membership over a 3-year period of the NC3Rs international working group that considered optimal approaches for head-fixation and controlled fluid intake for motivating animals to perform sensory tasks). Relevant items from NC3Rs newsletters and Animal Welfare and Ethical Review Body meetings will be circulated to group members and discussed at lab meetings.

### **A retrospective assessment of refinement will be due by 6 September 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. Targeting compartmentalised cAMP for precision therapy

### Project duration

5 years 0 months

### Project purpose

- Basic research

### Key words

Precision medicine therapy, Hypertrophic cardiomyopathy, Myocardial infarction, Heart failure, cAMP

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 explore the possibility of precisely aiming at a specific part of heart cells to improve the treatment of heart disease by working with a special cellular pathway called cAMP signaling.

### A retrospective assessment of these aims will be due by 20 September 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 heart is a complex organ which relies on messages from a signalling pathway to tell how to function. This pathway is known as the cAMP pathway. This messaging pathway has been shown to be malfunctioning or not working efficiently in cases of heart disease in the human patient. Our research aims to improve or repair this messaging mechanism to improve heart function. Currently,  $\beta$ -blockers are a mainstay in the treatment of cardiac disease. However, they are associated with side effects and they are not effective in hypertrophic cardiomyopathy (HCM) patients or patients affected by diastolic dysfunction.  $\beta$ -blockers interfere with cAMP signalling at its site of synthesis at the surface of the cells, thereby affecting the entirety of the myriad functions carried out by this signalling pathway. The work proposed here will investigate the possibility to target the cAMP pathway at specific locations within the cardiac myocyte cell, with the aim of reducing side effects and increase efficacy in the treatment of cardiac disease. As an example, we will investigate the benefit in reducing the remodelling and diastolic dysfunction associated with the development of heart failure of disrupting the interaction between troponin I and PDE4 at the myofilament. We have accumulated extensive in-vitro evidence that cAMP signalling is uniquely regulated at the myofilament by the formation of a troponin I/PDE4 complex. We have found that this interaction is enhanced in failing cardiac myocytes. Our previous in vitro work also demonstrates that disruption of this complex using a disrupting peptide affects local cAMP levels, troponin I phosphorylation, force of contraction and enhances cardiac myocyte relaxation, providing a strong rationale to extending the work to live animals.

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

We will characterise specific protein-protein interactions (PPI) that are involved in cAMP signalling and may provide novel therapeutic targets for precision therapy. We will establish their role in the physiology of cardiac myocytes and the effects of their disruption in normal and disease conditions. We will develop disruptors (peptides or small molecules) of these PPIs and characterise their properties.

The results of these investigations will be published in peer-reviewed journals.

Disruptors of relevant PPI that show beneficial effects in cardiac disease will be further developed for potential commercialisation.

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

The scientific community: New information, knowledge, and new strategies  
Patients: Novel precision therapeutic interventions for the treatment of cardiac disease.  
NHS and doctors: New therapeutic strategies for treatment for cardiac disease.

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

Dissemination of the new knowledge generated from the project via national and international media of newsletters, presentations of results at conferences and through social media platforms (Linkedin, Facebook.)  
National and international collaboration with academic researchers.



Publication in international peer-review articles.

### **Species and numbers of animals expected to be used**

- Mice: 6500

### **Predicted harms**

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

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

Types of Animals: Mouse heart disease models including surgery-induced cardiac hypertrophy and myocardial infarction leading to heart failure have been very useful for both mechanistic research and the assessment of pharmacological therapies for decades. In addition, genetically modified mouse models also have been widely used in heart disease research. The modified genes based on the mutations leading to human cardiac diseases have proved to be a highly valuable tool for studying genetic cardiomyopathies.

Mice in adult and juvenile stages are required for studying the disease process and the effects of treatments as these stages are relevant to disease progression in humans.

To better understand the protection efficacy of our intervention treatment in inherited hypertrophic cardiomyopathy, we need to administer the substance at the neonatal stage as the chronic disease progression in genetically altered mice may initiate in the early stage.

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

Genetically altered mice will be bred, which will not cause any harms. Some of the mutations will not cause any harm, however, some will result in the animals developing cardiac disease as they age. This may lead to sudden heart failure and as in the human subsequently death. We would expect these animals to be humanely killed or undergo an assessment of the heart disease before this stage of their lives.

Some mice will undergo up to eight echocardiography sessions performed under general anaesthesia to assess the progression of the heart disease.

Some mice will be given therapeutic substances on a single occasion or in combination with cardiac and vasoactive agents for acute pharmacological stress that are expected to affect disease progression, by either the intravenous, intraperitoneal, or subcutaneous routes. The routes and substances are not expected to cause any additional harm to the animals.

Some mice may be given therapeutic substances in the neonatal stage (up to day 3) via temporal vein injection as the chronic disease progression may be initiated in an early stage.

Wild type animals will have heart disease created by either aortic banding or by ligating one of the coronary arteries.

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



## **project?**

Echocardiography requires the use of anaesthesia to keep the animal restrained during the procedure. This is not expected to cause any adverse effects as we will ensure that the animal is fully recovered between each procedure.

The substance administration routes are not expected to cause adverse effects and the proteins we plan to administer should help limit the effects of the heart disease.

In those models where we create heart disease there is a risk of sudden death due to cardiac rupture whilst still under anaesthesia or up to seven days post-surgery.

We will increase the monitoring of the animals during the first seven days post-surgery and again towards the end of the experiment when the risk of heart failure is increased due to the damage to the heart. It is difficult to identify such animals as it is in humans. Typically, these experiments last up to 6 weeks but as an extreme it can be as long as 10 weeks.

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

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

62% subthreshold

19% mild

14% moderate

5% severe

### **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 20 September 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 study animals with heart problems because just studying this disease in the laboratory wouldn't give us the full picture of what happens inside a living body.

Using animals is important because it helps us see how manipulating the cAMP system



affects the whole body, not just individual cells or parts.

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

For some more mechanistic studies we will explore the possibility to use human iPSC-derived cardiac myocytes (iPSC-CM) to replace mouse cells.

### **Why were they not suitable?**

Current protocols to generate iPSC-CM do not achieve complete matured and the endpoint maturity does not recapitulate the mature phenotype. We will use these cells whenever possible to address a number of more basic, mechanistic investigations.

### **A retrospective assessment of replacement will be due by 20 September 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 required has been meticulously determined based on previous experiments and is the minimum required to achieve statistically significant results in our investigations.

Informed decisions to carry out several or all procedures on any given strain of interest will be made based on the data obtained from one or several of these procedures in conjunction with in vitro, cellular, molecular and electrophysiological data to reach scientific significance.

The minimum numbers of animals required have been carefully reviewed by the funding agencies.

We've used our annual return of procedures data to estimate the number of animals that we will need to use for breeding.

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

A thorough literature search was conducted to ensure that experiments that will be undertaken do not duplicate reliable data that is already published or present in data/resource repositories. Principles of experimental design have been applied in order to minimise the number of experiments required to achieve reasonable statistical significance



and data reproducibility.

Three steps for optimising experimental design have been taken:

The study design (justification for how experimental and control groups were chosen) for each project. By using Experimental Design Assessment (EDA), we have made the estimation of the calculation of studies.

We have refined our procedures for the isolation of cells from the rodent heart, so that we get a larger number of cells per heart. This means that we can perform many experiments from each animal.

Furthermore, we have developed a procedure that limits the surface area covered by the cells during culture meaning that less cells are necessary for each sample to be analysed.

Avoidance of bias when two or more treatment groups are compared, the animals in the groups will be in identical environments and be similar in every way apart from the applied treatments. Bias will be minimised by:

- Randomly allocating animals to the treatment groups
- Ensuring that all subsequent treatments (including housing) are applied in a random order
- Ensuring that researchers analysing experimental outcomes are unaware of the treatment received (blinded) until the final statistical analysis.
- Using appropriate number of animals (sample size)
- Controlling inter-subject variation (e.g. using randomisation)

### **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 rigorous measurements to optimise experimental design, pilot studies and computation to minimise unnecessary over-use of animals. For the new lines required, where possible we will import previously generated genetically modified mouse lines from colleagues/collaborators/resource centres. 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.

Tissues/embryos, DNA/RNA and protein samples obtained from experimental animals/embryos will be preserved, usually by storing in liquid nitrogen or at -80oC, to allow, where scientifically justified, use in other experiments by ourselves or other scientists who may request such samples.

Computational models of cardiac myocytes will be used whenever possible to test hypotheses in silico.

### **A retrospective assessment of reduction will be due by 20 September 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.**

Rodents are the lowest vertebrate group in which cAMP signalling is involved in the control of critical physiological functions such as heart performance and for which models of heart pathology have been established. Compared to rats, where models to assess cardiac performance are well established, mice have lower neurophysiological sensitivity and therefore are expected to suffer less than rats. The mouse is also the only appropriate animal model for experimental genetic modification as applied to mammals. So we will use mice as this species provides the unique combination of easy genetic manipulation with cardiovascular system and disease models sufficiently similar to humans. We will also introduce two of the most clinically relevant rodent surgery models, thoracic aortic banding and Coronary artery ligation to mimic human heart failure.

To better understand the protection efficacy of our intervention treatment in inherited hypertrophic cardiomyopathy, we need to administrate the substance in the neonatal stage via temporal vein injection as the chronic disease progression in genetically altered mice may initiate in the early stage.

Most of the breeding and maintenance of genetically modified mice is not expected to suffer any more than mild severity.

The administration of substances will be carried out via intraperitoneal injections, intravenous injection or subcutaneous injection as the most refined methods.

Physiological analysis: Animals are expected to suffer no more than moderate harm due to the nature of the non-invasive echocardiography technique which is the most refined method for this project.

Echocardiography will be conducted under anaesthesia and some animals may experience up to 8 anaesthetic administrations. This is the most refined method as, although conscious echocardiography is possible, the animals would be under extreme stress during the imaging and we would not be able to measure many of the parameters we intend to monitor. For the anaesthesia we will use isoflurane which is well tolerated and the recovery is very quick, so cumulative effects are not expected. All invasive procedures will be performed under anaesthesia and the animals will be continuously monitored post-surgery and will always receive analgesia.

We will conduct analysis of cardiac function in mice models of cardiac disease before any symptoms can be appreciated. It is therefore possible that, to detect a positive effect of our





treatment, it will be necessary to induce a temporary stress on the heart, similar to analysis of cardiac function under exercise conditions in humans.

For this purpose, we will administer vasoactive agents, such as dobutamine, during the echocardiographic imaging. These substances have only temporary effect and are well tolerated.

We will use both male and female mice in both the protocol 2 and protocol 3; for the myocardial infarction model (coronary artery ligation, protocol 4), will use female only in the first instance. In our experience, 25% of the male undergoing this protocol die by cardiac rupture (compared to 5% of female). We will include a male cohort if evidence indicating a sex difference will emerge. A body condition scoring sheet for rodents will be used to assess health and establish endpoints for mice where body weight is not a viable monitoring tool, such as young growing animals.

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

The cardiac physiology varies considerably from the less sentient non-mammalian animal models, the most obvious difference being the incomplete separation of both ventricles with the consequence of completely different circulatory physiology. Hence, use of a small mammal is the only viable option.

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

All the experimental techniques we propose to employ in this project are well-established for this species within our own group and by our collaborators both internally and externally.

A key component of refinement is the implementation of humane endpoints. Welfare assessment protocols and score sheets will be useful tools to monitor adverse effects and determine when humane endpoints have been reached.

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

NC3R's – for advice on animal welfare during and after procedures  
ARRIVE guidelines so that our experiments are performed in a way that is acceptable for publication and are reproducible.  
LASA – for guidance on substance administration and aseptic surgery.

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

By regularly attending the NC3Rs days available at the establishment.

Regular contact with groups working with transgenic mice within our institution allows us to update each other with advances in techniques and training.

Departmental animal welfare meetings are held termly and allow us to communicate any 3Rs implemented within our group and with invited members including Named Animal Care and Welfare Officers (NACWOs) and Named Veterinary Surgeon (NVS) to ensure that any new advances are disseminated, we will also seek the advice from the Named Information



Officer.

**A retrospective assessment of refinement will be due by 20 September 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. Cardiac disease, recovery, regeneration and ageing

### Project duration

5 years 0 months

### Project purpose

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

### Key words

Cardiovascular, Ageing, Heart Failure, Heart Attack

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

## Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is 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 programme of work aims to better understand cardiovascular disease (disease of the heart or blood vessels), including those diseases that are associated with ageing to enable the development of treatments to prevent or reverse these diseases.

### **A retrospective assessment of these aims will be due by 25 September 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?**

We are facing a worldwide increase in people suffering from heart failure; transplantation is currently the only effective treatment. The number of people suffering from heart failure has increased by 33% in the last 5 years, a trend that is likely to continue, and it is estimated that currently, 920,000 people in the UK live with heart failure. The number of people suffering from heart failure is increasing for several reasons including 1) the improved survival following acute disease such as a heart attack, 2) the increased survival of cancer patients who have been treated with anti-cancer drugs which can poison the heart and 3) The progressive ageing of the world's population, as ageing is associated with increased risk of cardiovascular diseases including, coronary heart disease that causes heart attacks, high blood pressure (hypertension) and age-related heart failure. Through a better understanding of the processes that lead to cardiovascular disease and cardiovascular ageing across the life course and into old age, we hope to identify new treatments that prevent, slow, stop or even reverse these diseases.

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

We will have a better understanding of why the heart ages and why we get heart disease as we get older. This will help us identify new drugs that may be able to prevent, slow, stop or even reverse these heart diseases. In addition, during this project, we will be able to test these new drugs to see if they are effective in animals. If they are, these could then be moved toward helping patients suffering from heart disease.

Publication of data in academic journals.

Oral and poster presentations e.g., conferences, seminars, lectures and workshops.

Talks to the public during engagement events.

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

In the shorter term, we expect the scientific community to benefit from our studies. The data obtained from our studies will identify important processes that contribute to disease and ageing which will guide future research, both our own and that of the international research community. Further, we work closely with the British Heart Foundation (BHF), and we have been involved in several engagement events, in which we have shared our data, which has helped them to raise awareness of cardiovascular disease and helps to fundraise. We aim to continue contributing to such events in future. In the longer term and even beyond the duration of this licence, we ultimately aim to identify new treatments which will help patients suffering from cardiovascular diseases, and given there are common processes involved in ageing, our studies could also benefit people suffering from other age-related diseases.

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

We collaborate with national and international collaborators and will share our information with these groups before publication.



We will aim to publish our data including "negative data" in peer-reviewed journals. When this is not possible we will publish in pre-print repositories e.g., bioRxiv.

We will upload our data sets to publicly accessible online data repositories.

We will present our data before publication as oral and poster presentations e.g. at conferences, seminars, lectures, and workshops.

We will continue to engage with the public and present our data at engagement events. For example, we have a close relationship with the British Heart Foundation (BHF) and regularly present our data to BHF donors, workers and patient groups.

### **Species and numbers of animals expected to be used**

- Mice: 4000

### **Predicted harms**

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

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

As we are interested in diseases that effect the adult heart, we will use adult mice which we will obtain from commercial suppliers, or which have been bred and aged in the local establishment. We have chosen to use mice for several reasons 1) Mouse hearts are structurally and functionally very similar to the human heart. They are four-chambered, do not repair themselves well after an injury and are made up of cell types including muscle cells, stem cells, blood vessels and nerves. In both mice and humans, these cells work together to allow the heart to function correctly. 2) Mice age similarly to humans and display many of the same age-related diseases including heart failure. 3) Although mice age quicker than humans this is an advantage as it allows us to perform ageing studies in a shorter timeframe than other larger animal models including rats. 4) Genetically modified mice are available that we can use to identify different cell types involved in disease processes, or which can be used to induce disease. 5) Mice are a well-established disease model, therefore many tools that we use for our studies are already available, for example, antibodies and protocols to model a heart attack are already established. 6) While we have considered using models such as Zebrafish, the fish heart is significantly different from the human being only 2 chambered and there are no methods currently available to study heart attack in fish. 7) We have also considered using non-animal alternatives such as cells in a dish. However, these are unsuitable as they usually contain only one type of cell and cannot model the complex 3D structure of the human heart.

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

Mice will be obtained from an established licensed commercial supplier or bred and aged in the local establishment. In some studies, a surgical model of a heart attack will be performed. This will involve anaesthetising the animal with a drug or substance that causes loss of feeling and awareness and then performing surgery in which a stitch is placed around one of the blood vessels that deliver blood to the heart causing a heart attack. In total, this procedure will take approximately 2 hours. Mice will be given pain relief medication and allowed to recover. The animal will be checked regularly, to make sure it is



not suffering and if the animal is suffering and this cannot be stopped it will be culled. Animals will be randomly assigned to an experimental group, for example, mice may be provided with a drug that we think will improve recovery from the heart attack or reduce age-related cardiovascular disease or given a non-active substance (similar to a placebo used in human drug trials). The drugs will be provided in a way that causes the least distress and discomfort possible for example, orally or by injection. The drugs will include those used by other groups that have been shown to have benefits for other diseases or drugs that have been identified in our research, using cell cultures and computer modelling that target cells or processes involved in cardiovascular disease. The animal may be imaged under anaesthesia to investigate heart function and shape to see if the drug had any effect.

Animals may be kept alive up to a maximum age of 35 months, to see the long-term benefits of the drug and to make sure there are no side effects. Although the age limit for animals in our studies is 35 months, we will only perform surgery on animals up to a maximum age of 24 months. However, the majority of surgeries are expected to be conducted on animals aged approximately 15-18 months. This specific age range is chosen because animals at this stage are biologically comparable to 60-70-year-old humans, which is representative of the average age at which patients experience heart attacks (the average age for humans having a heart attack is 65.8 years old). In a few select studies, we may perform surgery on animals that are 24 months old. The inclusion of these older animals allows us to study animals with an intensified ageing physical composition, which should reduce the number of animals required overall to detect biological differences and assess the effects of various treatments. At the end of the experiment, the animal will be humanely killed, and tissues will be collected. We will use these tissues to further investigate if recovery was improved and to identify how the drugs worked.

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

Some of the mice we will use for our studies are genetically modified and therefore we will need to use DNA analysis to identify these mice. This will be done by extracting DNA from an ear clip from each mouse. This procedure is quick and should only cause mild, short-lived pain. For the studies testing new drugs to treat cardiovascular disease or ageing, the drugs will be given by the least stressful method possible e.g., via food or drinking water. Alternatively, where an injection is required multiple times, we will perform the injections at multiple different sites or use an alternative delivery system such as an implanted device that delivers drugs slowly over time, termed a minipump. Mice may be allowed to age naturally, or we may use compounds to accelerate ageing (for example mice can be treated with anticancer drugs, which are known to speed up the ageing process and cause cardiovascular disease). Aged animals may suffer from normal age-related conditions such as hair loss or arthritis. Blocking a coronary artery (a blood vessel that delivers blood to the heart) will be used to model a heart attack. However, the surgery is complex and causes extensive damage to the heart; therefore, some animals may develop problems with breathing or symptoms of heart failure (for example, rapid weight loss, laboured breathing, and unresponsiveness). If this occurs, the animals are culled. After surgery, mice will be monitored closely and if they do show signs of suffering, which cannot be quickly alleviated or reversed, they will be culled. Consistent with several other groups who perform this type of surgery, up to 40% of young adult mice, and up to 60% of aged animals may die following injury to the heart. Collectively to date, approximately 40% of these deaths occurred during the operation while the mice were anaesthetised, approximately 30% of losses were mice that were culled due to health concerns and





approximately 30% of the deaths were spontaneous, likely due to sudden cardiac death (cessation of the heart beating, an abnormal heart beat or a rupture in the heart), which is effectively instantaneous and associated with minimal suffering. Some animals will be imaged using magnetic resonance imaging (MRI), electrocardiography (ECG), ultrasound or fluorescent methods. The animals will be under anaesthesia for this imaging and similar imaging is used routinely on patients in hospitals and is not normally associated with 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)?**

Mouse:

Non-Recovery, estimated ~10% Sub-threshold, estimated ~10% Mild, estimated ~10% Moderate, estimated ~60% Severe, estimated ~ 10%

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

- Killed

#### **A retrospective assessment of these predicted harms will be due by 25 September 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 human heart is a complex organ consisting of multiple cell types including muscle cells, stem cells, blood vessels and nerves. These cells work together to allow the heart to function correctly. Similarly, ageing is a process in which interactions between multiple organs and tissue, in particular the immune system, contribute to the ageing process. Non-animal alternatives such as cells in a dish do not accurately model the heart as they usually contain one type of cell and do not represent the 3D structure of the heart or the ageing process. We have also considered using Zebrafish; however, the fish heart is significantly different from the human being only 2 chambered and there are no methods currently available to study heart attack in fish.

Furthermore:

Mice are the best-characterised and most widely used experimental model.

Mice represent the simplest organism with a comparable heart and blood vessel (circulatory) system to humans, and they show many of the same age- related diseases as



humans including cardiovascular disease and decreased heart function.

Mice allow genetic modifications, which enable the investigation and identification of biological processes. For example, mice are available in which aged cells contain a label that allows us to identify aged cells and also allows these aged cells to be removed. This allows us to quantify these cells in different diseases and by removing them, we can see if they play a role in different cardiovascular diseases.

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

Wherever possible, non-animal alternatives will be used to complement animal work and contribute to reducing the total number of mice in this project. Further, if we are testing a new drug that has not previously been studied in animals, we will use non-animal alternatives first to make sure these drugs have the expected effects. When possible, we will use human and non-animal alternatives so that the data we obtain is the most clinically relevant. Non-animal alternatives that we will use include:

Human and mouse cell lines.

Human and mouse primary cells (cells isolated directly from human and mouse tissues). These primary cells will be either isolated in our lab or bought from commercial suppliers. While in some cases this still requires the use of animals it will reduce animal suffering as the animal will be humanely culled with no other procedure.  
Human and mouse ultra-thin heart tissue slices.

Human and mouse 3D cells in a dish.

Computer modelling of a biological pathway.

Clinical tissue banks from patients will be used to establish the relevance of the findings.

### **Why were they not suitable?**

The human heart is a complex organ consisting of multiple cell types including muscle cells, stem cells, blood vessels and nerves. These cells work together to allow the heart to function correctly. The above non-animal models cannot model this complexity. Further, to assess the effects of age and possible therapies we need to investigate heart function, which is not possible in the non-animal models.

Similarly, ageing is a process in which interactions between multiple organs and tissues, in particular the immune system, contribute to the ageing process. Even more complex culture models that contain more than one cell type lack a working immune system and therefore are unsuitable for our studies.

### **A retrospective assessment of replacement will be due by 25 September 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 estimated is based on the programme of work described and the existing experience of the minimal number of animals needed in each experimental group to provide statistically robust outcomes.

Group sizes are constantly being re-evaluated and updated when necessary. Control animals will also be required; in the case of drug studies, a “vehicle” control group will also be used. This control group will be given the inactive solutions that the drugs are made up with, similar to using a placebo in a clinical study. In our studies involving a surgical model of a heart attack, we will incorporate a control group known as "sham controls." These animals will undergo the same surgical procedure but will not experience a heart attack. By including sham controls, we can ensure that the outcomes of our study are not influenced by the surgical procedure itself and can confidently attribute them to the modelled heart attack. Animals will be randomly assigned to an experimental group, e.g., heart attack and sham, drug treatment or vehicle control. All studies will be analysed by someone who doesn't know which group the animal is from, this reduces the possibility of unintentional bias during the analysis. In situations in which new methods are used, for example, if animals are to be provided with drugs and the information regarding the doses is not available from prior published studies, small pilot studies will be undertaken to confirm the drug's effectiveness, check a studies feasibility to measure the appropriate outcomes and calculate how many animals are required for each experimental group.

We will work with the colony management team to find the most efficient breeding strategy for each mouse line used, and animals of both sexes will be used in our experiments.

We have consulted with the Animal Welfare & Ethical Review Board (AWERB) statistician and the Colony Management Team in estimating these numbers.

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

We have discussed our project with the establishment's colony management team to ensure we obtain the appropriate number of animals through the in-house breeding of mice or via purchasing animals from commercial suppliers.

We use the NC3Rs experimental design assistant (<https://eda.nc3rs.org.uk/>) to provide feedback on randomisation and blinding of experiments, as well as sample size calculation to determine the minimum number of animals required that are consistent with our scientific objectives. We also work with statisticians and use additional published online tools (e.g. G\*Power (Faul et al., 2007 Behaviour Research Methods 39(2):175-191) and SISA (<http://www.quantitativeskills.com/sisa/>)) to calculate approximate experimental group sizes based on existing experimental and published data.

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



We will work with the colony management team to find the most efficient breeding strategy for each mouse line used and when possible, we will use animals of both sexes in our experiments.

Breeding colonies will be monitored carefully to avoid the over-production of animals.

Breeding colonies that are not required in the medium/long term will be stored as frozen embryos or frozen sperm, to minimise the continued production of genetically modified animals.

Where specific genetically modified mice are available from academic or commercial sources, mice will be acquired for each study, to avoid maintaining a breeding colony. We will share tissues with multiple groups for example the establishment participates in several projects for archiving and sharing mouse lines and experimental data (e.g., EMMA, MRC Mouse Network) as well as maintaining informal arrangements for sharing mouse models.

When possible, we will perform studies that take data at multiple time points rather than use individual animals for each data collection point and we will take as many tissues and organs as possible either for our analysis or to share with other researchers.

### **A retrospective assessment of reduction will be due by 25 September 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.**

### **Mouse models**

For our projects, we need to be able to identify and quantify aged cells in the heart and elsewhere in the body. For this, we will use genetically modified mice in which these cells are labelled. These genetic modifications are considered non-harmful.

### **Delivery of bioactive substances**

For our studies, we will use several substances that interact with biological processes or the bioactive compounds found in living things. These include drugs that may prevent cardiovascular disease, substances that label cells, and compounds that activate genes in genetically modified mice, which will allow longitudinal studies and reduce the overall number of mice required.



Bioactive substances will be provided using the least invasive route possible, for example orally by voluntary intake of food/water.

When a new bioactive substance is to be used, we will first perform small pilot studies with group sizes of 3-5 animals that will be very closely monitored and carefully checked to detect whether there are unanticipated harmful effects before treatment begins in larger groups.

### **Blood sampling**

The leg vein and tail vein are more refined and appropriate routes of sampling for most studies and strains of mice.

### **LAD-Ligation model of a heart attack**

This model involves stopping the blood supply to the heart to induce a heart attack. To make sure the animals are subjected to a minimal amount of pain and suffering:

All operations will be conducted under full asepsis and performed by an experienced surgeon competent in all the techniques required throughout the procedure including pre-operative and post-operative care and monitoring.

Any anaesthetised animal that is deemed unlikely to recover will be culled.

Pain relief drugs will be provided prior to the animal waking from the operation, for a minimum of two days following the surgery and whenever required thereafter if suffering or pain is suspected and it is believed this could be alleviated with pain relief.

Following the operation, we will monitor the animal closely and frequently until the animal becomes responsive and mobile. Then for the duration of the study, the animal will be monitored daily to check for signs of ill health or distress. If at any point, alleviation of suffering is not possible or humane end-points are met the animal will be humanely culled. After the operation mice will be weighed daily for at least 7 days and then weekly for the remainder of the study. This means we can check that mice are recovering well in the first week and starting to regain any weight lost immediately after the surgery, and then check that their weight is being maintained at regular intervals to reduce any stress that being weighed may cause the animal.

Food that is easy to eat will be provided post-operatively.

### **Non-invasive imaging**

Non-invasive longitudinal imaging will lead to a reduction in animal numbers because mice do not need to be culled for tissue collection at different time points to monitor gene repression or activation.

For all models and studies, we will liaise with the Named Information Officer (NIO), Named Animal Care and Welfare Officer (NACWO), Named Training and Competency Officer (NTCO) and Veterinary team regularly to update them on the condition of our animals and to ask for advice if we observed any unexpected adverse effects. Animals will be humanely culled if they exhibit the adverse effects listed above and a full and rapid recovery is deemed unlikely.

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

We have considered using Zebrafish; however, the fish heart is significantly different from the human being only 2 chambered and there are no methods currently available to study heart attack in fish.

Worms and flies have been used to study ageing however, the hearts of these animals are



very different and therefore they cannot be used to address our research question.

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

All animals, regardless of the line used, will be checked regularly and supportive care will be provided to minimise distress or suffering and to improve welfare, for example, low-stress handling techniques will be used and habituation periods allowed when new mouse strains are brought into the animal unit. Regular meetings with the NACWO will be held to discuss animal welfare issues. For our breeding procedures, we can ensure that uniformly high standards of animal care and welfare are applied as the staff involved have extensive experience in this field.

Early socialisation to human contact of mice in our care to reduce the stress of being handled to an absolute minimum. Tunnel handling is our method to pick them up. Before each experiment, we will submit a study plan which will be assessed by a NACWO (surgical plans will also include an NVS assessment). This will allow the NACWO to inform us of any possible refinements. Following the study, any refinements can also be included in subsequent study plans. Study plans include:

A statement of the objective(s)

A description of the experiment, covering such matters as experimental treatments, the size of the experiment (number of groups, number of animals/group), the duration of the experiment and the experimental material.

An outline of the adverse effects, the end-point and special requirements.

Following invasive protocols, all animals will be carefully monitored by trained staff. Group sizes will be increased to accommodate for the loss of animals and to avoid single housing due to animal losses. Animals will be monitored for adverse effects such as changes in weight, dermatitis, piloerection (hair standing on end), paleness, changes in mobility, lumps, eye defects, abnormal respiration, or changes in stools. If these are observed animals will be treated accordingly, and animals that develop severe effects will be humanely culled.

Several experimental procedures in place for the assessment of cardiovascular function will be continuously refined:

#### **Administration of tamoxifen for gene deletion:**

Tamoxifen, a drug used to activate genes in our transgenic mice, will be administered orally via voluntary intake whenever possible or else delivered via I.P. injection and dissolved in corn oil, known to reduce peritoneal inflammation. Peanut oil can also be used as an alternative.

#### **Ageing model:**

Ageing animals will be carefully monitored by trained staff. Group sizes in ageing experiments will be increased to accommodate for the loss of animals and to avoid single housing due to animal losses due to old age. Animals will be monitored for adverse effects using a standardised score sheet, which will include changes in weight, dermatitis, piloerection (hair standing on end), paleness, changes in mobility, lumps, eye defects, abnormal respiration, or stools. If these are observed animals will be treated accordingly, and animals that develop severe effects will be humanely culled.





### **Delivery of bioactive substances:**

Delivery of bioactive substances will be specific to the compounds used. We will be guided by the available literature (if these compounds have been used by other researchers) or manufacturer instructions when using commercially available compounds. If compounds are used that have not been used previously in animal studies, we will perform small pilot studies which will be informed by the structural biology of each compound i.e., stability, dose required, what the compound can be dissolved in etc.

### **Potential routes include:**

Injection into the abdominal cavity. This is the most used method as the amount of administered compound can be better controlled.

Voluntary oral intake of substances avoids handling stress and can replace injections or the use of a feeding tube (oral gavages). When using a voluntary ad-lib water or food consumption route, it must be determined before any compound is added to the drinking water or food that intake will not be reduced during the experiment, because of the addition. The mean daily (24-hour) water and food consumption of the animals will be determined (strain, sex, age and weight differences taken into account) and compared against intake after the addition of any bioactive substance. If rodents reduce food and water intake by more than 10% of their previous intake, become dehydrated, or lose weight, the researcher must adjust the drinking water or food with additives to make it palatable for the animal (e.g., instillation with honey or peanut butter).

Advances in pain relief administration will reduce welfare costs (e.g., oral pain relief in jelly form replacing the necessity of daily injections).

### **Blood sampling:**

The leg vein and tail vein are more refined and appropriate routes of sampling for most studies and strains of mice.

### **LAD-Ligation model:**

We will consider hosting the mice in the facility where the imaging device is housed whenever it is possible. If transfer between facilities is required animals will be permitted a minimum of 5 days recovery prior to imaging.

Additional monitoring will be included for aged mice.

A standardised post-operative score sheet is used to monitor recovery following surgery.

We have invested in a new ventilator to improve the control of anaesthesia.

### **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.

We will follow the published guidelines including:

PREPARE guidelines: <http://journals.sagepub.com/doi/full/10.1177/0023677217724823>.

Code of Practice for Housing and Care of Animals Bred, supplied or Used for Scientific Purposes.

RSPCA Animals in Science guidelines

UFAW Guidelines and Publications



### NC3Rs and Procedures with Care

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

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

#### **Stay informed:**

Researchers and technical teams are educated, trained, assessed and supervised through the local establishment's competency assessment processes. This system ensures good and up-to-date competencies to keep negative effects on welfare to an absolute minimum for every individual working with animal models.

The local Animal Welfare and Ethical Review Body (AWERB), NIO, NACWO, NTCO and Veterinary team regularly inform and disseminate improvements and recent studies involving reduction, replacement, and refinement.

Workshops/seminars/researcher group meetings organised by host establishments throughout the year to inform and share ideas to develop the 3Rs further.  
Internal and external newsletters.

The information available on the 3Rs through different websites such as the Home Office, NC3Rs and the many journals with the relevant authority in animal biology. Alongside external resources including (but not limited to), collaborators, peers, conferences and lab animal and animal welfare bodies.

#### **To implement these advances:**

Prioritise grant opportunities from Research Councils advancing further improvements through the development, integration, and promotion of the 3Rs approaches.

Collaborate with Named persons within the establishment. For example:

I will work with the NACWO to develop the score sheet for aged animals and to assess recovery post-LAD-Ligation.

I will work with the NTCO to discuss new refinements and competency procedures.

I will work with the NVS to implement new refinement procedures.

### **A retrospective assessment of refinement will be due by 25 September 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. The role of inflammation in neurodegeneration

### Project duration

5 years 0 months

### Project purpose

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

### Key words

inflammation, brain, dementia, ageing, eye

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

## Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is 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?

For many years, it was believed that neurodegeneration is a disease of the brain, but we now know it is much more complex. Research has shown that inflammation can damage nerves. The inflammation occurs in the brain as a result of ageing, but is worsened as a



result of other diseases, which are commonly seen in elderly, such as bacterial or viral infections, arthritis, gum disease, gastrointestinal disease or diabetes. Some of these so-called 'co-morbidities' are a result of our lifestyle and our genes. The animal models proposed in this work are designed to study the inflammatory triggers that lead to the onset and/or progression of neurodegeneration and allow us to investigate how manipulation of the immune system may prevent, halt or treat age-related dementia or age-related vision loss.

### **A retrospective assessment of these aims will be due by 11 September 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?**

Worldwide, millions of people are affected by dementia, for example caused by Alzheimer's Disease or Parkinson's Disease. A similar number of people are affected by age-related vision loss, caused by macular degeneration. Currently there is no treatment to halt or cure these diseases. Better understanding of the biological process(es) that results in memory or vision loss, may result in novel ways to treat these devastating diseases of the central nervous system. When we age, certain proteins form clumps, known as plaques or tangles. These can cause damage to the brain, but we don't know how. Inflammation has been recognized as a key factor that can also cause damage to nerves. This may occur via the activation of specialized immune cells in the brain, called microglia. Alternatively, inflammation in the brain may be as a result of chronic systemic infections, or lifestyle choices which not only cause damage to the body, but also to the brain. Identifying the cells and proteins/molecules involved in nerve damage may result in a delay of memory loss, mood changes or vision loss. This will improve the quality of life of patients with dementia, and their families and carers.

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

This work is expected to provide novel information about the cellular and molecular events underlying deposition of misfolded proteins, synaptic degeneration, changes to the blood brain barrier, and inflammation, both inside and outside the brain, in chronic neurodegenerative disease. The results from this program of work would provide a biological explanation for the well-known clinical phenomenon that inflammation is associated with worsening and increased rate of progression of neurological disease. This



includes more recent observations that systemic inflammation or changes in gut homeostasis, are risk factors for earlier onset and/or progression of neurodegeneration.

Pathways and biological factors involved in neurodegeneration may be identified that could lead to the discovery of new therapeutic strategies for Alzheimer's Disease (AD), Vascular dementia, Parkinson's Disease (PD) and macular degeneration. The work should provide valuable information on how genes and proteins under investigation act on human brain or retinal cells. Genetically modified animals carrying human transgenes relevant for AD and PD and human brain tissue will be utilised for investigating the spread of misfolded proteins and pathology and information obtained from these animal models should have immediate application in patients. This program of work will also advance fundamental scientific knowledge of neurons and the genetic, cellular and molecular pathways that control degeneration of healthy, aged and the diseased brain.

Findings will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings. The transgenic animals utilized, combined with our models of neuro- and systemic inflammation are made available to other scientists.

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

Beneficiaries of our proposed program of work include:

Researchers, clinicians, patients, carers, pharmaceutical industry, policy makers. Our previous work has led to collaboration with Industry with the aim to translate the findings seen in mouse models of AD and PD to humans in clinical trials. Likewise, our finding that pro-inflammatory cytokines contribute to disease progression have led to clinical trials using cytokine inhibitors, in collaboration with industry.

The identification of the molecular basis by which peripheral inflammation might impact on synaptic signalling or degeneration processes could lead to identification of potential novel therapeutic avenues to prevent the exacerbation of the clinical condition by peripheral inflammation within the next 5-10 years. In the longer term we hope that these studies will lead to a better understanding of the mechanisms of neurodegenerative disease.

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

We will disseminate our findings in open access journals and conferences.

We will exchange knowledge with the general public using public engagement activities.

We will collaborate with other researchers in academia and industry to improve our understanding of the role of inflammation in neurodegeneration and/or to test novel therapeutic compounds generated by collaborators.

### **Species and numbers of animals expected to be used**



- Mice: 9000

## Predicted harms

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

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

We propose to model Alzheimer's (AD) and Parkinson's Disease (PD) via injection of misfolded proteins directly into the brain of mice, which is expected to cause disease similar as seen in humans. We will also use genetically altered animals that express human genes for the misfolded proteins, who spontaneously develop AD or PD-like disease. We do not expect any adverse effects of these injections or genes by themselves, but mice may in time develop dementia or motor symptoms, similar to humans. The injection of patient-derived or artificial misfolded proteins directly into the brain, may make the mice ill, or delay their recovery and has the risk of infection.

We are interested in the early and mid stages of disease to identify novel ways to prevent or treat age-related neurodegenerative diseases. We will use behavioural tasks that can detect changes in the brain before overt clinical symptoms. This will reduce potential suffering to moderate levels. Some animals will be exposed to inflammation, which will activate, or inhibit, their immune system and some animals receive treatment with a drug or antibody, to test if these may prevent or halt disease progression. We will also use aged mice to study how normal aging contributes to brain dysfunction.

Our main interest in this program of work is to study the role of inflammation in brain diseases. We will use mice that lack certain immune receptors, or mice that express the human version of these immune receptors, to better model human disease. We will generate cell-based models by isolating the brain cells from these 'humanised' mice and test aspects of disease in cell culture, before testing in live animals. We will use very young embryo's as it is easier to isolate the neurons when the brain is not fully developed.

Neurodegeneration does not only occur in the brain, the ageing eye is also affected. To model age related vision loss as a result of macular degeneration, we will use a laser to injure the retina; this procedure is painless in human and we do not expect this to be different in mice. Using this model we will test if drugs or antibodies that block inflammation may prevent or halt vision loss.

When the experiment is finished we will take brain or eye tissue, blood, and other organs and analyse them for signs of nerve damage and inflammation. We will also look at the gut and look in faecal samples, to get information about bacteria that can cause inflammation as a result of lifestyle and diet.

We typically use a range of techniques, and state-of-the-art microscopes to analyse the brain for disease markers, or measure genes following treatment of the animals.





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

In a typical experiment, we will inject into the brain a misfolded protein implicated in AD or PD. We will generate the misfolded proteins in the lab (prion, amyloid, tau, and synuclein) or isolate them from the brain of mice or humans that were affected by the disease. We will then monitor the disease progression by behavioural tasks, that measure memory, mood and motor and retinal function. Some mice will receive a bacterial infection or an altered diet (e.g. high fat), to mimic co-morbidities that increase the risk of early onset or progression of disease. Some mice will receive a drug or antibodies that modulate the immune system to test if these can alter disease progression. A typical experiment takes 4-24 weeks to reflect the chronic nature of a neurodegenerative disease; treatment with an immune modulator will typically be studied at the early or mid stage of disease, for example from 12-18 weeks. We will also use genetically modified animals that express human genes of the misfolded protein; these mice develop disease between 6-18 months, depending on the type of misfolded protein.

To investigate the cause of neurodegeneration or neuroinflammation in our proposed models, we will inject misfolded proteins in mice lacking certain immune receptors or factors (e.g. Fcγ receptors or complement factors), or we cross our AD and PD mouse models with mice that carry a fluorescent reporter that allows us to study the role of immune cells, and in particular microglia. We will also use tracers to track blood-derived proteins and immune cells into the brain or eye and take blood samples to study how effective immune modulators are in our models.

Neurodegeneration and neuroinflammation also occurs in the ageing eye. We will model damage to the retina and/or choroid after laser-injury and monitor the changes to photoreceptors and immune cells using life in-vivo imaging techniques. Our laser-induced injury to the retina results in progressive damage to photoreceptors, with similar neuropathology as seen in humans. We will use genetically altered mice, and drugs and antibodies that modulate the immune system to investigate the underlying biological mechanisms. We will test these treatments under normal and systemic inflammatory conditions to mimic co-morbidities seen in humans.

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

Injection of misfolded proteins into the brain or genetically altered mice may in time develop dementia or motor symptoms, similar to humans. A small number of the genetically altered mice that carry human APP/PS1 gene may die prematurely of seizure activity, similar as described in patients with certain types of dementia; when this occurs it appears to cause sudden death rather than repeated seizures.

Surgical procedures may induce infections and this may make the mice ill, or delay their recovery. Animals will be carefully monitored for any signs of infection and sterile equipment is used to prevent them.



Introducing misfolded protein and/or activation of the immune system may induce sickness symptoms and reduced memory and/or reduced motor strength. We will carefully monitor the mood, memory and motor function behaviour and any mouse in distress will be killed.

Aging mice may show signs of frailty, which may be exaggerated by misfolded proteins and/or inflammation. We will use a mouse frailty index, which takes into account, body weight, appearance, mobility, general activity/nest building, hair loss and any mouse showing adverse clinical signs will be killed.

The loss of photoreceptors in the retina may result in vision loss. We will carefully monitor the retina with state-of-the art imaging techniques and any mouse in distress will be killed.

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

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

The expected severity of mice undergoing stereotaxic injection is moderate. It is expected that ~50% of animals will undergo this procedure

The expected severity of mice undergoing systemic inflammation is moderate. It is expected that ~25% of animals will undergo this procedure.

The expected severity of mice undergoing immune modulation is mild. It is expected that ~25% of animals will undergo this procedure

The expected severity of retinal imaging is moderate. It is expected that ~10% will undergo this procedure.

The expected severity of aged mice is moderate. It is expected that ~10% of animals will be aged.

Even if the procedure is not causing any harm to animals, mice will be maintained for a prolonged period of time, handled multiple times for behavioural assessment and exposed to immune modulatory treatments; these cumulative steps will result in moderate severity.

The expected severity of breeding genetically altered mice that carry human APP/PS1 gene is severe; this is due to a small number of mice (~15%) may die prematurely of seizure activity.

Proportions of animals in each category is based on the total of animals expected to be used in this PPL (total = 9000) over a 5 year period.

summary of the overall estimated severity for the project as a whole: 9000 animal for the duration of the project

Protocol 1 (misfolded protein spread, n=2000) 100% moderate



Protocol 2 (misfolded protein spread + systemic inflammation, n=2000) 100% moderate

Protocol 3 (GA models of dementia +/- systemic inflammation, n=2000) 90% moderate; 10% mild

Protocol 4 (retinal inflammation, n=1000) 95% moderate; 5% mild

Protocol 5 (aging, n=1000) 100% moderate

Protocol 6: (ME7 prion propagation, n=10) 100% moderate

Breeding: GA breeding with harmful phenotype (n=1000) 15% severe, 85% mild Mild: 12%

Moderate: 86%

Severe: 2%

### **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 11 September 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?**

This work is aiming to understand the role of inflammation before clinical symptom occur and it is unethical to conduct experiments on humans where removal of parts of the brain or eye is required for investigating the role of inflammation at the early stages of disease. Due to the complex interaction between the immune and the nervous systems there is no alternative that would entirely replace the use of living animals.

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

We intend to use cells grown in cell culture, where possible, for example neurons or microglia or cells derived from the gut. We also consider developing 3D cultures, of organoids, to better mimic the complex cellular interactions as seen in a live animal. For



example, organoids can be generated using cells from selected organs, for example brain or gut. We also intent to use brain and eye tissue from people that have died with late-stage dementia or vision loss or use skin cells from patients, which can be turned into organ specific cells to generate human organoids. We will adhere to the robust ethical and governance policies when working with human tissue.

### **Why were they not suitable?**

This program of work aims to investigate the link between the immune system, the nervous system and environmental risk factors that contribute to onset and/or progression of neurodegenerative diseases. Alternative methods, such as cell lines, iPSC cell models and organoids are increasingly used to study aspects of neurodegenerative disease, but they do not fully model the interaction between two complex multi-organ systems. Further, age is the most common risk factor for dementia, and aging in cell based models does not fully recapitulate the complex process of aging as seen in whole animals or humans. An increasing body of evidence shows that the gut-brain axis significantly contributes to normal brain function and development of disease. The latter may be driven by diet and microbial composition of the gut microbiome. Cell-based models will not fully mimic the complex interaction between the host and its environment, and take into account the various cell types involved in the process underlying immune-to-brain communication.

### **A retrospective assessment of replacement will be due by 11 September 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?**

To provide a better understanding of the processes of how local and systemic inflammation affect neurological disease this project will over a five year period use about 9,000 mice. We have used knowledge from our own previous research and from other scientists to calculate the number of mice required for a reliable scientific experiment.

From analyzing our previous work, and applying statistical analysis methods, we have found that groups of 8-10 animals provide enough data for reproducible behavioural, histological and biochemical analysis. A typical experiment will contain four groups (control



vs disease, with/without immune modulation. This experimental design means we will use ~40 animals per experiment. Some experiments will be repeated to allow us to include two or three points for behaviour and tissue collection; this allows us to investigate different stages of disease progression and investigate the effect of immune modulation and/or treatment. Our team has 8 PIL holders that use my current PPL.

We anticipate new PIL holders to join the team over the 5 years (e.g. 2 additional users). On average PIL holders carry out 4 experiments per year (=160 animals per year/PIL holder (=~1600 animals per year for the whole team). The license application is 5 years, and I added 200 mice/ year to allow breeding/flexibility and to allow growth of our research portfolio/team.

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

We use the Experimental Design Assistant (EDA) of the NC3Rs to inform our experimental design. The design of the individual experiments will be optimized to ensure that the maximum amount of data is obtained from the minimum amount of animals, without causing additional harm to them. We have already optimized many of our methods which means we can use small amounts of tissue for analysis. This allows us to collect tissue to different techniques (e.g. histology and molecular biology), which reduces 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?**

Inbred mice with common genetics reduces variation between individuals, so fewer mice are needed. Genetically altered mice will be bred to meet experimental demand.

We keep a bank of animal tissue and offer to and have access to tissues produced in collaborators labs.

We isolate cells (e.g neurons, microglia, immune cells, gut cells) to allow in-depth analysis using in- vitro models. Multiple experiments can be done with cells for a single mouse, which therefore reduces the number of animals needed.

Human brain tissue and in-vitro neuronal cultures will be utilised for 'misfolded proteins spreading' experiments and information obtained from them should have immediate application in patients.

**A retrospective assessment of reduction will be due by 11 September 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 mouse models which are proposed in our program have many features in common with human neurodegenerative diseases, including loss of nerves, the activation of the immune system and typical behaviours such as memory loss and mood changes. We already showed that bacterial infections in mice induce symptoms seen in humans and that a high fat diet results in subtle inflammation in the blood, similar to those seen in humans. Previous studies using mice have provided a wealth of information on the role of inflammation in disease and these have resulted in novel treatments.

The knowledge obtained from these mouse studies can be used in our models of dementia and vision loss. The immune system of the mouse has been well investigated and the parallels with the immune system of humans are well known. Importantly, mice are also the only species where genetic manipulation has been widely carried out to permit further comparison and this allows us to investigate how genetics risk factors of dementia and vision loss are related to inflammation, life style choice or infection.

To reduce lasting harm we will restrict our methods to induce mild systemic inflammation and regularly monitor for pain, distress and sickness behaviours using a well-being score. If this score is exceeded, we will terminate the experiment. Mice showing early clinical signs or signs of frailty will be closely monitored for well-being and adverse effects and will receive additional nesting material and wet mesh to provide more easy access to food/water.

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

The immune system is divided in two arms: the innate and the adaptive immune system. The latter is unique to vertebrates and therefore rules out invertebrate models, such as worms and flies. Our program of work includes physiological and behavioural readouts, including motor function, cognitive behaviours and mood changes; for these behaviours higher functional brain networks are required, which requires mature animals. Where possible, we will utilize cells/organs from more immature life stage, for example 15E embryos, to generate neural networks using primary neurons, or to isolate primary cells to generate brain and gut organoids, but these cell-based models will not allow us to measure higher brain function or complex immune-to-brain communication pathways. We





believe that a system based approach, combined with the use of human-derived misfolded proteins and low-grade real life microbial infection is the most appropriate method to investigate the role of the immune system in neurodegenerative diseases, which urgently require novel therapeutic intervention and diagnostic tools.

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

We propose to use models of neurodegeneration, which are selected based on well-described characteristics and their association with human disease. We have selected immune modulatory agents, including real-live microbial infections and dietary interventions to mimic comorbidities seen in man. We will only use methods that induce mild or low grade inflammation to minimize suffering during the experiment. Following surgery and/or immune intervention we will apply the Wolfensohn scale to monitor well-being and pain, and formal behaviour tasks and/or frailty index to monitor clinical signs. Animals will be humanely killed if severity limits are exceeded. Monitoring for well-being are applied weekly at the minimum to limit suffering. We will handle mice prior to behavioural task or when multiple procedures are carried out, for example, when mice are exposed to a systemic infection and receive an immune modulatory treatment, which requires handling, for example to monitor body weight.

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

Where available, we will follow the NC3Rs guidelines for the most refined way of carrying out our experiments. For example, we will use refined capture techniques to habituate mice prior to starting, so mice are familiar with this procedure when daily checking of body weight loss is required. We will also monitor published manuscripts on refined techniques around behavioural assessment and induction of neurodegeneration using misfolded proteins.

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

My team and I are in regular contact with the NACWO and NVS to review current approaches and whether there are any new 3Rs opportunities.

My team and I subscribe to the NC3Rs e-newsletter to keep up to date with the latest 3Rs developments.

We attend the regular user group meetings for advice and guidance around good practice and use of 3Rs.

In addition to the NC3Rs portfolio and gateway and their webinars, we will regularly check use the following guides and databases on alternative approaches:



- The EURL ECVAM Search Guide has been developed to help users find information on alternative strategies and methods to animal-based research.
- FRAME has put together a resource on the basic principles of searching for 3Rs information.
- The Animal Welfare Information Centre has tips for searching for alternatives, as well as links to relevant databases.
- The Duke University Library and UC Davis Library also provide advice on how to search for non- animal alternatives and link to a number of freely available databases, most of which are specifically designed to search for alternative approaches.
- NORECOPA has a series of databases, including on alternatives to the use of animals and information about implementing the 3Rs.

We will also regularly review neuroscience specific databases that are available at the Coldspring Harbour Laboratory Library, including Human Connectome Project, Human Brain Project, Brain

Architecture Project, The Brain Architecture Management System, which provide insight into the latest knowledge about the nervous system, in mouse and human.

### **A retrospective assessment of refinement will be due by 11 September 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. Acute Inhalation Toxicology

### Project duration

5 years 0 months

### Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man or animals

### Key words

Acute, Inhalation, Toxicity, Chemicals, Pharmaceuticals

Animal types	Life stages
Rats	adult
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?

This project assesses the toxicity of test substances (industrial chemicals, agrochemicals (including microbial pesticides), biocides, pharmaceutical intermediates, food additives, food contact materials and household products (ingredients, where permitted), for effects following administration via the inhalation route. Methods used will comply with



published/accepted international test guidelines with methodology utilised not exceeding those specified in the relevant OECD (Organization for Economic Cooperation and Development) test guidelines.

These tests are required by governments worldwide to show that substances the public are exposed to are either safe, or classify the harms they may cause if inhaled.

Such studies are only required when it can be demonstrated that the substance in question has the ability to present an inhalation hazard to humans and suitable data do not already exist.

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 12 September 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 results of these studies allow regulatory bodies around the world to control the manufacture, transport and supply of new products and to make the necessary risk assessment for human and environmental exposure. The results of these risk assessments enable the appropriate risk management strategy to be enacted and this can include the appropriate classification, labelling, hazard communication, transport limitations or banning of substances.

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

The output of this project will be the provision of high quality GLP (Good Laboratory Practice) certified regulatory safety data that allow regulatory bodies (who look after the safety of substances) to control the manufacture, transport and supply of new products and to make the necessary risk assessment for human and environmental exposure. The results of these risk assessments enable the appropriate risk management strategy to be enacted and this can include the appropriate classification, labelling, hazard communication, transport limitations or banning of substances.

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



The short-term benefits will be to clients who have these substances tested, as it will enable them to classify hazards associated with them and allow them to develop and market their substances.

Longer term, this work will benefit the public by enabling these substances to which they will be exposed, to be classified in terms of any dangers they may pose to human health and enable precautions to be taken when they are being used.

### **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 substances being submitted for marketing approval. 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 safety of chemicals and pharmaceuticals that can be administered to humans, or that humans are exposed to.

### **Species and numbers of animals expected to be used**

- Mice: 320
- Rats: 3600

## **Predicted harms**

**Typical procedures done to animals, for example injections or surgical procedures, 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 may be used in this project. The adult rat is the preferred species defined in the regulations these studies are carried out under, and mice may be used if rats are for some reason unsuitable.

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



Animals are dosed by inhalation (either in a tube or in a larger chamber) usually on a single occasion for 4 hours. Food and water will be unavailable during dosing but will be returned afterwards.

After dosing the animals are returned to their cages and closely observed for signs of adverse effects caused by the test substance. This usually occurs for up to 14 days. If we see any adverse effects, the frequency of the observations will increase until the signs reduce.

On rare occasions, blood samples may be taken from an accessible vein for analysis to see if any change indicative of damage has been caused. Animals may be placed in a temperature-controlled warming cabinet to help with this. This makes it easier to take a sample as the heat makes the vein bigger (vasodilation) and easier to sample from.

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

Confinement in a tube or a chamber may cause temporary mild to moderate distress but would not be expected to cause lasting harm. Blood sampling would cause a similar amount of pain as a human patient would experience if they had a blood sample taken by a doctor or nurse.

The majority of harms experienced will be due to exposure to the test substances delivered by inhalation. Exposure to test substances can cause a range of effects ranging from mild, transitory respiratory rate effects to mortality (death), moderate to severe signs of toxicity are expected, with the majority of animals exhibiting mild to moderate severity (cumulatively) during each study period.

These harms will vary between test substances but can include, for example, a loss of bodyweight, underactivity, low body temperature, dehydration, lack of alertness, no response to an external stimulus (animal doesn't move when touched), changes in posture and movement, breathing issues (wheezing, shortness of breath, fast or slow breathing) leading to blue extremities due to a lack of oxygen in the blood (cyanosis), reduced food and water intake, and changes in excretion (urine and fecal output).

These signs can start shortly after dosing, and last for several days. Animals are observed over 14 days after dosing and any animals that display signs that cannot be resolved will be humanely killed.

All animals are humanely killed at the end of the observation period, and further tests may be carried out after death.

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

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





Under the last licence around 60% of animals were deemed to have displayed mild severity, around 35% moderate severity and less than 5% severe severity. We would expect a similar spread on this licence, although this will depend on the type of test substances being evaluated.

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

- Killed

### **A retrospective assessment of these predicted harms will be due by 12 September 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?**

There are currently no non-animal (in vitro) methods suitable for the replacement of these protocols nor are there, as yet, any accepted protocols to enable further reduction in the severity of these studies or in the number of animals used. If the data are not required for formal submission as part of a regulatory package, it is sometimes possible to perform a screening study using reduced animal numbers in order to show that the test item is/is not harmful/toxic.

The studies can be used to investigate a wide range of endpoints (including pathological examination of direct tissue or cellular effects in a wide range of target tissues each composed of multiple cell types) and to examine subtle toxic effects (including behavioural modifications and neurotoxic changes). The complex interactions involved in systemic toxicity are such that these effects can, currently, only be effectively studied in live animals.

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

None, as there are no non-animal tests that are accepted by regulators to assess the safety and toxicity of inhaled substances.

### **Why were they not suitable?**

There is no way to replicate an inhaled dose of a substance in a test tube, or another non-animal model, and non-animal methods are not acceptable to regulators, hence test



substances could not be assessed for hazard classing and safety, and therefore couldn't be awarded market authorisation for use by the public.

## **A retrospective assessment of replacement will be due by 12 September 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 usage in these studies is prescribed in the regulatory guidelines they are run under.

For all dose groups for the OECD 433 test guideline, the total number of animals (five animals of one sex per exposure for the main study) that are used (in order to determine classification of the test item) is reduced in comparison to the other test guideline (OECD 403) and as such the OECD 433 test guideline is now the preferred method to be followed within this establishment.

When the test item is expected to be relatively non-toxic (not expected to cause death), the OECD 403 test guideline allows the use of a reduced number of animals at the limit test concentration (3 males and 3 females or six animals of the most susceptible sex (either males or females)).

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

Mortality data obtained is processed using validated toxicity software. As this software can utilise many methods to determine a lethal concentration, this allows flexibility with regard to the data required. This can represent a reduction in the number of animals used as a meaningful result can be calculated for a variety of different study designs and may result in additional exposure groups not being required.

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



Variables that may affect the study are kept constant wherever possible to make sure the experiments stay the same time after time (e.g. all animals dosed using the same parameters). This actually means that the data is more reliable and meaningful, and easier to make assumptions about.

### **A retrospective assessment of reduction will be due by 12 September 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 general, acute inhalation test guidelines specify the use of young adult rats, other species may be used (mouse), if scientifically justified, but the rat is the species of choice.

The OECD 433 test guideline not only uses a reduced number of animals but also does not require the use of lethality as an endpoint; it instead uses evident toxicity (where moving up to the next highest concentration would be considered to cause death and as such testing would not take place at the higher concentration) in order to classify the test item. For these reasons this test guideline is the preferred method for use in this establishment.

The OECD 403 test guideline establishment of an LC50 (a dose that is lethal in 50% of dosed animals) lower than the limit concentrations outlined in the regulations will require exposure of some animals to lethal concentrations of the test item. Mortalities and marked signs of toxicity are, therefore expected in a certain number of studies performed under this protocol, although the aim is to avoid death as an endpoint as far as possible. Monitoring will be such that the expectation is that as few deaths as possible will occur as processes are in place to intervene with animals that are considered unlikely to survive.

As studies progress, better estimates on the possibility of recovery from the effects of exposure, based on previous exposure groups can be made and, therefore, it becomes easier to determine humane endpoints.



Refinement is also achieved by systems of care and accommodation that enhance the animals' welfare. Environmental enrichment by the use of fun tunnels and gnawing material is provided. Group housing of animals is encouraged unless precluded on scientific grounds.

One of our protocols has a severe severity classification due to the potential for animals to die after exposure to the test materials. This protocol covers regulatory requirements mandated by law to classify chemicals which may be hazardous to health by inhalation exposure in non-European jurisdictions. The OECD 433 guideline will be used to achieve these regulatory requirements if possible, as this is a more refined procedure. If, however, an LC50 is required by a regulatory authority, prospective authority will be sought from the Home Office to run an OECD 403 study, prior to study start.

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

The use of adult animals, usually rats (the preferred species), is detailed in the OECD 433 or OECD 403 guidelines. Other species cannot be used unless specifically justified.

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

During dosing and restraint, animals are closely watched for signs of distress.

If we have to repeatedly withdraw blood using a needle and syringe (rare), we would choose different sites to do this where possible to minimise local adverse effects. If we can take blood samples when an animal is 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 and an inhalation specific group) with a permanent brief to identify potential measures to improve animal welfare, and to trial such measures

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

OECD Guidelines for Testing of Chemicals 07 September 2009 No. 403 "Acute Inhalation Toxicity"



OECD Guidelines for Testing of Chemicals 25 June 2018 No. 433 "Acute Inhalation Toxicity – Fixed Dose Procedure

Method B2 of Commission Directive 92/69/EEC, 2014. Method B52 of Commission Directive 92/69/EEC, 2017.

USA Environmental Protection Agency (EPA) Health Effects Test Guidelines, OPPTS 870.1300, Acute inhalation Toxicity, August 1998.

Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF), Testing Guidelines for Toxicology Studies, 12 Nousan No.8147 (2-1-3) Acute Inhalation Toxicity, November 24 2000 (partially revised on

June 26, 2001).

EU worker safety, environmental safety and material safety data sheet legislation, e.g. the UK Control of Substances Hazardous to Health Regulations

Japanese Poisonous and Deleterious Materials Control Law

REGULATION (EC) No. 1907/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 18

December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No. 793/93 and Commission Regulation (EC) No. 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 200/21/EC

**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 12 September 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. Control of movement in health and disease

## Project duration

5 years 0 months

## Project purpose

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

Movement, Stroke, Spinal cord injury, Motoneuron disease, Tremor

Animal types	Life stages
Rhesus macaques	adult
Cynomolgus macaques	adult
Rats	adult
Marmosets	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?

This project has the overall aim of improving our understanding of the different nerve centres in the brain and spinal cord involved in the control of movement, and translating





the knowledge into improvements in treatments for patients recovering from injury, such as after stroke or spinal cord injury.

### **A retrospective assessment of these aims will be due by 28 August 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?**

This project will deliver new understanding of how different centres in the brain and spinal cord work together to control movement, and how this changes in disease. This will advance our understanding of the fundamental neuroscience of the control of movement by the nervous system, and also of diseases such as stroke, spinal cord injury, motoneuron disease, essential tremor and chronic pain.

**Stroke** is currently the leading cause of disability in the UK. There are >100,000 new strokes annually, one quarter in individuals aged under 65. The UK has 1.3m stroke survivors, around half of whom live with a disability that affects their everyday life (all figures taken from The Stroke Association).

Treatment options for improved rehabilitation are limited, especially for hand function – one reason for this is a poor understanding of the scientific basis for control of movement, and the processes underlying its recovery after injury. The information gained by this project will allow us to devise principled new treatments to improve rehabilitation. If this leads to even small improvements in function, it will translate into major social and economic impact.

In the UK, around 2,500 people per year suffer a spinal cord injury; 50,000 people are living with disability as a consequence of spinal injuries. Similarly to stroke, treatment options to improve hand function are limited, because of poor understanding of the recovery process. In this project, we seek to improve our understanding of how spinal circuits respond to injury, and to test new therapies intended to strengthen surviving pathways to improve function.

**Motoneuron disease** is a rapidly progressing, fatal disease which produces degeneration of the cells in brain and spinal cord which control movement. Most animal models for this disease use rodent models, but these lack an important connection seen only in primates, which may be critical to the biology of the disease. We recently developed a primate model of motoneuron disease, and in this project will characterise the model to understand more



about disease biology, and to test possible treatments aimed at slowing the spread of the disease

**Essential tremor** is a common movement disorder, which produces excess shaking of the limbs. Recent results from human patients have suggested that the disease arises from problems in a part of the brain. We recently developed an animal model of essential tremor by selectively damaging a particular set of connections in this part of the brain in primates; in this project we will use this model to understand features of the disease.

When an animal or human is in pain, voluntary movements are modified to protect the injured body part. We will study the brain circuits which allow this to occur; this could benefit human patients with pain caused by disrupted processing of sensory signals, as well as inform the use of movement scoring to assess pain in animals.

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

This project aims to learn more about control of movement by the healthy brain, and how that control goes wrong and then possibly recovers in diseases of the nervous system. The project will lead to new information, which will be disseminated via peer-reviewed publications and presentations at national and international conferences.

Multiple different parts of the brain cooperate to control movement; these can be divided into the cerebral cortex, which lies on the outside of the brain, and the sub-cortical structures which are deeper. This project will learn more about the relative contributions of cortical and sub-cortical systems to movement control. When a part of the brain is damaged (e.g. after a stroke), people normally show some recovery as undamaged parts of the brain take over some functions. In this project we will define the relative contributions of cortical and sub-cortical systems to this process. Sometimes, recovery leads to new deficits, which are a result of brain structures changing their function to try to improve movement - an example would be the spasticity which often develops after a stroke. This project will also learn how cortical and sub-cortical structures contribute to such harmful changes.

Because so many separate structures are involved in movement control, communication between them is essential to coordinate movements. This project will add to our knowledge of the interconnections between areas (both cortical and sub-cortical), and also within an area. In disease, these connections can be disrupted. The project will also help us to understand how altered connections between areas can lead to movement disorders.

Studies of movement control in humans often use non-invasive methods to assess brain function (e.g. transcranial magnetic brain stimulation). These are powerful approaches, but the measurements are often indirect. This project will use the same non-invasive methods typically used in humans in animals, to provide information about what the techniques actually measure that could not be discovered in human studies alone.



Brain connections are not fixed; they change continually in response to experience as we learn new skills. This project will reveal detailed knowledge of the rules which govern how connections are changed; these rules are likely to be different for different parts of the brain controlling movement (cortical vs sub-cortical structures). We will then go on to use these rules to develop new protocols for changing brain connections using non-invasive stimulation. The project will test these new protocols, providing definitive knowledge about what connections are changed which could not be obtained in human studies alone. These protocols may then find utility in augmenting recovery after damage, e.g. after a stroke or spinal cord injury.

One important type of activity which the brain displays is neural oscillations. Different brain regions exhibit oscillations in their activity at different frequencies. Oscillations are important, as they can often easily be recorded in humans using electrodes on the scalp (electroencephalography, EEG). This project will provide information about how particular brain regions generate oscillatory signals, which could not be discovered using non-invasive human recordings alone. Diseases of the nervous system often lead to reduced or enhanced oscillations - for example, in tremor, when excess oscillations in brain activity spill over into movement to produce debilitating shaking of the limbs. The project will reveal how oscillations become abnormal in diseases like tremor.

Motoneuron disease is a degenerative disorder which leads to progressive weakness; it is ultimately fatal and has no cure. In this project we will further develop and validate a new model of motoneuron disease in primates. The project will provide increased understanding of how the disease spreads from one part of the nervous system to another. It will also test whether certain drugs can slow disease spread; any successful findings would lead to a clinical trial in human patients with motoneuron disease.

When a person or animal is in pain, this alters their movement (e.g. walking with a limp). This project will improve our understanding of how pain interacts with parts of the brain which control movement to modify instructions to produce movements.

Animal procedures for this project often last more than one year. The project will use analysis of cellular features and also brain scans to determine if such long-term participation in experiments has a cumulative impact on the animals' welfare, or whether increasing familiarisation with procedures over time leads to decreasing welfare impacts.

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

Understanding the different contributions of each brain area to movement control, how these intercommunicate, and how different areas contribute to recovery after damage will benefit in the short term researchers interested in the control of movement by the brain and spinal cord. This includes those working only in humans (healthy subjects or patients), where results can often be difficult to interpret without more direct information such as will be provided by this project. The fact that our results will be obtained in macaque monkeys, a primate species with brain systems for movement control very similar to humans, will make them especially relevant to human findings. In the medium to long term our results



may lead to new treatments for disorders of movement, which could improve movement control or enhance recovery of function (e.g. after a stroke). This would benefit patients with these disorders.

Understanding better how non-invasive methods for recording or stimulating the brain work will benefit in the short term researchers using these methods, typically in humans. Because connections within the brain are often different between primates and lower species (e.g. rodents), it is important that any results validating non-invasive methods are obtained in a primate species similar to humans. In the longer term, these findings may lead to the development of new non-invasive approaches, which may not only advance science and treatment of clinical conditions but also allow the reduction of animal usage.

Improved knowledge on how brain connections can be modified is important to develop theoretical models of brain function; this will therefore benefit computational scientists. Because the types of cells and their connectivity is often different between primates and lower species, the fact that our data will be gathered in monkeys will make it especially valuable for developing models relevant to the human brain. Developing new non-invasive stimulation protocols to modify brain connections will be of immediate benefit to scientists interested in changing the brain, and in making changes to treat disease. In the long term, such protocols could lead to effective treatments for disease, which would benefit patients by improving their quality of life.

Providing information on the types of oscillations generated by different brain areas, and how these are generated, will be of great and immediate interest to scientists recording such oscillations non-invasively in humans (e.g. using scalp electrodes). Once again, because brain connections and functions are so different between primates and lower species, our results will be especially significant in interpreting experimental data from humans. Improved understanding of the role of oscillations in malfunctioning brain systems, such as in disease, will benefit clinicians who seek to understand, diagnose and treat these diseases. Ultimately, better understanding of the source and generation of these signals may lead to improved treatments for diseases, such as tremor, which would benefit people who suffer from these conditions.

Our results on motoneuron disease will be of immediate benefit to scientists studying the biology of this disease, as well as other neurodegenerative diseases which may share some features (e.g.

Alzheimer's). The connections from brain to spinal cord are quite different between primates and lower species, and these connections are important for how the disease spreads. Our results are therefore likely to be especially relevant to the human disease because they will be gathered in a primate species with similar connectivity to humans. If our experiments testing the ability of drugs to modify disease spread provide evidence of efficacy at reducing spread, this could benefit patients with motoneuron disease in the medium term as these drugs are progressed to clinical trials. Even if the particular drugs



we test are not effective, this negative information will be important in suggesting the next candidate substances, and could thus accelerate the development of effective treatments.

Understanding how pain affects movement will be of short term benefit to scientists interested in this interaction, as well as movement scientists with a more general interest in how sensory input can influence movement. In the long term, unravelling this interaction may suggest new approaches to therapy to treat pathological conditions such as neuropathic pain, which would benefit patients.

Because the systems for movement control are so different between primates and lower species, providing evidence for how pain influences movement in primates has especially high potential to generate clinical translation.

Providing evidence of whether there are cumulative impacts on animals of scientific procedures will benefit ethics committees and regulators in performing harm-benefit analysis of such work.

Longer term benefits described above for patients may be realised by other unrelated groups, using the information from this project by reading our papers. They may also partly be realised by our own group and our collaborators, as we are actively seeking to develop new treatments in our work with human patients, informed by our results in animals.

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

All findings will be published in peer reviewed journals to ensure effective dissemination of findings. We regularly give presentations at conferences, both of specific findings and of more general overviews of our recent work. This includes at meetings attended by clinicians, increasing the opportunity for our findings to be translated by those directly involved in patient care.

### **Species and numbers of animals expected to be used**

- Marmosets: 3
- Rhesus macaques: 75
- Cynomolgus macaques: 40
- Rats: 250

### **Predicted harms**

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

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

The brain and spinal systems for controlling movement are quite different in primates (such as humans) compared to lower species. For example, we know that in higher primates there are direct connections from the brain down to motoneurons in the spinal



cord which activate muscles. These direct connections are thought to give primates their uniquely fine control of the hand. Such connections are not present in lower animals such as rodents. For this reason, many of our studies need to use primates (macaque monkeys), so that our results can be directly related to human patients. For many studies we can use either rhesus macaques or cynomolgus macaques interchangeably, because their nervous systems are so similar. Both types of macaques can be used in work that does not require behavioural training. Both types can be trained to perform simple behavioural tasks, but rhesus macaques are better suited to behavioural training on the more complex tasks. The choice between rhesus and cynomolgus macaques often is thus determined by availability, although for studies requiring training on complex behavioural paradigms rhesus would be mandated.

Some experiments which look at very basic circuit properties can use rodents with validity - for example, when looking at cell types and connectivity within the brainstem, a structure which is conserved from rodents to primates. However, often detailed studies in rodents might need to be validated with a small number of studies in primates, to confirm that connections have not been altered by the substantial differences in other systems for movement control within primates.

Finally, we plan one study using marmosets. These are primates, and hence have very similar biology as humans. In particular, one protein which is important in motoneuron disease is similar between marmosets, macaques and humans, but differs in rodents. However, because marmosets are a New World primate they lack direct connections from the motor cortex (a major brain centre controlling movement) to motoneurons (which send connections out to muscles to activate them and generate movement). We will compare our primate model of motor neuron disease in marmosets and macaques. This allows us to reveal the importance of the direct connections to the spread of motor neuron disease, because the only difference between the motor system in these two primates is the existence of the direct connections.

This work is concerned with the function of the mature nervous system, and the control of movement in adults. For this reason, we will use adult animals in all of our studies.

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

### **Experiments in Macaques**

In most experiments, macaques will be trained to accept some restraint (a neck collar and arm restraint), and to perform a behavioural task. They are motivated to perform the task by having restricted access to food, and occasionally fluid; food and fluid rewards are then given for correct task performance. In some cases, animals obtain all of their daily food intake as rewards for task performance. Fluid restriction is only used overnight; animals always have free access to water after their daily training session. Fluid restriction is always combined with food restriction. At sometime during training, animals undergo an MRI scan under anaesthesia to show us the location of different brain regions in that animal, and to allow us to build a 3D model of the animal's skull. After training is complete,





they are surgically implanted with a headpiece to allow head stabilisation and electrodes in the arm to record muscle activity. The headpiece is shaped to fit the skull using the 3D model produced from the MRI scan. It includes ports, placed over openings in the skull, allowing access to record from different brain regions. Implantation of the electrodes in the arm requires multiple incisions over the upper limb. Recordings of muscle activity may also be made using an adhesive electrode with many contacts placed on the skin over a muscle. In order to maintain this in a fixed location from one day to the next, animals may have small alignment marks tattooed on the skin. In some animals, electrodes may be implanted in reward centres to motivate them to perform the behavioural task; this can be a refinement, as it allows us to use a reduced food restriction protocol while the animal maintains the same level of motivation. For this reason, reward centre stimulation is typically combined with food but never fluid restriction. Recordings will then be made from the central nervous system, whilst the animal is awake and performs the task. Animals may then be surgically implanted with a device on the back allowing access to the spinal cord, and further recordings made from spinal neurons during task performance.

In some animals, we will modulate activity within the nervous system by injecting tiny quantities of drugs directly into a particular brain or spinal region with a cannula. Another way to modulate cell activity is to inject a virus into a part of the nervous system carrying genes making a cell responsive to light. We then insert a light source (e.g. optical fibre) into that region, and use it to illuminate the cells with the correct colour to make them respond. Finally, we may inject a virus carrying genes making cells responsive to a drug, which does not normally affect nerve cells. We can then inject that drug to alter activity.

In some monkeys, we will use controlled heat applied to the skin to produce pain linked to a particular movement. Alternatively, high intensity electrical stimulation may be used, also linked to a particular movement. This mimics the common situation where movement produces pain, such as when a muscle is sprained; this allows us to study the ways in which the brain changes movement to avoid further tissue damage. In other studies, pain will be induced using injections of small amounts of high- strength salt solution, or a substance which is known to activate nerve fibres which sense pain. This mimics pain which does not appear only with movement, but which may also change movement to avoid further damage.

In a small proportion of animals, a specific small area on one side of the brain will be damaged using surgical techniques. Some animals will have such lesions in two specific small areas. MRI scans under anaesthesia may be used after the lesion to check on the lesion location. In other animals, the spinal cord will be damaged to mimic a spinal cord injury. This allows us to study how brain and spinal cord circuits reconfigure after damage, a process which may allow recovery but may also be unhelpful and lead to further disability.

In other monkeys, viruses will be used to insert genes into nerve cells. These genes will make the cells produce excess levels of protein thought to be involved in motoneuron disease. This permits us to study the biology of the disease spread, and to test ways of



reducing spread using drugs. Some of these animals may be implanted with a catheter in a vein, to allow administration of the drug and blood sampling to check on its metabolism within the body. MRI scans in the awake state, of the brain and the arm, may be used to assess disease spread with time.

In other monkeys, we will inject a drug into the brain which damages nerve fibres which use a particular chemical. We have shown that this creates a primate model of essential tremor, which is a common human disease. This allows us to study the changes to processing of information by the brain, and to understand how this leads to tremor.

In some animals, we may make measurements of their behaviour in their home cage, using instrumented tasks which they can access continually to earn small food rewards. Some of these animals may be implanted with identity chips in each arm (the same as used to identify pets), to allow the task system to identify which animal (of a pair housed together) is performing the task, and with which arm.

Animals will typically undergo a maximum of three major surgeries (e.g. a headpiece implant), and up to a further five more minor surgeries. Minor surgeries are typically required to affect repairs to implants. In addition, animals may be sedated (typically no more than monthly, but could be as often as twice per week for a short period), for example to allow cleaning of skin margins around the headpiece, or checking on a wound where this is not possible in the awake state.

### **Experiments in Marmosets**

In marmoset monkeys, viruses will be used to insert genes into nerve cells. These genes will make the cells produce excess levels of protein thought to be involved in motoneuron disease. After waiting for a period (around 7 months) where this protein is produced, the marmosets will be humanely killed and their brains and spinal cord examined to measure the spread of the disease.

### **Experiments in Rodents**

To answer some questions about connections between parts of the brain or spinal cord, or the types of cells found in a given location, and where systems are not expected to differ between rodents and primates, we will use rats. Rat experiments may also be used to provide comparative data, to illuminate how primate and rodent systems for movement control differ. Rats may be prepared for recording by a surgery to inject substances into the nervous system. These substances will either be tracers, which allow us to track where connections travel to, or novel genes, which confer new properties on nerve cells (e.g. making certain specific types of cell responsive to light). After these injections the rats are allowed to survive for a few weeks. Subsequent experiments are carried out under anaesthesia, and involve making electrical recordings from the nervous system or removing brain samples for analysis in a dish. The animal is killed before it can recover from this anaesthetic.



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

### **Experiments in Macaque Monkeys**

The most common adverse effects are associated with wound infections associated with the long-term implants (e.g. for head fixation). These are managed by medical or surgical treatment (e.g. with antibiotics, or by cleaning and resturing the wound), under the advice of the named veterinary surgeon. Most animals (<100%) will experience some infection. Individual incidences of infection are usually treated successfully and last <2 weeks, but infection may recur at a later date. Rarely (<10%) a headpiece may loosen its attachment to the skull, requiring a repair surgery to restabilise it. Loosening of a spinal implant is more common (<40%).

Some animals (<20%) may fail to adapt to the laboratory environment, or fail to learn a given task. In these cases, we usually switch the animal to a simpler task for a different project. Some animals (<20%) may show weight loss as a consequence of food restriction; this is typically associated with failure to adapt to the laboratory or learn a task. In these cases, switching to a simpler task usually reverses the weight loss; ad libitum food is given to ensure that they have sufficient.

All animals show initial stress when neck collar and arm restraints are first introduced, but this usually rapidly declines (within 2 weeks) as they learn that training sessions are associated with positive rewards. Occasionally (<10%), rapid movements against restraints can lead to mild bruising or marking of the skin.

In some animals (<10%) after implant of identification chips, the implant comes out. This could cause mild pain and discomfort for a few days until the residual wound heals.

Sometimes (<20%) animals show a reaction to implants in the arm to record or stimulate from muscles and nerves; this is treated medically to reduce inflammation, and lasts less than 2 weeks. In animals implanted with a catheter for intravenous injection or blood sampling, there is the potential for a reaction against the catheter (<20%); this is also treated medically to reduce inflammation, and lasts less than 2 weeks. There is also the potential for bleeding around the catheter implant site (<10%); this would be treated surgically to ensure a good seal between catheter and blood vessel, and will last no longer than a day.

Where an animal wears a jacket to protect wounds from interference, it is possible that the jacket may rub or chaff (<5%). This is prevented by careful sizing of the jacket to the animal.

In monkeys where we study the impact of pain on movement, the monkeys will experience painful stimuli on some trials. However, just as in humans exposed to this procedure, we expect that the monkeys will rapidly change their movements to avoid activating the stimuli, and thus the pain will only occur on a few trials.



In monkeys following brain lesions, in the days immediately following the animals may need nursing help with feeding due to impaired movement ability, which may include a loss of movement in one limb. However, as in human stroke patients with little damage they often show a rapid recovery (1-2 weeks), although there is likely to be a residual impairment in limb function on one side. Some monkeys (<70%) may show spasticity.

After lesions of the spinal cord, some animals (<10%) may experience bladder or bowel dysfunction. This will last no longer than 3 days, with supportive treatment given to empty bladder and bowel over this time if necessary.

In monkeys used to create a model of motoneuron disease, there will be some loss of motoneurons and weakness in the targeted muscle. The weakness may then spread to other muscles, although the experiment will be terminated before this impacts significantly on welfare (e.g. interfering with self-feeding or self-care, or ability to move around the home cage).

In situations where a lumbar puncture is performed, or electrodes are implanted close to the spinal cord, there is a possibility of a leak of cerebrospinal fluid which will cause a headache (<10%). This usually resolves with treatment within a few days.

Sometimes when electrode penetrations are made into the brain or spinal cord, swelling or damage can result in weakness in the limb controlled by that area. This is rare in the cortex (10%), but more common for the brainstem (30%) or spinal cord (80%). Rarely (<10%) irritation by the electrode can lead to an epileptic seizure.

After any surgery, all animals show mild effects of recovery from the anaesthetic, but this is usually resolved by the day after the surgery. Rarely (<10%), and animal may show stiffness of a limb due to fixed posture during the surgery; this resolves within 2 weeks. Rarely (<10%) the facial nerve can be damaged by the equipment used to hold the head during surgery; this can reduce the ability of the monkey to blink. This resolves within 4 weeks.

### **Experiments in Marmosets**

Rarely (<10%), injection of viruses into the marmosets may cause swelling of the brain, infection or a leak of cerebrospinal fluid causing a headache. This is resolved within 2 days.

All animals will show mild effects of recovery from the anaesthetic, but this is usually resolved by the day after the surgery. Rarely (<10%), and animal may show stiffness of a limb due to fixed posture during the surgery; this resolves within 2 weeks. Rarely (<10%) the facial nerve can be damaged by the equipment used to hold the head during surgery; this can reduce the ability of the monkey to blink. This resolves within 4 weeks.

### **Experiments in Rats**



Recovery from the initial surgery is unlikely to show adverse effects, other than mild effects of recovery from the anaesthetic which is resolved by the day after the surgery. There is a chance of weight loss and abnormal post-operative behaviour (<10%), wound breakdown or infection (<10%), or weakness following neurosurgery on important structures involved in the control of movement (<10%).

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

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

The macaque experiments will almost all (>90%) have moderate severity, although the licence limit of 'severe' may be reached for short periods in some animals associated with the period immediately after surgery-induced brain or spinal cord damage. No more than five animals (<7%) are likely to experience an overall classification of 'severe' over the entire five year licence.

Marmoset experiments will be of moderate severity (100%).

Rat experiments which include a recovery surgery (up to half of the animals, 125 rats, 50%) will all be of moderate severity. Some rat studies will require only an experiment under anaesthesia where the animal is not allowed to recover (50%, non-recovery).

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

- Killed

#### **A retrospective assessment of these predicted harms will be due by 28 August 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?**

This project investigates the complex interplay of nerve circuits in different regions of the brain and spinal cord, and as such must be carried out in intact organisms. The laboratory does run a substantial programme of experiments in healthy human volunteers and patients; however, these can only produce indirect data. Detailed understanding at the level of single nerve cells and their connections can only be achieved using the invasive approaches possible in animals. I maintain a knowledge of the literature by checking



PubMed and Web of Science databases, to ensure that I am aware of any replacements if they are developed.

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

We have an active programme of research in humans, both healthy subjects and patients. Using non-invasive methods such as transcranial magnetic brain stimulation, a procedure that uses magnetic fields to stimulate nerve cells in the brain, we can activate brain circuits and make a conclusion reached on the basis of evidence and reasoning about function. Human studies have many advantages over animal work. Experiments are substantially lower cost, and human subjects can rapidly follow instructions to perform complex motor tasks with little requirement for training. It is also feasible to recruit large numbers of subjects, allowing population-level statistical inferences. Finally, human patients provide the most valid model to study human disease.

### **Why were they not suitable?**

Despite these advantages, human studies have major limitations. Non-invasive methods are unable to tell us which part of the brain is active, or when, to the level of precision we need. For example, transcranial magnetic stimulation is a method used to activate the brain non-invasively in humans, This stimulates all cells within a region of cortex around 1cm<sup>2</sup>; for comparison, a single neuron is around 10um in diameter. Likewise, electroencephalogram is a method which permits recording of brain activity from the scalp non-invasively in humans, but averages over populations of many thousands of cells. In addition, non-invasive methods are capable of activating or recording only from superficial structures; they cannot work with the systems deep within the brain of importance to this project. The only way to overcome these limitations of resolution is to insert electrodes directly into the brain, which requires animal work. Finally, in humans we can only study lesions which have occurred naturally, such as stroke. This prevents a careful dissection of the impact of different areas on disability. In animals, we can make precisely delimited lesions, leading to understanding of exactly how damage to each area produces specific functional loss.

### **A retrospective assessment of replacement will be due by 28 August 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?**

This project will use no more than 75 macaque monkeys in total; this will be comprised of a mixture of cynomolgus and rhesus macaques, depending on availability. These two species can largely be used interchangeably in this work, because they have very similar nervous systems. Both can be trained on simple behavioural tasks, but rhesus macaques are more suitable for training on the more complex behavioural tasks. This total number of animals (for both rats and monkeys) has been determined based on the amount of work, across different individual projects, that we anticipate performing over the five year course of this licence. The number of animals per project is determined as follows.

In most of our studies in monkey where we record activity from nerve cells, we are making comparisons of how cells are active in different conditions. These comparisons are made within one animal. We repeat studies in a second animal, to demonstrate that results are reproducible and not affected by individual characteristics. A full study thus needs only two animals. A similar approach is used in our lesion work, where individual lesions are tested in just two monkeys and the behavioural effects measured.

In some studies in both rat and monkey, we are looking at fundamental properties of cells and neural circuits. In these cases, the key experimental unit is the number of cells (combined across animals). In this case, we must use sufficient animals to record sufficient cells. In experiments under anaesthesia it is typically not possible to gather as many cells as in the awake state; such experiments may therefore require 4-6 monkeys, or 10-20 rats.

In some of the work on motor neuron disease, we are comparing the effect of different treatments on spread of disease. For this work, we have used statistical models based on our previous data to estimate the number of animals per group required to yield statistically significant results. Nine animals are expected to be used for three groups based on this calculation.

The estimate of 75 macaques is therefore made up of: 9 animals for the effect of treatments on MND; 10 animals for two experiments under anaesthesia; 50 animals used in 25 sub-projects in the awake state, using two animals per project; 6 animals as spares, to be used to supplement data from a sub- project where insufficient data were gathered from two animals.

For the marmoset experiment, we expect to use 2 animals for one sub-project; the estimate of 3 allows for one spare in the case of a technical failure.

For the rat experiments, the estimate of 250 animals will allow us to perform around 15 sub-projects.



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

A power calculation has been performed for the study into the effect of drugs on spread of motor neuron disease which is the only study for which this is relevant. In that study, we will compare multiple drugs against a control in one study, allowing us to make maximal use of the control and reduce animal numbers. Analysis of tissue from these animals will be blinded - the experimenter will count stained cells in sections without knowledge of which animal they came from.

In most other cases when using monkeys we often only require two animals, a number chosen to allow us to confirm that results are reproducible between animals; all statistical analysis is performed within the same animal (e.g. comparing cell firing rate in two different tasks). More animals may be required if, for example, recordings are especially challenging so that the yield per animal is low; in that case, we would combine recordings across animals but perform analysis after each animal is completed, to ensure that we stop recording once sufficient data are gathered. Rat experiments typically have a lower cell yield than studies in monkey, and hence larger animal numbers per study are typically required; once again, analysis is performed as we go along, to ensure that we can stop recording once sufficient data are gathered. Control animals are not needed for such studies, where we compare recordings in the same animal under different conditions (e.g. during different movements, or before and after a lesion).

With such small numbers of animals per project, the experimenter cannot be blinded. However, all analysis is performed automatically by computer algorithms, so that bias due to knowledge of categories cannot influence the analysis.

The NC3R's Experimental Design Assistant will be used where appropriate. We have extensive statistical expertise within the group, having previously published on novel analysis methods for neurophysiological data. Quantitative analysis of tissue will follow advice from the university's Bioimaging Facility.

**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 sophisticated multi-electrode recording methods, which ensure that the maximum of data is gathered from each animal. Experiments under terminal anaesthesia use advanced anaesthetic methods to maintain the animals in good condition for extended periods (around 70 hours for macaques); this again enables us to gather extensive datasets from each animal. Animal numbers are minimised by performing analysis mid-way through an experimental series, ensuring that only sufficient data is gathered as required to address the scientific question.

When one of our monkeys is humanely killed at the end of an experiment, we seek to share tissue as widely as possible to maximise the benefit from these valuable animals. Tissue will also be shared from rodent experiments where possible.



## **A retrospective assessment of reduction will be due by 28 August 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.**

Some short-term studies will be performed in rats or monkeys. The animals may be anaesthetised, and injections made into the brain or spinal cord of neural tracer or virus to introduce foreign genetic material to modify the genes to express proteins such as those able to make specific cells respond to light. After a period of recovery, the animals are anaesthetised and electrophysiological recordings made from their nervous system; the animals are humanely killed before recovering from anaesthesia. These types of experiment cause minimal distress to the animals, as they involve just one recovery surgery and then a non-recovery anaesthetic.

Some longer term studies will be carried out in monkeys. In these experiments, animals are first trained to perform a behavioural task. They are then implanted with a headpiece to allow head fixation, electrodes in the limbs to record muscle activity, and chambers to allow us to record from the brain or spinal cord. These implants are all performed under general anaesthesia, with a full programme of post-operative care including pain relief and antibiotics. Once the animal has recovered from the implant and they have been trained to accept the head restraint, we can then record from the brain or spinal cord in the awake state, without causing pain, suffering or distress.

In some of the longer term studies, we make specific lesions (damage) to parts of the nervous system, to study the processes involved in recovery or to create a model of a human disease. Examples would be damaging a small part of the cortex which controls movement, which models a stroke, or the spinal cord, modelling a spinal cord injury. We may also cause a small number of neurons to make a protein involved in motor neuron disease; this will model the spreading of motor neuron disease. In all cases, we choose the extent and nature of the lesion to produce the scientific result that we need, but to keep the impact on the animal's welfare to the minimum. Often, this impact is so slight that an



observer at the cage side a week after the lesion surgery would not notice anything different from a healthy monkey.

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

An important feature running through this project is to study the interaction between the cerebral cortex and lower neural systems. This requires studying mammals, which have a cerebral cortex, unlike lower animals such as fish or reptiles.

Some basic nerve circuit properties can be investigated in rodents such as rats. However, the nerve centres and connections controlling movement differ in key aspects in primates compared with non- primate species.

For a small number of experiments studying motoneuron disease, we will use marmosets. Rats would not be suitable for these studies, as a protein of key importance to the disease differs between rats and primates.

For most experiments, we will use macaque monkeys. Macaques have a direct connection from the cerebral cortex to motoneurons which control muscles. This is the same connectivity as in humans, but differs from both marmosets and rats. These connections underlie a highly developed ability to produce fine control of small groups of muscles, especially those of the hand; this provides Old World primates with their high level of manual dexterity. It thus essential to use Old World primates such as macaques for these studies to ensure results will translate to human patients. In addition, the intelligent, inquisitive and social nature of macaques means that they are capable of learning complex behavioural tasks, which would not be possible for either marmosets or rats.

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

Our techniques have been refined over many years, and we continually seek to improve them – for example, by improving the design of our headpiece and electrode implants to make them more tissue- friendly, which allows better healing of skin margins and integrates wires which run under the skin into the headpiece structure. We have also improved the way in which we repair wounds after spinal cord implant surgeries, which has reduced the incidence of wound complications. All recovery surgeries are carried out under full aseptic conditions, with advanced anaesthetic regimes which produce rapid and uneventful recovery. Full programmes of post-operative pain management are in place. Stress involved in behavioural training to accept restraints is reduced using anxiolytic medications. Analysis methods are constantly being optimised: for example, by advanced statistical methods, we can achieve the same scientific benefit with shorter durations of recordings and fewer trials performed.

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



Our facility and experiments follow the following NC3Rs guidance and guidelines:

- Non-human primate accommodation, care and use
- Training animals
- Chair training of non-human primates
- Blood sampling: vascular catheters
- Anaesthesia
- Refining food and fluid control in macaques

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

I, and members of my research group, regularly attend national and international conferences where others using chronic implant methods present their work. This ensures that we are up to date about advances in the field, and able to discuss new approaches with other scientists. We also regularly visit other laboratories, and host visits by scientists to our laboratories, which allows us to discuss practical refinements in the context in which they are used.

**A retrospective assessment of refinement will be due by 28 August 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. Immunotherapies for the prevention and treatment of neurodegenerative diseases

### Project duration

5 years 0 months

### Project purpose

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

Alzheimer's disease, Immunotherapy, Tau, Amyloid, Dementia

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?**





Our project aims to deepen our understanding of how amyloid and tau proteins contribute to the progression of Alzheimer's disease (AD) and to evaluate the effectiveness of new vaccine candidates against amyloid and tau proteins as potential treatments for AD.

### **A retrospective assessment of these aims will be due by 28 August 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?**

Alzheimer's disease is the most common form of dementia, which affects over 55 million people worldwide and is projected to affect 139 million people by 2050. Although great strides have been made recently in the development of new immunotherapy treatments for Alzheimer's disease, there are still no treatments that can reverse the course of the disease. Furthermore, the treatments that have succeeded in phase 3 clinical trials have shown only modest disease modifying effects. Therefore, it is important to continue development of new, more effective, immunotherapy treatments.

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

Our translational research will lead to data which will be published in peer-reviewed scientific journals extending our understanding of the underlying pathogenesis, and hopefully see a candidate vaccine for Alzheimer's disease advanced to clinical trials by our pharmaceutical industry partner.

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

Our pharmaceutical industry partner will benefit from our translational research, as our data will aid in the selection of a vaccine candidate to take forward into clinical trials.

Other researchers will benefit from our replication of the human Alzheimer's disease tau seeding model used in this project via data which will be published in peer-reviewed scientific journals.

In the long-term our translational research may lead to improved treatments for AD and other tauopathies.

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

We routinely present our research data at scientific conferences.



We attempt to publish our research in open access journals wherever possible.

Our data will be shared with our collaborators to aid in the refinement of immunotherapy approaches and development of new vaccine candidates.

### **Species and numbers of animals expected to be used**

- Mice: 2000

### **Predicted harms**

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

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

This project aims to test the effectiveness of new immunotherapies (vaccines) designed to use the body's immune system to stop the spread of abnormal amyloid beta and tau proteins, which are thought to be responsible for damage caused to the brain by Alzheimer's disease. It is therefore necessary to use animal models that can replicate the harmful build-up of amyloid and tau proteins, as seen in the brains of humans with Alzheimer's disease.

Mice are the animals of choice to study neurodegenerative diseases such as Alzheimer's disease because the rodent brain is very similar to the human brain, both anatomically and on a cellular level. This project requires the use of genetically altered animals, and mice are ideal for this role as the scientific community know a great deal about their genetic code and have produced many strains of genetically altered mice which are available to researchers around the world. Mice are also small enough to be easily handled by experimenters, reproduce quickly, and have a relatively short generation time that allows us to study the consequences of ageing.

Our study will use three different experimental models to test vaccine candidates:

1. Wild-type mice which will be injected with tau protein derived from the brains of post-mortem human Alzheimer's patients (human AD brain extract).
2. Genetically altered mice that overproduce tau protein (THY-Tau22) which may or may not be injected with human AD brain extract.
3. Genetically altered mice that overproduce amyloid beta protein (APP<sup>swE</sup>/PSEN1<sup>dE9</sup>) which may or may not be injected with human AD brain extract.

Model 1 (WT mice) slowly develops Alzheimer's disease pathology over 9 months, and so we will start with mature adult mice aged 3 months and keep them until a maximum of 12 months old.



Model 2 (THY-Tau22 mice) develop Alzheimer's disease pathology much more rapidly; therefore we will use mice between 1 and 12 months old.

Model 3 (APP<sup>swe</sup>/PSEN1<sup>dE9</sup> mice) begin to develop Alzheimer's pathology between 6 and 9 months of age. We will keep these mice up to 18 months old, which is considered old-aged, to better replicate Alzheimer's disease in humans.

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

Mice will typically be housed in groups of 5 individuals with cage enrichment and nesting material. Mice will have free access to food and water throughout the project.

Mice will undergo vaccination against tau and/or amyloid beta. The vaccine will be injected into the thigh muscle of the hind leg, typically every 2-3 weeks, with a maximum of 10 doses per mouse. Mice will be initially handled with a tube, then restrained for injections. The mice will experience mild and transient discomfort from the injection and restraint.

Immediately prior to each vaccine dose, mice will be placed in a restraining tube and blood samples will be withdrawn from the tail vein. Mice will experience mild and transient discomfort from blood sampling.

Some mice will undergo a surgical procedure to inject human AD brain extract into the brain. During surgery, the mice will be anaesthetised and a hole will be drilled in the skull. Prepared human AD brain extract (maximum volume 10  $\mu$ L) will be slowly delivered into the brain via a thin needle. The mouse will be given a dose of a painkiller and allowed to recover in a warm chamber after the surgery. Once the mouse has recovered from the anaesthesia, it will be transferred to its home cage and monitored regularly for any signs of adverse effects. The mice will experience some discomfort after surgery and some mild to moderate pain which will be treated with painkillers.

After the surgery, mice will be given further booster doses of vaccine and blood samples will be taken as described above.

Some mice may undergo up to five sessions of whole-body computerised tomography (CT) imaging under general anaesthesia, lasting up to 30 minutes. The mice will experience mild and transient discomfort from restraint before being given anaesthesia.

Some mice may undergo one or more behavioural tests to measure cognitive or motor function. These tests are non-invasive, but mice may experience mild and transient discomfort from restraint and handling, and during the test.

At the end of the experiment mice will be killed under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain.

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



Intramuscular injections will cause slight distress due to the handling and restraint, as well as mild and transient discomfort from the injection. The cumulative effect of a regular program of injections will cause a small amount of additional distress to the mice. The injected vaccines are not expected to cause any additional adverse effects to the mice. No signs of pain or discomfort related to this type of vaccine have been observed in previous studies.

Blood withdrawal will also cause slight distress due to the handling and restraint, as well as mild and transient discomfort from the puncture of the tail vein. The cumulative effect of a regular program of blood withdrawals will cause a small amount of additional distress to the mice.

The surgical procedure will be conducted under general anaesthesia where the animals will only be aware of the anaesthetic being administered. This will cause mild distress due to handling and the induction of gaseous anaesthesia. Upon withdrawal of anaesthesia mice will experience some discomfort from the surgical procedure and some mild to moderate pain which will be treated with a suitable analgesic. To our knowledge, the potential adverse behavioural effects of injecting human AD brain extract into wild-type mice have not been reported in the literature. Our pilot experiments have shown that the injections lead to development of tau pathology which might be expected to cause cognitive or motor deficits, however we have not observed any significant behavioural changes or signs of pain or distress in pilot studies using this model. We intend to perform behavioural tests to better measure any possible changes in cognitive or motor functions.

Some of the genetically altered mice may show severe adverse effects including epileptic behaviour and sudden death within the first 6 months, possibly related to seizure activity. Such seizure activity is found in other mouse models of Alzheimer's disease, and increased seizure susceptibility is associated with Alzheimer's disease in humans. The animals will be carefully monitored for any seizure activity and if observed these mice will be killed to prevent undue suffering.

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

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

Mice: 89.5% moderate, 10.5% severe

#### **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 28 August 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?**

To evaluate the effectiveness of a vaccine, the experimental model must have an immune system which can recognise a foreign protein and produce antibodies against it. The experimental model must also replicate other features of the human central nervous system which may affect vaccine performance, such as the blood-brain barrier, immune cells and waste clearance pathways. Finally, the model must closely resemble the human brain to allow comparable development of Alzheimer's disease pathology, so any effects of the vaccine can be reasonably extrapolated to humans. Mice are the least complex organism which meet these criteria.

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

Prior to this project the following non-animal alternatives were considered:

### **Tau**

Computer modelling has been used to examine the spread of tau protein throughout mouse brain, but not without the initial use of animals. Tau spread has been replicated using cultured cells. Brain organoids have been developed, for example using human stem cells which replicate some features seen in Alzheimer's disease brains. Tauopathy fruit fly and roundworm models exist for the study of tau accumulation; these have mutations in genes analogous to the human equivalent which cause tau pathology.

### **Amyloid**

Computer modelling has been used to investigate conformational changes in amyloid structure. Brain organoids have been developed, for example using human stem cells which replicates the amyloid accumulation seen in Alzheimer's disease. Fruit fly and roundworm models which overexpress amyloid have been developed.

Human studies can aid our investigation of both amyloid and tau. For example, the use of human brain tissue from Alzheimer's disease patients can complement our studies by allowing us to test the specificity of antibodies produced after animals are inoculated with a vaccine.

### **Immune Response**



Computational models have been developed to model the immune system response to infection. These are most useful at simulating the viral or bacterial load. Methods have been developed which mimic the immune system, and human lung slices have been used to screen vaccine candidates. The immune system is difficult to mimic but some systems have been developed, using cultured spleen cells.

### **Why were they not suitable?**

The alternative methods mentioned capitulate some of the criteria required for this project. However, no alternative method encompasses all of the necessary criteria which are needed to test a vaccine's effectiveness, by modelling both the immune response to a vaccine and resultant effect on amyloid or tau pathology in the brain.

Computational models are most effective at determining the shape of the tau or amyloid fibrils, rather than predicting whether a human is likely to produce appropriate antibodies in response to a vaccine which can remove the target protein. Cell-based models, lacking a complete immune system, cannot reliably give an indication as to the effectiveness of a vaccine. Animal models using less complex organisms such as fruit flies and roundworms do not have an adaptive immune system or blood circulatory systems. These methods also do not allow the collection of vital data which is invaluable prior to testing in humans to ensure safety, such as post-mortem analyses of inflammatory markers and analysis of blood antibody titres to determine the potency of a vaccine. Finally, models of immune response allow some modelling of the immune system and the possibility of identifying lead vaccine candidates but do not give any indication of how efficacious a vaccine candidate is likely to be, necessitating the use of larger animals such as rodents.

Cell-based and non-animal testing is essential for the development of drugs and vaccines; these tests are performed by our industry partners to select the best vaccine candidates.

### **A retrospective assessment of replacement will be due by 28 August 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?**





Each test vaccine will be a separate experimental group. Up to 3 vaccines will be tested in a single study. Our sample size calculations, based on pilot studies, suggest that up to 26 mice will be required per group. We will also use an unvaccinated control group that will be injected with an equivalent volume of adjuvant. Together this amounts to 104 mice required for a single vaccine experiment.

We anticipate running up to 3 parallel vaccine experiments per year with different mouse models. Over the 5-year project this will give a total of 1560 mice.

Additional genetically altered mice will be required for breeding purposes but not used in experiments. We therefore estimate a total of 2000 mice.

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

Where possible up to 4 test vaccine batches will be tested together in the same assay to maximise the use of the reference vaccine and control groups. Thus, minimising the use of animals overall.

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

At the end of the experiment, we will harvest blood samples and brains from the mice to ensure we are using as much tissue as possible. The parts of the brain which we do not need for analysis will be kept ready for future analyses or made available to other researchers who might need them.

### **A retrospective assessment of reduction will be due by 28 August 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.**



Animals will be vaccinated using methods similar to human vaccination, e.g., injection of substances into the muscle. A subset of animals will undergo surgery to inject tau protein derived from the brains of post-mortem human Alzheimer's patients (human AD brain extract) into the brain.

Our study will use three different experimental models to test vaccine candidates:

- Wild-type mice which will be injected with human AD brain extract.
- Genetically altered mice that overproduce tau protein (THY-Tau22) which may or may not be injected with human AD brain extract.
- Genetically altered mice that overproduce amyloid beta protein (APP<sup>swe</sup>/PSEN1<sup>dE9</sup>) which may or may not be injected with human AD brain extract.

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

Mice are the model organisms of choice as the least sentient animal that can be reasonably used for these studies. There is a considerable body of data on the neurobiology of the rodent that is directly relevant the functioning and pathology of the human central nervous system. Zebrafish are used for Alzheimer's disease research, but they are unsuitable for this study as they cannot receive stereotaxic injection.

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

Mice will be monitored by researchers for signs that may indicate systemic inflammation or neuroinflammation for the first 7 days after a vaccine dose. Mice will be monitored weekly thereafter. Signs of pain will be treated with administration of appropriate analgesics.

Following surgery, mice will be weighed and monitored by researchers each day for signs of dehydration, weight loss, pain and distress for the first 14 days after surgery. Mice will be monitored weekly by researchers thereafter.

Signs of pain will be treated with administration of appropriate painkillers. Signs of weight loss or dehydration will be treated with addition of soft food to the diet.

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

We have used guidance from the NC3Rs website, including guidance on minimising aggression in group-housed male mice, blood sampling: mouse, housing and husbandry: mice, refining rodent stereotaxic surgery webinar.

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

The researchers will stay informed about advances in the 3Rs by following updates through the NC3Rs and Norecopa websites and newsletters, by reading peer-review articles on different replacement techniques, by regularly discussing refinement and best practices



with colleagues and animal facility staff and by actively implementing the latest relevant techniques.

**A retrospective assessment of refinement will be due by 28 August 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. Detection of Bacterial Toxins

### Project duration

5 years 0 months

### Project purpose

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

### Key words

Botulism, Diagnosis, Prevention, Treatment, Public health

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?

This is a service licence and the aim of the project is to provide a diagnostic service for patients with suspected botulism. This project also helps with the effective investigation, surveillance and control of outbreaks of disease, including the rapid detection of food contamination.

### **A retrospective assessment of these aims will be due by 13 August 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?**

Botulism is a rare but fatal disease, It is important to diagnose and treat it as early as possible. In the case of infant botulism immunoglobulins (BABYBIG) must be purchased from the USA and will not be released until there is a definitive diagnosis confirming botulism. There are non-animal methods of detecting the disease that can be unreliable such as PCR, and on occasion, this technology will generate conflicting or spurious results that conflict with the presenting symptoms. In these cases, it is prudent and justified to utilise the mouse bioassay which is still the 'gold standard' and is reliable but it is not the first resort. Our organisation has the capacity and expertise in the detection of bacterial toxins which is why we extend the service to other countries where they do not have the capacity or expertise to diagnose botulism.

Work provides standard and internationally recognised 'Gold Standard' techniques for the detection of neurotoxins from *C. botulinum*. This will facilitate the rapid and effective diagnosis of patients suspected of having botulism and the investigation, surveillance, and control of outbreaks of botulism.

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

**IT MUST BE EMPHASISED THAT ANIMALS ARE NOT USED FOR RESEARCH PURPOSES AND SOLELY USED FOR THE CLINICAL DIAGNOSIS OF C. BOTULINUM.**

The primary role is for the rapid and effective investigation, diagnosis, surveillance, and control of outbreaks and incidents of botulism.

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

Results of the tests for botulism are returned to clinical colleagues involved with the management of diseased patients and allow confirmation of the clinical diagnosis and evidence for their most appropriate clinical management and treatment. Results of tests for botulism are also used for the identification of sources of infection and are of special importance to those involved with control of the food chain i.e. environmental health officers and staff of the food standards agency. These results provide vital informed and evidence-based information for the identification of toxic food and allow its removal from the food chain to prevent further cases of the disease. Botulism is a rare disease and the diagnosis and surveillance of botulism are centralised at our laboratory where the work will be undertaken.

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

**IT MUST BE EMPHASISED THAT ANIMALS ARE NOT USED FOR RESEARCH PURPOSES AND SOLELY USED FOR THE CLINICAL DIAGNOSIS OF C. BOTULINUM.**

Clinical diagnosis of botulism to provide the most appropriate clinical management and treatment of patients.

### **Species and numbers of animals expected to be used**



- Mice: 1000 (600 Primary test & 400 Secondary test)

## **Predicted harms**

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

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

The mouse is the animal of choice for the test and this species has been proven historically to be the most appropriate species for this test. This means all validated tests for botulinum diagnostic purposes use mice. Any strain of laboratory mouse can be used for the mouse bioassay but as a first choice, we use the Balb/c model because it is an ideal mouse model for toxicology work as it is an inbred strain which as a result has an underdeveloped immune system due to decades of strategic inbreeding but it is not severely immuno-compromised so does not have to be kept in high or special containment.

The scientific data does not indicate that any other species other than the mouse can be used for the clinical diagnosis of botulism.

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

Test preparations are injected into mice, and those samples which produce characteristic symptoms of botulism are pre-incubated with panels of antitoxins and injected into additional mice to establish the toxin type. Due to the large volumes of clinical samples suspected of containing *C. botulinum* (this could be up to 0.5ml), once injected, mice are frequently observed, and on recognition of specific endpoints are immediately humanely killed. Characteristic symptoms of botulism in mice become evident from about 5-18 hours post-inoculation depending on the toxin type and concentration present. Mice will be observed at the time of inoculation and at 30 minutes then every hour for at least seven hours for symptoms of botulism. If any of the symptoms of botulism are detected, observation will be made at 30 and 15-minute intervals until the defined endpoints (wasp waist, laboured breathing, and general paralysis) have been reached. If no signs of botulinum intoxication are apparent, observations will continue to 18 hours post-inoculation, and then at regular intervals for up to 4 days.

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

The characteristic symptoms of botulism are ruffled fur/piloerection, laboured breathing, “wasp” like waist, limb weakness/ paralysis. Symptoms of botulism in mice become evident from about 5-18 hours post-inoculation (depending on the toxin type and concentration present), and include ruffled fur, pinching of the waist (“wasp abdomen”), laboured breathing, limb paresis, and general paralysis.

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

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





When the test is positive for botulism, animal will suffer considerable pain and distress and the severity category for positive tests is severe. Over the tenure of the licence approximately 30 percent of animals are likely to experience severe levels of severity. This is because they will succumb to botulism or toxicity of a non-specific origin. The remaining 70 percent of animals are likely to experience mild/moderate severity because they will not suffer the adverse effects of botulism or the non-specific toxicity will be mild to moderate.

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

- Killed

### **A retrospective assessment of these predicted harms will be due by 13 August 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?**

Currently, the mouse bioassay (MBA) or mouse test is the only laboratory test authorised by an accrediting body for the detection of biologically active *C. botulinum* neurotoxins.

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

Over the years, there have been several tests developed to detect botulinum toxins from clinical specimens and food. Until recently, none of them have been sensitive enough to replace the mouse bioassay (MBA) completely. Scientists in the USA have carried out trials using the ELISA test, However, this initial Enzyme-Linked Immunosorbent Assay (ELISA) test was not accurate and it lacked sensitivity, especially when used with clinical/medical specimens and did not identify whether the toxin was biologically active, which is essential for food testing. More recently, another ELISA test was described but it was still not sensitive enough and could be used only for preliminary screening of foods. The Endopep-MS test has been under development for some time in the USA. This test's specificity and sensitivity have been constantly improved over the years with the aim of reaching results comparable to the mouse test.

We have made considerable progress in the use of the polymerase chain reaction (PCR) as an alternative to animal tests, but these detect the presence of neurotoxin genes and the potential of strains to produce neurotoxin; they do not detect the presence of active neurotoxin itself. Thus, there is an occasional need for the mouse test to detect the actual toxin in clinical specimens, foods, and cultures as the PCR may not detect organisms with unusual toxin types or toxin variants.

We regularly check databases for novel/new toxin gene sequence variants and ensure our PCR detection tests are continually updated so that all known novel/new toxin variants can be detected.



A new PCR test detecting genes that are present in botulinum and bacterial strains that produce a toxin, including those producing unusual toxin types or toxin variants and in toxin-producing strains of toxin-producing bacteria is being developed. This should allow PCR detection of all strains capable of producing neurotoxin regardless of toxin type or if it is a new toxin variant.

### **Why were they not suitable?**

None of these tests are commercially available and if they had been used instead of the MBA there would have been serious consequences for patients because these tests are not sensitive enough to make an accurate diagnosis.

Regarding the Endopep-MS (MALDI-TOF) test described above, we are currently in the process of setting up and implementing this assay which has the potential to reduce and possibly replace the mouse test in the future. However, this will depend on the approval from the relevant authorities and the commercial availability of the test. Due to the impact of the COVID pandemic and the unavailability of critical reagents and components for the test, work on the validation and implementation of this method has unfortunately been delayed over the last 3 years.

### **A retrospective assessment of replacement will be due by 13 August 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?**

As a result of previous years of experience and the fact that the number of mice estimated is based on the unpredictable nature of botulism outbreaks, the numbers estimated would be sufficient and serve as a surge capacity, although we hope very few mice will be used over 5 years. Throughout a project, less than 100 animals have been used in previous years, but outbreaks are unpredictable, so we need to be prepared to respond to them. Unfortunately, there are no statistical or mathematical calculations/formulas to predict or accurately estimate animal numbers due to the nature and unpredictability of a disease outbreak. **However, we have estimated the number of 1000 mice (600 for the primary test and 400 for the secondary test)** over 5 years over two protocols based on past outbreaks, and bearing in mind the potential use of 1000 mice over 5 years is still a relatively low number. Based on the data that we have used 100 mice over previous years in a non-outbreak situation we can safely assume that we will potentially require 10 times that amount in an outbreak situation to efficiently deliver the diagnostic service.

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



Since 2004 we have been using an in-house, real-time PCR assays to detect neurotoxin genes in broth cultures inoculated with clinical specimens likely to contain *C. botulinum* for identifying bacterial isolates. This has resulted in a 90% reduction in the use of animal bioassays. However, as some clinical samples do not contain bacterial cells e.g. serum, and PCR assay do not detect biologically active toxin in foods, detection of the *C. botulinum* neurotoxin by mouse bioassay cannot be completely eliminated. Whenever possible, we also prioritise alternative samples for PCR, however, in situations when toxin detection is the only option, the bioassay is unavoidable. In food samples, where botulism toxin detection is essential, PCR is also used to identify the type of *C. botulinum* present which reduces the number of animals otherwise used for toxin typing by further neutralisation tests. In recent years we have modified our SOP so that all faecal and food specimens are now directly treated with trypsin for the detection of toxin produced by nonproteolytic types of *C. botulinum*, and this also reduces the number of animals used.

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

**IT MUST BE EMPHASISED THAT ANIMALS ARE NOT USED FOR RESEARCH PURPOSES AND SOLELY USED FOR THE CLINICAL DIAGNOSIS OF C. BOTULINUM.**

Two mice are used for a standard bioassay and there are two specific reasons for this; the first is the welfare aspect taking into consideration that mice should not be singly housed unless there is a very strong justification to do so i.e. it is a necessity of the test, whereby if the mice were not housed singly it would impact negatively on the outcome of the test and therefore potentially adversely affect clinical results. In addition to this, mice are grouped housed prior to the commencement of a bioassay, two mice is the minimum number that is used during a test in order to prevent undue distress to the animals. The second reason for using a minimum of two animals for a test, is that one mouse can show symptoms of the effects of *C. botulinum* while the other shows no obvious signs or symptoms of the disease. Using two mice ensures that the test is standardised and accredited. The demand for the bioassay is arbitrary and hard to predict.

**A retrospective assessment of reduction will be due by 13 August 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 are the animals used for the diagnostic assay for botulism. Mice will be injected with either blood serum, human stool, or food extract to diagnose botulism and the animal will experience clinical signs if the test is positive for the presence of botulinum neurotoxin. All



animals will be housed and tested in pairs and never used alone. The test is carried out by staff who are highly trained and participate in regular competency checks to ensure they are proficient in carrying out regulated procedures and recognising the signs and symptoms of positive cases of botulism at the earliest possible time. All animals will be observed frequently to assess whether they are showing signs of botulism. If signs of botulism are observed, then the tests will be halted at the earliest opportunity.

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

Based on scientific literature the mouse is the animal of choice for the test and this species has been proven historically to be the most appropriate species for this test. This means all validated tests for botulinum diagnostic purposes use mice. No other animals have been validated for botulism **clinical diagnostics**.

An accrediting body carries annual audits based on the above.

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

All animals will be housed and tested in pairs and never used alone. The test is carried out by staff who are highly trained and participate in regular competency checks to ensure they are proficient in carrying out regulated procedures, recognising the signs and symptoms of positive cases of botulism at the earliest possible time. All animals will be observed frequently to assess whether they are showing signs of botulism. If signs of botulism are observed, then the tests will be halted at the earliest opportunity. All animals are made as comfortable as possible for the duration of the test. Moistened food will be provided for animals that may have difficulty accessing food or water in a conventional manner and extra bedding is always provided to keep the animals warm.

Taking the welfare aspect into consideration that mice should not be singly housed unless there is a very strong justification to do so i.e. it is a necessity of the test, whereby if the mice were not housed singly it would impact negatively on the outcome of the test and therefore potentially adversely affect clinical results. In addition to this, mice are grouped housed prior to the commencement of a bioassay, two mice is the minimum number that is used during a test in order to prevent undue distress to the animals. The second reason for using a minimum of two animals for a test, is that one mouse can show symptoms of the effects of C. botulinum while the other shows no obvious signs or symptoms of the disease. Using two mice ensures that the test is standardised and accredited.

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

- 1) Paper published by the Home Office describing the current process used by the home office to conduct Harm-Benefit analysis (Advice Note: 05/2015, Animal in Science Regulation Unit, December 2015) required under the animals (Scientific Procedures) Act 1986.
- 2) 'Delivering our commitment to Replace, Reduce and refine the Use of Animals in Research' developed in between the Home Office Animals in Science Regulation Unit (ASRU), the Department for Business, Innovation and skills (BIS) and Government Office for science (First published: February 2014, URN BIS/14/589, ISBN 978-1-78246-264-4)



3) Article Source: Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research:

Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting; The ARRIVE Guidelines for Reporting Animal Research. PLOS Biology 8(6): e1000412. <https://doi.org/10.1371/journal.pbio.1000412>

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

Registered and subscribed to the e-newsletter to stay informed about the latest scientific advances and development methods for the detection of botulism from clinical, food, and environmental samples. We are closely following the project by a team at a respected organisation to develop a novel portable bioluminescence assay (BLD-Test) for detecting botulinum neurotoxin (BoNT) which could in the future replace the mouse bioassay. We are currently in the early stages of working towards implementing a Mass Spectrometry endopeptidase-MS assay developed by an agency in the USA as a potential method to reduce our dependency on the mouse bioassay for the detection of botulinum neurotoxin in clinical samples. The Endopep-MS test has been under development for some time in the USA. This test's specificity and sensitivity have been constantly improved over the years with the aim of reaching results comparable to the mouse test.

**A retrospective assessment of refinement will be due by 13 August 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?