Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted April – June 2024

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1. Assessment of abuse potential and evaluation of novel entities to treat substance abuse

Project duration

5 years 0 months

Project purpose

- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Pharmaceuticals, Abuse, Rodents, Safety, Efficacy

Animal types	Life stages
Rats	adult, juvenile

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To determine the abuse potential of novel pharmaceuticals and to study the effects of experimental treatments for substance abuse disorders.

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

Why is it important to undertake this work?

Psychoactive substance abuse poses a significant threat to health and the social and economic fabric of families, communities and nations. In an attempt to limit and control the drug abuse potential of novel drug candidates entering the marketplace, specific preclinical studies are mandated by the world regulatory authorities (e.g. European Medicines Agency EMA and USA Food and Drug Administration FDA) for all novel compounds in development which enter into the CNS, irrespective of their

therapeutic indication. In addition, novel drugs for the treatment of substance abuse are required urgently.

The overall project aim is to provide highly specialised preclinical services to the pharmaceutical and biotech industry to evaluate the abuse liability of drugs which enter into the CNS irrespective of their therapeutic focus and evaluate the efficacy of novel drugs for the treatment of substance abuse. This is achieved by the following three objectives:

1. To assess the abuse liability of novel drug candidates in development which enter the CNS regardless of therapeutic indication, as mandated by the regulators. This includes determination of the pharmacokinetics of the drug candidate, as required by the regulators, so the exposure in the rodent model can be related to the human exposure. These studies will allow the regulator to determine if the drug candidate should be scheduled (yes or no) and if yes what schedule it should be placed into thereby limiting its distribution in the wider population.

2. To assess the abuse liability of new chemical entities in preclinical development for abuse liability. These studies will enable clients to determine if their new chemical entity is advantaged over existing therapies and hence worthy of further preclinical and clinical development.

3. To assess the efficacy of new chemical entities to treat drug abuse. These studies will enable clients to determine if their new chemical entity is worthy of further preclinical and ultimately clinical development.

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

One of the outputs from this project licence will be Regulatory Abuse Liability packages in the form of comprehensive, audited Study Reports. These will be used by sponsors as part of their Regulatory

Submissions in support of the licencing of new drugs.

Studies may also be carried out at earlier stages of the drug development process in order to assist sponsors with an assessment as to whether their novel molecules, or drug targets are likely to have abuse liability issues.

In addition, some studies under this licence will examine the potential of novel pharmaceuticals as treatments for substance abuse disorders, i.e., drug addictions, potentially leading towards the development of new therapies.

Work performed under this licence may be published in the scientific literature or presented at conferences.

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

To assist sponsors to develop, perform and report a pre-clinical Regulatory Abuse Liability package which will meet the world-wide regulators' requirements, and provide the appropriate scientific support throughout the process. This will allow the sponsor to move rapidly through the clinical development programme without the

assessment of abuse potential becoming a rate limiting factor. In addition, these studies will allow the regulators to decide whether a drug candidate should be scheduled or not and if scheduled which schedule the drug candidate should be placed into thereby limiting its distribution to the wider population. In addition, to allow clients to make decisions in regard to their novel compounds in preclinical development. Does the compound show reduced potential for abuse potential over a marketed product or compound in clinical development? Does the novel compound exhibit efficacy in a model predictive of the ability to treated substance abuse. The medium benefit is the discovery of compounds with the propensity for reduced abuse potential or to treat substance abuse and the long-term benefit (likely to be subsequent to completion of the licence) may be a clinically effective drug (since regulatory agencies expect a drug's sponsor to have screened the new molecule for pharmacological activity, prior to assessing its therapeutic potential in humans).

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

Where client confidentiality allows, work performed under this licence may be published in the scientific literature or presented at conferences.

Early-stage studies in particular are collaborative in nature, being run as part of a sponsor's drug development programme. Data and best practices are exchanged in written reports and in regular meetings.

Species and numbers of animals expected to be used

- Rats: 4200

Predicted harms

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

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

Adult rats will be used for the studies under this licence. The rat has been chosen since its anatomy, physiology, behaviour and genetics has been well-documented. Rats display many of the behavioural and cognitive characteristics of humans and are easier to train than mice and, therefore, they are typically used in the more complex behavioural models, such as those included in this project licence. There is a large literature detailing the use of rats in these abuse models and methods proposed in this licence have all been validated in these species. The long-term nature of the studies necessitates the use of adult animals.

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

Rats will be kept in cages either in groups or, when scientifically justified, singly. They will have free access to water and, in most studies, food. In some long-term studies, rats will have their food intake restricted to approximately 80% of their normal intake. The main reason for this is to improve the animals' health as long-term free access to food can lead to obesity and related health conditions.

In all studies rats may be dosed by oral, intravenous, subcutaneous or intraperitoneal routes. Dosing requires a brief period of restraint and may cause mild, transient discomfort.

In some studies, rats will be dosed, typically by the oral route, and then have their body temperatures measured using a rectal thermometer, followed by a period of behavioural observations. Body weights, and the food and water intake of these rats will be measured daily.

Other studies involve the dosing of rats then withdrawal of blood samples from the tail vein at a range of timepoints afterwards (up to 24h).

Three studies under this licence require rats to be trained to press levers within operant chambers into which they are placed for between 10 minutes and 4 hours per day on 5-7 days of the week. Two studies require surgical implantation of intravenous catheters which are exteriorised at a port on the animal's back. Surgery is carried out under general anaesthesia and rats are administered analgesics to reduce postoperative pain. The intravenous port is connected to an infusion line protected by a spring tether for typically 2h per day, but on occasion up to 4h per day. Compounds of interest are dosed via the infusion line.

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

The majority of the animals are likely to have a to experience only brief periods of mild discomfort, generally on a daily basis. The majority of studies will involve the administration of drugs which enter the central nervous system (predominantly orally or via an indwelling catheter) for behavioural testing. Drug treatment might be once or repeated. Some compounds will have been extensively evaluated prior to assessment. In such circumstances no adverse effects are expected. Some compounds may not have been tested extensively in animals and unexpected toxic effects might arise which could cause pain, suffering, lasting harm or in extreme cases death if humane end points were not applied.

Some studies involve general anaesthesia, with an associated low risk of harm or death, as in humans. The animal models employed may involve training in specialised equipment which may produce transient discomfort/stress. Upon completion of procedures animals 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)?

Rat, 8% mild, 92% moderate.

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

- Killed

Replacement

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

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

The project objectives each require investigation of candidate substances to be tested in an integrated behavioural/physiological/pharmacological model that requires the whole animal which cannot be replaced by in vitro or ex vivo studies. Prior to testing, it is expected that candidate substances will have been selected on the basis of extensive efficacy and safety evaluation (compounds assessed as part of the abuse liability regulatory package) or in silico, in vitro, ex vivo and in vivo experiments for more novel compounds undergoing evaluation prior to moving into development.

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

Non-animal alternatives are unsuitable for studies under this licence. Regulatory guidelines require the assessment of compounds in animal models.

Why were they not suitable?

The in vivo Abuse Liability package is a requirement of Regulatory bodies worldwide. The acceptable studies to be included in Regulatory submissions are clearly defined within published guidelines and are largely non-negotiable.

Reduction

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

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

The estimated animal usage is based upon a figure of 2000 rats per year over 5 years, sufficient to run approximately 6 abuse liability packages and 3 substance use disorder discovery phase studies. The exact number of animals used will be dependent upon external factors such as the number of clients and the success of those clients in designing suitable drugs. Additionally, all studies are designed on an individual basis dependent upon the scientific questions being asked, so use differing numbers of animals.

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

1. The expertise of the company's statisticians who are able to perform power calculations to ensure that studies are suitably powered to detect the difference of

interest. We also have a large historical data set that we can draw upon for this purpose.

2. Use of animals where appropriate to evaluate multiple test compounds or reinforcing drugs (e.g. heroin, cocaine, etc.). This not only reduces the number of animals used but has the following advantages: allows comparison of effects of related drugs in the same animals (including pharmacokinetic evaluation), minimises the number of animals that need training in a particular task, and surgical intervention.

3. When evaluating drugs that potentially possess sedative euphoriant properties, the rats need to be trained to self-administer a positive control. Opiates and opioids, e.g. heroin, remifentanil and oxycodone, are often used as the positive control when training rats to self-administer opioids. These drugs cross the blood-brain barrier very easily and very quickly, and consequently, are highly rewarding (reinforcing) which means that a large proportion of the rats given access to these drugs develop strong self-administration responding. Morphine is not an ideal choice as a training reinforcer because it does not cross the blood-brain barrier quickly and it has relatively poor brain penetration. Thus, in comparison with compounds like heroin, remifentanil and oxycodone, morphine has much less rewarding effect. Additionally, some rats find the effects of morphine aversive, particularly at higher doses. Therefore, it takes more sessions to train rats to self-administer morphine and the proportion of rats in an experimental study that achieve successful acquisition of drug-self-administration is much lower (40-50% typical responder rate with morphine, 80-90% typical responder rates with heroin).

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

We make extensive use of pilot studies where a small group of animals (typically 3) is run ahead of the main study. This allows doses of test compound to be optimised and adverse effects minimised during the main study phase.

Another approach that we take to optimise doses prior to committing a large number of animals to a study is to run pharmacokinetic experiments in advance and use the data from these to set groups for the main studies.

In some cases, results from pilot and pharmacokinetic studies facilitate a decision to no longer proceed with further work in larger groups of animals.

Refinement

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

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

The rat has been chosen since its anatomy, physiology, behaviour and genetics has been well- documented. Rats display many of the behavioural and cognitive characteristics of humans and are easier to train than mice and, therefore, they are typically used in the more complex behavioural models, such as those included in this project licence. There is a large literature detailing the use of rats in these abuse models and methods proposed in this licence have all been validated in these species. Rats are social animals and will be group housed unless single housing is believed to be preferable for the animal's wellbeing, or the scientific validity of the study.

The models detailed in this project licence have been established, used and refined by the company over the past 15 years. They have all been validated using suitable compounds and widely used by the pharmaceutical industry.

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

The tests to be performed under this project licence require a species that expresses complex behaviours, such as the rat. Less sentient species cannot be trained to perform the operant tasks (e.g. lever pressing), that are required. Similarly, a conscious state is required for the expression of operant behaviours, so anaesthetised animals are unsuitable.

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

Following surgical implantation of jugular vein catheters into animals for IVSA work, a member of staff is designated to monitor the animals throughout their recovery from anaesthesia and for the first few hours after recovery. This allows for rapid intervention in the event of problems arising, including humane culling if necessary. Animals undergoing surgery are pre-treated with an analgesic (e.g. meloxicam), with a second dose being administered on the day following surgery. As IVSA animals are housed singly to avoid damage to their catheter ports being caused by cage mates, rats are allowed to socialise in groups of up to three for an approximately 15-minute period at the end of their daily experimental sessions.

Rats on IVSA and DD studies are monitored via closed-circuit television while they are in the operant chambers each day. This allows scientists to monitor animal behaviour and welfare, intervening if necessary.

We make extensive use of pharmacokinetic (PK) and pilot experiments prior to committing large numbers of animals to a study. The PK studies allow us to determine that the compound doses that are proposed to be used produce the correct plasma exposures, ensuring against under- or overdosing of rats, the former leading to a possible repeat study, and the latter risking adverse events.

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

When planning studies, we will make reference to the PREPARE and ARRIVE guidelines and checklists. We also utilise the guidelines on the LASA and NC3Rs

website with respect to techniques such as dosing, blood sampling and animal handling.

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

Our studies are based within an accredited animal facility. As such, we can discuss 3Rs related changes to techniques with staff and the named persons (NVS, NACWO and NTCO) to ensure that we are familiar with current best practices in in vivo research. We also work collaboratively with study sponsors, many of whom have in house research teams with which we can discuss and share best practices.

We also participate in the facility's AWERB system, and our project licence applications and amendments are discussed at the regular AWERB meetings, offering the chance to discuss refinements.

As stated above, we have an in-house team of highly experienced statisticians and involve this group at the experimental design stage. This group keeps up to date with relevant changes in study planning and analysis.

We regularly monitor relevant scientific literature to stay up to date with the abuse-liability field of research. We attend scientific meetings and, where client confidentiality allows, publish on our work.

2. Breeding, production, archiving and the application of assisted reproductive techniques of genetically altered zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Zebrafish, Genetically altered, Breeding, Cryopreservation, Recovery

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This licence will permit the archiving, recovery and maintenance of genetically altered Zebrafish in a leading research institute for the duration of 5 years and will facilitate the efficient and ethical management of Zebrafish throughout the duration of various research programmes in the facility.

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

Why is it important to undertake this work?

It is important to undertake the work under this project licence to;

1. To maintain breeding colonies of genetically altered lines for distribution to various research projects.

2. To provide the application of assisted reproductive techniques for the purposes of efficient colony management.

3. Facilitate archiving and recovery of zebrafish lines.

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

In addition to breeding and healthy maintenance of animals, we can use this licence to cryopreserve sperm to ensure that genetically altered fish can be stored for future use without having to keep breeding animals. We will also use this licence to carry out IVF to recover genetically altered fish by inseminating fish with unfrozen sperm.

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

The wider research community will benefit from the outputs of this project licence by ensuring they have the genetically altered zebrafish they need to enable them to carry out novel and cutting edge research. Researchers will also benefit from not maintaining excess numbers of zebrafish in situations where they can be cryopreserved, which is also good for reducing the overall numbers of animals used.

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

We have a dedicated facility for the work carried out under this project licence with a number of research groups to support. Where possible, work under this project licence will be presented at national or international conferences and would be likely to reflect refinements in practice that have been developed during the course of the licence. To further maximise the outputs of the work, we would publish any findings that would promote good practice within the industry and would actively support the dissemination of information on unsuccessful approaches.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 201000

Predicted harms

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

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

This project will allow us to provide an efficient service to researchers who use genetically altered Zebrafish. Zebrafish will be used because they are the least sentient genetically altered species that are used for biomedical research. We need to use Zebrafish from the point of fertilsation through to adulthood. This enables us to carry out in vitro fertilisation (IVF) with sperm in order to create the genetically altered animals that we require and maintain them through to adulthood for use in scientific procedures in other project licences or for breeding purposes under this project.

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

The majority of zebrafish (approximately 60%) used in this project will be bred and maintained and will have subthreshold or mild symptoms resulting from their genetic alteration.

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

Due to the nature of this project, the anticipated adverse effects within this licence are likely to be very mild i.e. through breeding and maintenance of genetically altered Zebrafish. All Zebrafish on this project will either move onto another project licence, or will be culled by a schedule 1 method.

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

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

All Zebrafish are expected to experience a maximum of mild severity. Many animals used for breeding only will experience a subthreshold severity.

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

- Killed
- Used in other projects

Replacement

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

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

Zebrafish can only be bred and genetically altered through the use of live animals. This project provides a service to support the production and preservation of zebrafish that have genetic alterations that are essential for research purposes.

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

Non-animal alternatives are not possible because of the nature of this project and so could not be considered.

Why were they not suitable?

They were not suitable because there is no alternative to live zebrafish for breeding purposes.

Reduction

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

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

We have estimated the number of animals that will be used based on our experience of zebrafish usage in previous years and the predicted service provision over the next 5 years.

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

This project does not require experimental design because the purpose is not to use animals for experimental procedures. However, some animals may be humanely killed for tissue only and used for experimental purposes during the course of maintaining zebrafish colonies.

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

The centralised production, breeding and archiving of genetically altered zebrafish lines provides an opportunity to analyse working practices in order to use the fewest animals to achieve the intended aims. Archiving zebrafish lines will inherently reduce the numbers of animals required for any given project and when completed pre-emptively, will allow for efficient line removal and, if necessary, expansion.

Refinement

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

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

All of the animals bred and maintained under this project licence will experience only mild (or subthreshold) symptoms of their genetic alteration. The diseases or bodily systems that they will be used to study will vary widely and these zebrafish will move to other projects for the study of these diseases or systems.

Gamete collection should only cause transient, mild harms to the zebrafish and they will be fully recovered quickly after the procedure.

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

It is not possible to use less sentient animals than zebrafish for this project because its purpose is specifically to support research on zebrafish.

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

Current methods used are the most refined where advancements are made we will seek to actively implement them into our procedures. This includes the use of analgesia for regulated procedures such as fin clipping and when carrying out sperm freezing we ensure we use young fish, typically around 12 months of age. This ensures less fish are used to successfully achieve freezing down a line and when carrying out IVF.

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

Guidance will be obtained from several resources such as NC3Rs, RSPCA and UFAW.

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

We will stay informed of the most up to date procedures and advancements within the field by utilising updates from ASRU, NC3R's, IAT and LASA. Where there are advances that will directly benefit the 3R's, these will be assessed and utilised where possible.

3. Creation, Breeding and maintenance of genetically altered mice

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Genetic alteration, Breeding, Mice

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The purpose of this licence is to support the generation, breeding and maintenance of mice with genetic alterations and supply them either directly, or as provision of tissues, for work that supports both basic research into pathways and processes, and also the causes and treatment of disease. It also supports the cryopreservation of genetically altered strains for future use.

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

Why is it important to undertake this work?

In order to better understand the pathways underpinning both normal homeostasis and also disease, the various genes and/or targets involved must be manipulated. The use of genetically altered animals permits the manipulation of these genes/targets in order to study the effects within a whole living organism.

These models are now sufficiently sophisticated that they allow control over when and where the genetic alteration will occur in the animal. This level of precision means that the genes and pathways involved can now be explored at different life stages and/or at different stages of disease progression to aid in the advancement of knowledge, the discovery of medicines and potentially provide cures for serious diseases.

There are many research groups within the Establishment that require the use of genetically altered mice as a fundamental part of their scientific studies. These include areas such as cancer, the immune system, infectious diseases, and ageing and metabolism.

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

The aim of this project is to generate and maintain genetically altered animals for use either as live animals, or for the supply of tissues, by the research community at the Establishment and elsewhere.

Having supplied the animals or tissues required for downstream use, this will lead to publications of scientific value to the research community. This is expected to lead to advances in both basic research and also in the understanding and treatment of disease.

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

In the short term, researchers at the Establishment will benefit by receiving animals generated under this project licence to be used for their own research.

In the short-to-medium term, those researchers and their collaborators will benefit from the data obtained from these animals.

In the long term, the findings and conclusions drawn by the research community using animals generated under this licence are expected to lead to significant advances in scientific knowledge and the potential identification of disease treatments.

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

By offering this work as a centralised service, a central database is held of all strains available. This helps to maximise use of the strains available and avoid duplication of efforts and the associated waste of animals in generating lines already held by others (either currently, or as frozen stocks).

Genetically altered strains required by more than one research group will also be maintained under this licence, as this avoids duplication of breeding pairs, whilst ensuring the maximal number of offspring are used.

Species and numbers of animals expected to be used

- Mice: 51000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 licence is for the creation, breeding and maintenance of genetically altered animals and so involves animals at all life stages, up to the age of 15 months.

Mice are required as they are the species for which the most genes have been targeted for genetic alteration. This means that the majority of strains can be brought in from external sources. If a different species were to be used then it would require large numbers of animals to create the required genetic alterations in the new species, and new baseline scientific data would also need to be obtained, also requiring more animals.

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

Most animals used under this licence will be for the purpose of breeding and maintenance. This will involve standard natural mating, birth and subsequent weaning of the pups. Almost all animals will undergo an ear biopsy for identification purposes, with the tissue by-product used to confirm genetic identity. A minority will undergo a second ear biopsy if the genetic identification requires confirmation.

Some animals generated will undergo no further procedures and will be humanely killed for the supply of tissues only.

A smaller proportion of animals will be used to either create new strains, re-derive pre-existing strains from archives/stocks, or cryopreserve strains held in the facility. In order to achieve this:

- Some female animals will undergo injections to cause superovulation (i.e. to stimulate the ovaries to produce more eggs than normal). This will allow the generation of larger numbers of blastocysts, or embryos for rederivation, IVF and/or cryopreservation.
- A minority of male animals will be vasectomised under anaesthesia. These sterile males will be mated to females to create pseudo-pregnant females.
- Pseudo-pregnant females will be used as recipients for embryos that are implanted either surgically, or by non-surgical methods.
- Embryos generated as a result of the above may be allowed to develop to term to create genetically altered animals to be used in subsequent natural matings.

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

Animals being used for standard breeding and maintenance will not experience any adverse effects as part of the natural breeding process. Whilst genetically altered, the majority of animals do not appear any different to a normal mouse. A minority of animals however may experience some adverse effects as a result of the genetic modification.

Whilst dependent upon the strain in question, these adverse effects include development of intestinal polyps that could progress to intestinal tumours if left unmonitored, however the animals are humanely killed prior to this when they start to indicate the early signs of disease progression such as pale feet (the animal itself does not experience any adverse effects at this stage, but it is a valuable marker for underlying disease progression). Other animals may have reduced immunity, in which case measures will be put in place to prevent opportunistic infection, and the animals closely monitored for signs such as reduced activity. Some strains used in arthritis-related research may develop signs of the disease such as swollen and painful joints.

Those animals undergoing injections such as for superovulation will experience transient pain, but no other adverse effects. Animals that undergo surgery such as for vasectomy or as an embryo recipient will receive peri- and post-operative analgesia to mitigate any surgical-related pain. These techniques will cause no additional adverse effects other than those associated with routine surgery.

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

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

70% subthreshold
10% mild
20% moderate

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

- Killed
- Kept alive
- Used in other projects

Replacement

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

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

Although non-animal methods will be used wherever possible, animal models are still required to provide a setting in which a particular gene or pathway can be manipulated within a whole body system. Only in this setting can the impact of this

manipulation be understood both across systems and within individual tissues upon which various body systems have impacted over time.

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

The use of non-animal alternatives very much depends on the downstream research area in question, however some alternatives include the use of in-silico data and models, the use of in vitro data obtained from pre-existing cell lines, and also the use of models such as organ-on-a chip using patient derived cells.

Some of these approaches may be used to replace elements of the whole body system (for example organ-on-a-chip), whilst others may be used to replace aspects of the planned animal work such as early screening to identify potential candidate genes for further investigation.

Why were they not suitable?

These non-animal alternatives will be used wherever possible, however these alternatives cannot fully model the complete array of molecular, cellular, physiological and behavioural interactions necessary to fully understand how genetic modifications result in normal or abnormal processes.

Whilst these methods will not currently replace all in vivo research, they will be used where possible to provide the scientific evidence to determine what, if any, in vivo research is required and what direction this needs to take.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 in this project is based on previous experience, and an awareness of the volume and type of work undertaken in the Establishment that this project is designed to support.

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

When animals are to be brought into the Establishment under this licence, discussions will take place to understand the need for the strain and the downstream experimental plans (either under this licence if tissue is required, or following transfer to another licence). This ensures that the minimum number of animals are used to achieve the required outcome, including assisting in designing appropriate breeding strategies where needed.

Where genetically altered strains are available elsewhere, they will be brought in as this requires significantly less animals than re-creating the strain in-house. Cryopreservation banks and databases will be used for this purpose (for example the MMRC: https://www.mmrrc.org/)

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

The animal care staff overseeing the breeding programmes have expertise in breeding colony management and so will ensure there is no overbreeding of animals. The breeding performance will be monitored and intervention levels pre-determined depending on the strain and breeding strategies. A thorough understanding of laboratory animal science will ensure careful management of colonies, matching supply to demand and thus reducing the production of surplus stocks.

We will ensure that the breeding matches the scientific demand so that there is no wastage of animals. The breeding programme will be subject to periodic review to optimally meet anticipated demand.

We have an in-house cryopreservation facility to ensure that strains are held as frozen embryos or sperm thereby reducing the need to keep live colonies if there is no scientific requirement. We archive strains as sperm where possible as this requires less animals than to archive as embryos.

A full list will be maintained for all existing strains, new strains and crosses of existing strains for production of new combinations under this service licence, this will detail the nature of the mutation, any known adverse phenotypes, breeding and husbandry requirements.

The facility also deals with all requests for animals for tissues and so tissue sharing is facilitated and encouraged through this route.

Refinement

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

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

This service licence will be used to generate, supply, maintain and cryopreserve genetically altered mice for research.

Mice are a well recognised species for work involving genetic alterations and the protocols in this licence are standardised, using approaches that have been optimised for this species.

The majority of mice used under this licence will only experience natural mating and a small sample of ear taken for identification purposes which can subsequently be used for genotyping purposes, so as to avoid the need for a second sample wherever possible.

The remaining animals will either experience injections of drugs to induce superovulation, and/or surgery (e.g. laparotomy and intra uterine embryo introduction for female recipients, or vasectomy to produce sterile stud males). These will be performed using the most refined approaches, and where possible and appropriate, the use of non-surgical methods will be explored.

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

The purpose of this licence is the generation, supply, maintenance and cryopreservation of genetically altered mice for research.

Where the research purpose allows, a less sentient species will be used under alternative licence authority (if required). However the mouse is the species most commonly used for studies involving genetic alterations, and is often the least sentient species in which the research question can be answered.

All life stages are required for this licence, and terminally anaesthetised animals cannot be used due to the need to breed, maintain and supply animals.

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

Where possible, new strains will be brought into the facility as frozen stocks so as to avoid the stress associated with transporting live animals.

Mouse passports will be used where available when importing new strains into the facility. These will be reviewed to ensure that staff are aware of any potential phenotypes and that if needed, husbandry practices are adjusted to best support the animals. All new strains will be monitored closely for any unexpected emerging phenotypes.

The use of non-surgical methods of embryo transfer will be explored where appropriate and possible to avoid the need for surgery.

Where surgery is performed, peri and post-operative analgesia will be used, and animals will be kept warm using additional support measures such as heated operating stages, blankets and/or recovery cabinets. Once recovered animals will be returned to group housing unless there are husbandry-based reasons as to why this is not appropriate.

Refined handling methods (tunnel or cup handling) are used when picking up mice.

One of the chief benefits of undertaking this work under a service licence is that all the techniques to be used are undertaken by a small group of highly experienced technical staff which minimises the potential for suffering.

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

Mouse passports will be used where possible to ensure that maximum information is captured prior to the strain being established in-house.

LASA guidelines for dosing routes and volumes will be used where drugs are to be administered (for example to induce superovulation).

LASA blood sampling guidelines will also be used.

Downstream recipients of the mice generated under this licence will be reminded of the need to publish according to ARRIVE2.0 guidelines (https://www.nc3rs.org.uk/arrive-guidelines)

The NC3Rs resource library (https://nc3rs.org.uk/3rs-resources) will be consulted for the latest information on best 3Rs practice

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

We actively engage with the NC3Rs, and receive their newsletters and other updates. Facility staff regularly attend 3Rs-related events and other opportunities for Continued Professional Development.

The Establishment also has a 3Rs Focus Group with active participation from the facility. One aim of the focus group is to scope for new 3Rs-related actvities.

4. Environmental and anthropogenic drivers of migration success in fish

Project duration

5 years 0 months

Project purpose

- Basic research
- Protection of the natural environment in the interests of the health or welfare of man or animals
- Research aimed at preserving the species of animal subjected to regulated procedures as part of the programme of work

Key words

migration, ecology, behaviour, environmental change, conservation

Animal types	Life stages
Salmon (Salmo salar)	juvenile, adult
Brown Trout (Salmo Trutta)	adult, juvenile
Pike (Esox lucius)	adult, juvenile

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To determine the migratory patterns and behaviours of fish in both freshwater and marine waters. In addition, this project aims to examine the environmental cues for navigation and orientation during migration, and how environmental factors (both natural and man-made) could be altering or impacting fish migration.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 population sizes of a number of migratory fish species have declined markedly in the last few decades. This is particularly true for the Atlantic salmon and sea trout (the sea migrating form of trout) to the extent that it is predicted that several populations of these species could be extinct in Scotland within the next 20 years. Atlantic salmon and sea trout are a vital component of rivers and are essential components to the lifecycle of freshwater pearl mussels, whereby juvenile mussels parasitise the gills of these salmonids. Atlantic salmon undertake a high-risk migration from their home river to their marine feed grounds in the North Atlantic before returning to spawn in their home rivers. There are significant knowledge gaps around the migratory behaviour and patterns of salmonids in the waters around the UK and Ireland, including but not limited to their migratory pathways, navigational cues, swimming behaviours, environmental drivers of migration and how the environment modified by human activities might be impacting this migration. There are also knowledge gaps in the behaviour of predatory fish species such as pike and whether their behaviour changes during the period of migration of salmon and sea trout. As such, a greater understanding of the processes of migration by Atlantic salmon and sea trout is urgently needed to support the conservation and management of these species.

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

The outputs from this project include:

New information on the migration pathways of salmonids (i.e. Atlantic salmon and sea trout) and how these migration routes interact with offshore developments such as windfarms and aquaculture.

Peer reviewed scientific publications which contribute to our understanding of the complex migratory patterns and behaviours of salmonids and how these are driven by environmental factors, predation pressures from species such as pike and altered by pressure driven by human activity.

Results from this project will inform future mitigation measures to manage impacts from human activities undertaken by non-governmental organisations (NGO) and government.

Information will also be disseminated through formal and informal scientific briefings to government agencies, NGOs, river managers and through dissemination of information to raise public awareness.

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

The imperative for urgent evidence-based management of migratory fish and in particular Atlantic salmon have been recognised by the Scottish Government. In addition to benefiting the scientific community through publications on the fundamental processes involved in fish migration, this work will benefit governmental agencies and NGOs by providing an improved understanding of the impacts of pressures driven by human activity on the migration of salmonids. Thus, this work will likely have a direct impact of current and future policy decisions and direct management by these organisations.

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

The output of this project will be maximised by:

publications of the studies conducted with as many studies as possible published as open access in journals.

Results will be presented at scientific meetings and at sector management and policy meetings with government agencies, environmental charities and industry.

Data sharing with other scientific projects, management and policy organisations.

Collaboration with NGOS, Governmental organisations and industrial partners with interest in this project.

Species and numbers of animals expected to be used

- Brown Trout (Salmo Trutta): 4000
- Salmon (Salmo salar): 34000
- Other fish: No answer provided

Predicted harms

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

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

Wild Atlantic salmon and sea trout in the UK and Ireland are in decline and it has been shown that a major component of this decline occurs through losses during migration. Some losses during migration are natural but some are the results of environmental changes resulting from human activity (e.g. instream barriers, construction, aquaculture, offshore windfarms). Therefore, understanding the fundamental processes of migration is essential to conserve and protect these species. Both juveniles and adult life stage of salmon and trout undergo migration. Juveniles migrating out from freshwater to marine waters and vice versa for adults. In addition, it is important to understand the relationship between predator (pike) and prey (salmon and sea trout) during their migration in freshwater. Migration through freshwater lakes is challenging for juvenile salmonids, with previous research highlighting that salmonids take much longer than expected to migrate through these bodies of water and their movement often appears to be random. Understanding the relationship between pike and salmonids in these bodies of water will further our understanding of the migratory patterns and behaviour of salmonids and pike in lakes.

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

Tagging and marking – Fish which are tagged to allow for future identification of individuals and allow their movements to be tracked over time. All fish will be anaesthetised using an anaesthetic which contains analgesic properties. Where

possible fish will be tagged with a minimally invasive method. The exact method used will depend on the type of tag used and the fish species and size.

There are two different tagging techniques which will be used: a – a tag inserted or attached externally using a minimally invasive technique. These tag types usually provide information via either the recapture of an individual or the detection of individuals at fixed points in rivers. b – a tag is inserted by a surgical procedure which is more invasive.

The majority of fish in this project will have a tag attached externally or inserted internally using a minimally invasive technique (a). This involves using a tag applicator to insert the tag which will cause momentary pain to the animal (total time taken once the fish is under anaesthesia is usually less than one minute).

However, for a smaller proportion of fish in this project the tag will be surgically inserted (b). This more invasive technique, involves a small incision being made into the abdomen, the tag is inserted and the wound is closed with sutures (total time taken once the fish is under anaesthesia is usually 3 – 5 minutes).

Once the fish have been tagged (by either technique), if suitable following trials and in discussion with NVS, would treatment will be applied to all wounds. Where legislation permits, and withdrawal periods can be guaranteed, for post-operative pain relieve, analgesics will also be added to this wound treatment. Fish are then returned to a holding container with fresh well oxygenated water to recover. Once fully recovered, fish will be returned to the river.

Tissue collection for genetic analysis – genetic samples will be collected from wild fish for analysis of for example, population genetics, sex ratio, introgression and genetic markers associated with success. This involves the wild fish being anaesthetised and a small piece of fin tissue or a small number of scales being removed. The fish are then allowed to recover in a holding container with fresh well-oxygenated water. Once fully recovered fish will be returned to the river.

Experimental quantification of navigational behaviour – either wild or hatchery reared fish (type used will be dependent on their appropriateness for the experiment) will be used to investigate the fundamental processes underpinning migration by testing environmental variables (for example water velocity and electromagnetic fields) encountered by fish during their migration. Before laboratory experiments begin, fish will first be anaesthetised and a tag inserted by a minimally invasive technique

(a). These experiments will be conducted over months and involve fish being held in experimental tanks. During this time fish will be periodically anaesthetised and exposed to a range of environmental conditions that fish would normally expect to encounter in the wild.

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

The adverse effects of the minimally invasive tagging technique, described above, will involve momentary pain which will last no more than a few seconds. Whereas, the adverse effects of the more invasive tagging technique, described above, will

involve pain and / or discomfort which is likely to last a few hours to a few days. Previous experience has shown that fish tagged in this way express normal behaviour in less than one day. For both tagging techniques, the anaesthetic used has analgesic properties and will provide pain relief to individuals. The fish may experience short term pain and loss of appetite (minutes).

For fish held for laboratory experiments, are likely to include adverse effects from minimally invasive tagging technique which will involve momentary pain which will last no more than a few seconds. The anaesthetic used has analgesic properties and will provide pain relief to individuals. In addition, if suitable following trials and in discussion with NVS, would treatment will be applied to all wounds.

Where legislation permits, and withdrawal periods can be guaranteed, for post-operative pain relieve, analgesics will also be added to this wound treatment. The fish may experience short term pain and loss of appetite (minutes). In addition, both wild and hatchery bred fish may experience temporary elevated levels of stress which may last a few hours to a few days.

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

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

75% mild, 25% moderate

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

- Set free
- Killed

Replacement

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

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

The overall aim of this study is to quantify the migratory patterns and behaviours of migratory fish and investigate the fundamental processes of migration in fish. To achieve this, it is necessary to study animals in their natural (and human modified) environments to understand their migration pathways, the navigational cues of migration and how environmental (natural and anthropogenic driven) factors which could impact this migration. For a few environmental effects we will be able to test hypotheses about how environmental variables act as navigational cues during migration on species using controlled experiments in the laboratory.

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

For some questions it is possible to use statistical modelling in the study design. For example, we can predict how environmental variables (natural and modified) may

impact the migration of salmon and trout. However, to be able to adequately model these effects we need to quantify these effects from wild animals. It is therefore, necessary to use animals in this project to collect the important empirical data needed to inform modelling.

Why were they not suitable?

Modelling will be used alongside studies using animals to reduce and replace animal studies where possible. However, some animal studies are required to gain important data that will be used to inform and adequately model the environmental effects being examined.

Reduction

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

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

Numbers have been estimated for studies planned over the next 5 years based on previous experience conducting similar studies and previous published studies. A large proportion of the salmon used in this study will be to examine the lifetime migration success (the number of adult fish returning to rivers following migration to sea), in response to differing environmental variables. These salmon will be tagged as juveniles with a tag type that is long lived but has a very low impact on the fish after the tagging procedure is complete. It is expected that 1- 1.5% of fish might return as adults. Therefore, a large number of juvenile salmon need to be tagged in order for any findings to be statistically robust.

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

Current planned studies were designed on the basis of experience of minimal sample sizes from similar published studies, as well as, from previous experience and using statistical analysis to determine how large samples should be to find an effect.

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

For some of the studies, pilot studies will be run to look for a possible effect (if there is no indicative signs of an effect we will not proceed further) and to allow early analysis of how large a sample size is needed to robustly detect any effect; thus allowing us to reduce the sample size to a minimum that will still yield results. For studies where it is not possible to run a pilot study, the results of the project each year will be used to refine the number of animals used in subsequent years.

Refinement

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

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

This project uses fish models to investigate the effects of environmental change and human modified environmental pressures on the migratory behaviours of wild species. The method has been chosen to provide a clear answer to our questions whilst causing the least pain, suffering, distress or lasting harm to the study animals.

The method involves using tags to identify individual animals and to track their movements between different habitats. This enables us to determine the potential impacts of anthropogenic induced pressures on the migratory behaviours and patterns of individuals.

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

The drivers for the work described here is to improve policies around the management of wild migratory salmonids. Using other species would not address the questions necessary to achieve this.

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

The fish used in the studies planned in this project will be subject to high levels of behavioural monitoring by experienced staff at the point where they are captured, during the procedure and before release into the wild to ensure that any change in the low welfare costs anticipated are identified quickly and steps taken to manage that.

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

We will follow the rapidly emerging literature to ensure experiments are conducted in the most refined ways and make changes to the study design to take into account any changing best practices.

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

Using the abundant online resources, such as NC3Rs, which are updated regularly on advances in the 3Rs, the expanding scientific literature and the regular information circulated by the establishment.

5. Environmental and dietary manipulations: Effects on fish physiology and locomotor activity

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Nutrition, Fish performance, Health, Environment, Locomotor activity

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, juvenile, adult, aged
Rainbow Trout (Oncorhynchus mykiss)	embryo, juvenile, adult, aged
Medaka (Oryzias latipes)	embryo, juvenile, adult, aged

Animal types	Life stages
Salmon (Salmo salar)	embryo, juvenile, adult, aged
Brown Trout (Salmo Trutta)	embryo, juvenile, adult, aged
African catfish (Clarias gariepinus)	juvenile, embryo, adult, aged

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The main objectives of this project licence are to advance our understanding of how fish function and how changes in their diet and/or their environment influence their physiological responses.

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

Why is it important to undertake this work?

New fish diets are continually being developed because of changes in the raw materials being used, such as the switch from marine ingredients to terrestrial based ones such as plants, animals or microbes. In addition, there is a need to determine the requirement levels for nutrients across new and existing species being farmed. As such these nutritional responses need to be studied to ensure they correctly support growth and health, across a range of environmental conditions.

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

Communication activities for a specified target audience will be planned in order to distribute information generated in the project. Outputs will include scientific publications in specialized peer reviewed journals in the field such as Aquaculture, British Journal of Nutrition, Chronobiology, Fish Physiology and Biochemistry etc. Attendance to conferences such as International Symposium of Fish feeding and Nutrition or Aquaculture Europe. Additionally, popular articles in the national or international magazines, such as Hatchery International or IntraFish are also expected to be published with results generated from the project. Furthermore, information generated from the project will likely contribute to constructing dossiers about innovative feeds.

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

As a result of this project there will be improved animal health and welfare (benefit to the animals). This will further contribute to improved industry performance through a reduction in poor-health associated losses (benefit to humans) and also improved industry sustainability (benefit to humans and environment). Additionally, refined husbandry protocols and environmental tolerance through stress studies and programming at an early stage of development (nutritional/environmental programming and epigenetic effects) to optimise fish welfare and performance in established and emerging species.

Ultimately the outcomes of this project will also allow for a reduced environmental impact associated with aquaculture (benefit to environment) by underpinning a more efficient use of resources, such as improving the feed conversion ratio, and an ability to include more sustainable raw materials in feeds.

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

Several strong collaborations exist at the moment with Academia and Industry (aquafeed producers, raw material providers, fish producers) that will ensure the adequate dissemination both in peer review (Aquaculture, Comparative Biochemistry and Physiology, Fish and Shellfish Immunology...) and dissemination journals (e.g. Fish Farmer, Fish Focus...) as well as quick translation into farming/feed formulation practices. While it is tempting not to publish disappointing results, we will aim to

publish them as the information gathered from null results and failed trials serve deeper, long-term learning.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 1000
- Medaka (Oryzias latipes): 1000
- Brown Trout (Salmo Trutta): 5000
- Rainbow Trout (Oncorhynchus mykiss): 5000
- Salmon (Salmo salar): 20000
- Other fish: No answer provided

Predicted harms

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

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

A range of freshwater, anadromous and marine species are expected to be used, including Atlantic salmon (Salmo salar; 20,000), Atlantic halibut (Hippoglossus hippoglossus; 5,000), lumpfish (Cyclopterus lumpus; 5,000), ballan wrasse (Labrus bergylta; 10,000), tilapia (Oreochromis niloticus;

10,000), African catfish (Clarias gariepinus; 5,000), rainbow trout (Oncorhynchus mykiss; 5,000), brown trout (Salmo trutta; 5,000), zebrafish (Danio rerio; 1,000), medaka (Oryzias latipes; 1,000), gilthead sea bream (Sparus aurata; 1,000) and European sea bass (Dicentrarchus labrax; 1,000). Over the five-year period of this project licence we anticipate using the following numbers of animals; fish: 74,000. These species were chosen because they are: 1) commercially produced in Europe (Atlantic salmon, halibut, lumpfish, rainbow and brown trout, gilthead sea bream and European sea bass; 2) commercially relevant species in third countries we collaborate with (tilapia and African catfish) or 3) model species for human and animal studies (zebrafish and medaka).

Different life stages will be used as dietary requirements and the effect of dietary additives have a different impact depending on the life stage. In addition, the plasticity to induce a positive long life change (e.g. nutritional programming) has several windows and therefore needs to be investigated along the whole life cycle.

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

This project licence involves various procedures classified as "mild" or "moderate" in terms of their impact on welfare. Many of the techniques employed are standard in fish management, such as adjusting diet, temperature, salinity, oxygen levels, and assessing weight-length relationships. The duration of experiments varies widely, ranging from one week for digestibility trials to 18 months for whole life cycle trials in Atlantic salmon.

Commonly collected in vivo data includes fish identity, weight, length, feed consumption, behavioural observations, and water quality parameters like temperature, dissolved gases, suspended solids, pH, hardness, alkalinity, salinity, water turnover rate, and flow rate. In vivo samples may include blood for endocrine analyses, gametes for histological assessment, faeces for digestibility analyses, and mucus for biochemical analyses.

Post-mortem data or samples may involve weight and length measurements, blood for endocrine analyses, tissue samples for histopathology, microbiome studies, DNA for genotyping, and RNA for gene expression. Procedures such as X-ray radiography, blood sampling, and external observations may be conducted under terminal anaesthesia to minimize the delay between death and sampling.

X-ray radiography is used for feed intake assessment, and ultrasound is employed for morphological assessment, both procedures being repeated every second day in short-term studies. The study of feed intake involves feeding with feed containing radio-opaque glass beads, not exceeding 1% by weight.

Blood withdrawal is done via superficial venepuncture of the caudal vein, with the volume not exceeding 1% of the fish weight (up to a maximum of 2.5 ml). Stripping of faeces for digestibility studies follows specific guidelines, and stripping of gametes is performed based on the spawning pattern of the species. Catheterisation of gonads is conducted for reproductive assessment, and immune challenges involve intraperitoneal injection with adjuvants containing pathogen-associated marker patterns (PAMPs), with a control treatment of sterile saline.

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

Few adverse effects from the allocation of a specific dietary or environmental treatment are anticipated. During the instigation of any treatment the fish will be closely observed with any moribund fish being euthanised. Additionally, most of the protocols described in this project licence involve techniques that are used in routine fish management (e.g. alteration of diet, temperature, salinity, oxygenation, weight-length assessment, stripping). At the end of each experiment, those fish which have only been subjected to the mild protocols from this project licence may be returned to stock with the approval of the Named Veterinary Surgeon (NVS), provided they are not suffering and not likely to suffer. This would not apply to those fish that have been subjected to manipulation of the immune status, which will be euthanised by an appropriate schedule 1 method as part of the protocol. Similarly, those fish that have been subjected to “moderate” harm will also not be returned to stock.

Additionally, a variable frequency of minor health problems that also affect stock animals and are not procedure related are expected. Following transfer from freshwater signs may include handling marks, retention of parr colouration and poor adaptation to saltwater. Physical signs occurring at any time may include local scale loss, fin erosion and lesions resulting from fin nipping, jaw injuries, eye injuries or exophthalmia and associated regional darkening of appearance, calluses on the ventral midline, pinpoint haemorrhaging on the skin, naturally occurring gill infections such as amoebic or complex gill disease, occasional external parasites, or

precocious sexual maturation. Behavioural signs may include reduced feeding, erratic, fast or uncoordinated swimming, or rapid breathing. Animals showing any of these signs will be assessed by experienced staff and may be culled. Animals showing lesions on their flanks that can occur during periods of stress will be culled. If animals undergoing procedures for quantitative experiments are culled for non procedure related health problems, the validity of continuing the experiment using remaining animals will be reviewed.

Every effort will be made to anticipate and prevent mortality but we do expect a low background mortality rate due to non procedure related congenital defects, physiological maladaptation, disease or injury in line with normal commercial practise.

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

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

This project licence involves procedures which are all categorised as “mild” (approximately 70%) or “moderate” (approximately 10%) in terms of the severity, while approximately 20% will be subthreshold (control groups). Notably, good handling techniques by trained and experienced staff with appropriate anaesthesia will be used throughout.

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

- Killed
- Used in other projects

Replacement

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

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

The physiological responses we are investigating in this program of work and the associated biological responses (e.g. growth) require experimentation on live animals. Additionally, the direct extension of experimental outcomes to commercial practices requires the use of live animal studies that are accepted by industry as the most reliable basis for underpinning evidence for change.

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

A variety of resources (e.g. literature and scientific networks, in-vitro models) have been used to identify potential alternate models to the use of animals in these studies. Although most of the work in this project licence requires animal-based experimentation, in certain circumstances isolated discrete tissues (e.g. a liver cell culture) may be utilised. In addition, to date, of over 43,400 cell lines deposited at the American Type Culture Collection (ATCC) and The European Collection of Cell

Cultures (ECACC) only 43 are fish derived cell lines which are usable and available for dissemination to researchers globally. In digestibility trials where several ingredients were to be tested, the use of in vitro digestibility can be used as a screening tool to identify ingredients with poor digestibility. Where possible, non-invasive monitoring of fish behaviour will be used to further assess the welfare of the animals in response to variations in diet and environment

Why were they not suitable?

The mechanisms we are interested in are a product of a complex interaction of various specialised tissues within the animal and the result of environmental manipulations. As such, there exists no isolated cell based model that can replicate the outcomes achieved in live animals. While primary and continuous cell lines could be used for basic studies on specific cellular processes, they are not suitable for most of the research activities of this project.

Reduction

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

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

The numbers of animals required for each experiment will be defined by the use of appropriate statistical pre-tests (e.g. power analysis) to define the minimum potential number of tanks of fish required, in conjunction with any logistical constraints due to behaviour of fish within a tank (e.g. number of fish within a tank to avoid social hierarchy formation) and the required sample size (e.g. amount of faeces) to undertake a robust analysis of the samples collected.

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

All studies will be designed with a minimum number of animals used within a statistically valid experimental design (e.g. Experimental Design Assistant; https://www.nc3rs.org.uk/our- portfolio/experimental-design-assistant-eda) to define the minimum potential number of tanks of fish required, in conjunction with any logistical constraints due to behaviour of fish within a tank (e.g. number of fish within a tank to avoid social hierarchy formation) and the required sample size (e.g. amount of faeces) to undertake a robust analysis of the samples collected.. When fish are killed at the end of studies, additional tissue samples will be collected post-mortem and archived for future potential use to reduce the requirement for any subsequent experimentation.

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

Where procedural harm is minimal (e.g. short-term digestibility study with faecal stripping alone, mucus collection from skin), fish may be re-used up to four times to further reduce the total number of fish required for any single experiment. Only fish that have not lost weight and are free from physical damage will be considered for re-use. Multiple tissues will be collected from the same animal in order to perform different analysis (e.g. samples of liver will be collected for fatty acid, molecular biology and eicosanoid analysis).

Refinement

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

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

Work will be carried out on commercially important fish species farmed mainly in Europe, but also in two model species (zebrafish and medaka). Due to the diversity of the species, farming systems and environments used in aquaculture it is important to target a range of species in this project licence application to ensure project objectives can be attained for the desired relevant species and industry sector. The two model species (zebrafish and medaka) will be used as their genetic tractability, rapid development, and physiological similarities with other fish species makes them a valuable model organisms for advancing our understanding of aquaculture-related research. These species will be particularly helpful in instances where, for example, there is not yet enough material to produce feeds for a larger species due to technology development (eg initial development of a single cell protein).

Most of the methods described in this project license have a mild or moderate severity and are not expected to cause lasting harm to the animals.

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

Animals from different life stages will be used as the physiological response to different nutrients varies with fish age, therefore early and later life stages will be used. When possible, environmental trials with yolk sac larvae will be carried out rather than with free feeding individuals.

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

Throughout the work all fish will be housed in conditions that comply with the welfare standards of the target species of interest and monitored by the animal care technicians, Named Animal Care Welfare Officers (NACWOs) and Named Veterinary Surgeons (NVS). To further refine these procedures fish will be housed in facilities that are constantly monitored for temperature and oxygen, allowing further

refinement of critical environmental constraints. Local anaesthesia (e.g. benzocaine) will be applied during PIT-tagging.

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

The ARRIVE guidelines will be followed. In addition, scientific literature, RSPCA recommendations, commercial certifications relevant to aquaculture (e.g. Global Aquaculture Alliance Best Aquaculture Practices) and in house discussion will also be used for best practise.

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

I am subscribed to the NC3Rs e-newsletter which provides monthly updates about 3R's publications and different events. If any advance (e.g. technological advance that allows to detect welfare markers in the water) is deemed suitable to be included in this project licence, an amendment will be performed and the advance adopted by me. Whenever possible I will also attend he RSPCA "Focus on fish" meetings or listen to te recording produced to keep up to date with cutting-edge knowledge and approaches to refining fish use, enhancing both animal welfare and translatability.

6. Generation and screening of companion animal therapeutics

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
- 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

Companion animal, monoclonal antibody, Veterinary medicine, Cancer, Atopic dermatitis

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To complete genetically engineered mouse platforms containing a collection of genes (immunoglobulin loci) from companion animals that are responsible for the generation of species-specific antibodies that would normally recognise and eliminate harmful substances like bacteria, viruses or toxins. The mice will be used to generate antibody sequences against a range of companion animal diseases. Once antibody sequences with desirable characteristics have been identified the antibodies will be produced using stable cell line culture techniques and molecules will be assessed in a range of studies using rodent models to test how well they remain detectable and/or functional over a defined period of time. The information

gathered here will inform us which drug is best suited to take forward to test in the target animal species.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 programme of work is expected to discover new drugs which can be used to treat a range of diseases that companion animals suffer from. Companion animals are medically underserved compared to humans with only a handful of antibody drugs available compared to more than 120 human FDA approved antibody (mAb) drugs available.

Cell based efficacy tests provide valuable information, however it is necessary to confirm their anticipated characteristics in an animal by using mouse or rat models before progressing to the target animal species.

This project aims to generate drugs which address a range of diseases such as cancer, skin conditions, disease induced weight loss and pain.

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

Genes which are used in the creation of antibodies are well understood. By introducing these genes from a companion animal species into a mouse model, we expect to complete a genetically altered mouse which contains the vast majority of either cat (felinised), dog (caninised) or horse (equinised) immunoglobulin heavy and light chain genes. We will introduce enough genes so that each species specific platform can reflect the naturally occurring diverse antibody repertoire.

The companion animal platforms will then be presented with different antigens to create a range of therapeutic drugs to address prevalent and regularly occurring diseases where there is a clinical veterinary need, such as cancer, skin conditions such as atopic dermatitis, pain, and weight loss associated with long term disease.

Once therapeutic candidate drugs have been generated they will be tested extensively in vitro (in cells) and the best and most likely to succeed candidates will be administered into rodent models to understand how they behave in a whole animal, we can also gather information on how safe these candidates are too prior to administration in their target species. For some indications such as cancer or anaemia we can use rodent models that reflect critical elements of the target disease to determine how likely our therapeutics are to succeed before testing in companion animal species.

Summary;

- New veterinary drugs addressing 5-10 disease targets such as cancer, atopic dermatitis, pain, anaemia and cachexia.

- Genetically engineered mouse models which produce species specific antibodies for companion animals that can be used to address additional therapeutic targets.

- Publications in the peer reviewed scientific literature   Presentation of scientific data at meetings

- We will file patents, thereby placing in the public domain detailed knowledge of the discoveries we have made.

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

Antibody therapeutics being identified in this license will be used to validate and eventually treat companion animal disease indications. Candidate therapeutics that prove to be safe and effective may progress from this license into a preclinical testing phase in their target species. Following a demonstration of efficacy and safety, candidate therapeutics discovered under this license will progress into field studies and clinical trials in compliance with market authorisation standards detailed by the United States Department of Agriculturem, the European Market Authorisation and the Veterinary Medicines Directorate. Once market approval is obtained, therapeutics discovered under this license will progress towards use in general clinical veterinary practices.

Short term:

Monoclonal antibody therapeutics being discovered in this license that prove to be safe and effective may progress through to preclinical and clinical trials within the field of clinical veterinary medicine.

Therapeutics discovered under this license will also progress towards obtaining USDA (United States Department of Agriculture) approval so that they can be used in the United States and EMA approval (European Market Authorisation) to enable their use within Europe. Following Brexit additional market authorisation is expected to be required after 2025 through the Vetereinary Medicine Directorate in order for therapeutics to enter veterinary practices within the United Kingdom.

Medium term:

Therapeutics discovered under this license (or in future using the genetically altered mouse models) are expected to become available for use in clinical veterinary medicine to improve the health and welfare of our companion animals, treating a range of potentially serious and debilitating illnesses, such as cancer and atopic dermatitis. Monoclonal antibodies are expected to become a new standard of care in veterinary medicine.

Long term:

We are in a unique position to be able create therapeutic antibodies to address new targets more quickly than in the human pharmaceutical industry. It is anticipated that the success or failure of novel companion animal therapeutic areas may infuence

target selection and prioritisation within human medicine in cases where the biological mechanisms are similar.

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

Drugs discovered under or due to this license will likely become available for use in clinical veterinary medicine. Appropriate business development will result in these drugs being administered internationally.

Data obtained under this license will be published in the form of patents and it is expected to be published in scientific journals so that some of the knowledge gained under this license can be utilised by other research groups.

Where possible technical or ethical refinements may be published in open access journals.

Species and numbers of animals expected to be used

- Mice: 42450
- Rats: 2400

Predicted harms

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

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

Well established and sophisticated technologies are available to create genetically altered mouse models, where their genetic composition has been manipulated. Technology capable of introducing large amounts of genetic material (BACs) will be used to substitute part of the immunoglobulin loci in the mouse genome, which is responsible for generating antibodies. Companion animal immunoglobulin genes will be introduced so that they can be used to generate species specific antibodies. These methods are very efficient and have been refined over many years so that less animals are needed compared to achieving the same goals in other species. When challenged with a particular foreign molecule, these mouse models will produce companion animal antibodies against it. These antibodies will then be identified and screened for desirable characteristics.

The canine transgenic platform that we are creating is the most complete to date, and displays similar immunological characteristics to dogs (Beagles) with very similar V(D)J recombination. This process shuffles and combines Variable (V), Diversity (D) and Joining (J) gene segments to create trillions of possible antibody receptor sequences which could recognise new disease target molecules. The canine mouse platform reproduces gene fragment combination and usage that is very similar to the target species, this provides excellent validation that our approach with a transgenic platform will be successful.

In some cases, the disease target molecule (antigen) will be similar in to one which occurs naturally in the mouse, in order to avoid inducing an immune response against self, some mice will have the mouse version 'knocked-out' or deleted so that it is recognised as foreign. This will only be completed if no negative side effects are expected form 'deleting' the specific gene.

All life stages of mice will be used to generate the genetically altered companion animal mouse models.

Rodent models represent the least sentient species which still demonstrate the physiological and genetic similarities to the companion animal species that we are generating drugs for. There are currently no better animal model steps that could validate our therapeutic drugs before progressing into target species preclinical testing.

After screening of new candidate drugs in cell lines (non licensed), they will be injected into mice or rats to gather information on how well they remain functional and available in the blood or organs. Also, what impact they have on the injected animal. Blood will be collected at established time points to obtain this data.

New drugs will also be tested in adult mice or rat disease models where the animals will recapitulate a particular target disease.

Examples of disease models include using mice in tumour engraftment studies. There are already well established protocols available to grow tumours under the skin (subcutaneous) on the flanks of mice, which are easy to monitor and measure, and which cause very little discomfort to the animal. The tumours generated from specific cell lines will be engineered to present companion animal versions of the disease target antigens so that new companion animal drugs can be assessed first in mice before progressing to target animal studies. Rodent models will also be used to assess how well new therapeutic antibodies perform which target energy intake disorders.

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

Superovulation (stimulate the release of additional oocytes)- female mice over three weeks will normally receive two substance administrations over a period of three days. Substances will be injected into the abdomen (intraperitoneal) or under the skin (subcutaneous). To conduct this procedure mice will be scruffed and will remain conscious.

Vasectomy (to generate sterile mice) - male mice over the age of four weeks will be anaesthetised using gaseous anaesthesia and small 3mm incision will be made to the scrotum. The duct through which mature sperm pass, will be identified and then severed to remove a 4mm section. The small incision in the skin will be closed with one or two sutures (stitches). This process takes roughly 5 minutes. Pain relief is given before the procedure so the male mice will not suffer as a result.

Embryo transfer - Female mice which have been time mated to vasectomised males will be anaesthetised using gaseous anaesthesia. A small 5mm incision will be made along the midline of their back to allow access to either side of the reproductive tract.

On each side of the mouse an incision will be made through the body wall to expose relevant reproductive organs (ovary, oviduct and uterus).

Early stage preimplantation embryos will then be transferred to the section of reproductive tract which best matches their stage of development. Following embryo transfer the exposed tissues will be replaced and the incisions closed either by surgical glue, or using wound clips that will be removed 7- 10 days later. This process takes up to 7 minutes. Pain relief is given before the procedure so the recipient mice will not suffer as a result.

Breeding and maintenance - Genetically engineered mice will be mated together in either pairs or in trio/harem breeding systems and the resulting offspring may carry genetically engineered genes.

Blood withdrawal - Mice which have a suitable combination of engineered genes may receive several blood withdrawals all from a superficial blood vessel by puncture with a needle whist they are conscious. Veins such as the saphenous (leg) or lateral tail vein will be used. Guidelines for blood withdrawal will be followed - no more than 10% in 24 hours or 15% in 28 days.

Immunisation - Mice may receive up to 5 administrations of an antigen over the space of 4 months. Injections may contain mild adjuvants (substances) that will induce a stronger immune response.

Substances will normally be injected into the abdominal cavity (intraperitoneal), under the skin (subcutaneous), into a blood vessel (intravenous), rarely substances will be injected into muscle (intramuscular). Occasionally hydrodynamic tail vein injections will be used as a route of immunisation, where a large volume (8 to 10% of the body weight as a volume) will be injected into a mouse tail vein over a period of 8 to12 seconds. This solution will contain DNA which encodes for the target antigen, that will integrate primarily into the liver after organ swells and it will begin to express the target antigen. This procedure is conducted under anaesthesia and pain relief is provided. Mice will remain anaesthetised for 4 minutes after the injection so that the most significant side effects wear off. Mice should be fully recovered within 2 to 4 hours.

Tumour studies - Some mice will have tumour cells injected under the skin (subcutaneous) on their flank and be left to form tumours potentially until they reach a mean diameter of 15mm. Normally this will take 4 to 6 weeks after administration however the experiment may persist longer if they are given a drug which decreases the size of the tumour or delays the growth.

Pharmacokinetic, pharmacodynamic and safety studies - Novel drugs will be administered under the skin (subcutaneously) or more commonly into a vein (intravenous) of mice or rats. By performing routine scheduled blood withdrawals we are able to monitor how well the drug remains available in the blood and in certain organs of the animals over time. Antibodies typically have a long half life so the animals may be monitored for several months.

Metabolism targets - Mice or rats will receive a substance either in their food or drinking water, or given through oral dosing (oral gavage) or as an injection into the

peritoneal cavity (Intraperitoneal) that will begin to replicate a weight loss disease up to a maximum of 10% weight loss. Some animals will be given a drug as an injection under the skin (subcutaneous) or into a blood vessel (intravenous) that aims to increase weight. In some cases a high fat diet may be given to the animals so we can assess a difference between drugs over a shorter time period. Experiments are not expected to last more than 6 weeks.

Pain studies - Mice or rats will receive an injection into one knee joint that will simulate the onset and progression of arthritis. Candidate drugs will then be administered with the aim of alleviating pain (measured by limb weight bearing ratio and joint swelling) and slowing or preventing disease progression. Experiments will last up to 28 days.

Terminal blood withdrawal - In some cases where a terminal blood sample is required, animals will be placed under terminal anaesthesia and blood will be withdrawn using a needle and syringe either directly from the heart or from an internal easily identifiable blood vessel (caudal vena cava). Animals will then be killed by an approved method without regaining consciousness.

Animals will not be reused during this project.

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

Some female mice will receive a couple of injections to stimulate egg release for time point mating/harvest. This is not expected to exceed the minimum level of suffering associated with the insertion of a hypodermic needle following good practice and will have no lasting harm.

Most genetically altered mice used under this license will be used to breed for 3 to 6 months and are not expected to experience any adverse effects associated with any genetic modification.

Some of the animals will experience transient pain from the minor surgery required for embryo transfer and vasectomy. These procedures will take a roughly 5 minutes and will always be conducted under anaesthesia with a suitable pain management program in place to support the recovery over a few days.

The majority of mice which are immunised under this license will experience transient discomfort from a series of injections which are likely to occur every 2 to 4 weeks for up to 5 occasions. Adjuvants may also be administered alongside a target antigen to stimulate an immune response. Mild, non-ulcerating adjuvants will be used but mild injection site reactions may occur, such as the formation of small granulomas, localised inflammation. Rarely animals may exhibit reduced movement, hunched posture, piloerection (erect hairs over their body) for a period up to 3 to 4 hours following immunisation. Blood will be collected following a needle stick to a superficial vessel at predetermined points. This is expected to cause mild discomfort that will resolve within minutes.

A small proportion of immunisations will be conducted under anaesthesia to inject DNA directly into a vein. Appropriate pain relief will be provided and mice will be

recovered from anaesthesia when the majority of adverse effects have resolved after approximately 4 minutes. Mice will be subdued and lethargic after injection but will recover fully within 2 to 4 hours following injection.

The procedures used for tumour cell implantation under the skin will involve needle pricks. They will be conducted under anaesthesia to ensure refined placement and the mice will be expected to be subdued and lethargic for 30 minutes after the injection whilst they recover from anaesthesia.

The tumours will be allowed to grow to a size where they may cause some discomfort and may cause other clinical signs. We will carefully monitor mice with tumours to make sure that the tumour is not having a major impact on the health of the mice. We monitor weight, mobility and general condition of the mice. We also routinely measure tumour size, which may involve feeling it through the skin or using imaging techniques.

Tumour growth will occur from roughly two weeks after injection however it is unpredictable and they make take up to two months to grow. Monitoring will be regular but will increased during critical periods of growth. Tumour bearing mice will be humanely killed at the point that tumours reach a predetermined size in line with current UK guidance.

Some genetically altered animals which are being produced may have an altered immune system that may make them more susceptible to infection. To keep mice with immune deficiencies healthy, they are maintained in a very clean facility where they are not exposed to harmful viruses or bacteria.

Mice and rats used in dose ranging studies will experience several blood withdrawals, they are expected to cause mild discomfort that will subside very quickly after the procedure and have no lasting harm. Due to how well antibodies persist within an animal it is not expected that we will require blood withdrawals frequent enough to warrant the use of catheters.

For some disease model studies we need to induce a metabolic intake pathway disease. Whilst the administration regime is not expected to cause any adverse effects it is likely that a small proportion of control animals will lose up to 10% of their body weight, and may experience discomfort as a result.

Clinical signs may include becoming less active and adopting a hunched posture and their coat quality deteriorating. Animals on this protocol will be weighed up to 3 times a week to monitor weight loss.

The pathway induction step will commonly coincide with the provision of a high fat diet - this will ameliorate weight loss but may also cause animals to gain weight and become obese. The experiment duration is not long enough to expect significant adverse effects as a result of the weight gain however measures will be in place to regularly change food to lower excess levels in the home cage environment. Animals on high fat diets will often develop greasy unkempt coats partly due to residual levels of diet around the cage. In rare cases this may cause irritation or skin reactions.

Mice and rats used in osteoarthritis like pain studies will experience localised pain and inflammation to a single knee joint. The vast majority of animals will receive a type of drug to alleviate this pain however some animals will not receive any treatment so we can understand how much of an effect each drug has. These animals are expected to show weight bearing preference to the unaffected limb and may move less. If ant any point mice or rats exceed the end points of extreme limb preference or significant weight loss then will be killed.

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

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

Mice
Mild: 90%
Moderate: 10%
Rats
Mild: 75%
Moderate: 25%

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

- Killed
- Kept alive
- Used in other projects

Replacement

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

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

Within each animal trillions of different antibodies are presented on the cell surface of B cells, which circulate in the blood. Naturally they recognise and kill bacteria and virus infected cells by binding to a foreign substance known as an antigen. Because each antibody is different, once it binds an antigen it is already quite specific, but other antibodies will evolve from this sequence so that they become more and more specific and bind more tightly. This specificity, affinity and ability to recruit an immune response to destroy the target antigen presenting cell makes them useful as a therapeutic drug.

Depending on how antibodies recruit a killing response from the immune system, they may also act to bind and block a target antigen so to prevent further activity in a particular biological pathway.

The immune response is highly complex and the adaptive immune response we are interested in matures over several months. It involves the interaction of many different cell types and migration of these cells to a variety of sites in the body. The

intricate interaction between the different cell types simply can't be reproduced in a laboratory. Antibodies which have matured through this process are more likely to have better characteristics which are key to creating viable therapeutics such as thermal stability, solubility and specificity. By introducing companion animal immunoglobulin genes into a mouse platforms we are able to fully utilise this biological mechanism to make species specific therapeutic antibodies.

There are already more than 100 antibody therapeutic drugs approved by the FDA/USDA/EMA for the treatment of human diseases which target specific antigens present on disease-associated cells, such as cancer.

Regardless of their quality in cell based tests, it would be unethical to use experimental therapeutics on target animal species or patient owned animals without first performing some safety studies in a controlled laboratory setting, in this case using mice and rats. Before any candidate therapeutic is administered to an animal under this license a comprehensive selection and screening cascade is completed using at least two cell based tests.

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

Display technologies

An alternative, artificial antibody discovery process known as 'phage display' uses bacteria. As part of the phage display process, viruses which naturally infect bacteria are modified in such a way that they carry antibody fragments. A 'library' of different sequences is then generated which may contain millions of combinations. It is then possible that some will bind the particular drug target molecule (antigen). Yeast display libraries can also be used in a similar way.

When monoclonal antibody combinations generated in this way bind to an antigen of interest, there are associated issues which are problematic when creating therapeutic antibodies. The binding is normally not very strong, because the pairing is immature and they also often lack specificity which could result in off target binding, which can cause serious side effects.

When antibody sequences are obtained from our transgenic mouse platform they are considered to be mature. They will have gone through rounds of 'optimisation' against the target antigen by repeat antigen administrations (immunisations). Biological processes responsible for this adaptive response have been highly conserved across species over millions of years indicating how fundamental this process is. Mature antibodies typically make much better therapeutics, they will bind to a target antigen up to a thousand times more tightly than those generated through display technologies and they will have much better specificity so that they can be developed as drugs without further modification.

Modifying antibody sequences is more likely to result in side-effects when injected into their target species.

Artificial intelligence & machine-learning

In recent years, artificial intelligence and machine-learning (AI/ML) for both the prediction of unknown protein structures and also the design of protein-protein interactions has received a high profile in mainstream media and in specialist areas of research. Many factors contribute to this, including a number of academic breakthroughs, advances in computing power and, arguably the most important contribution is due to the increased availability of large datasets for AI training.

In order to predict protein structures and design new proteins, large language models (LLMs) are used. These are based on neural networks which are a series of pattern-recognising algorithms. These algorithms are loosely modelled on the current understanding of how neurons function in the brain.

Generative artificial intelligence for the de novo or novel generation and design of proteins (including monoclonal antibodies) has developed significantly over the past 5 years. The ability to rationally design binding properties in silico for a protein of choice has immediate therapeutic applications, this has been led by small molecule drugs for over two decades. However, large biological molecules such as an antibody are significantly larger and more complex than small molecules, they are therefore more difficult to model and design in a rational way. For an antibody to be a therapeutic candidate, it must satisfy a set of stringent criteria or “drug-like properties”. For example, it must usually bind the target in a precise place (epitope) which is determined by the biology of that target, it must bind to the target with an affinity that permits standard medical doses (usually <10 nM), it must be amenable to manufacturing at scale (“developable”), sometimes cross-reactivity with different protein families is necessary or conversely be highly selective of a single protein and it must fail to be recognised as foreign in the body.

To achieve these properties, selecting a therapeutic antibody from a discovery campaign has meant screening hundreds or thousands of candidate antibodies in order to find one which displays the complete set of characteristics. AI/ML promises to design-in many of these features in silico instead of relying on discovering them “by chance” during antibody discovery.

Why were they not suitable?

Display technologies

Artificial phage display technologies are a viable option for some fields such as toxicology and anti- venom research and in some cases, they can be used in experimental applications such as tool antibodies used for cell based tests such as generation anti-idiotypes, biomarker detection, sandwich ELISAS or FC/IgG isotype detection. However, when trying to develop therapeutic drugs unfortunately, this technology has significant limitations which restricts its usefulness.

The EURL ECVAM publication which provided the controversial recommendation that "animals should no longer be used for the development and production of antibodies for research, regulatory, diagnostic and therapeutic applications. In the EU, the provisions of Directive 2010/63/EU should be respected and EU countries should no longer authorise the development and production of antibodies through animal immunisation, where robust, legitimate scientific justification is lacking". The report acknowledges it did not consider the field of therapeutic applications despite

including the field in their recommendation, particularly the use of transgenic mouse platforms. This is surprising and concerning given the lack of successful market authorisations for monoclonal antibodies generated by display technologies.

Monoclonal antibodies generated by transgenic platforms achieve twice the revenue of monoclonals generated by display technologies and have four times as many blockbuster drugs (>1 billion USD in sales annually). It is worth noting that one of the earliest display technology generated monoclonal antibodies accounts for over 80% of revenue for all display generated monoclonal antibodies. Despite the investment into display technologies over a comparable time period, market authorisations have been lacking. It is noteworthy that this first blockbuster display generated monoclonal antibody was not isolated de novo from a proprietary company library, but instead it was isolated using a “guided selection” approach in which a human antibody was derived by switching the original murine antibody binder chain for a human equivalent.

In vivo antibody maturation in an immunocompetent mouse allows antibodies to develop and mature in their natural biological context, which provides the necessary selection pressure and interactions with immune cells to refine the antibody's binding properties. In vivo maturation involves a process called affinity maturation, where B cells that produce antibodies with higher affinity for the target antigen are preferentially selected and undergo further rounds of mutation and selection. This process of continual improvement gradually improves the antibody's binding affinity after repeat exposure to an antigen.

Immunisation also takes advantage of the interaction between B cells and T cells within the complete immune system. T cells provide signals which help B cells optimise their antibody production, leading to the creation of antibodies with improved specificity and functionality. In vivo maturation generates antibodies with natural modifications, such as somatic hypermutations, glycosylation patterns, and alternative splicing. These in vivo modifications impact an antibody's efficacy, stability, and interaction with other immune components which are fundamental for a therapeutic antibody.

The generation of phage display antibodies only uses in vitro selection, which lacks the complexity and intricacies of the complete mammalian immune system, in some cases the display prokaryotes will not be able to correctly fold an antibody protein in the same way as a Eukaryote cell (used for therapeutic antibody production). These processes cannot fully replicate the complex affinity maturation process occurring in a whole organism nor the complex interactions between B cells and T cells. For example it is difficult to generate antibodies with long HCDR3 regions (which are more appropriate for certain antigens and certain species) and it is common that phage display libraries are unable to express certain immunoglobulin gene families which limits their ability to capture natural diversity and pairing, something that is crucial when generating therapeutic antibodies.

In summary, when used to address native targets (such as a virus) their lack of initial specificity (binding more than one target), have weaker binding and associated developability issues which can make it difficult to formulate into a drug that can be given (in our case) to a companion animal. To fix these issues requires a huge

laboratory resource and has a low success rate. Rounds of additional biopanning can be used to affinity mature phage candidates however artificially modifying antibody sequences is also more likely to result in serious side effects associated with immunogenicity, the likes of which in some cases have led to fatalities within human clinical trials.

It is also worth considering that phage display libraries are not truly animal free as they are often attained from a target species after they have been immunologically challenged.

Artificial intelligence (AI) & machine-learning (ML)

A US-based drug-discovery company at the forefront of antibody generation de novo (from nothing) using AI/ML techniques and their recent publication provides a good summary of current capabilities in this space. Authors used AI/ML to design new antibodies which bind the cancer target antigen HER2 at a site which neutralises its function with therapeutically-relevant characteristics. 3 antibodies which have a slightly higher affinity than an existing therapeutic 'blockbuster drug. This is a significant achievement. These antibodies had been designed to contain high likeness to human antibodies ("humanness") so that the likelihood of them being immunogenic after administration is lowered. A closer inspection of the paper reveals that for the design of these AI-generated antibodies they first required structural knowledge of both the target antigen HER2 and the ideal place for the antibody to bind (epitope). This is identical to the existing therapeutic. Unfortunately for most novel antibody discovery projects this information is simply not known or available when generating therapeutics and importantly it is not a requirement to discovery antibodies by other means such as through animal immunisation. The AI-designed antibodies were only fully novel in one small region of the antibody variable region (heavy chain CDRs which account for about 10% of the antibody variable sequence) and the rest of the antibody sequence including the whole light chain was taken from the pre-existing therapeutic antibody. In typical novel discovery campaigns, a template sequence is not usually available. Furthermore, there was no indication of the developability properties of these AI-designed antibodies.

In summary AI/ML algorithms are currently capable of producing which given the complexities of designing an entirely new protein capable of binding another, is remarkable. However, this relies on a wealth of pre-existing information that just isn't available in novel drug discovery campaigns. There has so far been no demonstration that in silico approaches can generate a fully-novel, fully drug-like monoclonal antibody.

Summary

The ethical and cost arguments against animal-derived antibodies are strong, however this must be balanced against bringing the best antibodies possible to a clinical setting. At the moment, the best and most likely avenue for generating therapeutic grade antibodies combining the required developability profile is clearly from in vivo-derived immunisations. However given the speed of development in the area of artificial intelligence and machine learning, its limitations and weaknesses

should be thoroughly re-investigated in line with the anticipated end of this project in 5 years time.

Until replacement technologies are truly equal in their capability to generate therapeutic antibodies we must focus on reduction and refinement by selecting therapeutic targets with as much scientific diligence as possible and continue to optimise immunisation protocols, by ensuring the best and most appropriate immunogens are used and lastly by fully extracting, analysing and exploiting all data generated.

Reduction

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

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

The majority of the companion animal discovery platform colonies have already been completed so we are able to estimate the number of mice required quite confidently based on existing experience.

However generating mice from highly engineered embryonic stem cells can be challenging and somewhat variable.

The complex breeding regimes to create the multi-allele discovery platforms has been calculated using in-house developed algorithms which are used routinely to predict which mice and how many of themto use in breeding. These calculations have also been applied when considering the generation of genetically altered disease models.

The number of animals required for screening studies has been calculated on the number of anticipated new drugs expected to be tested under this license.

Following harm/benefit analysis, re-using animals is not considered.

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

Control groups provide an essential contrast to the drug treatment groups. They will provide critical baseline data that drugs can be assessed against. Animals will be allocated randomly to experimental groups by using a randomisation tool however social housing will be taken into consideration where possible.

Technicians and veterinary staff will also be blinded to the candidate therapeutics being administered as part of the experimental design strategy.

Quality control will be performed on all materials used with in vivo studies, such as cell lines and proteins, to make sure that when they are administered into mice or rats there is the highest likelihood of producing good data.

Standard operating procedures will be used to ensure that we get the maximum amount of useful biological information from each animal.

For every study plan type the NC3R's EDA tool will be used to help validate and provide clarity on the structure of each study.

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

In silico and in vitro screening programs will be completed first for any therapeutic being administered under this license. Those with obvious liabilities will be excluded at the earliest time point possible so that only the most likely to succeed will progress into animal studies.

Colony management software will be used to coordinate study plans and to monitor rodent colonies. This database will link with in-house developed algorithms to predict the most beneficial mating so that the fewest animals are used to achieve a desired genotype or colony size. The software records a comprehensive amount of data including pedigree, regulated procedures and an electronic health record for individual animals from birth until death.

Mouse colonies will be cryopreserved and removed as a live resource at the earliest opportunity when they are no longer required.

Pilot studies will be conducted using a small number of animals to confirm the expected performance of particular antigen materials or disease models before initiating experiments with larger numbers of animals (or not if they are unsuccessful).

Experimental noise will be limited by controlling as many variables as possible, including genetic background, age, sex, the environment. Microbiome screening will also be utilised to this end.

Refinement

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

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

Both Mice and rats will be used in this project

Genome Engineering

Most of the genome engineering steps required in this project will be for the replacement of mouse antibody producing genes with corresponding companion animal genes. These replacement genes will work as the mouse genes do so that the mice will maintain a fully functional immune system.

Where mice are used to model disease they may be genetically engineered so they express a companion animal version of a protein that will interact with a therapeutic drug so we can simulate interactions which may occur in the target species.

Some gene knock out models will be created if the drug target antigen is highly conserved between species. By deleting a conserved gene, the mouse will then recognise the drug target antigen as 'foreign' and mount an immune response against it. Literature and databases will always be consulted to confirm whether the deletion is viable and that it wont have an associated harmful phenotype.

Immunisations

The immunisation methods in this license are refined and widely used and a standardised approach to each discovery target is adopted. State-of-the-art technologies such as B cell isolation and sequencing are well established to insure that we gather as much information as possible from every immunised mouse.

A significant amount of work is performed in the laboratory to maximise the chance that an antigen has the correct composition to bring about a good and meaningful immune response. This includes ensuring that the antigen is pure, free of toxins and has not been damaged during its preparation and that it has not been degraded or aggregated together.

Thorough preparation of the antigen will also ensure that each mouse experiences the fewest and mildest adverse effects necessary whilst still producing the desired immune response.

Dose ranging and safety studies

Following the administration of a therapeutic drug into mice or rats, blood withdrawals are crucial and will be taken at set time points to understand how long our drugs remain active in an animal. This procedure is simple and well established for both species that will be used under this protocol.

Disease models

When addressing cancer targets, genetically engineered or wildtype mice may be used to conduct experiments where cancer cell lines presenting the disease target are injected into mice. In some cases the mice may have immune defects so that we can manipulate their immune response. Typically the tumours will be injected under the skin which allows us to monitor the size of the tumour to limit suffering. We can then confirm how well our drugs modulate the disease progression.

Wildtype rats will also be used alongside or instead of mice in some disease models where their larger size offers a scientific or ethical advantage, for example if they can reduce the variability and number of animals required in a study.

Well established protocols exist in both mice and rat species which use chemicals to create localised pain and inflammation like that experienced from arthritis. Rats will be used when there is a need to perform more tests on the fluid from within the knee joint because it is not possible to recover a large enough volume from mice. Using a chemical to create this type of pain and inflammation is more consistent and refined method when compared to surgical methods which physically damage or cut ligaments or membranes in the knee joint. After administering the particular chemicals into the knee joint, the speed of cell death is well established and we plan to use the lowest possible dose to simulate arthritis. Also, lower concentration injections of chemicals may better reflect real companion animal arthritis pain and inflammation.

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

The immune system requires several months to develop a robust immune response against a particular target antigen. This process only happens in mature adult animals so it is not possible to use younger animals or those which have been terminally anaesthetised.

Organisms deemed to be less sentient are not suitable either due to the lack of genome editing technology available and because they do not possess the same immune system mechanisms.

When assessing how well a drug persists within an animal, or testing the effects upon the animal it is necessary to use a species which has a physiologically similar immune system to our target animal species.

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

The production of genetically altered mice is very refined and streamlined. One recent refinement that I will also implement on this project is the co-housing of excess female mice with stud males which have been vasectomised who would otherwise would spend ~70% of their time singly housed.

When starting immunisation campaigns the mildest adjuvants and antigen presentation methods are used, only if these are not successful will stronger adjuvants and methods be used. Where mice already produce a protein which is very similar (highly conserved) this escalation of adjuvants can in some cases be circumvented by producing mice which have a genetic 'knock out' of the target so that the mouse will recognise the antigen as 'foreign' and mount an immune response against it. Resources such as the international mouse phenotyping consortium and the international mouse strain resource will be used to identify any viability issues of 'knock out' models, also whether they already exist to prevent duplicating mutations.

Wherever regulated procedures are completed the use of pain relief will be carefully considered and an appropriate management regime implemented under the direction of the Named Veterinary Surgeon.

In addition to comprehensive pain management, animals recovering from surgical procedures or those on disease model studies will be provided with floor food and soft diet if necessary. A programme of more frequent monitoring will be implemented for all animals on experimental procedures so that any adverse effects are quickly identified.

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

Where appropriate we will act in best accordance with the ARRIVE (2.0) and PREPARE guidelines from norecopa/RSPCA particularly with regard to the equivalent methods sections.

The Experimental design assistance (EDA) developed by the NC3Rs will also be utilised to help validate study design and also to enhance understanding of overall study design by providing useful visualisations of experimental groups and commissions.

The NC3Rs good practice guidance documents will also be utilised when performing administrations and blood withdrawals.

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

Subscription to regular newsletters from groups like the NC3Rs, LASA and the RSPCA as well as attending relevant courses and conferences will help to stay informed on best practice. Close liaison with the Named Information Officer at the establishment will also facilitate efficient passage of information.

I am a member of several transgenic technologies groups where advances in genome engineering techniques and best practice are frequently discussed.

The NC3Rs 3Rs resource library will also be referred to during the project to help maintain best practice.

7. Genes and therapies for inherited blindness

Project duration

5 years 0 months

Project purpose

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

Key words

Eye, Retina, Therapy, Vision, Stem cells

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To understand why genetic changes lead to inherited blindness and to develop potential new treatments.

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

Why is it important to undertake this work?

Inherited retinal dystrophies are the major cause of blindness in the working age population. Yet we do not fully understand why these genetic changes lead to blindness and there are very few approved therapies. Therefore, there is an unmet need to increase our knowledge of how these genetic changes affect the eye and to use this understanding to develop new potential treatments.

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

We will gain new insights into how genetic changes lead to blindness. These results will be published in open access journals and/or posted as preprints. We will communicate our findings to other researchers at scientific conferences and to the public through public engagement events. Potential new treatments that are identified will be moved towards clinical trial.

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

Our outputs will be important for the broader understanding of vision and how genetic changes can affect this, which will be relevant to the community of scientists and clinicians that are actively researching these questions. It will also benefit patients through a better understanding of how their genomes are leading to disease and how their disease progresses. In the future, new therapies will be important to the pharmaceutical industry and for patients.

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

Our publications will be in open-access journals and posted on preprint servers. The data will be presented at scientific meetings. We will collaborate with other scientists in the UK and internationally to maximise the potential of our findings. We will also make resources available to other researchers (e.g., data, animals, tissues).

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

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

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

We have chosen to use mice as they are the best characterised model for studying vision in a lower mammal, whose eye resembles the human eye to help us model human disease. Furthermore, mice can be genetically altered relatively easily and many lines are already available to use to investigate how genetic changes affect vision.

Mice will be used at all ages and genetically altered (GA) mice will be bred and maintained. Young mice (from birth to weaning) and young adult mice (from weaning to 3 months of age) will be the main experimental subjects, although in some instances breeding mice and experimental mice might be kept for up to 15 months.

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

Animals will be maintained in a specialist facility with regulated temperature and lighting and will have food and water freely available. They will be bred with other GA mice by natural means.

Typically, animals will experience mild, transient pain and no lasting harm from administration of substances by orally or by injection using standard routes (intravenous, subcutaneous, intraperitoneal) to alter the rate of retinal dystrophy. Where local administration of substances to the eye is required the animal will be anaesthetised and an injection made into the eye. These animals will experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics. Animals will experience mild and transient discomfort from blood sampling. Animals will have their vision tested by behavioural methods and/or will be anaesthetised to enable vision tests and their eye to be imaged and then recovered. These tests and treatments might be repeated several times. The final procedures will be undertaken under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain.

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

Mice will have general anaesthetic for eye injections and visual tests. They are expected to recover quickly from these injections and will be given painkillers and post-operative care just like people recovering in hospital. Other injections are expected to only cause transient pain and no lasting harm.

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

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

Total project. 34% moderate, 33% mild, 33% sub-threshold

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

- Killed
- Used in other projects

Replacement

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

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

The eye is a complex organ that is essential for vision. The human eye resembles the eye of other mammals, including mice. It is composed of many cell types that interact to enable the detection of light and the transmission of that signal to the brain. We need to be able to study how genetic changes in individual cells affect how this complex organ works and how the cellular changes progress over time, as well

as how they affect the ability to detect light. We also need to study how drugs and genes affect this complex network. This is not currently possible without using animals.

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

Our investigations start with human genetic data and phenotypes.

We study genetic changes using computer modelling and then progress to studies in cell culture.

We can now use patient cells and/or gene editing to make stem cells and produce a 'retina in a dish' to study how these changes affect the human eye in a dish.

Non-protected species like flies have eyes that are very different to human eyes.

Why were they not suitable?

Patient data are very valuable but limited in how it can be used to understand the molecular basis of disease. Potential treatments need to be tested and shown to be safe and efficacious before they can be used in patients.

Computer modelling and studies in cell culture can predict the consequences of genetic changes but the actual consequences might be different in the eye, so we still need to test these predictions in other systems.

The stem cell derived retina in a dish, retinal organoids, can reproduce the major nerve cells found in the retina but other cells are lacking and some of the cells do not fully develop and mature. Therefore, we cannot use them to test how genetic changes affect the interplay with all the cells in the eye. At the moment it is also not possible to fully model the degenerative disease we see in patients or to study how well the cells respond to light.

Reduction

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

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

We have used the typical variations from our own earlier experiments to calculate minimum numbers of animals to be used whilst ensuring that the results are statistically significant. Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of 6-8 to achieve the quality of results we need. 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?

In addition to our experience of performing this type of experiments in the past, we have used the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We will also use the EDA diagram and report outputs to support experimental planning with animal users.

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

We will maximise the efficiency of our breeding and use pilot studies as needed. Studying the vision of the same animals over time with non-invasive tests of visual function and using ocular imaging will enable us to use fewer animals.

At the end of the experiment, we will harvest tissues at post-mortem. If we don't need to analyse the tissues immediately, we will freeze them and make them available to other researchers working on similar questions.

Refinement

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

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

We will use GA mice to model human blinding disease. Blindness in mice does not cause suffering and distress and can be naturally occurring in laboratory mice with no clear effects on behaviour.

The methods we will use are designed to cause the least distress using the most appropriate and least distressing route of administration. Local delivery will be used to reduce effects on the whole animal and will be given after anaesthetic to reduce suffering.

Non-invasive tests of visual function and eye health using contemporary technology will be used to monitor how the genetic changes and any interventions affect vision and degeneration.

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

Non-mammalian animals are limited in their use because they either do not have the right type of eye structure and the cells within their eye function in a different way from the human eye to provide relevant results.

We cannot use embryos or very young animals to study how vision is lost, as their eyes are still developing and do not open their eyes until they are at least 2 weeks old.

We will perform some experiments on terminally anaesthetised animals that will be killed at the end of the procedure, but other animals we will need to recover and study over time to help reduce numbers.

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

Animals will be monitored carefully post-anaesthetic by staff to ensure full recovery and pain killers and antibiotics used for pain management and infection control at injection sites.

Non-invasive tests of eye health will be used to reduce any distress and produce measurable, reproducible outcomes.

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

We will follow the PREPARE guidelines (https://norecopa.no/prepare), as well as guidance and publications from the NC3Rs and Laboratory Animal Science Association.

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

We are a multidisciplinary group that uses a wide range of approaches to study inherited eye disease, including stem cell derived retinas in a dish, and we are constantly checking for technological and methodological improvements in these approaches. In addition, we will check information on NC3Rs website, we have signed up to the NC3Rs newsletter, we will meet the NC3Rs contact, and attend Regional 3Rs symposia.

8. Identification of feeding deterrents for grey squirrels (Sciurus carolinensis) by bioassay- guided fractionation of broadleaf tree bark

Project duration

3 years 0 months

Project purpose

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

Key words

Grey squirrel, Bark-stripping, Tree injury, Antifeedant, Bioassay-guided fractionation

Animal types	Life stages
Grey squirrel (Sciurus carolinensis)	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To identify an antifeedant present in trees causing them to be resistant to squirrel damage, with a view to develop humane prevention methods for bark-stripping.

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

Why is it important to undertake this work?

In Britain, eastern grey squirrels (Sciurus carolinensis) cause extensive damage to broadleaf trees and woodlands as a result of their bark-stripping behaviour. By

exposing the inner bark tissue layers of the tree, bark-stripping prevents the flow of nutrients within the stem, ultimately leading to tree death.

Squirrel damage additionally undermines the UK Government's ambitious targets to increase woodland area and mitigate the effects of climate change. With the UK committed to planting 30,000 hectares of woodland annually from 2030 to 2050, the establishment and successful growth of these trees is incredibly important for reaching national and global climate targets. Accordingly, grey squirrels were recently identified as 'the number one threat' to broadleaf woodland health and creation by the Royal Forestry Society (RFS), above other threats such as woodland and tree damage by deer, as well as introduced diseases. Squirrel damage threatens woodland creation initiatives and diminishes timber quality through staining and structural deformities, reducing both timber yield and value in the process as well as killing trees directly. Effective management is needed to increase incentives for landowners to plant native broadleaves, which are often highly susceptible to squirrel damage (e.g., Quercus spp., Fagus sylvatica). Although the impacts of bark-stripping on biodiversity are unclear, complex and older forest canopies are known to support a rich diversity of invertebrates. Effective management strategies against squirrel damage are required to protect these irreplaceable habitats and the biodiversity and ecosystem services that they provide. This project will provide us with knowledge of a deterrent chemical compound naturally occurring within trees resistant to bark damage, which will in turn inform the development of targeted, humane methods to prevent bark damage.

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

The largest benefit for this project is to inform the development of a humane and targeted management approach to prevent damage to trees from grey squirrel bark-stripping. Outputs will include a PhD thesis, scientific publications, patents to be filed for any identified compounds, and the identification of antifeedants for grey squirrels isolated from broadleaf trees. The project will also be presented at international conferences, which could inform approaches to managing other mammalian herbivores in human-wildlife conflicts involving crop damage across the globe. Long-term benefits include using the knowledge of a resistant trait to develop tree breeding or targeted genome editing techniques for wider woodland use, so that we can plant desired native broadleaves without the threat of squirrel damage.

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

In the short-term, this project will be supporting an early-career researcher as a project licence holder, in identifying a unique and targeted approach to prevent grey squirrel damage. This project will also provide the foundation for further future work in investigating how tree chemistry mediates bark- stripping by grey squirrels. Additionally, the wider research group including supervisors and collaborators will also benefit from the outputs and patent filings of this project.

In the long-term, the welfare of wild free-ranging grey squirrels in Britain will benefit from the outputs as we can identify an approach that is not invasive, lethal or harmful to squirrels, and only aims to exploit an already existing interaction between squirrels and trees. The outputs of this research will provide more targeted management to

reduce bark-stripping damage, and could be used alongside current and future population control efforts. Forestry practitioners and conservation NGOs will see immense benefits from the outputs of this project, as they will be able to confidently plant broadleaves with wider ecosystem service benefits that will not be damaged by grey squirrels. The public will also benefit from these outputs as they will be able to support a management approach that does not involve lethal methods of population control, which they generally find unacceptable.

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

We will communicate to policy leads and makers' the findings of the project. This allows us to have immediate policy impact, and inform the way in which we manage squirrel damage directly. We are also collaborating with forestry professionals and non-governmental organisations (NGOs), allowing us to work closely with the environmental sector in informing management options. Further, the results from this project will be communicated via open-access journals and presentations at international conferences.

Species and numbers of animals expected to be used

- Other rodents: No answer provided

Predicted harms

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

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

This project will use adult wild-caught grey squirrels in captive feeding experiments. Although other rodents can bark-strip such as voles, the context under which grey squirrels bark-strip is specific to their nutritional, ecological and behavioural requirements. Compared to other sciurid species which bark-strip as a result of lack of food availability, grey squirrels bark-strip in the spring and summer months when there is a variety of food sources available. The context under which grey squirrels in Britain bark-strip is unique, as they also don't bark-strip to the same extent in their native range. It is hard to replace grey squirrels in such an experiment, when the causes for the behaviour are currently unknown. Using wild squirrels will allow us to detect any attractants or deterrents that may be present in the bark of broadleaf trees, which would be specific to the requirements of this species.

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

Free-ranging grey squirrels will be live-trapped by trained and competent individuals at the secondary establishment. After a health assessment, the squirrels will be housed in same-sex pairs in outdoor enclosures. Any squirrels deemed not fit for study will be killed via a humane method. This is due to the invasive status of squirrels in the UK (The Invasive Alien Species Order 2019, Schedule 2), it is illegal to release grey squirrels back into the wild. Enclosures will contain appropriate enrichment and nest boxes for the animals. The squirrels will be given an

acclimatization period of at least 1 week prior to commencing experiments, where they will be pit tagged under anaesthetic for identification purposes. During this acclimitisation period and in-between experiments, individuals will be provided with a standard captive diet. During experiments, squirrels will be offered feed treated with bark extract, a control, or both. The squirrels will be monitored using a welfare assessment grid, approved by the NVS and NACWO at the secondary establishment. The feed of each treatment will be weighed after each experiment, and they will be provided with the standard captive diet in between experiments. At the end of the protocol, squirrels will be dispatched via an appropriate Schedule 1 method.

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

Squirrels will be offered crude or fractionated extracts made from two different tree cultivars, one that is known to be susceptible to squirrel bark-stripping damage and the other that is observed to be resistant to damage. We do not expect to observe any adverse effects or clinical signs as a result of consuming extracts made from the two tree cultivars.

Expected severity categories and the 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 the protocol is mild as we are not expecting to observe any adverse effects as a result of feeding squirrels bark extracts.

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

- Killed

Replacement

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

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

We need to use animal models, and specifically grey squirrels, to understand the behavioural responses that are generated in response to chemical compounds naturally occurring within tree bark. No in vitro system will be able to simulate the behavioural response of grey squirrels in this instance.

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

We could not consider non-animal models for this work due to the nature of investigating animal behaviour, which cannot be substituted with computer modelling, in vitro methods or using invertebrate models.

Why were they not suitable?

The aim of this project is to determine a feeding deterrent specific to squirrels and their bark-stripping behaviour. No other animal or non-animal model would be suitable for achieving the aims and objectives of this project. The study species, grey squirrels, cannot be replaced with non-wild laboratory model alternatives such as mice or rats, as they do not perform the behaviour under investigation, bark-stripping. Further to this, computer models would also not be a suitable substitute for wild-caught grey squirrels as the project relies on observing the feeding behaviour of real animals, to produce informed recommendations for the development of preventative measures targeting bark- stripping behaviour.

Reduction

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

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

We will conduct two studies in this project, a pilot study to assess the safety of the extracts when fed to squirrels and a fully powered study to identify a chemical antifeedant.

Initially, we will carry out pilot trials of the resistant cultivar crude extract on a small number of animals (two) to ensure no adverse effects occur from consuming the extracts. This may be carried out at varying concentrations, before proceeding to a fully powered study with the extracts. Adverse effects from the extracts will be monitored against a welfare assessment grid provided in this licence. This welfare assessment grid may be subject to change on the advice of the NVS and NACWO at the secondary establishment.

The experiments for the fully powered study will be split into three rounds, as fractionation (separation of extracts into smaller chemical components) will depend on which of the extracts squirrels do not prefer. We anticipate 7 trials using up to 4 pairs of squirrels for each trial, which will allow us to test for up to 7 extracts of our resistant cultivar. For the fully powered study, in total we need 24 squirrels to conduct the trials (8 squirrels per round), and have set maximum numbers at 32 to account for individuals used in pilot trials, and any squirrels that may not acclimatise to captivity and cannot continue in the study.

The purpose of this project is to determine the feeding preferences of squirrels when offered various bark extracts. This will be measured by how much food is consumed by the squirrels of each treatment and control. Because we are measuring relative palatability, the squirrels must be offered each treatment with a control so that we can compare the palatability of each treatment. There will be no random allocation of control and treatment groups. Previous bioassay-guided fractionation studies have

employed this same method for controls (for example, Pass et al. 1998 and Reichardt et al.

1984). We will limit confounding factors by standardising the time of day procedures are performed, and when data are collected (i.e. provide treated food at the same time each day, and weigh the remaining food at the same time each day).

References:

Pass, D. M., Foley, W. J. & Bowden, B. 1998. Vertebrate herbivory on Eucalyptus—Identification of specific feeding deterrents for common ringtail possums (Pseudocheirus peregrinus) by bioassay- guided fractionation of Eucalyptus ovata foliage. Journal of Chemical Ecology, 24, 1513-27.

Reichardt, P. B., Bryant, J. P., Clausen, T. P. & Wieland, G. D. 1984. Defense of winter-dormant Alaska paper birch against snowshoe hares. Oecologia, 65, 58-69.

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

The experimental procedures outlined in this project are unique to animal behaviour studies, and are uncommon within clinical science projects. As such, we informed the experimental design from previous studies investigating similar behaviours in other mammalian species that consume the bark, twigs or leaves of trees (e.g. Reichardt et al. 1984, Reichardt et al. 1990a, Reichardt et al. 1990b, Pass et al. 1998, Ukeh et al. 2009).

We will carry out initial pilots with a small number of animals to ensure no adverse effects are observed from the crude extracts before increasing numbers to a fully powered study.

References:

Pass, D. M., Foley, W. J. & Bowden, B. 1998. Vertebrate herbivory on Eucalyptus—Identification of specific feeding deterrents for common ringtail possums (Pseudocheirus peregrinus) by bioassay- guided fractionation of Eucalyptus ovata foliage. Journal of Chemical Ecology, 24, 1513-27.

Reichardt, P. B., Bryant, J. P., Anderson, B. J., Phillips, D., Clausen, T. P., Meyer, M. & Frisby, K. 1990a. Germacrone defends Labrador tea from browsing by snowshoe hares. Journal of Chemical Ecology, 16, 1961-1970.

Reichardt, P. B., Bryant, J. P., Clausen, T. P. & Wieland, G. D. 1984. Defense of winter-dormant Alaska paper birch against snowshoe hares. Oecologia, 65, 58-69.

Reichardt, P. B., Bryant, J. P., Mattes, B. R., Clausen, T. P., Chapin, F. S. & Meyer, M. 1990b. Winter chemical defense of Alaskan balsam poplar against snowshoe hares. Journal of Chemical Ecology, 16, 1941-1959.

Ukeh, D. A., Birkett, M. A., Pickett, J. A., Bowman, A. S. & Luntz, A. J. 2009. Repellent activity of alligator pepper, Aframomum melegueta, and ginger, Zingiber officinale, against the maize weevil, Sitophilus zeamais. Phytochemistry, 70, 751-8.

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

Due to the novel nature of the behavioural experiments in this project, pilot studies will be employed to determine the toxicity of the resistant cultivar extracts. Adverse effects from the extracts will be monitored against a welfare assessment grid provided in this licence. This welfare assessment grid may be subject to change on the advice of the NVS and NACWO at the secondary establishment. This will ensure that we provide extracts that are at less-than-toxic concentrations.

At the end of the experiment at post-mortem, we will take and store as many tissues as possible and make them available to other researchers.

Refinement

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

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

The bark-stripping behaviour performed by grey squirrels in the wild is a species-specific issue, making the likely solution species-specific, too. Because of this, no other species or non-animal models will be sufficient in reaching the aims and objectives of this project. Further, grey squirrels are unique in their ability to process a level of plant secondary metabolites in their diet such as tannins, and are to some extent familiar with the taste and smell of these compounds due to their seed-predominant diet (Barthelmess, 2001). With this in mind, the squirrels are expected to recognise aversive chemicals that may be present within the resistant cultivar extracts, and avoid eating them due to the pre-ingestive effects (i.e. smell). By using an alternative animal model, we would not be able to answer the main objectives of this project and they would likely not be familiar with these compounds which would risk the alternative model more pain, suffering and distress due to their naivety to the compounds under investigation.

References:

Barthelmess, E.L., 2001. The effects of tannin and protein on food preference in eastern grey squirrels. Ethology Ecology & Evolution, 13(2), pp.115-132.

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

Non-mammalian animals, embryos and young animals are limited in their use because they do not perform the behaviour under question, bark-stripping.

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

The squirrels will likely be familiar with the smells and tastes of the extracts offered to them, and may be able to determine the palatability of the food prior to ingestion. This has been observed in other mammalian herbivores, such as common brushtail possums, that consume leaves and bark of poplar and willow species (McLean et al. 2001). In these cases, phenolic glycosides produced by such tree species (e.g. salicin) are avoided due to smells animals detect in plants. We conducted preliminary chemical analysis of volatiles (i.e. fragrant and gaseous compounds) that are produced by the bark of the resistant and susceptible cultivars to be used in this project. This analysis determined the presence of a compound called salicylaldehyde. The phenolic glycoside salicin is a precursor for the volatile we identified, salicylaldehyde, and is found within trees of the Salicaceae family, the taxonomic family of the tree cultivars we will be using. We may expect to identify a compound such as salicin in our resistant cultivar, and are expecting the squirrels to avoid these compounds due to known volatile cues that they receive to avoid ingesting the bark or leaves of these trees.

Other refinements include conducting pilot studies prior to experiments to assess the toxicity of extracts, and concentrations will be adjusted accordingly. Further, we will be monitoring the behaviour of the squirrels throughout the experiments through a live feed of CCTV, which is available for all squirrel enclosures used in the experiments. Where it is necessary to handle individuals (e.g. removal from traps), signs of stress will be monitored by competent individuals at the secondary establishment who have extensive knowledge of ‘normal’ squirrel behaviour. To avoid over stress occurring, the squirrels will be handled as minimally as possible.

References:

McLean, S., Pass, G.J., Foley, W.J., Brandon, S. and Davies, N.W., 2001. Does excretion of secondary metabolites always involve a measurable metabolic cost? Fate of plant antifeedant salicin in common brushtail possum, Trichosurus vulpecula. Journal of chemical ecology, 27, pp.1077-1089.

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

The published PREPARE guidelines will be followed to assist with planning the experiments, conducting the experiments, and reporting all relevant results.

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

We will regularly check information on NC3Rs website and we’ve signed up to the NC3Rs newsletter. Additionally, we will consult other colleagues and researchers on their work with captive grey squirrels to refine husbandry and handling practices.

9. Immune responses to TriTryp parasites

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Autoimmunity, Immune cells, Protozoan parasites, Trypanosomes, Leishmania

Animal types	Life stages
Mice	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this study is to evaluate if the quality and magnitude of parasite-reactive and autoreactive immune responses triggered by Trypanosoma cruzi, Trypanosoma brucei and Leishmania spp. (TryTrip parasites) determine the overall outcome and level of pathology caused by these infections, and to identify potential molecular targets to modulate these immune responses to favour the host.

Studies will primarily focus on Trypanosoma cruzi infection, which causes Chagas disease, with the long-term goal of translating the knowledge generated here to further extend the study into Leishmaniasis (Leishmania spp.) and African trypanosomiasis/sleeping sickness (Trypanosoma brucei).

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

Why is it important to undertake this work?

Chagas disease, sleeping sickness and Leishmaniasis affect over 20 million people in Latin America, Africa and Asia, and cause extensive suffering and deaths. These three diseases are part of the 20 World Health Organization neglected tropical diseases that mainly affect people living in poverty.

Currently available drugs for these diseases are limited, inefficient and produce extensive side-effects. No protective vaccines are available.

In particular, Chagas disease (caused by Trypanosoma cruzi) is the highest-impact parasitic disease in the Western Hemisphere and the leading cause of infectious heart disease worldwide. Chagas heart disease (CHD) is an inflammatory cardiomyopathy that develops in approximately one-third of chronically infected people. Here, whilst both protective T. cruzi-specific as well as autoreactive cardiac-specific immune responses have been identified, there is a lack in understanding of how these immunomodulatory mechanisms impact this disease phenotype and patient outcome.

The knowledge generated through this research will contribute to the production of novel treatments and vaccines to help people suffering from these infections.

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

We expect to have a better understanding of how our immune system reacts to the parasites that cause Chagas disease, sleeping sickness and Leishmaniasis. This knowledge will be publicly available through publications in scientific journals and openly available data sets. The outputs of this research may also include knowledge that will help in the design of new drugs and vaccines for the treatment and prevention of these parasite infections.

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

In the short-term, this research will benefit the scientific community studying infectious diseases by providing novel tools and approaches to look at immune responses to parasites. In the medium term, this research will benefit clinicians in charge of monitoring and treating patients suffering infectious diseases. In the long-term this research may benefit patients in Latin America, Africa and Asia by providing novel drugs and vaccines to treat Chagas disease, sleeping sickness and Leishmaniasis.

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

Knowledge, data sets and reagents generated in this project will be freely available to the scientific community and the general public. This knowledge will be shared via scientific publications, oral communications in scientific meetings, press releases and institutional websites.

In addition, we hold strong links with clinicians in charge of monitoring and treating individuals affected by Chagas disease, sleeping sickness and Leishmaniasis. This will help to translate the knowledge generated during this research to new clinical tools.

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.

Adult mice will be used to study the immune response to parasites. The mouse's immune system closely resembles that of humans, and immunological reagents are widely available.

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

Mice will be injected with parasites to initiate an immune response or to monitor the course of parasite infections. As we aim to study immune responses during chronic infections, some of the experiments will be relatively long (e.g. up to 10 months). In all cases, the most refined monitoring and housing techniques will be in place to assure the welfare of the animals. At the end of the experiments, mice will be euthanized using methods involving the least stress and discomfort required to successfully achieve our goals.

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

All procedures in this project are well established. Most mice will undergo procedures that cause only mild and short-term discomfort (e.g. needle injections and/or temporary restrain). Most mice receiving parasite infections may experience transient mild symptoms such as hunched posture, piloerection, oedema and constipation; from these, a few days of piloerection and mild oedema just after the peak of infection that completely resolves 4-8 weeks after infection will most commonly be observed. ~40% of mice receiving parasite infections will experience moderate symptoms that can manifest either during acute or chronic infection, including more prolonged mild piloerection, oedema, transient constipation, transient diarrhoea, transient urinary incontinence, mild anaemia and slightly reduced level of activity. Every effort will be made to keep discomfort to the very minimum, and following the most up-to-date and refined procedures.

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

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

Mild > 50%

Moderate < 50%

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

- Killed

Replacement

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

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

The immune system and infection dynamics are highly dependent on the structural integrity of organs and tissues. Therefore, in vivo animal models are required to fully understand the whole complexity of the immune response.

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

Available databases of immune responses and in vitro cultures with cell lines or human blood.

Why were they not suitable?

We cannot accurately reproduce and capture the whole complexity of chronic parasite infections and associated immune responses using in vitro cell cultures. Available databases are limited in the knowledge we aim to generate during this research.

Reduction

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

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

We have estimated numbers by first designing every single experiment involved in this research, and then calculating the minimum number of mice per experimental group required to arrive at meaningful, reliable and reproducible results.

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

We have used quantitative data from our previous research and information available through collaborators and from published literature, and by applying mathematical and computational tools.

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

When required, pilot studies will be performed generate data to calculate the number of mice required to obtain meaningful, reliable and reproducible results. Small pilot studies will also be used to validate novel research techniques before performing larger experiments. Moreover, analysing as many organs as possible from each mouse and sharing tissue with in-house collaborators will reduce the number of mice required for pilot studies, and maximise the output of data per mouse.

Refinement

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

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

The model used will be adult mice. In all cases, we will use the most refined immunisations and parasite infection models required to arrive at meaningful results. These are in general very well tolerated by mice with only mild or moderate transient discomfort. In all cases, strict monitoring and humane endpoints will ensure maximum reduction of pain, suffering, distress, or lasting harm to the animals.

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

Laboratory adult mice are the lowest sentient animal models that can faithfully reproduce the immune response to parasites in humans.

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

The methods adopted in this project are state of the art with respect to analysis of immune function in mice and are continually evolving to reflect technological advancements that may reduce animal usage and suffering.

The animal facility has a rigidly enforced health policy, including additional bacterial pathogens above FELASA guidelines. Any changes in breeding success or animal behaviour are rigorously investigated. Facility staff are trained to the highest international standards, and every effort is made to maintain updated both refinement methods used and technical skills of the staff.

Any unexpected changes to animal health will be investigated and interventions agreed with animal welfare experts to prevent any avoidable suffering.

Specific examples of refinements used in this programme include: Daily monitoring assisted by a detailed welfare assessment scoring system, implementation of anaesthesia and analgesia to minimise distress, enriched housing, tube picking and single-use needles.

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

The published best practice guidance that will be followed include those from the NC3Rs (e.g. https://www.nc3rs.org.uk/the-3rs, NC3Rs ARRIVE guidelines, NC3Rs 2015 Responsibility in the use of animals in bioscience research) and UK government (e.g. https://www.gov.uk/guidance/research-and- testing-using-animals, https://www.gov.uk/government/publications/animals-in-science-regulation-unit-newsletters).

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

In addition to keeping up to date with the latest NC3Rs and LASA newsletters, our establishment organises mandatory and frequent training as well as expert users' forums with the latest advances in the 3Rs.

10. Investigating the cellular drivers of lung fibrosis

Project duration

5 years 0 months

Project purpose

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

Key words

Lung, Inflammation, Autoimmunity, Fibrosis, Fibroblasts

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We aim to examine the role of tissue resident fibroblasts and immune cells in the progression and persistence of lung fibrosis.

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

Why is it important to undertake this work?

Interstitial lung disease (ILD) is a group of respiratory diseases which together account for over 4 million deaths per year globally. These diseases begin with inflammation of bronchioles, alveoli, and/or microcapillary beds. Once inflammation breaks immune tolerance and becomes persistent or chronic, this eventually leads to an exacerbated tissue repair response termed as lung fibrosis. Fibrosis is a result of increased deposition of extracellular matrix (ECM) proteins which alters the lung

tissue cellular landscape and ultimately causes scarring or tissue destruction inhibiting normal lung function by reducing its capacity to oxygenate blood.

The mechanism by which lung fibrosis occurs is unclear. Immune dysregulation is considered a major contributor of lung fibrosis with involvement of both innate and adaptive immunity. However, the significance of these responses is controversial and therapeutic efficacy of available immunosuppressive drugs is only partial, suggesting there are unidentified mechanisms that support a hyperactive tissue repair response within the lung.

Tissue resident fibroblasts play an important role in repair and structural modification in a normal lung environment. In response to inflammation-induced growth factors, these cells proliferate and form pathogenic fibroblastic foci which drive fibrosis through the release of collagens and metalloproteinases. Despite these findings little is known how fibroblasts govern fibrosis. If we can understand the mechanisms that underlie this process in the target tissue of ILD we may be able to develop novel ways of therapeutically modulating these cells and alter the disease course to restore lung function.

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

We anticipate that this project will lead to the generation of novel data which will give rise to mechanistic insights into diseases with a significant unmet clinical need.

Project outputs will include:

- advances in scientific knowledge on the role of fibroblasts in tissue fibrosis.
- scientific and lay publications of our findings and methodology.
- presentation of our data and/or methodology to the wider scientific community through conference oral and poster presentations.
- identification of novel therapy targets or agents which can be taken forward to develop new therapies.

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

Short-medium term

Scientific developments and innovations leading to enhanced understanding of the role of ECM remodelling fibroblasts on the development of fibrosis. The project aims to improve our understanding of the processes driving pathology in chronic disease, specifically disease pathways mediating tissue fibrosis that we anticipate will underpin the development of the next generation of therapeutic agents.

Medium-long term

We intend to invite external seminar speakers who have an interest in the role of fibroblasts in chronic inflammatory diseases to our organisation, with the view of fostering further collaborations based on the concepts and ideas incorporated in this proposal. We envisage that these collaborations will occur during and following the

completion of this project, and therefore represent a medium to long-term impact of this work. Using these collaborations, we will develop the infrastructure needed to translate these findings into experimental medicine studies and if appropriate, clinical trials, therefore fully exploiting the translational potential of this work.

Long-term

Clinical academics, pharmaceutical companies and patients directly will benefit from advances in our understanding of disease pathology and identification of novel targets which can be taken forward to develop new therapies. We will develop collaborative networks to realise the translational potential of our findings over the subsequent 5-10 years following the completion of this project.

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

Dissemination of information

We will work with the relevant teams within our establishment to facilitate communications and resulting impact. We plan to use several routes to disseminate our findings to the wider scientific community and the public that will facilitate end-user engagement, namely:

(a) Peer-reviewed publication: we aim to publish high impact papers based on the findings generated from this project licence. In addition, we have a strong tradition of publishing methodology papers; and negative data to ensure that others do not unnecessarily repeat experiments that either technically are flawed or biologically yield the null hypothesis.

(b) Presentations: we and our collaborators will present data at internal seminars along with national and international conferences, such as the European Society Respiratory Congress.

(c) Dissemination via international societies: we and our collaborators are active members of various scientific societies including European Society Respiratory Congress, allowing our findings to be disseminated to the wider scientific community in societal magazines and training workshops.

Clinical networks and translational collaborations

My team and I are active members of several multi-institute research centres and will be able to present our findings at least twice annually at ongoing Centre seminars. We will also attend clinical conferences where we will present our data and foster collaborative opportunities for translational research across the fields of fibrosis, ageing and chronic inflammatory diseases. As a team of both clinicians and scientists we have access to unique patient cohorts and therefore the expertise and ability to translate findings rapidly to early clinical studies.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

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

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

Mice are the best vertebrate model for the study of persistent disease because:

1. the main components of their immune system are shared by humans; this is essential where immune responses as opposed to the function of individual genes is being studied and thus will produce satisfactory results.

2. a wide range of wild type and genetically manipulated strains of defined genetic make-up are available

3. an extensive range of reagents is available for analysis of the cellular and molecular interactions occurring during immune responses

4. as supportive data was generated in mice, using the same species will inform our research rather than repeating studies in an alternative species

For the fibrosis experiments we will use mice aged 6 weeks onwards. This is to ensure that adult lungs have developed. Furthermore, interstitial lung disease is very rare in human children and therefore, none of the clinical, radiological, or histological descriptions used in adults can be transferred to a paediatric setting.

Typically, what will be done to an animal used in your project? Breeding and maintenance protocols:

Animals will be grouped or paired, mated and subject to such other non-painful procedures as may be required for the conventional breeding and maintenance of animals with specific genetic alterations.

Animals may be marked and genotyped by appropriate husbandry methods which cause no more than momentary discomfort. Some animals will be humanely killed with tissues taken for analysis.

For lung fibrosis experiments using bleomycin aspiration:

To model lung fibrosis, bleomycin will be applied to the back of the tongue whilst the animal is anaesthetised. This is then inhaled into the lungs during the normal breathing process. Animals are then allowed to recover from the anaesthetic.

Some of these animals will then be administered substances via injection or orally, in order to study the effect on disease progression. These may be administered up to twice a week for up to four weeks (8 injection in total).

Some animals may undergo blood sampling, and/or a large sample taken under a terminal anaesthetic without recovery. Animals will be humanely killed at the end of the study with tissues taken for processing and analysis.

All procedures will be undertaken using the most appropriate anaesthetic and analgesia will be given. The mode of substance administration will be chosen to cause the least harm and distress to the mouse. Any new substances or route of administration will be tested in a small pilot study and the mice monitored daily for signs of adverse effects.

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

As animals may experience pain and discomfort from bleomycin aspiration this will be done under anaesthesia. Mice will experience mild transient pain for all other procedures: injections, ear notching, blood withdrawal and restraint. Oral aspiration of bleomycin causes periods of lung injury within the first 14 days which results in acute inflammation. Animals will lose weight during this period but are expected to recover weight loss when the lung has begun to repair. Mice will be monitored and scored daily during this time using an established scoring system which includes assessment of respiration rate, laboured breathing, hunch posture, piloerection and dehydration. Frequency of scoring will only lessen once an animal has begun to regain weight on two consecutive days, and there are no indications that condition is otherwise deteriorating. Animals will be humanely killed if they demonstrate signs of ill health such as pronounced piloerection and hunch posture, inactivity, or inappetence for a period greater than 24h. Any animal with >18% weight loss, diarrhoea or making a significant effort to breath such as breathlessness laboured breathing will also be humanely killed.

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

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

~10% sub-threshold

~20% mild

~70% moderate

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

- Killed

Replacement

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

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

This program of work is aimed to address the complexity of tissue fibrosis, such as the cellular drivers of this lung scaring and the trajectory over which fibrosis occurs and when to therapeutically intervene. To study tissue fibrosis three components need to be examined: time, place and cell type. While place (organ) and cell type (leukocyte or stromal) can be examined relatively easily in humans, it is difficult and, in some cases, unethical to perform multiple biopsies and adoptive transfer experiments in human patients with chronic diseases such as interstitial lung disease (ILD) without underpinning preclinical data that support therapeutic utility. Furthermore, as manipulating stromal cell biology has the potential to affect systemic immune function, we will need to determine whether manipulating these cells as a therapeutic target affects the innate and acquired immune response. This step is essential to ensure that the translation of our findings to humans go through the appropriate clinical regulatory stages before use in patients with ILD.

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

We considered a range of co-culture models and organoid experiments as non-animal alternatives, using patient samples and archived tissue. Co-cultures will be used to induce and characterise the myofibroblast phenotype as well determine important interactions between these fibrotic cells and immune cells as well as understanding how our cellular transfer suspensions specifically target these cells. Organoids will be used to understand the timing of fibrosis within a 3D in vitro tissue state. From this we can improve the timing in which we therapeutically intervene in the mouse model. Together, these techniques will supplement where possible animal experimentation and therefore reduce the overall number of animal experiments needed.

Why were they not suitable?

We and our collaborators have pioneered a range of in vitro fibrosis models such as scratch assays and myofibroblast differentiation culture systems that have furthered our understanding the importance of stromal cells in fibrosis. However, we have now reached a point where we cannot proceed to test our ideas without resorting to animal models of fibrosis. A key strength of our work is that it combines both human and animal models so that each can be used to inform the other and therefore minimise an over reliance on mouse models of disease. While there are no in vitro alternatives to this work, we will use cell culture experiments and target validation experiments on human samples alongside the program of work explained in this licence to support and validate our findings. We are confident that this approach will prevent us from developing a large program of work in vivo that is void of a functional relevance in human disease.

Reduction

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

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

We have used specific mathematical calculations based upon previous studies and the likelihood of our interventions producing positive results, to estimate the number of animals we will use in our study. In addition, we have attended an NC3Rs experimental design assistant (EDA) workshop and will use this free online tool to use the lowest possible number of animals will be tested whilst ensuring that the experimental result is robust.

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

A key strength of our work is that it combines both human and animal models so that each can be used to inform the other and therefore minimize an over reliance on mouse models of disease. This will give us the option to stop the line of research at any stage where our findings fail to show any significant increase in our understanding of lung fibrosis. We are running these studies in parallel with studies that explore the same cell populations in immune mediated inflammatory diseases in humans.

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

Pilot experiments will be used to estimate the standard deviations of the groups and these data used in a power analysis to calculate optimal numbers of animals for a definitive experiment. In addition, through previous work we have identified specific time points that we can analyse which define critical steps in fibrosis establishment. This preliminary work will allow us to limit the number of observations and therefore animals required in each experiment. To maximise the information gained from a single animal, we aim to perform multiple ex vivo analyses on each individual.

Where possible, new interventions will first be tested for efficacy using in vitro models prior to use in vivo. Where new routes of administration or new interventions are being examined, pilot studies will first be established in 2-3 mice prior to full experiments. Subsequently these pilot data will be used in the specific mathematical calculations described above to ensure that we use the minimum number of animals needed to obtain statistically significant results.

Refinement

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

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

Building upon previous work, we will use a well-defined and published mouse model of lung fibrosis – bleomycin. We will induce lung fibrosis in mice by oropharyngeal aspiration of bleomycin (a derivative of streptomyces verticillatus).

1. The procedures listed in this licence have been optimised to minimise discomfort for the animals. The oropharyngeal route leads to pulmonary fibrosis persisting for up to 6 months, while requiring shorter-duration animal sedation, eliminating a surgical procedure and associated post-surgical analgesia, and allowing more rapid post-procedure recovery than the intratracheal route of administration. This model of lung injury mimics idiopathic pulmonary fibrosis is well characterised and highly reproducible (sharing key pathological features with pulmonary fibrosis) and models the progressive nature of fibrosis within 14-28 days. Our experiments are short-term, taking place over hours or a few weeks, with clear and defined humane endpoints. Mice that undergo procedures will be monitored closely and appropriate action taken if they are deemed to be suffering.

2. We have refined the protocol to take minimal time, with short duration anaesthesia and bleomycin aspiration procedures. Other non-invasive methods such as nasal inhalation are available however, as bleomycin is a type of chemotherapy it targets and kills dividing cells. Therefore, bleomycin inhalation will cause inflammation and damage to the nasal passage and ultimately unnecessary pain and discomfort.

3. We have put in place strategies to reduce the clinical symptoms of pain or discomfort in mice by treating them with analgesics if appropriate.

4. Where possible we will use established reagents and protocols that we have developed and refined over time. Where the compounds have not been tested in vivo we will perform small pilot experiments to ensure there are no unexpected adverse effects. The lowest doses of agents that are well tolerated will be used.

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

Less sentient animals do not possess the same sort of glandular structure, and often their vascular tree and immune system do not fully represent that of humans. Small rodents are the lowest mammals that can be used to recapitulate the response of the human immune system to localised inflammation. Mice at a more immature life stage cannot be used due to their lungs not being fully developed. The model requires the use of live animals since our aim is to track changes in the immune response and tissue repair over a course of hours to weeks, therefore terminally anaesthetised animals cannot be used.

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

The oropharyngeal bleomycin lung fibrosis model is well established, and lung injury and inflammatory induced fibrosis is highly reproducible. We will also continue to seek advice from the NVS regarding best use of anaesthetics and analgesics, particularly for any mouse strains which have never previously undergone bleomycin aspiration. Pilot studies with enhanced monitoring will be performed on any strain which has never received bleomycin aspiration. A single dose of bleomycin (60IU)

has been shown to be well tolerated in mice. Once damage to the lungs has been inflicted using bleomycin, soft bedding, long drinking spouts, soaked diet, and hydration gel will be applied to cages as needed to reduce weight loss and prevent dehydration.

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

Animal welfare is a key consideration in all of our protocols, and we will be guided by our NACWOs and NVS in always ensuring that we are using best practice and the most refined techniques. All staff involved in animal experiments will review the literature on animal welfare and following every experiment and regularly during group meetings we will review our procedures from a welfare standpoint to identify any potential for refinement.

Finally, we will follow the LASA guidelines on the administration of substances Administration of Substances (researchanimaltraining.com) We will also follow the PREPARE and ARRIVE 2.0 guidelines for our planning and reporting of our experimental findings.

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

I or a member of my research team will attend local 3Rs relevant events and sign up to the NC3Rs newsletter. We will also be reviewing the literature on regular basis in our journal clubs and through this network we will discuss any refinements that could be applied to our own work.

11. Investigating the impact of stress during pregnancy

Project duration

5 years 0 months

Project purpose

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

Key words

pregnancy, cardiovascular, placenta, oxidative stress, obesity

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim is to understand how different stresses (e.g. obesity, heat, oxidative stress) during pregnancy can impact on pregnancy outcome, placenta function and maternal cardiovascular function. In particular, focusing on physiological changes in maternal and fetal cardiovascular function to identify molecular pathways that could be targeted for future therapy.

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

Why is it important to undertake this work?

The underlying molecular reasons which contribute to pregnancy complications (maternal hypertension, foetal growth restriction, still birth) are largely unknown. This makes detection and treatment difficult. The scientific community and governments are calling for research into women's health. A recent editorial in the Lancet states there is a deficit in understanding health and disease in females extending from clinical trials to pre-clinical animal models. The UK Government Women's Heath Strategy 2022 points that the male has been used as default for research leading to major gaps in knowledge and treatment for women. The report prioritises pregnancy as a key strategic area to develop more understanding.

Pregnancy complications such as preeclampsia, a severe hypertensive disorder during pregnancy, is the leading cause of maternal and foetal deaths worldwide. Preeclampsia often leads to other pregnancy complications such as foetal growth restriction and pre-term birth. It is also associated with gestational diabetes. Preeclampsia is difficult to predict, there is no treatment other than delivering the baby and most anti-hypertensive treatments lack information for use in pregnancy.

The risk factors for complicated pregnancy are increasing, such as lifestyle (age) environmental (increasing global temperatures), as well as disease pandemics (obesity, diabetes, covid). Yet we don't fully know how these risk factors effect the body function (physiology) or the biological pathways during pregnancy. In 2014, the annual report from the Chief Medical Officer identified that over half of women in the UK are overweight or obese during pregnancy. Exposing mothers and offspring to an immediate and long-term health risk. Research for treatments in this area offers an early opportunity to improve the health of the pregnant women and the babies. Likewise, the global climate changes will put more women at risk, as an increase in maternal core body-temperature increases the risk of still birth, pre- term birth and pregnancy complications.

Elevation in global temperature is expected to have worldwide impact on human health. Linkage studies have shown that elevation in maternal body temperature by 1-2oC during different stages of pregnancy correlates with pre-term births, gestational growth restriction and increased numbers of gestational hypertension. All of which have significant impact on long-term health of both mother and child. Yet, our understanding of the impact of heat during pregnancy are woefully inadequate.

The health problems do not stop once the baby has been delivered. Women with pregnancies complicated by preeclampsia, foetal growth restriction, gestational diabetes and preterm birth have a 2-fold increased risk of cardiovascular disease in later life. Similarly, the babies born are also at high risk for cardiovascular and diabetes later in life. Cardiovascular disease is the leading cause of death in women, however is under-diagnosed and understudied.

Together this shows a great need to explore the underlying molecular pathways and link these to physical changes (placenta function, maternal heart) during pregnancy. In this project licence we will look at common hallmarks of pregnancy complications and cardiovascular disease to ultimately improve maternal and foetal health. These

include understanding the role of faulty blood vessels; how and why cells produce cellular chemicals (reactive oxygen species) that can cause blood vessel damage.

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

We expect to:

- publish several research articles about our investigations into gestational stress and the impact on maternal and fetal physiology.

- provide foundation studies in how stresses such as climate change, obesity can impact pregnancy in mammalian pregnancy.

- identify the pathways that underpin gestational hypertension and growth restriction which can be further explored or utilised as biomarkers (measuring a particular product in e.g. urine or blood samples) in human pregnancy.

- produce large molecular (gene and protein) databases from different maternal and fetal organs that have undergone gestational stress, which will be publicly available.

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

The main output of this project is to publish several research articles about our discoveries in stress during pregnancy, placental function and consequence on maternal cardiovascular function.

We expect our research of maternal physiology coupled to understanding how biological pathways interact will advance our understanding in how, when and why disruption in maternal cardiovascular system impacts placental function leading to complications in pregnancy. Our findings will be relevant to clinicians and our collaborators running clinical trials since our physiological measurements will match clinical measurements. Our findings with biological pathways will help identify clinical biomarkers that can be confirmed in human studies.

We will present our findings at international science conferences.

We will engage with patient group, specialised key workers (e.g. sonographers, midwives) and public to share our discovery.

Our findings will help shape in vitro replacement models such as 3D placenta on a chip models.

A major training output will provide early-stage researchers to engaged and be trained to carry out physiological techniques.

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

The scientific outputs will benefit researchers including clinical and basic scientists interested in maternal and fetal medicine. Validation and characterisation of mouse models will enable other researchers to interrogate their molecular mechanism of interest.

In the medium-term outputs will benefit human studies. Our potential research on impact of climate in pregnancy will inform policy makers and public health assessment. A wider scope of researchers could also benefit for example those researchers that are conducting field research into climate change on pregnancy require physiological and biological signals to measure the impact.

In the long-term identification of biological pathways that are crucial in stress during pregnancy can be used to design future therapy. Moreover, the development of well characterised pre-clinical models matched closely with the clinical situation will allow for efficient future pre-clinical drug target studies.

Species and numbers of animals expected to be used

- Mice: 4500

Predicted harms

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

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

We aim to study stresses which cause complications in pregnancy. In particular we are focusing on changes in cardiovascular physiology of the mother, foetus, and the placenta. We need an organism that has a placenta. Common models in embryology that use lower organisms like the zebrafish and chick embryo are not suitable as they lack the placenta.

We are investigating pregnant mice and look at mid- and late-stages of pregnancy as this has clinical relevance for growth restriction and gestational hypertension in humans.

The mouse is a good model as we can manipulate the genetics to control how much of a particular protein is produced, either increasing or decreasing how much is made.

We can do this in particular cell types and also change the amount made over time . In some cases we can change just one part of a protein, for example preventing an enzyme from being able to work but still allowing the cell to produce it. This control of the genes allows us to work out in detail what the protein does and so to determine how important it is in a particular disease setting. We also have cutting edge equipment that can make accurate measurements of how particular body systems are working in the mice in a very similar way to the measurements in hospitals of patients. This includes a high-tech ultrasound imaging that can see inside the body of the mouse making measurements the size of 10th of a mm, so we can see, for example, how the heart is pumping and how healthy it is. This allows us to match the clinical signs in the mice with the same clinical signs in a person.

In the mouse, pregnancy only lasts around 21 days. This short gestation period allows us to assess the effects of changes that could be caused by stress responses

(for example to changes in diet or in temperature) in different stages of pregnancy quickly. It is very hard to look at this in human pregnancy because it is much longer and also there are ethical concerns because we don't know if being exposed to a stress during pregnancy might cause life-long damage to the baby. For example, a recent international clinical trial (STRIDER)to reverse foetal growth restriction by increasing placenta blood flow during pregnancy had to be stopped. In this trial they used a drug that could increase blood flow and had already been shown to be safe in millions of patients (sildenafil/Viagra) Unfortunately, the trial was halted due to more foetal deaths in the treatment group. This underlines the need for careful validation of molecular targets in animal pregnancy models before testing in humans.

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

Pregnant mice will be monitored by non-harmful techniques such as specialised ultrasound or assessment of body-composition at different stages of pregnancy. Imaging of maternal, foetal and placenta, and other essential organs under light anaesthesia, will provide an understanding of maternal and foetal cardiovascular function, foetal and placenta size, and maternal metabolic measurements.

Finally, the mice will be euthanised before the end of pregnancy (<day18.5). Under terminal anaesthesia a catheter will be inserted into the heart to provide highly accurate heart and vascular pressure and volume measurements

The effect of real-world stress in pregnancy will be investigated under different conditions, such as;

- obesity, where pregnant females (Dams) will be fed a westernised diet (e.g. high in fat and sugar) for a number of weeks prior to pregnancy (approximately 10-weeks);

- climate, where pregnant Dams will be exposed to elevated ambient heat to raise core body temperature for a limited time (~6h) to mimic the effect of heat waves; or

- pre-existing cardiovascular disease such as hypertension e.g. through genetic modifications or drug interventions.

We will take sequential highly accurate measurements that tell us about heart and blood vessel health over the course of the pregnancies, to match clinical measurements in patients to provide information on how changes to the body caused by the above conditions could affect pregnant women. To maximise the information we get, we take blood samples and, after the animals have been humanely killed, we will take organ and further blood samples so that we can intensively study to how tissues, cells and how cells work have been changed compared to animals that didn't have these stressors.

Using animals with genetic modifications will aid our understanding of the role of different biological pathways.

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

The impacts and adverse effects on the pregnancy will be minimal. The majority of the measurements will involve repetitive non-invasive imaging such as ultrasound. For these to take place, pregnant mice are anaesthetised. Anaesthesia can cause short-term abnormal behaviour such as hyperactivity (<20mins). The amount of anaesthesia will be closely monitored using low flow rates (~20mls/min).

This will minimise the impact on the pregnant mice and to prevent impact on developing embryo. Periods of anaesthesia will be limited to 3-days apart.

In some cases we will surgically implant small devices (telemeters) into the blood vessel of the mouse, which “radio-in” the readings back to a receiver plate. This is used to take very-accurate blood pressure measurements when the mouse is free to move around its cage. There is a risk that blood clots can form after this procedure causing stroke or paralysis, but we take steps to reduce any risk of this, so these are very rare and animals showing signs of these would be immediately euthanised.

Animals get pain relief that has been recommended by the vet at the time of surgery and for as long as is needed to keep them comfortable afterwards.

The non-invasive measurements often require hair removal for imaging which sometimes induces irritation of the skin. Cream and ultrasound gel will be applied to provide moisturisation as a preventive measure.

Mice fed a westernised diet gain weight but do not show any abnormal behaviour. Stress such as heat and obesity during pregnancy can cause an increase in foetal defects or still births, mimicking the real- world situation. The mother and foetuses will be euthanised before delivery so neither will feel any pain.

To accurately administer drugs we may surgically implant a small device that is a few millimetres long and slowly releases the drug at a set rate over time. This “minipump” is placed into a “pocket” made under the skin. In some cases, the pump rubs if the pocket is too tight and can cause the skin to break down causing the pump to begin to stick out and requiring a quick extra anaesthetic to repair the skin, but this is rare and the people doing the surgery are trained and experienced to help prevent this from happening. The advantage of pumps are that the mice do not have to go through a daily procedure like injection or gavage (where a tube is gently placed into the stomach), which can be stressful.

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

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

80% are expected to reach moderate severity. This is an estimate to account for multiple times the mice need to be anaesthetised, in case the anaesthetic makes them feel unwell for a bit afterwards, or they find having several anaesthetics unpleasant.

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

- Killed

Replacement

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

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

We are studying pregnancy and the effect of placenta not working, therefore we need an animal model with a placenta. The mouse is considered as one of the least sentient animals with a placenta.

At present there are no lab-based models that can replicate the interaction between mother-placenta and foetus.

We want to link measurements in our animal model with humans, we can achieve this in a mouse as we have the equipment to make ultrasound measurements in mice.

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

- 3D multicellular in vitro organ on a chip models
- Human studies
- In silico models

Why were they not suitable?

We have developed a 3D Organ on a-chip model using human stems cells changed into placental cells (trophoblasts). This model can have been shown to mimic some of the functions of the placenta in a 3D system using human stems cells changed into placental cells (trophoblasts). In this model we can measure placenta barrier function, placenta development, and interaction between placenta and mothers' endothelial cells (the cells that line blood vessels).

However, it is not possible to measure blood flow of the mother of foetus, how the heart of either is affected or how the foetus is developing, the maternal and foetal cardiovascular function in this model. Also, the development of the foetus.

Human studies were considered as an alternative to the use of animals. Human studies are limited to observational studies requiring large numbers to be able to control for certain conditions (gestational age, ethnicity). It is not always possible (or ethical) to conduct extensive physiological measurements on pregnant women. Furthermore, there is no way to get samples of the potential to gain biopsies is limited to other than after the baby is born, which means that we can only get term placenta. This limits us to only look at placenta at the end stage of pregnancy and not at the time it is not working properly. Also, placenta from because healthy pregnancies generally last longer than pregnancies with complications, the placenta from a healthy pregnancy is usually delivered (38-40wks term) later (38-40 weeks), which is quite different than placenta during complicated pregnancies which is usually delivered earlier (<35wks).

Mathematical modelling can provide an alternative to animal studies. However, this requires datasets from different animal models and human pregnancies to be integrated.

Reduction

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

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

We have estimated the numbers of animals based on our extensive prior experience studying cardiovascular disease using mouse models. In addition we have information from prior studies and technical experience that help inform the amount of mice needed.

For the majority of the techniques the information from prior studies provide a baseline measurements enabling us to plan the study and predict the appropriate number of mice that would enable us to test a hypothesis, understanding that we are expecting to see a change that would be clinically important.

This will minimise the amount of mice needed for a study.

In studies that we do not have prior information that we can base our numbers on we will either perform a small pilot study in a small number of mice to get an understanding of the numbers that would be required.

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

NC3Rs experimental design assistant is used to calculate the numbers, providing accurate power analysis, graphical representation of the study and defining the optimal control conditions. This ensures that we use the correct number of animals to get reliable results for any study.

Where possible the in vitro replacement model (placenta on a chip) will be used to assesses molecular pathways before conducting animal studies.

Non-invasive imaging will be used to allow sequential measurements in the same mouse. This makes the results more reliable because we can follow changes over time in individuals and it also means that we can use fewer animals. It also reduces the requirement to cull mice at a range of times points that would need to happen if we used more invasive measurements.

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

Efficient breeding strategies will be used to reduce the number of animals bred for the experimental work. We regularly attend national seminars which can advise on best practice.

Statisticians will provide advice on experimental design before the study. Using more complex types of study design, such as random block design and sequential measurements, means that more information can be gained from fewer animals.

Pilot studies and previous data from our group or others in the division will be used in order to carry out power analysis.

In all experiments, once we have humanely killed the animals, a wide range of organs will be carefully collected, logged, and preserved for future use and sharing with other researchers, minimising the need to repeat experiments.

Data from our project will be clearly labelled and stored on data repositories so other scientists worldwide can use the data.

Refinement

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

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

We will take measurements in the mice using advanced technology that allows us to take measurements in small animals similar to what can be done in humans. These techniques e.g. ultrasound are non-invasive and either require the mouse to be restrained or lightly sedated. For example we can use ultrasound to image the size of the placenta and foetus. This is a refinement as the alternative would be invasively open the mouse or kill the mouse to take measurements of the placenta at multiple time points.

We use mouse specific anaesthetic equipment that allows automatic anaesthetic flow rates specific for mouse size.

To provide continuous blood pressure measurement or body core temperature in freely moving mice we will implant a telemeter. Although this involves a one time surgery, this prevents invasive surgeries at multiple time points.

We will look at emerging technology or techniques to implement more refinement.

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

The work requires an animal with a placenta. Development studies regularly use zebrafish and chick embryo which are less sentient but lack a placenta.

The mouse is one of the least sentient mammals to investigate the placenta function.

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

I will continue to seek funding to improve refinement and replacement of animal models and work with tech-companies to advance these.

We will use new technology (where feasible) such as anaesthetic machine which delivers low flow rates specifically for mice.

Study plans are in place and monitored by the NVS (Named Veterinary Surgeon) to ensure the use of techniques with minimal severity to enable the best scientific outcome. For new studies, pilot studies using a small number of animals are designed to provide the knowledge necessary so that we can implement the lowest harm protocol for studies using a larger cohort larger number of animals. Mice that are taking part in a study are regularly and carefully monitored by experienced researchers and animal technicians to ensure any possible welfare issues are detected and dealt with promptly.

For surgical procedures, animals will be provided with pain killers as required and recommended by the NVS to minimise the harms.

The resource unit regularly introduces new procedures to promote welfare, such as increasing environment enrichment, improved handling methods and behavioural conditioning.

Where possible we will investigate training the mice so they can be restrained in order to take the measurements. For example, the ability to conduct basic ultrasound without sedation will be explored.

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

Best practice guidance for experiments will be acquired from discussions and attendance at webinars/workshops with local and national experts, NC3Rs, local user group, RSPCA, the Research Animal Training online programme, and other appropriate websites.

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

Our local animal users' group is kept updated with recent advances and organises regular webinars and workshops from other research institutes.

We will continue to attend webinars and workshops organised by NC3Rs and RSPCA. We regularly review the NC3Rs monthly newsletter and articles to ensure we are aware of any advances.

I am in contact with sales and scientific representatives from various tech-companies that provide updates on technological advances that can have refinement benefits.

12. Mechanisms of Cardiovascular Remodelling

Project duration

5 years 0 months

Project purpose

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

Key words

cardiovascular disease, atherosclerosis, restenosis, therapy, imaging

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Diseases (such as heart attack and stroke), which are the biggest single cause of death in developed (and increasingly in developing) countries are caused by remodelling of the heart and blood vessels. This project aims to improve our understanding of the processes that cause changes in the heart and blood vessels, so that we can identify new therapeutic targets and test promising new treatments.

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

Why is it important to undertake this work?

The suffering caused by cardiovascular diseases is considerable and involves populations across the World, affecting both men and women. Cardiovascular diseases (which include any disease involving the heart and blood vessels) are the leading cause of death globally. For example, cardiovascular disease was responsible for 17.9 million deaths (32.1% of total) in 2015. In westernised countries, deaths from cardiovascular disease have been falling since the 1970s, showing that an improved understanding of the causes of these conditions, combined with better treatments, can dramatically reduce the burden they place on society. This is balanced by the observation that deaths from cardiovascular disease are increasing in developing countries. Furthermore, lifestyle issues in many societies, including the current increase of cardiovascular risk factors such as obesity and diabetes mellitus, have contributed to the prediction that by 2030 more than 23 million people will die every year from cardiovascular diseases. Consequently, it is vital that we improve our understanding of how risk factors contribute to the development of cardiovascular disease, develop new types of treatment, and identify new therapeutic targets.

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

This work is based on the use of small animals to model cardiovascular disease. These models will be used to show whether targeting specific causes of disease, using new or existing drugs, or alternative approaches to therapy (e.g. administration of stem cells), will improve treatment by altering the way blood vessels remodel. Specific outputs expected would be new approaches to treatments for a number of conditions (including atherosclerosis, neointimal proliferation, calcific aortic valve disease, peripheral vascular disease, pulmonary arterial hypertension), as well as for clinical problems such as blood vessel remodeling and constriction caused when a tube is advanced into the blood vessel to allow treatment to take place. These models will be investigated using imaging techniques equivalent to those used for patients, which can allow detailed investigation of the cardiovascular system without the need for invasive surgery.

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

New treatments for cardiovascular disease (including new types of drugs to reduce cholesterol), often aimed at new targets (such as reducing inflammation to combat heart disease), are being developed at a remarkable pace. These promise benefit in the immediate future to the large number of patients with cardiovascular disease. The research described in this licence is aimed at identifying new targets and new therapeutic approaches and has the potential to produce new treatments of direct benefit to patients in the short-to-medium term. Furthermore, the use of state-of-the-art imaging procedures, similar to those used in patients, has the potential to develop new ways of assessing the mechanism of action and effectiveness of drugs in a way that could be applied rapidly to patients.

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

This work will incorporate important collaborations between clinicians, vascular biologists, pharmacologists, physicists, bioengineers, and bioinformaticians. This will ensure targeted use of state-of-the-art technologies, applied to appropriate small

animal and of disease and imaging techniques (including ultrasound and Magnetic Resonance Imaging), in research of immediate relevance to the development and treatment of cardiovascular disease.

Dissemination of results will be achieved by presentation of data at local, national and international conferences and seminars. It is also essential that the work completed is published in relevant peer- reviewed journals; this will include publication of data whether it supports or disproves the underlying hypothesis.

Wherever relevant, protection of intellectual property and/or patenting/ commercialisation will be considered in collaboration with the Establishment's commercialisation service.

Species and numbers of animals expected to be used

- Mice: 12500
- Rats: 2700

Predicted harms

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

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

Investigation of the causes(s), diagnosis, and treatment(s) of cardiovascular diseases is challenging in humans as many of these conditions develop slowly (over several decades) without producing any clinical symptoms. Detection and diagnosis may also require state-of-the-art (often invasive) imaging, whilst acquisition of relevant biological samples for analysis is challenging (and sometimes not possible).

Consequently, small animal models provide an accessible, clinically-relevant way of investigating the development, imaging, detection and treatment of cardiovascular disease. Many of the cardiovascular diseases of interest (e.g. atherosclerosis, peripheral vascular disease, calcific aortic valve disease, pulmonary arterial hypertension) do not develop spontaneously in these animals but can be induced (using modified diets, surgical manipulation, genetic modification; or a combination of these approaches). Surgical procedures can involve directly damaging an artery, removing/ blocking an artery to reduce oxygen supply to tissues (including the heart), or implantation of devices/ compounds to modulate arterial thickening and/ or growth of new blood vessels. These approaches can be applied to both male and female animals (which can be particularly important for improving our understanding of the impact of sex hormones on cardiovascular disease).

The availability of these pre-clinical models of human disease enables investigation of the impact of different interventions (e.g. new drugs, new devices, stem cells) on disease development. Similarly, combination with genetically-altered animals opens up the possibility of assessing the role of specific signaling molecules in causing, or contributing to the response to treatment of, a particular disease. These disease

models can also be combined with surgical manipulation (e.g. surgical removal of glands that produce important hormones) to assess the impact of important regulatory systems on development of the disease.

The use of animal models of disease allows tissue to be harvested at appropriate times to explore the development of a disease process or its response to treatment. This can be combined with state-of- the-art, clinically-relevant imaging to improve the power of investigations and to help develop use of clinical imaging in identifying/ diagnosing disease and in monitoring response to treatment.

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

The underlying approach to the work described in this licence is to induce a cardiovascular disease in a small animal and use novel treatments and/ or genetic modification to shed light both on the factors that cause the disease/ condition to develop, and on potential new approaches to treatment. In many studies, this approach will be combined with key, non-invasive imaging modalities. This enables us to:

(i) increase the power of the investigations; (ii) reduce the number of animals required for the investigation; (iii) provide additional information on the development of the disease and the effectiveness of treatment; and (iv) confirm the accuracy of the non-invasive imaging procedures.

In order to advance this work, it may be necessary to:

(i) breed animals that have been generated with specific alterations to genes that have been linked to processes that alter arterial remodeling. Mice may also be bred with modifications (e.g. incorporation of fluorescent markers) that allow identification of specific cells or molecules. These markers may need to be activated by administration of a chemical to the animal.

(ii) find the appropriate dose of a new drug to administer.

For the protocols designed to investigate models of arterial remodeling, the standard basic protocol in a typical animal will be:

(1) Induce disease (e.g. using a diet/ drugs/ viral vectors/ environmental manipulation/ surgery/ or a combination of these).

(2) Apply a treatment for the disease (by injection, in diet, by gavage, or with an in-dwelling device).

(3) Maintain the animal for sufficient time to allow arterial remodeling to take place before ending the experiment and retrieving tissue samples. The amount of time required for remodeling will vary (generally 2-4 weeks for angiogenesis or arterial thickening after injury, 8-18 weeks for development of atherosclerosis in response dietary manipulation. For most of the protocols, the animals are not expected to experience any symptoms related to the arterial remodeling process or any treatments applied.

During this procedure, small samples of blood may be taken for analysis, and repeated non-invasive imaging will be performed. Surgical procedures and some imaging are performed under general anaesthesia and appropriate analgesia is used. It is important to have properly controlled experiments using appropriate control/ sham conditions. For this purpose, some experiments may be performed in animals who have not had the cardiovascular disease induced and some animals may undergo relevant sham operations.

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

It is not expected that animals used under this licence will experience sustained adverse effects because:

(a) The conditions of interest (arterial thickening, new blood vessel formation, pulmonary arterial hypertension, catheter-related thrombosis) develop slowly without producing any symptoms in the models used.

(b) The techniques used to generate these conditions in animals have been used for many years and have been shown to produce few adverse effects.

(c) Genetically-altered animals used to investigate these processes do not exhibit any adverse effects caused by the genetic alteration and, similarly, activation of genetic markers is not associated with adverse effects.

(d) Any surgical techniques will be managed appropriately to reduce the likelihood of adverse effects.

(e) Experiments over many years have shown that drugs used to target arterial thickening or new artery produce few, if any, adverse effects in rodents.

Dietary manipulation to induce or alter cardiovascular disease in small animals has been in use for many years and the effects of relevant diets are recognised. Novel diets are routinely administered for up to 18 weeks and, whilst they may cause some physical changes (e.g. mild weight gain), they are not expected to cause pain or behavioural changes.

Some animals may be genetically altered to increase their likelihood of developing cardiovascular disease. The alterations used to promote lesion formation in rodents generally produce no symptoms and do not increase the likelihood of the animal dying prematurely. Similarly, processes used to 'switch on' or 'switch off' targeted genes are well established and have been shown to produce few adverse effects in rodents.

Surgical procedures may cause temporary pain but this will be kept to minimum by performing operations using good surgical technique, under appropriate (general or local) anaesthesia, and reducing pain after surgery by administration of pain killers (analgesics). Surgery may also cause mild, temporary weight loss, and brief irritation at the site of skin incision. There is a small possibility of infection but this is reduced using aseptic technique. Effective post-operative monitoring will be used to ensure

appropriate recovery from any techniques. The impacts of surgery are expected to last for less than 7 days.

Surgical techniques used to cause arterial thickening or new arterial growth can cause temporary symptoms (such as lameness after manipulation of a blood vessel in the leg, impaired exercise tolerance). This will be managed using good surgical technique, under appropriate (general or local) anaesthesia, and reducing pain after surgery by administration of pain killers (analgesics). It is expected that animals will have recovered from these symptoms within 3 days of surgery; subsequently, animals are expected to show no adverse effects in response to these procedures.

Some strains of rodent are more likely to suffer adverse effects in response to surgical techniques used to cause new blood vessel growth (e.g. Hindlimb Ischaemia). This will be avoided by using strains that are known not to be susceptible to these adverse effects. Where a strain being used has not previously been exposed to this procedure, adverse effects will be be avoided by performing initial studies in a small number of animals to assess the impact of less extensive reduction in blood flow before moving to the full procedure.

It has also been noted that some strains of mice have structural differences in the blood vessels that supply the brain, which makes them more likely to suffer a stroke if the main blood vessel from the heart to the brain (the carotid artery) is blocked. If there's any doubt about blood supply to the brain in a strain of mouse not used previously, preliminary investigations will be performed to determine whether they are at increased risk of stroke.

In order to model Pulmonary Arterial Hypertension rodents need to be housed in a reduced oxygen environment for up to 35 days. Experience shows that they undergo a short period of acclimatisation where they exhibit temporary weight loss, reduced activity, and signs of hypothermia. However, this can be managed (e.g. using additional nesting material and reducing condensation) and the animals resume normal activity and eating behaviour and weight gain proceeds normally (although hypoxic animals always remain leaner than controls).

Models used to cause blood clots on tubes (catheters) placed within blood vessels are designed so that the clots to stick to the implanted tube. Experience shows that neither implantation of the tube nor formation of clots cause adverse effects in these animals.

Any imaging used in the protocols described in this licence will be performed under general anaesthetic and is not expected to cause adverse effects.

In summary, the models used in this licence are designed to mimic disease by stimulating processes (arterial thickening, new blood vessel growth) that do not cause adverse symptoms. The adverse effects of any surgical techniques are expected to be temporary, and will be kept to a minimum using appropriate management. Drugs used to target the processes under investigation are not expected to produce adverse effects in these models and any imaging will be performed under anaesthesia.

Therefore, any adverse effects experienced by animals used in this licence are expected to be short- lasting, no more than moderate in severity, and reduced using temporary administration of pain killers.

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

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

It is expected that the procedures used in this licence will be only of Mild and Moderate severity.

Mild: 40%
Moderate: 60%

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

- Killed
- Used in other projects

Replacement

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

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

Cardiovascular remodeling in humans is a slow (decades long), symptomless process. This makes it difficult to detect onset of disease, to predict (often life-threatening) cardiovascular events, and to determine the role and significance of factors that cause disease. Animals provide essential models of cardiovascular disease, as they can develop components of the remodeling process, with changes occurring over much shorter timescales. They can also be used to provide tissue samples at planned intervals during disease progression, which is not possible when studying patients.

Mice will be the main species used, primarily because genetic modification is more common in mice (thus makes it easier to use genetically-altered animals to investigate of pathways of interest).

However, the use of rats is becoming more practical with the development of new methods for genetic manipulation (CRISPR/ Cas9) and for targeting pathways that influence cardiovascular disease development (e.g. using viruses to alter cholesterol levels). Manipulation of relevant genes will be used to refine experiments (e.g. by deleting a gene of interest) or by producing animals which more closely mimic aspects of human disease.

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

Replacement is considered wherever possible. This can be achieved by the use of techniques in which relevant cells are grown in the laboratory. In many cases, these experiments use human cells and tissue (e.g. arterial ring) culture instead of cells from animals. These techniques are also being developed to produce 'body-on-chip' technology to model the cardiovascular system, and which can make screening of new drugs faster ans more efficient. Body-on-chip techniques can also be combined with new imaging methods to increase the power of the data generated and increase the likelihood that results are relevant for use in patients.

Collaboration with colleagues in physics has advanced our use of mathematical modelling to assess the stress applied to teh blood vesel wall by circulating blood. This helps improve our understanding of the influence of blood flow on thickening of the blood vessel wall and on the development of new blood vessel networks.

Our research has also addressed the use of human cells to grow complex (multi-layered), 'bioartificial' blood vessels outside the body. These will help clarify the mechanisms that control the formation of blood vessels and the processes that lead to them becoming blocked in patients with cardiovascular disease. In addition, these bioartificial blood vessels have potential in the treatment of symptoms of cardiovascular disease (for example, to bypass blocjkages in important blood vessels).

Why were they not suitable?

All the techniques described provide information relevant to the development and targeting of arterial remodeling. However, none of these approaches predict, reliably, the responses that would be seen in an intact animals, as no single model comprises all relevant factors that regulate arterial remodeling in an intact individual.

Reduction

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

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

Breeding colonies are now closely monitored using an electronic, on-line database which ensures detailed control of numbers and optimum use of animals. Colonies are managed by dedicated colony managers. In addition, collaborative groups have been developed to make best use of lines designed to address the role of particular mechanisms/ cells. Most genotyping is now outsourced to accelerate mouse identification and usage. Where possible, breeding is optimised to produce only the mice required for experimentation; for example, cell–specific androgen receptor deletion was designed to produce litters containing a combination of the animals required for investigations (i.e. with selective deletion of the receptor from either the

lining cells or the muscle cells of the blood vessel wall, plus mice with deletion of the receptor from both types of cell, and control mice without the receptor deleted).

Optimum use of each individual, without compromising animal welfare or data quality, is achieved by careful study design. This includes studies using appropriate imaging (such as ultrasound) and analysis (remote measurement of blood pressure) methods that allow repeated measures whilst minimising impact on the animals used. These provide detailed insights into the diseases being studied whilst also reducing variability in the measurements taken; thus reducing the number of animals needed for these experiments. We have developed, and continue to develop, the use of imaging (and novel tracers) to allow repeated imaging in the same subject. In addition, detailed analyses can now be performed on small repeat samples (blood, tissue) removed under light anaesthesia/ analgesia.

Harvesting tissue at the end of experiments allows detailed analysis to complement data generated earlier in the experiment.

It is standard for the effects of treatment to be tested against appropriate controls, vehicle administration, and/or sham procedures. Many of the models used to investigate blood vessel remodeling can act as their own controls or sham groups. For example, many blood vessels (such as the femoral artery which supplies blood to the leg) are present in pairs (on the left and the right side of the body). Thus, in a model which uses surgical damage to cause thickening of the vessel wall, thickening can be induced in both versions of the artery; one artery can then be used to test the effect of direct application of a novel treatment, whilst the other artery is used as an untreated control.

Similarly, investigation of new blood vessel formation caused by implantation of a sponge beneath the skin can provide its own controls by insertion of two sponges; one receiving the novel treatment, the other acting as a control). This halves the number of animals required for investigations and reduces data variability, thus increasing the power of the experiment. In addition, suitable blinding and randomisation procedures are used to prevent bias and improve the power of data generated.

Data analysis is performed using appropriate statistical methodology and, where required, discussion with professional statisticians. Group sizes are determined based on previous experience and from use of relevant data.

The research group is fully aware of the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines and the principles guiding the Replacement, Refinement and Reduction of Animals in Research (www.nc3rs.org.uk/ARRIVE/). All experiments will be executed adhering to the ARRIVE guidelines, the principles governing the NC3Rs, and the PREPARE guidelines.

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

In the experimental design stage of the project, we have made plans to perform pilot studies where possible before planning for large cohorts. All experiments will be conducted according to the ARRIVE guidelines.

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

Efficient breeding procedures are used. These are supported by well-designed pilot studies, where required, to assess the potential for publishable results. Use of repeated, non-invasive imaging in live (anaesthetised) animals increases the statistical power of the studies performed and reduces the number of animals used for individual experiments. Computational modelling and analysis of datasets will be performed to maximize the information extracted from data generated.

Refinement

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

Which animal models and methods 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 small animal model of choice for study of cardiovascular remodeling as they can be used to recapitulate key features of clinical disease, can be used for genetic manipulation, and are suitable for testing novel treatments. The use of mice increased dramatically with the availability of genetic manipulation to produce models with greater susceptibility to remodelling and/ or that allow investigation of the role of specific mediators or pathways, or to enable cell labelling and tracking. The advances in transgenic techniques over recent years has introduced ever more sophisticated models with cell-specific and inducible deletion/ overexpression of genes providing increasingly powerful research models. Combination of these models with environmental manipulations (e.g. high cholesterol diet, salt-induced hypertension) provide arguably the best, most versatile approaches for investigation of cardiovascular remodeling. Immunocompromised mice can also be used to prevent rejection when human cells/ tissues (e.g. stem cell derived endothelial cells) are used to investigate cell fate or cell- based therapies. These developments have been augmented by the adaptation of surgical techniques for use in small animals and the increasing power of imaging in small animals that now allows serial, non-invasive measurement with excellent resolution.

Rats have also been used extensively as models of blood vessel narrowing and new blood vessel growth and to assess the effects of hormone manipulation (before and after birth). They do not, however, readily develop the characteristic blood vessel narrowing (atherosclerosis) of cardiovascular disease. They also provide useful genetic (e.g with spontaneously increased blood pressure) and environmental (high salt diet) models of high blood pressure and are a more practical size than mice for some surgical procedures. Their size also means they provide a better option for some of the imaging techniques used in living (anaesthetised) animals and can also provide larger tissue samples, enabling more detailed analysis. The introduction of new techniques for gene editing (e.g.

CRISPR/Cas9) promises improved availability of transgenic rat models in the future.

The work described uses wild-type and transgenic rodents without harmful phenotypes which will experience no harmful effects unless a regulated procedure is applied. Most of the models used generate cardiovascular conditions (arterial thickening, new blood vessel formation, high blood pressure) that have no adverse effects on the animals.

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

The purpose of this research is to understand the complex mechanisms that lead to blood vessel thickening and growth, particularly in cardiovascular disease, and to identify potential new treatments with relevance to humans. It is not possible to examine this in less sentient species

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

All studies involving surgical or invasive intervention will adopt appropriate pain management and post- operative care.

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

I will stay up to date on best practice guidelines set forth and regularly updated from the NC3Rs website (Guidance on the Operations of ASPA - https://www.nc3rs.org.uk/).

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

I will stay informed about the advances in the 3Rs by attending informational events provided locally and provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

13. Modelling heart development and function in zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Heart development, Heart disease, Zebrafish, Disease models

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to maintain and/or generate zebrafish models that can be used to investigate how the heart and blood vessels forms in embryos. This will include using and making transgenic zebrafish that have fluorescent proteins in cells and tissues or interest, allowing us to see these cells and tissues as the embryo develops. It will also require us to use or make zebrafish with mutations in genes required, or hypothesised to be required, for heart development or function, since this will allow us to understand why these genes are important for formation of the heart.

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

Why is it important to undertake this work?

Cardiovascular disease (diseases affecting the heart and/or blood vessels) is one of the most common causes of mortality (25% of all deaths in the UK are due to heart or circulatory disease). Congenital heart defects (CHDs) are a type of cardiovascular

disease where the heart hasn't formed the right shape by the time a baby is born. These occur during formation of the heart in the womb, and are the most common birth defect (1% of live births) and highest contributor to infant deaths. Understanding how the heart develops normally, and why specific genetic mutations can lead to abnormal shape or structure of the heart (which can also cause problems with heart function), is important for understanding the origins of these diseases. This helps us provide counselling for individuals with CHDs or who are at risk of having babies with CHDs, and provides important basic knowledge that could be used in development of future therapeutics or interventions.

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

This project will provide new information into the genetic mechanisms driving heart development in zebrafish, which can be applied to other vertebrates.

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

In the short term this work will benefit:

Academics investigating heart development in zebrafish, as well as other model organisms, and those interested in heart development in humans.

Academics interested in tissue formation during development, in particular those working on the shaping of epithelial tissues.

Academics with an interest in the intersection between tissue function and tissue remodelling, in both development and disease.

While we expect the studies carried out under this project to take the form of fundamental knowledge underpinning developmental processes, this work may in the long term inform translational approaches, or clinical practice. For example, identification of novel genes or regulatory genetic regions that promote heart development could drive expanded or targeted screening of additional genetic regions in individuals with heart defects of unknown origin.

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

We will take several approaches to maximise the output of this work.

We will disseminate knowledge derived from the project prior to publication at (intern)national conferences, in a variety of scientific fields (heart development and disease, zebrafish, disease models, etc).

We will publish our findings in a timely manner in open access journals. We plan to preprint our work (and have done with all previous papers from the group) to ensure both the public and academics can access the work as early as possible.

We will encourage collaborations with other laboratories, sharing our zebrafish resources and any other resources deriving from this work. We have previously set up collaborations with other laboratories, sharing both fish lines and software developed in-house prior to publication so they can benefit from the resources we

have generated. This approach is also good 3Rs practice, ensuring that laboratories worldwide do not duplicate generation of new animal lines if not necessary.

For lines in which we do not see overt phenotypes, there is a new initiative on the public zebrafish reference site Zfin.org where academics can publish zebrafish lines they have generated that have no phenotype in the specific tissues they have analysed (ZebraShare). Sharing this kind of ‘negative data’ can be difficult via traditional publishing routes, but this resource provides a mechanism by which to share this valuable knowledge, which can help inform other academics as to whether they should generate specific mutant lines or not, helping 3Rs considerations.

Species and numbers of animals expected to be used.

- Zebra fish (Danio rerio): 10950

Predicted harms

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

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

We are using zebrafish in this project, We will use adults to maintain lines and to generate embryos for experimental analysis. Most experimental analysis will be performed in embryos younger than the age of protection (< 5.2 days post fertilisation). This is because we are primarily interested in understanding the origin of structural heart malformations. These typically have their origins during very early stages of heart development, which in zebrafish is within the first 5 days of development. Occasionally we may investigate the hearts of larvae within the first 7-10 days post fertilisation, or we may analyse hearts in juvenile or adult zebrafish. This would typically be in situations where we are working with zebrafish models of human cardiac disease where cardiac phenotypes are too mild in embryos to detect (thus we need to analyse larvae slightly older than age of protection), or that may result in progressive remodelling of the heart (which may arise due to early structural defects that were too mild to detect).

Zebrafish are an excellent model in which to study heart development and disease. This is because zebrafish eggs are fertilised and the embryos develop externally, meaning that one breeding pair of fish can over their lifetime contribute thousands of embryos for experimental analysis of heart development, without needing to sacrifice the mother.

The transparency of the embryos is very advantageous for understanding heart development. This is because we use transgenic lines to visualise specific tissues and cell types, which means we can often visualise the developing heart in live embryos. This allows us to understand heart shape in development with minimal processing or disruption of the tissue, providing a very accurate analysis of heart development.

Due to the small size and external development of zebrafish embryos, they are also able to survive the first few days of development prior to the age of protection with compromised heart function, which is not possible in amniotes. This provides a unique opportunity to investigate the important relationship between heart function and heart development.

Zebrafish have very good genetic and developmental conservation with more neurologically advanced vertebrates, with conservation of over 70% of the genome, and over 80% of disease-causing genes in human having an easily-identifiable ortholog in the zebrafish genome. This makes them a good model to investigate the function of these disease-causing genes.

Zebrafish currently represent the model organism with lowest neurological complexity that combines all these advantages, as is thus an excellent model to understand heart development in line with 3Rs principles.

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

The typical experience of a zebrafish in this study is likely to be as follows:

Prior to the age of protection the larvae may have a tail fin biopsy taken at 3 days post fertilisation for genotype identification. At the age of protection (5.2 days post fertilisation) the larvae of the required genotype will be transferred to the aquarium, and raised to adulthood in a tank with up to 60 siblings. If a fin biopsy was not taken at larval stages, at around 4 months of age the fish may be briefly anaesthetised and a small biopsy of the fin taken. While awaiting the results of genotyping the fish may be solo-housed for between 24 hours and 1 week, after which it will be returned to a tank with siblings. If a fish needs to be solo-housed for longer it will be provided with distinguishable tank-mates (e.g. fish with a different pigment pattern). The adult fish will be used for breeding to collect embryos, either through placing a marble collection pot in the housing tank, or through individual pair mating. Breeding will be performed in general no more frequently than once in a 2-week cycle. The animal will be culled at 30 months old.

On occasion, the zebrafish larvae may have been injected with genetic material to introduce a transgene or a mutation before being grown to adulthood. These fish will require pair-mating only to identify founder fish who have incorporated the desired genetic changes.

Infrequently, zebrafish may be grown to adulthood that harbour mutations which may affect heart function or morphology. These larvae will be closely monitored when growing, and the adults may be subject to additional analysis, such as video monitoring of swimming behaviour or exercise tolerance tests, to asses the impact of the mutation on their behaviour or exercise tolerance. These tests will be performed on no more than 3 occasions per fish, within a 2 week window (with 3 days between each testing).

Typically animals will be culled by a Schedule 1 method when no longer required for breeding, or experimental analysis has been completed, or have reached maximum age.

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

Most zebrafish on this licence will not be subjected to experiments that will have adverse welfare impacts or effects on the animal. Some animals may be solo-housed while genotyping, which prevents some natural social behaviours such as shoaling, but this is estimated to last between 24 hours and 1 week, depending on genotyping method.

A subset of adults will be genotyped, which may necessitate obtaining a fin biopsy. In this case fish will be anaesthetised and a small portion of the tail fin excised. This does not typically result in adverse effects for the animals (for example, infection at biopsy site occurs in less than 0.5% of cases).

A small number of zebrafish may harbour mutations that impact cardiac structure or cardiovascular function at juvenile and/or adult stages. This can manifest as some or all of: progressive dilated cardiomyopathy; reduced movement; increased respiration activity. We have previous experience with animals exhibiting these progressive adverse effects. Fish can exhibit dilated cardiomyopathy with no other adverse behaviours, and can be maintained for 3-5 months without any signs of distress.

Zebrafish displaying a combination of adverse behavioural effects (typically reduced movement and increased respiration, with/without dilated cardiomyopathy) are not maintained for more than 24-48 hours.

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

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

We expect the severity rating of most animals under this project to be sub-threshold (if they do not need to be genotyped as adults and are used only for breeding), or mild (if the animals do need to be genotyped as adults, and are used only for breeding). This will encompass over 95% of animals on the project.

We expect a small number of animals to exhibit a moderate cardiovascular phenotype as a result of mutations that impact heart shape and function as the animals moves through the life course. This will likely include dilated cardiomyopathy and failure to thrive.

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

- Killed

Replacement

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

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

Understanding the genetic regulation and origins of cardiac disease requires the studying of organ development in the context of an embryo, and can have important short and long-term implications in furthering knowledge, genetic counselling, and developing therapeutics. The embryonic stages at which many congenital heart abnormalities arise are very early – between around 2.5-5 weeks in human gestation. It is therefore not possible to study 'normal' heart development in human embryos. Furthermore, understanding gene function in heart development, and building suitable disease models requires the use of non-human (animal) models.

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

Heart development is a complex 3-dimensional process, involving multiple cell types, fluid flow, and mechanical contraction. Potential published (i.e. validated) alternative models can best be identified through PubMed (NCBI), using search phrases such as 'in vitro models of heart development', 'bioengineered embryo heart', or 'in vitro model embryo heart'. Identified in vitro models can utilise human iPSCs to generate cardiac cells (typically cardiomyocytes). The closest in vitro models to embryonic development with which to understand vertebrate heart development are gastruloids and cardiac organoids. These are 3D cultures that attempt to recapitulate early embryonic development, or the development of specific early embryonic structures. Other 3D in vitro alternatives are bioengineered tissues, 3D bioprinted cardiac or vascular structures, and heart-on-chip models, some of which can also incorporate some element of mechanical stimulation such as fluid flow.

Why were they not suitable?

Development of gastruloid and cardiac organoid models is in its infancy. To-date, these models are only able to generate some of the cell types encompassing the heart, and can only recapitulate limited aspects of tissue organisation found at stages significantly prior to those we analyse in our work, typically that of tissue reorganisation in the adult hearts. Validation of the accuracy of these models to recapitulate in vivo development is limited and not convincing. Heart development also requires a) complex signalling between the heart and adjacent cell types, and b) blood flow through the developing heart, neither or which are represented in these organoid or cardiod models. Bioengineered tissue, 3D bioprinted cardiac tissue, and heart-on-chip models also don't contain the multiple cell types found in the developing heart, do not recapitulate the correct structure of the developing heart, and do not undergo morphogenesis. Furthermore, the relationship between cardiomyocyte differentiation from hIPSCs and cardiomyocyte differentiation in vivo (i.e. stages of differentiation, relationship to morphology) are still unclear, and thus it currently not possible to ensure that stage-appropriate differentiation cell states are used in relevant morphological analysis. Thus, there is no current non- animal model that is suitable for understanding vertebrate heart development. None of the aforementioned models are suitable for understanding the impact of embryonic structural heart defects on juvenile and adult heart function.

Reduction

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

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

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

This is based upon the number of animals used in a previous project licence, which was for similar usage of animals. The zebrafish used in the project will be primarily used for breeding purposes, thus we need to maintain males and females. Sex determination in zebrafish is complex, and is influenced by some environmental factors such as tank density and temperature. We have extensive experience in zebrafish husbandry, and typically raise 30-40 animals per strain to ensure sufficient breeding pairs carrying the required genotypes or transgenes.

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

Where possible we will genotype embryos prior to the age of protection, allowing us to raise only those animals that carry the required mutation or transgene. For transgenes we can screen for the desired fluorescent marker between 1-4dpf and raise only those animals. For mutations, we can perform an embryonic fin biopsy or extract some genetic material from the epidermis of the embryo at 3dpf, genotype for the mutation of interest, and only raise those animals. As an illustration, for strains where embryonic genotyping is possible it reduces the number of animals that are used by up to half.

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

Where possible we will share breeding adults carrying specific transgenes or mutations that are of interest to other research groups with those researchers, to prevent duplication of zebrafish stocks.

For adults harbouring mutations that result in onset of cardiomyopathy and associated adverse effects at juvenile or adult stages, we will retain the hearts after Schedule 1 killing to allow us to perform more detailed analysis of heart structure at a later date if necessary, reducing the need to generate more animals.

For testing whether candidate genes play a role in cardiac development, we can use targeted genome- editing (i.e. CRISPR-Cas mutagenesis) to perform pilot screens in embryos, allowing us to select the most likely candidate genes or mutagenesis strategies that will lead to useful adult models.

Refinement

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

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

We are using zebrafish during this project. Refinements of procedures on these animals include in genotyping protocols – where possible we will perform embryonic genotyping (embryonic fin biopsy, dermal abrasion), which is a refinement compared to adult genotyping through fin biopsy. Where adult genotyping is required, we will perform skin swabbing if possible, to avoid the use of anaesthesia or fin excision (wounding).

Where it is necessary to perform tissue biopsies on adult fish, we can choose to administer analgesia post-operatively to reduce the likelihood of the animals experiencing short-term pain.

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

Zebrafish currently represent the model organism with lowest neurological complexity for understanding morphological cardiac development and cardiac function. Most of our analyses will be on zebrafish embryos prior to the age of protection, meaning that we need adult zebrafish for breeding.

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

For animals that harbour mutations which may result in decreased cardiac function (for example as a result of structural malformations) fish will be monitored closely twice a day. Where these models have been previously published, we will ensure genetic background is maintained to avoid background mutations that may be present in varied wild type backgrounds interacting with the mutation of interest to result in more severe phenotypes.

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

I will follow the guidance published on the NC3Rs website for use of zebrafish in research.

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

I am a member of the Animal Welfare and Ethical Review Board. Our regular meetings include updates and advances in 3Rs considerations and practices, which I can then apply to the projects I supervise. I subscribe to the NC3Rs newsletter for 3Rs updates, and will consult with the Resources Library on the NC3Rs website. I also have regular meetings with our aquarium team, who are extremely active in the zebrafish husbandry and welfare community, and who are keen to discuss implementation of current best practice.

14. Mouse models of ovarian cancer

Project duration

5 years 0 months

Project purpose

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

Key words

Cancer, Chemotherapy, Immunotherapy

Animal types	Life stages
Mice	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to use mouse models to investigate how ovarian cancer develops, grows and responds to treatment. We are also particularly interested in using these models to understand how ovarian cancer interacts with the immune system and why it become resistant to treatment.

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

Why is it important to undertake this work?

Cancer remains a major cause of death globally. In the UK, cancer is the commonest cause of death for those aged over 60. Although treatments have generally improved in recent years, conventional chemotherapy and radiotherapy still have limited effects against many forms of cancer, and also produce side effects that can be serious and permanent. Even in the era of novel types of anti-cancer treatment, which have revolutionised the treatment of some cancers, many human cancers

remain incurable. Therefore, there is still a major need for novel therapeutic strategies.

Ovarian cancer is the fourth commonest cause of cancer death in women in the UK, and survival has improved little over the past fifteen years. Although most women respond well to initial treatment with surgery and chemotherapy, over 70% grow back (also known as 'relapse') and require further treatment. All relapsed cancers eventually develop resistance to treatment, which is ultimately fatal.

In this project, we aim to develop new treatments for ovarian cancer, using a panel of animal models that will provide data that could lead to trials in patients, and ultimately to new treatments. Ultimately, these new treatments will resolve unmet clinical needs.

Cell line models have been used to great effect in cancer research but have limitations. In recent years, more sophisticated models, including growing cancer cells from tumours removed from patients (for example at surgery or a biopsy) have been developed. These allow more complex analysis of cancer growth and response to treatment without resorting to animal experiments. However, even these novel models have significant limitations, the most important of which is they do not allow us to investigate development and growth of cancer and response to treatment in the context of an intact immune system. There is increasing understanding that the body's immune system can regulate the development and growth of cancer and that immune responses are fundamental in determining how well a cancer responds to treatment.

Therefore, there remains a real need for well-designed animal experiments using models that are accurate representations of human disease.

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

The main outputs at the end of this project will be publications in scientific journals. The publications will allow the scientific and medical community to read and assess our work, allowing it to reach an international audience, and will expand overall knowledge about the biology and treatment of human cancers. We will also present our work at conferences and meetings. This is another medium through which we can demonstrate the quality and quantity of the work that we undertake. These meetings also allow discussion and debate.

Our ultimate hope is that the work we undertake will support the development of new treatments for patients with cancer. Therefore, a desired output of this project is one or more clinical trials of new cancer therapies based upon the work that we undertake. These trials could involve new therapies that we and our collaborators are developing, or the use of existing therapies in different cancers.

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

Our work will develop improved and more realistic animal models of human cancer and human cancer development. These models will allow more rigorous evaluation of new cancer treatments and improve our understanding of the role of various

factors, such as the immune system, in the growth of tumours and their response to treatment.

The short-term beneficiaries will be researchers in field of cancer, in particular ovarian cancer. They will benefit from our work as it will reveal new insights into how cancer starts, grows, spreads and responds to treatment. We will ensure that our results are presented at meetings and published in journals that are free to access (so-called Open Access), which will maximise the potential impact of the work.

We will also share any materials (e.g. transplantable tumours) developed in this programme with other researchers in the field. All materials will be made freely-available to other researchers as with previous models generated in the licence holder's lab.

The medium and long term beneficiaries will be patients with ovarian cancer. The ultimate aim of the work in this project (and the laboratory work that supports is) is to improve outcomes for patients with this type of cancer. The simplest way in which that will happen will be through evaluation of new anti- cancer therapies in mouse models, which will then move into clinical trials. All new treatments for patients with cancer need to be tested first in animal models - to confirm how effective they are, to demonstrate that they are safe and also as a prerequisite for any future testing in patients. The animal models that we will develop and evaluate in this project will be as similar as possible to cancer in humans. This will ensure that our results can be rapidly and confidently moved towards clinical trials.

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

As stated above, we aim to publish our work in medical and scientific journals, and present our data at medical and scientific meetings. These are two established methods for disseminating results. We will always publish in Open Access journals, reducing barriers and ensuring that all researchers are able to view our results. We will also make our data available - all raw data from complex analyses (for example DNA and RNA sequencing) will be deposited on publicly-accessible databases where it can be accessed free of charge. This has two immediate maximising benefits. Firstly, it allows others to repeat our analyses to ensure the robustness of our conclusions. Secondly, it allows researchers to perform new analyses without using any more animals. Thus, open data access supports the 3Rs by reducing unnecessary animal experimentation.

We collaborate widely. One of the mouse models that we have generated previously has been shared with over 160 laboratories worldwide. We will continue this practice in the current project - any new tool or cell line model will be shared freely with researchers.

We strive to publish all data, both positive and negative. Unsurprisingly, it is more difficult to publish negative and unsuccessful approaches in peer-reviewed journals. However, it is the policy of the host laboratory to upload results onto pre-print servers, which are freely available to all.

Finally, we shall use social media, judiciously, to present our data. Through the press office and webpages of the host institution, we shall announce our results publicly.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

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

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

The over-arching aim of our programme is to improve outcomes for patients with ovarian cancer. To do so, we need better understanding of how cancer starts, how it grows and spreads to other parts of the body, how it responds to treatment, why it recurs and then stops responding to treatment. Ultimately, these questions will only be fully answered through research using patients with cancer or who are at risk of developing cancer. However, animal experiments are essential in addressing these questions - these experiments will evaluate how effective new treatments are, they will evaluate how safe new treatments are, and they are an absolute regulatory requirement before any new treatment can be assessed in patients with cancer.

In this programme, we will only use adult mice as they have enormous advantages as model systems. They are mammals, and so are biologically similar to humans. We already have a huge amount of information about mice, and a vast array of experimental tools (for example antibodies, cell lines) are already available. It is also possible to alter the genetics of mice easily, which allows us to answer very specific questions more easily - indeed, there are many hundreds of genetically altered mice available for researchers to use already, which enable us to know precisely how individual gene influences the development, growth, spread and response of cancers.

This combination of information and experimental tools means that judicious use of adult mouse models significantly improves the sophistication of our experiments and allows us to address fundamental problems in human cancer that would otherwise be impossible to answer.

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

Adult mice will typically be bought from a commercial breeding establishment. Once they have been delivered to the host facility, they are allowed to acclimatise for a week, housed in cages with no more than four other mice. They will be free access to food and water.

A typical experiment will start with the injection of cancer cells into a site that is clinically relevant. Ovarian cancer cells are usually injected into the abdomen (which is where nearly all human ovarian cancers spread) or into the ovarian bursa (which is where nearly all human ovarian cancers originate) - the latter requires a surgical procedure. However, we will also sometimes inject the tumours under the skin (subcutaneous) - subcutaneous tumours have the advantage of being easy to measure and assess as they grow.

We will also use models in which mice have a genetic predisposition to develop ovarian cancer. In these models, tumours start to grow after administration of agents that induce specific genes to be activated or inhibited in the ovary. These agents are either given as a general treatment (for examples as an intravenous injection) or by a local injection into the ovary under anaesthetic.

The tumours are then allowed to grow for a period of time - usually several weeks. Mice are monitored regularly during this time to ensure their well-being.

After a pre-defined period, treatments are started. This usually requires injections of drugs into the abdomen or intravenously. However, drugs can be administered orally (either by a process called gavage, where a narrow tube is inserted into an animal's mouth to allow drugs to be adminsitered, or by addition of drug to feed or drinking water). The number of injections and the volume of fluid per injection is restricted, again to ensure animal well-being.

Any surgical operation will be performed under suitable anaesthetic by researchers who have been trained (and certified as competent) in that procedure. Mice that undergo surgery will receive suitable pain relief in the recovery period.

Mice may also be treated with radiotherapy - this will require either sedation or a general anaesthetic.

Mice may also undergo imaging, such as MRI or CT scans. Again, imaging will be performed under suitable anaesthetic by researchers who have been trained (and certified as competent) in that procedure.

Small quantities of blood may be taken during experiments to allow monitoring. No more that 10% of total blood volume will be taken at any one point and no more that 15% in a 28 day period. Experience indicates this volume can be removed without any adverse effects on the mice.

All mice will be killed at the end of the experiment. Sometimes this will be at a specific time point (for example 24 hours after last treatment). Other experiments will last until the mice reach a pre-defined humane endpoint, such as abdominal swelling or a maximum tumour size. Mice will be monitored regularly to assess whether they have reached that endpoint, and all decisions about endpoints will be made by staff who are not directly involved in the experimental outcome to ensure that animal welfare is paramount and to avoid experimenter bias.

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

In some experiments, we will use mice that have an altered immune system. This means that they are susceptible to infections. These mice will be maintained in individually ventilated cages under barrier conditions to avoid infections. Experience indicates that the rate of infection is very low. However, should any signs of infection appear, the mice will be removed from the study and killed.

We will also use mice that have specific genetic alterations that could result in a particular defect or disease. Mice will be closely monitored for symptoms relating to

that disease. The defect or disease will depend upon the genetic alteration of the mouse but most typically will involve development of tumours in the ovary. These mice will also be monitored for more general manifestations of ill health, such as hunched posture, separation from cage mates, reduced activity, failure to eat or drink, or weight loss of 15% or more. Any animal showing any of these signs will be killed.

The volume of tumour cells injected will be kept to as low as possible for the experiment to be successful. The cells will result in the growth of a tumour but will not otherwise affect the wellbeing or movement of the animal. The rate of tumour growth will be dependent on the cell type injected and the location of the injection.

As tumours grow, they may cause discomfort or abnormal signs suggestive of pain (e.g. hunching, reluctance to move, ruffling of coat). Mice showing any of these signs will be killed. The commonest site of tumours growing in this project will be the abdomen. For these animals, swelling of the abdomen is the most common adverse effect due to the build up of fluid within the abdomen (called ascites). In these cases, mice will be killed when abdominal distension greater than that seen in late pregnancy is observed particularly if accompanied by slow movement, pale feet, reduced activity or other signs of discomfort, with advice expert facility staff.

Surgery can cause pain and infection. Aseptic techniques will be used during any surgery and operations will be performed by experienced researchers to minimise the duration of the anaesthesia. Pain relief will be given before and after operations to minimise pain. To prevent heat loss during surgery, mice will be placed on a heat mat to ensure body temperature is maintained. They will also be allowed to recover for at least 45 min in a warmed cage to ensure recovery, and will be observed for any adverse signs. Infections are rare and wounds will be monitored for local infection such as reddening and crusting. In the event that a wound becomes infected, the opinion of a Named Veterinary Surgeon will be sought. Other adverse effects of surgery may include slow recovery from anaesthesia. In this case, the animal will be monitored closely following surgery and, if not recovered fully by the end of the day, then humanely killed.

Many mice will receive anti-cancer agents. The commonest adverse events are anaemia and diarrhoea. Anaemia will be recognised by pallor and confirmed by a full blood count – any mouse that reachest the human endpoint for anaemia (recognised by palor of mucous membranes accompanied by lethargy) will be humanely killed. Diarrhoea will manifest as unformed, watery faeces. Any mouse with diarrhoea that does not response to supportive measures within 48 hours will be killed by a humane method. Anti-cancer agents can also cause a general reduction in well being, with hunched appearance, reluctance to move, reduced appetite/weight loss and ruffling of the coat. If these signs appear, mice will be killed.

Mice may also receive radiotherapy. This too can cause many of the same effects as anti-cancer agents, including anaemia, diarrhoea and general reduction in well-being. This will be managed as for anti-cancer agents. Radiotherapy can also increase the susceptibility to infections. Mice will be maintained in individually ventilated cages under barrier conditions to avoid infections. Should any signs of infection appear, the mice will be removed from the study and killed.

Some mice may also receive injections of immune cells from other mice. This can cause diarrhoea as well as hair loss. Rarely, a side effect called cytokine release syndrome occurs with fatigue, loss of coordination and other general signs of poor well-being. Any animals displaying hunched appearance, reluctance to move, swelling around the neck, poor appetite or ruffling of the coat will be removed from the study and killed.

Some anti cancer agents may be administered by oral gavage, where narrow flexible tube is inserted into the mouse's mouth to administered fluids. Only experienced handlers will carry out procedures using aseptic technique, good restraint and slow delivery of small volumes. A maximum of two attempts daily may be performed for oral gavage.

Finally, we will use imaging. There are no adverse effects expected from the imaging itself as the technique is non-invasive. However, all mice will require anaesthesia and most require intra-peritoneal (ie injections into the abdomen) or intravenous injections prior to the imaging.

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

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

The greatest severity overall will be moderate - we expect 90% experiments to fall into the moderate category

There are no expected severe categories.

Approximately 5% of experiments will be categorised as mild and 5% as sub-threshold

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

- Killed

Replacement

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

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

The over-arching aim of our programme is to improve outcomes for patients with ovarian cancer. To do so, we need better understanding of how cancer starts, how it grows and spreads to other parts of the body, how it responds to treatment, why it recurs and then stops responding to treatment. Although, these questions will only ultimately be fully answered through research using patients with cancer or who are at risk of developing cancer, pre-clinical experiments are vital to answer these questions (ie how cancer starts, how it grows and spreads to other parts of the body, how it responds to treatment, why it recurs and then stops responding to treatment).

Many pre-clinical questions can be addressed using non-animal alternatives (see below). However, there are specific questions that cannot be addressed using non-animal models. These include how effective a cancer therapy is when treating cancer in multiple sites (because many patients have cancer that has spread to many sites, so called 'metastasis'), how well drug combinations work (because nearly all anti-cancer treatment in patients is administered as combinations of drugs), how rapidly anti-cancer agents are eliminated from the body (because the rate of elimination is important in deciding how much drug to administer and how often it needs to be given) and what effects anti-cancer drugs have on non-cancerous tissue (which is crucial in understanding likely side effects of treatment in patients). Crucially, it is not possible to assess the full effects of the immune system on cancer and its treatment using non-animal models.

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

We are already utilising sophisticated non-animal models that attempt to recreate the complexity of cancers as observed in patients. These include the following:

1. Ovarian cancer cell lines with specific known mutations that have the same mutations as are observed in tumours in patients

2. Three dimentional (so-called spheroid) cultures that aim to recapitulate 3D architecture of a tumour

3. Co-culture systems where we grow cancer cells and non-cancer cells together. This can include cancer cells and immune cells

4. Growth of primary tumour cells and whole tumours from patients. We have ethical permission to use fresh tumours removed at surgery to evaluate anti-cancer therapies and are utilising novel devices to perfuse these tumour samples ex vivo.

5. Whole tumour ex vivo culture.

We will continue to use all of these methods throughout the lifetime of this project licence.

In addition, we will also use publicly available datasets from patient samples to address key questions and utilise computational modelling where possible.

Why were they not suitable?

In the field of statistics, it was once stated that 'All models are wrong, but some are useful'. This is true of cancer research, where the aim of pre-clinical experiments is to try to predict what will happen in patients with cancer. No one model can give all the answers and it is necessary to utilise as wide a range of experimental models as possible.

Non-animal models are immensely important and support the large bulk of experiments that we perform. Indeed, non-animal models can address some questions that are far beyond the scope of animal experiments. However, there are

several critical features that cannot be modelled without the use of animals. These include the following:

1. Does this treatment work for cancer that has spread to multiple sites in the body, and does it work equally well at each site that it has spread to?

2. Can a treatment or intervention prevent cancer from developing in situations of high risk due to inherited genetic mutations?

3. How does resistance to therapy develop in patients? Although it is possible to create drug- resistant cancer cells in vitro, there is very strong evidence that this does not fully recreate the mechanisms of resistance that develop in patients. In particular, it is almost impossible to recreate the movement of drugs through the body in the laboratory setting.

4. How does the body's immune system influence the development, growth and spread of cancer? 3D co-culture systems of tumour and immune cells cannot fully recapitulate the complexity of the immune system. Similarly, these 3D systems cannot address how specific mutations within tumour cells influence the extent and nature of interaction with the immune system.

5. Does the body's immune system and other non-cancer cells influence how well anti-cancer treatments work? 3D co-culture systems of tumour and immune cells cannot fully recapitulate the complexity of the immune system. Similarly, co-culture systems of tumour cells with non-tumour cells cannot recreate all the cell interactions that exist in a whole tumour.

6. Do anti-cancer treatments alter normal organs such as the lung and liver?

7. How rapidly is a treatment eliminated from the body? Is the liver the primary site of elimination or the kidneys?

8. Can a new anti-cancer treatment be given as an oral medication or does it have to be injected?

The most novel non-animal models that we use, namely using tumours freshly removed during surgery, are limited by the inability to maintain tumours viable for more than 72 hours. Only in animal models is it possible to assess tumours over multiple days or weeks.

Reduction

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

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

This number is based upon the number we have used in the current licence. We have averaged approximately 500 mice per year for the past three years and do not envisage a major change in the coming five years.

Experiments fall into two broad categories - those assessing biology, where we use a small number of animals and analyse at fixed time points, and those assessing longitudinal tumour growth, where we induce tumours and monitor time to reach humane endpoint (with or without treatment with anti-cancer agents). We usually perform twice as many biology experiments as longitudinal experiments.

Most biology experiments require 10 mice, and we perform approximately 20 of these experiments per year (ie 200 per annum). Longitudinal experiments are usually larger, usually requiring 30 mice and we usually perform ten of these per year (ie 300 mice), making 500 per annum.

We envisage a modest expansion in activity in the coming five years; most expansion will be in biology experiments, where we expect to perform up to 30 experiments per annum, making 300 mice in that category. Thus, the expected total is 600 per annum, totalling 3000 over 5 years.

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

There are four main ways in which the number of animals used is reduced to the minimum possible.

1. Use of sophisticated in vitro models of cancer as detailed in the Replacement section.

2. Statistical power calculations. All experimenters are required to provide details of the statistical assumptions and power calculations for each experiment. The minimum number of animals required to address to a stated endpoint are used and a biostatistician within Division of Cancer is able to provide statistical advice where necessary. By undertaking studies with adequate statistical power, definitive positive or negative data can be generated from a single experiment, thus avoiding repeated small under-powered experiments that may be futile and wasteful.

3. Randomisation, blinding and third parties. We use randomisation to allocate treatments and all allocations are recorded on a database to allow verification. The person undertaking the randomisation is different from the person administering treatment to mice. Blinding of treatment is employed where possible – at a minimum, preparation and administration of drug is undertaken by separate individuals to minimise bias. In addition, tumour measurements and all decisions about endpoints are made by an animal technician who is not directly involved in the design or analysis of the experiment.

4. In vivo imaging enables small cohorts of animals to be observed prospectively throughout an experiment rather than relying on sacrifice of separate groups of animals at each time point. This is especially true in models of ovarian cancer, where measuring the amount of cancer growing within the abdomen is challenging.

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

Pilot studies are integral to our ways of working: When using a new strain of mouse or first administration of a new anti-cancer agent, a small number of mice (pilot study) will be used. These experiments are not statistically powered but mice are monitored carefully for adverse events and multiple tissues harvested at end of experiment to assess any adverse tissue effects. These pilot experiments can act to reduce overall mouse use by eliminating treatments that have no effect or have more toxicity than expected. They also an approximate estimate of how effective a treatment might be, allowing better planning of larger experiments.

We also utilise clear pharmacodynamic (PD) assays in these pilots. PD assays assess what effect a drug (or other intervention) has on the intended target tissue to address whether the drug is doing what it is supposed to do. Such PD experiments require a detailed knowledge of the intended drug effect and are based on non-animal experiments. Thus, we will not take a new anti-cancer agent into mouse experiments unless there is a robust PD assay available.

Some of our experiments require mice as donors. These animals are killed in order to isolate immune cells that can be injected into new animals (so-called recipients). Wherever possible, we use spare animals from breeding programmes. These animals would otherwise be killed and disposed of.

However, it is frequently possible to use the spleen or blood from these animals under terminal anaesthesia or immediately after Schedule 1 killing for immune cell isolation, thus reducing the total number of animals required.

In the host lab, many researchers work on similar projects and it is possible to use one experiment to address several questions simultaneously - certainly tumours and normal tissues from mice are shared across experimenters whilst data generated from one experiment (especially assessments of gene expression in tumours and normal tissues) can support multiple sets of experiments.

Finally, we also use data generated elsewhere - it is now standard practice to upload DNA and RNA sequencing data onto publicly available databases, where it can be mined to address critical questions. We have previously generated mouse models of ovarian cancer that have been shared with over 160 labs worldwide, and there are now multiple datasets available worldwide, either openly or through specific collaborative agreements, that we can interrogate without having to repeat mouse experiments.

Refinement

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

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

The focus of this project is ovarian cancer. In designing experiments, we have to balance the need to use models that are faithful recreation of ovarian cancer growing in patients (most women are diagnosed with extensive disease causing symptoms) with the desire and need to cause the least pain and suffering to the mice.

We will use models in which tumour cells are either injected directly into the abdomen, which is the site of spread of human ovarian cancers, or injected directly into the ovarian bursa (the space immediately next to the ovary), which is where nearly all ovarian cancer originate.

We now have extensive experience in monitoring mice growing intra-abdominal ovarian cancers. To ensure that we minimise pain and suffering, we monitor mice carefully, with daily assessment and weighing, careful inspection of the abdomen and close liaison with the named animal care and welfare officer (NACWO) and named veterinary surgeon (NVS). We have clearly-defined humane endpoints that we apply and all decisions about animal welfare are made by individuals who are not involved in experiment design/analysis, to ensure that welfare considerations are paramount. We now achieve great consistency, with <10% inter-experiment variation in median time-to-endpoint for our most commonly used model, showing that we are able to apply our humane endpoints reliably and repeatedly.

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

Non-mammalian and embryonic models lack ovaries nor do they have an omentum: this is an apron of fatty tissue that hangs in front of the intestines in mammals, and is the commonest site of metastasis in ovarian cancer. These models also either lack an immune system or have immune systems that are too different from human to permit meaningful comparison.

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

Our monitoring regime is adapted on the basis of previous experience and expected effects. Thus, mice are weighed twice weekly at the start of experiments where we do not expect significant tumour growth but the frequency of monitoring increases as the expected endpoint approaches. Similarly, if our routine monitoring reveals any unexpected toxicities, we can increase the frequency to twice daily or more if required, including weekend monitoring. In addition, we can utilise imaging to assess tumour burden: in addition to reducing total mouse numbers, this can allow us to end experiments before mice develop symptomatic tumours.

All animals undergoing surgery have pre- and post-operative analgesia. Operations are performed on heated mats to prevent heat loss and mice are allowed to recover in heated cages to facilitate recovery. Wounds are inspected regularly. Appropriate treatments will be administered according to veterinary advice and humane end points will be applied for any post operative complications.

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

The PREPARE guidelines (Smith et al Lab. Animals. (2018) 52:135-141) will be adhered to when planning experiments.

We will also adhere to the Workman guidelines (Workman et al BJC (2010) 102:1555-1577) for assessing tumours and tumour burden, and the Morton guidelines (Morton et al Lab. (2001) 35:1-41) on the administration of substances to mice.

Body condition scoring will also be used to assess overall health status (Ulmman-Cullere and Foltz. Lab Anim Sci (1999) 49:319-323) for all routes of administration.

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

The host institution has created a new 3Rs resource centre. As registered licence holders, we have signed up to the resource centre, which will ensure that we are updated on 3Rs activity. The primary aim of the resource centre is to promote the 3Rs principles across the university and influence the university's Culture of Care. The centre runs training programmes for new starters and courses to aid good experimental design, and hosts seminars that support the principles of 3Rs. The centre also runs initiatives including shared animal management software to allow researchers within the different campuses to share animal resources. The licence holder and their research team will also utilise resources available via NC3Rs website, including the large number of items in the resource library.

Other resources that will be utilised will include the Norwegian resource base, Norecopa.

15. Neural circuits of flexible behaviour

Project duration

5 years 0 months

Project purpose

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

Key words

Cognition, Neural circuits, Behaviour, Attention, Neural networks

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to elucidate the neural circuits that allow flexible, cognitive behaviour, with a focus on cortico-cortical and cortico-subcortical circuits.

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

Why is it important to undertake this work?

This project will advance our understanding of neural information processing, and will illuminate the logic behind the flexibility of neural circuits underlying cognition, such as seen during attention switching tasks. By understanding the detailed neural network basis of cognitive behaviour, we will be able to gain insight into the basic building block of complex intelligent thought.

The data obtained through this project will consolidate existing scientific research and bridge various disciplines ranging from synaptic physiology to psychophysics. We will thus provide a framework for the key determinants of cognitive behaviour. Elucidating the neural mechanisms that produce complex cognitive behaviours might shed light into the pathophysiology of cognitive disorders.

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

The primary potential benefits of this project relate to the generation of new knowledge. We will provide a framework for the key determinants of cognitive behaviour.

We will publish results from this work in peer-reviewed journals and disseminate the results in national and international meetings.

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

In the short term, our research will advance our understanding of neural information processing, and will illuminate the logic behind the flexibility of cortical circuits. By understanding the detailed neural basis of a simple cognitive phenomenon, we will be able to gain insight into the basic building block of complex intelligent thought.

In the longer term, a secondary potential benefit relates to the value of our results to clinicians. For example, cognitive deficits underlie several neurological and neuropsychiatric diseases including epilepsy, autism, and schizophrenia. Elucidating the neural mechanisms that produce complex cognitive behaviours might shed light into the pathophysiology of these disorders.

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

We will publish this work in high profile journals and disseminate the results in national and international meetings.

We will make the results freely available through open-access publication.

Species and numbers of animals expected to be used

- Mice: 16300
- Rats: 1000

Predicted harms

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

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

This research program aims to understand cognition, which is inherently a mental phenomenon, existing only in awake, behaving animals. For this reason it is not possible to perform this research without using animals. The mouse and rat have

emerged in recent years as ideal species to uncover the circuit mechanisms of sensory, motor, emotional and cognitive phenomena. Early and late developmental stages of the mouse and rat allow studying the development and execution of cognitive behaviours. Since the development of the neural circuitry underlying cognition begins in the embryonic stage, and continues into the neonatal and juvenile stages, we need to focus a substantial part of the research on these stages in addition to adults. The rodent cortical circuitry is relatively similar to human, allowing us to address many fundamental issues of function and dysfunction in the cortex without having to make use of higher mammals such as monkeys. Another advantage of rodents as an experimental organism is transgenic technology, which is used to express fluorescent markers or other genes to assess the activity, function or structural properties of neurons and their processes. These genetic tools will facilitate our understanding of the brain at the level of synapses, cells and neuronal networks. I will use a combination of wild type and transgenic mice and rats that enable the fluorescent labelling and optical recording/manipulation of specific cell types, including excitatory and inhibitory cells of different cortical layers and projection targets.

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

Typically, animals will undergo surgical procedures under general anaesthesia, intra-cranial injections and implants under anaesthesia, injections of substances such as viruses and gene altering agents, and occasionally more minor surgical procedures to resolve issues with the implants. Gene alterations will sometimes be done at the embryonic or postnatal stages. These will be followed by training on cognitive tasks which typically involves a cross-modal attention-switching task motivated by food reward in food restricted animals, which may be accompanied by recording and manipulating the activity of brain regions. Some experiments will involve other behavioural tasks such as odour trail tracking or rarely other subthreshold behavioural tests such as open field exploration. The duration and number of procedures will vary substantially depending on the experiment, but will typically last for 2-3 months.

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

There is a low risk of deaths occurring from anaesthetic or surgical complications (<5%), as well as an equally low risk of post-surgical infection (<5%). There is a low risk of complications during pregnancy due to manipulations of embryos and injection of gene alteration substances. The behavioural training procedure will typically involve motivating the animals to perform a task for food reward by keeping them food restricted at a target body weight lower than their free feeding weight. This will last for the duration of the behavioural training, typically 2-3 months. Some transgene inducing or deleting agents can have adverse effects on the pregnant dams resulting in foetal mortality leading to spontaneous abortion or an inability to induce parturition. For injection of substances, most animals will show no adverse effects other than transient pain at the moment of injection. Some transgene inducing or deleting agents such as tamoxifen, can be toxic if delivered at high concentrations and/or repeatedly over many days. There is a small risk of tumours developing over the life of the animal.

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

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

The majority of animals will be under mild severity (about 70% of mice and rats) or non-recovery severity (about 10%). The remaining will be under moderate severity (about 20%). No animals will be under severe severity.

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

- Killed

Replacement

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

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

This research program aims to understand cognition, which is inherently a mental phenomenon, existing only in awake, behaving animals. For this reason it is not possible to perform this research without using animals. Examination of the web site www.frame.org.uk confirms that there are no current alternatives to animal experiments for this research.

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

We considered computer simulations as an alternative for this project.

Why were they not suitable?

While computer models are capable of running algorithms that can perform various computations on information, these algorithms are far from achieving the performance of real brains in even the most basic of tasks such as object detection. Although computer simulations do have a role in advancing our understanding of cognition, we concluded that they can only meaningfully contribute after incorporating extensive experimental findings from animals.

Reduction

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

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

Animal numbers were calculated based on the known or anticipated success of surgical procedures, biological variability and measurement variability. For example, experiments relying on repeated imaging of neuronal structures during behavioural training require a number of experimental steps with relatively low chances of success.

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

We used the NC3R's Experimental Design Assistant to reduce the number of animals being used in this project.

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

We will use effective breeding programs to limit the numbers of animals born. We will regularly use longitudinal measurements of brain activity from the same animals. This will yield data with great intrinsic scientific value (how neuronal circuits generate complex behaviour). Specifically, it is impossible to obtain knowledge about the dynamics of the brain without time-lapse, real-time measurements. In addition, the data obtained in this fashion are highly statistically robust since they allow within animal measurements from the same set of neurons, which dramatically decreases variability of the results.

Refinement

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

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

We will use mice and rats during this project. We will take advantage of mice and rats as experimental organisms due to transgenic technology, which is used to express fluorescent markers or other genes to assess the activity, function or structural properties of neurons and their processes. These genetic tools will facilitate our understanding of the brain at the level of synapses, cells and neuronal networks. We will use a combination of wild type and transgenic animals that enable the fluorescent labelling and optical recording/manipulation of specific cell types, including excitatory and inhibitory cells of different cortical layers and projection targets.

Importantly, we will employ methods that maximise the amount of data collected from each animal. For example, with two-photon calcium imaging, one of the core methods of the proposed research, we can record up to several hundred neurons at the same time, thereby reducing the number of animals required by at least 10-fold

for key questions of how neurons encode sensory information. Similarly, by recording repeatedly from the same animals in longitudinal studies, we can obtain more valuable data about the dynamics of neuronal processes from individuals, thereby again reducing the number of animals needed, compared to single time-point experiments that require data comparison from large numbers of experimental subjects. Furthermore, the use of techniques such as viral transfection minimises the number of animals necessary for research, as it alleviates the need to make transgenic mice expressing or lacking candidate genes, which requires the breeding of many generations of mice. Mice and rats are also the most refined model for studying the questions in this project, since they are the simplest animals which possess the appropriate level of cognitive abilities required for this project. The procedures in this project have furthermore been refined to result in the minimum harm to the animals.

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

This research program aims to understand cognition, which is inherently a mental phenomenon, existing only in awake, behaving animals. For this reason, it is not possible to perform this research without using sentient animals. Alternative models such as fish or drosophila are not appropriate since they do not possess the sophisticated cognitive abilities required for this project, such as attentional task-switching and set-shifting.

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

To minimise animal suffering we will continually employ refinements to our techniques and procedures. For example, we will incorporate the use of motorised robotic arms for performing precise drilling of the skull during surgery to minimise damage from the drilling. We have developed a highly refined procedure for training animals on cognitive tasks in extremely short times (days and weeks rather than months as seen in other groups and monkey labs). We will also continuously refine the procedure for behavioural training, by incorporating automatic algorithms which modify the training parameters for each mouse individually to enable more efficient learning.

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

I will follow the following published guidelines, on one of which I am an author: LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments)

NC3Rs-funded video tutorials on the Research Animal Training website (https://researchanimaltraining.com/article-categories/aseptic-technique/)

Refinements to rodent head fixation and fluid/food control for neuroscience (https://doi.org/10.1016/j.jneumeth.2022.109705)

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

I am a member of a working group at the NC3Rs, which enables me to keep up to date with the latest advances in the 3Rs. I have participated in workshops and seminars organised by the NC3Rs and will continue to do so, in order to stay informed and implement advances in my lab regularly and effectively.

16. Optimizing pharmacotherapy using pharmacokinetic principles

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Pharmacokinetics, Biodistribution, Cancer, Autoimmune diseases, HIV

Animal types	Life stages
Rats	adult
Mice	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overreaching aim of this PPL is improvement of delivery of drugs based on their physicochemical properties and principles of pharmacokinetics. By utilizing pharmacokinetic principles and taking into account physicochemical properties of drug molecules, a right drug, at a right concentration, at a right time, could be delivered to a right body compartment. This will result in more targeted, safe and effective treatment outcomes of existing and new pharmacological therapies for conditions such as autoimmune diseases, infectious diseases or cancer.

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

Why is it important to undertake this work?

Therapeutic failures due to suboptimal pharmacokinetic and biodistribution properties of drugs are unfortunately very common. Examples include very low and variable bioavailability following oral and other routes of administration, such as in case of cannabinoids. In case of autoimmune diseases, poor permeability of immunomodulatory compounds into important immune system organs, such as lymphatic system, significantly limit their therapeutic benefits. Other examples include suboptimal distribution of the drug molecules to the site of action. That is the case of drugs against HIV infection that poorly penetrate into HIV reservoirs in key tissues, such as lymph nodes or brain. This poor permeability of drugs into viral reservoirs is currently recognised as one of the main obstacles to achieve cure for people affected by HIV. In case of solid cancers, poor permeability of anticancer and immune oncology drugs into tumour microenvironment and draining lymph nodes not only limits therapeutic efficacy, but also lead to higher systemic toxicity.

Therefore, it is important, for drugs with challenging pharmacokinetic and biodistribution properties limiting their therapeutic benefits, to study their pharmacokinetics and biodistribution with high accuracy and precision. Using this knowledge, it is then possible in many cases to optimize the physicochemical properties (by prodrug or co-drug approach), formulation, or route of administration to achieve more favourable pharmacokinetics and biodistribution, leading eventually to better therapeutic outcomes.

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

The work is expected to provide novel information and publications (journal articles and conference proceedings) about the optimal pharmacokinetic and drug delivery approaches to efficiently target anticancer drugs to tumour microenvironment and draining lymph nodes. Obtained data will include concentrations of anticancer drugs in relevant tissues, blood and blood plasma. The obtained data will be then subjected to advanced pharmacokinetic modelling and simulations, which will lead to optimization of treatment regimens and drug delivery platforms to improve treatment outcomes of cancer. This is of paramount importance for better treatment of gastrointestinal cancers, brain cancers and other tumours prone to metastasis through lymphatic system, as well as for antineoplastic and immune oncology agents with low oral bioavailability.

The work is also expected to provide novel information and publications (journal articles and conference proceedings) about the optimal pharmacokinetic and drug delivery approaches for immunomodulatory compounds. Obtained data will include concentrations of immunomodulatory agents and their derivatives in the lymph nodes, lymph fluid, as well as in plasma and various relevant tissues. The obtained data will be then also subjected to advanced pharmacokinetic modelling and simulations, which will lead to development of treatment regimens and drug delivery platforms of immunomodulatory agents and their derivatives to improve treatment outcomes of conditions associated with autoimmune diseases, such a inflammatory bowel disease or multiple sclerosis.

The work is also expected to provide novel information and publications (journal articles and conference proceedings) about the optimal pharmacokinetic and drug delivery approaches to efficiently target drugs against HIV to HIV viral reservoirs. Obtained data will include concentrations of antiretroviral drugs and latency reversal agents, as well as their derivatives in central and regional lymph nodes, lymph fluid, brain, plasma and other relevant tissues. The obtained data will be then also subjected to advanced pharmacokinetic modelling and simulation, which will lead to optimization of treatment regimens to improve treatment outcomes of HIV/AIDS at different stages of the disease, as well as to novel approaches to pre-and post-exposure prophylaxis of HIV infection.

The work is also expected to provide novel information and publications (journal articles and conference proceedings) about the pharmacokinetic properties and biodistribution of a novel broad spectrum antiviral compound thapsigargin and its derivatives following various routes of administration. We will Identify and quantify the main metabolites of thapsigargin and its derivatives in plasma and tissue samples. We will also develop and optimize the formulations of thapsigargin and its derivatives. The final aim and ultimate benefit is development of orally bioavailable broad-spectrum antiviral drugs, which can be used at the first signs of respiratory viral infections.

The outputs from this work will include publications in peer-reviewed journals, talks and abstracts at national and international conferences, and patents. Likelihood of success of the work in this PPL is strongly supported by our strong past performance under previous PPLs in regards to outputs and impact.

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

The publications in peer-reviewed journals, as well as conference proceedings will be used by other scientists in the fields of drug delivery, pharmacokinetics, oncology, immunology and infectious diseases to inform their work and to build on and improve the findings in this work.

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

Publications will be available in open access format (in compliance with REF and Research Councils policies).

Short-term benefits

The benefits to other scientists in the form of access to published articles in peer-reviewed journals, as well as to the abstracts and presentations at conferences will be available within the duration of this licence, and likely starting from the second year of this PPL.

Although clinicians will also be aware of the findings starting from the second-third year of this PPL, clinical decision and practice are unlikely to change in the short-term

Medium- and long-term benefits

We expect that toward the end of this PPL and after that the findings would bring to changes in clinical practice (in the form of optimized dosing regimens and delivery routes and systems) in order to optimize treatment outcomes in patients.

Species and numbers of animals expected to be used

- Mice: 1000
- Rats: 3800

Predicted harms

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

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

For pharmacokinetic assessment and biodistribution studies we choose rat as a model in most cases. Rats are the most widely used model for pharmacokinetic assessment of drugs and biodistribution and there is significant amount of previous work that has been done with pharmacokinetics, biodistribution and lymphatic transport of drugs using rat model. Although some groups use dogs as a model for intestinal lymphatic transport studies, it is the opinion of the applicant that the additional information obtained in dogs versus rats (e.g., slightly more accurate quantification of the lymphatic transport in regards to similarity to humans) does not ethically justify the use of dogs for this purpose.

The size of the rat allows multiple blood sampling of relevant volumes from the same animal– which results in lower number of animals used versus mice (the entire pharmacokinetic profile of a given drug can be followed in individual animals). Due to small size of mice, inserting cannulas into jugular vein or lymph ducts is not practical in these animals. In addition, for rectal administration studies, the rat rectum is about 80 mm in length and not prone to prolapse compared to the mouse rectum, which is 5 mm in length and is prone to prolapse.

However, in some cases we will perform pharmacokinetic and biodistribution studies in mice instead of rats, or both in rats and mice. The studies in mice will be performed (or added to studies in rats) in the following cases: 1. When the metabolism profile of the compound clearly indicates that metabolites profile in mice is more similar to humans than in rats, and therefore studies in mice are more relevant;

2. When relevant efficacy disease models are established in mice and not in rats and therefore accurate pharmacokinetic profile in mice must be known to design future efficacy studies.

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

In this project we will optimize the routes of administration (oral, rectal, subcutaneous injections, intramuscular injections, intravenous administration), formulations and physicochemical properties (by pro-drug or co-drug modification) of pharmacological

agents with an ultimate goal of improving their pharmacokinetics (concentrations of drugs in blood plasma over time) and biodistribution (distribution of drugs to different tissues and organs at different time points) to achieve better treatment outcomes. For each objective the work will begin with in vitro and/or in silico and/or ex vivo experiments aimed to narrow down the number of candidate molecules, formulations and routes of administration with the ultimate goal of reducing the number of subsequent in vivo experiments and number of animals used. In in vivo experiments we will administer candidate drugs and formulations to rats and/or mice by various routes of administration. For pharmacokinetic experiments in rats, in most cases we will implant cannula into jugular vein under general anaesthesia 2 nights before first drug administration. The cannulae is tunnelled subcutaneously once placed, and is exteriorised at the dorsal neck. In pharmacokinetics experiments blood samples will be taken from the cannula or from superficial veins at predetermined time points. The blood samples will be then processed for the determination of concentrations of the drugs and appropriate pharmacokinetic calculations. Most pharmacokinetic experiments will be for single administration of drugs, and in such cases the duration of experiments will normally not exceed 72 hours (3 days). In some cases we will perform pharmacokinetic experiments for multiple dose regimens, in which case the duration of experiments will normally not exceed 288 hours (12 days). Animals will be sacrificed by schedule 1 method following the completion of pharmacokinetic experiment. Not more than 15% of blood volume will be collected during the pharmacokinetic experiment. In biodistribution experiments the tested drugs will be administered by various routes of administration, then rats or mice will be anesthetised by general anaesthesia at predetermined time points, terminal blood collection will be performed under anaesthesia (from vena cava or by cardiac puncture), then the animals will be sacrificed by schedule 1 method, and then various tissues will be collected and processed for determination of drugs concentrations.

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

1. Restriction in food availability (fasting). Weight loss is inevitable outcome of fasting. We will limit the fasting to up to 16 hours in mice and 24 hours in rats. Rats will fast up to 16 hours in most studies, except for administration into rectum studies in which rats will fast up to 24 hours. The duration of the effect will be short due to single occasion of limit of access to food at or below 24 hours in rats and up to 16 hours in mice. The reasons or food restrictions are following: a. For drugs that are administered orally food affects the absorption, and we, therefore, will restrict food in order to minimize additional variable that food creates; b. For drugs that are administered by other routes of administration, food restriction is in order to maintain the same experimental conditions as in oral administration and therefore increase the quality of scientific data and reduce variability; c. For drugs that are administered into rectum additional reason for food restriction is to maintain rectum relatively free from faecal matter, and therefore reduce factors that affect the absorption of drugs from the rectum.

2. Oral gavage (administration of drugs by a tube through oral cavity that delivers the drugs directly into stomach). Minor discomfort from the procedure will be experienced by 100% of animals. Only compounds and doses believed to not lead to

adverse effects likely to exceed moderate severity or lasting above 1 hour, will be administered. Where any doubt exists, pilot studies will be conducted. Good animal handling will be applied to minimise discomfort and observation will be done after dosing. END POINT: Humane killing of any animal showing signs of miss-dosing or damage.

3. General & Terminal Anaesthesia. Throughout the duration of the procedure surgical pain and discomfort will be controlled with a help of general anaesthesia. Anaesthetic concentration will be carefully controlled and monitored following guidance from the named veterinary surgeon. The recovery from gaseous general anaesthesia is usually within minutes after the end of anaesthetic administration, and animals are expected to be free of any post-anaesthesia clinical signs within 30-60 minutes after anaesthesia. END POINT: Schedule 1 method killing if failure to maintain control of anaesthetic depth.

4. Surgical cannulation (implantation of a catheter) of blood vessels, ducts, tracts or hollow organs.

A number of possible adverse effects could be associated with this procedure, including:

● Inappropriate positioning leading to changes in arrhythmia (changes in heart rate). This is uncommon adverse effect. Care will be taken to ensure that the cannulae are located in a manner not to induce arrhythmia (up to 3cm length inserted). END POINT: If clinical signs indicative of arrhythmia are seen (drastic sudden change in breathing pattern), animals will be killed immediately by Schedule 1 method.

● Potential for post-operative pain could continue for 2-3 days post-surgery. Pre, peri and post- operative analgesic (pain killers) regimes will be used. Animals will be monitored at regular intervals. If there is any indication that the animals are in pain, the advice of the veterinary surgeon will be sought and further appropriate analgesic administered. END POINT: any animal showing signs of pain which exceeds or is likely to exceed a moderate severity will be immediately after detection schedule 1 euthanised.

● Post-operative infections are rare. Aseptic (free from contamination and harmful microorganisms) operating procedures and aseptic preparation of the surgical tools and cannula will be carried out as agreed with the NVS. END POINT: Schedule euthanasia if wound discharge or swelling occurs.

● Uncontrollable bleeding. This is uncommon adverse effect. Care will be taken to ensure that the procedures are performed carefully to minimise the chance for uncontrollable bleeding due to trauma of central blood vessels. END POINT: Schedule 1 method killing immediately after detection if uncontrolled bleeding occurs. Uncontrollable bleeding will be defined as massive bleeding from a major blood vessel that cannot be stopped.

● Single housing. At the moment all animal following jugular vein cannulation surgery are single- housed. We will be trying to eliminate the need in single housing,

or at least reduce substantially the duration of single housing by implementing advanced blood sampling access methods.

5. Post-operative recovery, handling of animals. Re-opening of the wound is an uncommon adverse effect. One attempt will be made for re-closure of re-opened wounds by appropriate means as agreed with the NVS. END POINT: S1 euthanasia if the wound opens again after the re-closure attempt during the pharmacokinetic experiment. In the case the cannula is disclosed the animal will be S1 euthanized.

6. Administration (by intravenous injection or continuous infusion) of substances, or withdrawal of fluids via previously implanted cannula, catheter, or delivery system. The dose volume and withdrawal will not exceed the LASA recommendations.

Only compounds and doses believed to not lead to adverse effects likely to exceed moderate severity or lasting above 1 hour, will be administered. Where any doubt exists, pilot studies will be conducted. END POINT: Schedule 1 method killing (immediately after detection) if irritant or toxic effects are observed that cannot be controlled within the moderate severity limit or last above 1 hour.

7. Substances administered by injection. 100% of animals will experience effect of the dose volume/compound. The dose volume will not exceed the LASA recommendations. The compound will be of an appropriate acidity level and free from pathogenic material. Only compounds and doses believed to not lead to adverse effects likely to exceed moderate severity, or lasting above 1 hour, will be administered. Where any doubt exists, pilot studies will be conducted. END POINT: Humane killing (Schedule 1) immediately after detection if irritant or toxic effects are observed that cannot be controlled within the moderate severity limit or last above 1 hour.

8. Rectal administration of fluids, drugs and nano/micro scale formulations. Rectal administration may be associated with short (15-30 minutes) and minor discomfort. There is a remote possibility of perforation (creation of a hole in the wall of the rectum) or prolapse of the rectum or lower colon. Good animal handling will be applied to minimise discomfort after dosing. To clear the rectum from faecal matter before the administration of tested compounds and formulations, orally administered laxative (leading to defecation) and/or anti-diarrhoeal (preventing defecation) compounds could be used, as well as suppositories and/or enema. This could have adverse effects of minor pain in the stomach area and diarrhoea. END POINT: Humane killing (Schedule 1 method) immediately after detection if irritant or toxic effects are observed that are likely to exceed the moderate severity limit or last above 1 hour.

9. Administration of anticancer agents and their derivatives or prodrugs. Due to the nature of these compounds adverse effects are potentially possible. Only compounds and doses believed to not lead to adverse effects likely to exceed moderate severity, or lasting above 1 hour, will be administered. Where any doubt exists, pilot studies will be conducted. END POINT: Humane killing (Schedule 1 method) immediately after detection if irritant or toxic effects are observed that cannot be controlled within the moderate severity limit or last above 1 hour.

10. Administration of cannabinoids (drugs derived from cannabis plant). Due to the nature of these compounds, minor sedation (feeling sleepy) is likely. Only compounds and doses believed to not lead to adverse effects likely to exceed moderate severity, or lasting above 1 hours, will be administered. Where any doubt exists, pilot studies will be conducted. END POINT: Humane killing (Schedule 1 method) immediately after detection if irritant or toxic effects are observed that cannot be controlled within the moderate severity limit or last above 1 hours.

11. Administration of antiretroviral agents (drugs that work against HIV virus) and HIV latency reversal agents (drugs that activate HIV virus) and their derivatives or prodrugs. Only compounds and doses believed to not lead to adverse effects likely to exceed moderate severity, or lasting above 1 hour, will be administered. Where any doubt exists, pilot studies will be conducted. END POINT: Humane killing (Schedule 1 method) immediately after detection if irritant or toxic effects are observed that cannot be controlled within the moderate severity limit or last above 1 hour.

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

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

In these experiments we will administer candidate drugs and formulations to rats and/or mice by various routes of administration. For pharmacokinetic experiments in rats, in most cases we will implant cannula into jugular vein under general anaesthesia 2 nights before first drug administration. Because of surgery under general anaesthesia, all pharmacokinetic experiments in rats are defined as MODERATE severity. In pharmacokinetics experiments blood samples will be taken from the cannula or from superficial veins at predetermined time points. The blood samples will be then processed for the determination of concentrations of the drugs and appropriate pharmacokinetic calculations.

In biodistribution experiments rats or mice will be sacrificed at predetermined time points and various tissues will be collected and processed for determination of drugs concentrations. Most biodistribution experiments will be under MILD severity limit. The exception is experiments with antiviral drug candidate thapsigargin and its derivatives. Because of the adverse effects of this drug and its derivatives, all experiments with thapsigargin are under MODERATE severity.

We expect about 60% of both mice and rats to go through experiments under MILD severity, and 40% under MODERATE severity.

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

- Killed

Replacement

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

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

The studies that aim to assess the pharmacokinetics (concentrations in blood, plasma or tissues over time) of drugs require the interplay between a chemical compound and physiological function of different systems in the body. At this time, no in vitro or in silico (computer-based) methods exist that are able to totally replace animal experiments for generation of reliable information about pharmacokinetic behaviour and biodsitribution of drugs in the body.

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

Where possible, alternatives to replace animal experiments will be used. The following non-in vivo alternatives will be implemented in this project:

1. In-silico (computer-based) model for prediction of absorption of drugs into the lymphatic system following oral administration will be used.

2. In silico (computer-based) prediction of absorption and distribution properties of thapsigargin and its derivatives will be used.

3. Modeling and simulation of the plasma concentration-time profiles of drugs (pharmacokinetic data) will be performed.

4. Ex vivo (outside the body) analysis of blank tissue and tissues obtained and stored from previous studies will ensure the techniques for measuring drug concentrations are feasible with this tissue matrix and confirm instrument conditions and sample manipulation.

5. In vitro and ex vivo assessment of drugs absorption, distribution and elimination properties.

We have considered using the following in-vitro and in silico methods instead of animal experiments:

1. Cell culture methods for prediction of drug absorption.

2. Liver extracts for prediction of drug metabolism.

3. In silico (computer-based) models for prediction of drug absorption.

4. Physiology-based pharmacokinetic modelling and simulation.

5. In vitro cell culture-based methods for prediction of drug delivery into brain.

Why were they not suitable?

1. Cell culture methods do not express the same transporters (that help absorption of drugs into cells or removal of drugs from the cells) as normal intestinal cells, and are not able to produce chylomicrons (large lipoproteins) in a quantitative manner (necessary for lymphatic transport of drugs).

2. Liver extracts are not able to address the changes due to route of administration and absorption mechanisms.

3. In silico (computer-based) models for prediction of drug absorption do not take into account diet and formulations (such as lipid-based formulations) and provide no information about post absorptive events such as distribution and elimination.

4. Physiology-based pharmacokinetic modelling and simulation is far from mimicking the systems in the living organism and therefore it does not allow at this moment replacement of use of animals for obtaining pharmacokinetic data.

5. In vitro cell culture models for prediction of drug delivery to the brain are limited due to the difficulty in acquiring human brain tissue regularly, so lines based on immortalized (cancer-like) cells are often used. These cells do not replicate the full barrier. Also, it is still unclear the level to which these models can replicate transporters, efflux pumps and receptors.

Reduction

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

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

Typical variations and differences between groups from our previous experiments under two previous PPLs have been used to estimate sample sizes. Since in most experiments more than 2 groups will be compared, and not always pair-like post-hoc analysis will be performed, we have selected (as recommended by EDA decision three) using G*Power software as EDA calculator currently does not support these options. Calculations (using G*Power software) typically show that we need group sizes of 6-8 animals for pharmacokinetic experiments and 6-8 animals/time point for biodsitribution experiments.

In a number of cases in which pilot studies are needed to assess the safe dose, additional n = 2 animals/pilot experiment/experimental group have been added to calculations.

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

We employed the NC3Rs experimental design principles and have used G*Power to calculate the sample size for each experiment.

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

The following measures to ensure that the minimum number of animals will be used for:

1. Following development of analytical methods for measuring of concentrations of test compounds in biological samples, for experiments involving intestinal lymphatic transport or targeting, an ex-vivo study of association of test compounds with plasma derived chylomicrons (largest lipoproteins that are assembled in the intestinal wall cells in presence of dietary lipids) will be performed. The ex vivo experiments with rats chylomicrons will be performed after in silico (computer-based) prediction of affinity to chylomicrons, and experimental association with artificial (non animal-derived chylomicrons- like lipid particles), thereby reducing the number of compounds that would reach the ex vivo study with rats chylomicrons. Due to presence of proteins on the surface of chylomicrons, the association of drugs with artificial lipid particles, or purely in silico predictions frequently substantially deviate from affinity of drugs to actual chylomicrons, making these ex vivo studies with rats chylomicrons still necessary with most promising compounds. Only compounds with actual affinity above 5% to these chylomicrons will proceed to in vivo experiments. However, in case of chemical changes to the drug molecule, the initial parent compound (even if it does not have association with chylomicrons above 5%) will also proceed to the in vivo step (as control group).

2. The intestinal lymphatic transport (absorption of drugs into the lymphatic system after oral administration) study will be only performed for those test compounds that their plasma pharmacokinetics (concentration in blood plasma over time) and biodistribution (distribuion into different organs and tissues) are altered as a result of structural chemical modification and co- administration with lipid-based formulations.

3. In silico (computer-based), in vitro (not using animals and animal tissues) and ex vivo (using animal tissues outside body) drug metabolism and pharmacokinetics experiments will be performed, leading to lower number of compounds proceeding to in vivo studies using animals and therefore lower number of animals being used eventually.

The experiments will be carefully designed and number of animals in each experiment will be assessed using biostatistical principles including power analysis.

In a number of cases in which pilot studies are needed to assess the safe dose, additional n = 2 animals/pilot experiment/experimental group have been added to calculations.

Refinement

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

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

Choice of species:

The ex-vivo model for association of drugs with chylomicrons was established using rat chylomicrons and therefore for the model to be predictive for the lymphatic transport potential it is logical to use chylomicrons derived from the same species. Chylomicrons are large lipoproteins (lipid-protein particles) that are assembled in the intestinal wall cells in the presence of dietary lipids or lipid-based formulations. These chylomicrons, together with co-administered drugs, are absorbed through intestinal lymphatic system rather than directly into blood circulation. We have previously demonstrated that association of drugs with rat chylomicrons is practically identical to association of drugs with human chylomicrons.

For studying delivery to the brain we choose rat as a model. The majority of studies on drug delivery to the brain use rats, as they are much larger than mice, and it is therefore logical to use the same species in our studies.

For pharmacokinetic (concentration of drugs in blood or blood plasma over time) assessment and lymphatic transport (concentrations in lymph nodes and lymph fluids) of test compounds we choose rat as a model in most cases. Rats are the most widely used model for pharmacokinetic assessment of drugs and lymphatic transport and there is significant amount of previous work that has been done with pharmacokinetics and lymphatic transport of drugs using rat model. The size of the rat allows multiple blood sampling of relevant volumes from the same animal– which results in lower number of animals used versus mice. For biodistribution (concentration of drugs in tissues and organs over time) assessment studies we chose to use both rats and mice. Rats will be used in order to compare the biodistribution results to the pharmacokinetics and lymphatic transport results. Mice will only be used for biodistribution studies when metabolism (chemical transformation in the body after drug administration) profile of the tested compound showed to be similar between humans and mice (but not in rats), or if in vivo efficacy models have been already established for diseases in question. In such cases we will perform biodistribution studies in mice in order for the work to progress efficiently to these established efficacy models.

For pharmacokinetics and tissue distribution of thapsigargin and its derivatives, mice will be used initially as our preliminary data of metabolism in hepatocytes (liver cells) of different species suggest that metabolic pathways of thapsigargin are similar in mice, dogs, non-human primates and humans (but different in rats). Once metabolic profile of thapsigargin in mice has been identified, subsequent pharmacokinetic and bioavailability studies on thapsigargin and its metabolites will be conducted in both mice and rats.

For rectal administration studies, rats will primarily be used due to having a longer rectum than mice, therefore, there is a lower chance of adverse effects. We will use a catheter with blunt end, and lubrication in order to minimize any adverse effects of

rectal administration. In addition, we now have good understanding of the anatomy and physiology of the lymphatic system around rectum in rats (but not in mice).

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

We have considered less sentient species, such as fish. However, the difference in the anatomy and physiology between fish and human is extremely high. The physiology and anatomy of rodents (rats and mice) closely resembles human physiology. This includes similarities in absorption from gastrointestinal tract, similarities in metabolism and excretion mechanisms (urinary and biliary). For this reason, using less sentient animals than rodents in studies aimed to understand drug pharmacokinetics, drug metabolism, as well as complex questions associated with drug delivery, is not going to provide required answers to the asked scientific questions.

Fish would also have to be anesthetised for most procedures, including accurate drug administration by different administration routes. General anaesthesia is known to affect the blood flow and metabolic rate, and therefore will affect the measured pharmacokinetic parameters. Moreover, it is not practical to anesthetize the animals for several days, which is a period commonly required to complete pharmacokinetic experiments.

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

Where any doubts of adverse effects exceeding the maximum stated severity level stated for the protocol exists, pilot experiment will be conducted to make sure the severity limit is not exceeded.

In all pharmacokinetic experiments involving recovery surgery, NVS advice will be followed for appropriate perioperative pain management (analgesia). Each animal undergoing the surgery will have a separate monitoring sheet to reduce any risk for any adverse effects/complications to go undetected. Two nights post-surgery recovery period will be included in design of all pharmacokinetic experiments involving jugular vein cannulation under general anaesthesia, before drug administration and beginning of blood sampling schedule. The mode of pharmacokinetic experiments involving surgery of jugular vein cannulation is on its own a refinement, as blood sampling from the cannula is pain-free. Potential adverse effects include post-surgical pain (managed by appropriate analgesia (based on NVS advice), bleeding from the canulae if it is damaged, post-surgical infection (minimized by aseptic techniques).

Despite these potential adverse effects, the pain-free sampling and the ability to obtain a complete pharmacokinetic profile from one animal (one full biological replicate) provides substantial refinement versus tail-vein blood sampling. The cannulae is tunnelled subcutaneously once placed, and is exteriorised at the dorsal neck; the external portion of the catheter could potentially be chewed by the rat post-surgery. Under this PPL we will also be working toward further refinement of blood sampling procedures, implementing more advanced blood sampling access ports. We will be also looking at eliminating, or, if impossible, reducing to minimum the duration of single housing (using the advanced blood sampling access methods).

The cannulae is maintained by heparinized saline (prevention of clots inside the cannulae). Despite this it is possible that the cannulae could be blocked before the administration of tested compounds or during the pharmacokinetic experiment. If the cannulae is blocked before the administration of the tested compounds and flushing the cannulae with heparinized saline does not help, the animal will be euthanized by schedule 1 method. If the cannulae is blocked after the drug administration, flushing the cannulae with heparinized saline does not help and there is no effect on animal welfare, the animal will be sacrificed at a later time point (or at the end of the pharmacokinetic experiment) in order to collect tissues for biodistribution purposes.

We will be also adding heat pads to maintain body temperature. We will be administering supplementary fluids (e.g normal saline) to replace those lost during anaesthesia and surgery.

All animals will have acclimatisation period of at least a few days following arrival to the facility and before any experiments or surgery will begin.

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

We will consult and follow published PREPARE: guidelines for planning animal research and testing.

Moreover, we will access and follow NC3Rs website in order to refine the procedure in accordance with NC3Rs principles.

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

We will make sure to regularly check information on NC3Rs website. We will also make an effort to regularly attend a Regional 3Rs symposia.

Moreover, our ongoing constant close contact with a team in animal facility will also contribute for us to stay informed and updated.

17. Prevention of bacterial infection

Project duration

5 years 0 months

Project purpose

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

Key words

bacteria, vaccines, resistance, immune responses

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aims of work in this project are to understand how bacteria cause disease and to identify vaccine candidates for preventing bacterial disease.

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

Why is it important to undertake this work?

Bacterial infection poses an increased threat to human health through the spread of antibiotic resistance.

We face the prospect of a return to the pre-antibiotic era when bacterial disease was largely untreatable and often fatal.

This project will be directed at studying bacteria which cause disease in humans. We will also develop and test vaccines designed to protect individuals from bacterial infection.

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

1) Testing vaccine candidates for preventing bacterial infection,

2) Development of models of bacterial infection,

3) Dissemination of results in publically available, open access scientific papers, and

4) Establish new intellectual property to enable clinical trials.

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

In the short term (i.e. during the first half of the project), the results of the immune responses from candidate vaccines (output 1) will enable selection of antigens and improvement of vaccines. The scientific community will benefit when we disseminate of our results (output 3), by allowing them to use our findings and prevent duplication.

In the short term, development of models of bacterial infection (output 2), and dissemination of our results (output 3) will enable us and others scientists to define bacterial factors necessary for infection. This information will benefit academics and the pharmaceutical industry as it will allow the assessment of vaccines and therapeutics.

In the medium term (i.e. during the second half of the project), establishing new intellectual property (output 4) is essential for any vaccine to be developed by a manufacturer; beneficiaries include the University, and a commercial partner. We will continue to assess vaccine candidates from a broader range of pathogens.

In the longer term (i.e. > 5 years), human populations will benefit if they are protected against disease with vaccines developed through this project, either directly or indirectly.

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

We will maximise the benefits by performing high level, peer reviewed research with input from available scientific literature, and by interacting with world-leading experts, either as advisors or collaborators. This will enable our outputs to be novel, relevant, benefit from latest advances/techniques, and to maximise their potential benefits for human health. We will disseminate our finding through presentation at leading international scientific meetings. This usually precedes open-access publication in which all results and datasets reach the widest audience possible.

Species and numbers of animals expected to be used

- Mice: 12,000

Predicted harms

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

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

It is not possible to replicate the complexity of the mammalian immune system using cell culture models. Therefore, assessment of the immunogenicity of vaccine candidates requires the use of animal models. Similar considerations apply for models of gastrointestinal infection.

We will use adult mice for this work. Mice are the lowest order of species routinely used to study immune responses. We will take advantage of the many available reagents for working with mice immune molecules and cells. Adults have a fully developed immune system so are likely to give more consistent results than young mice. This reduces the total number of animals that need to be included in experiments.

We may use genetically modified animals in a limited number of situations. For example, vaccine responses in mice lacking particular aspects of their immune system might tell us how they are protected against infection, and enable us to refine vaccine design. A similar approach will be taken to understand the host factors that are necessary for bacteria to successfully cause gastrointestinal infection.

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

1) To assess immune responses to vaccines, mice will typically be given vaccines by injection or by oral gavage on three occasions over a period of six weeks. Injections may be given just under the skin or in deeper sites (for example into muscles as used for some vaccines in children). Small blood samples may be collected throughout the procedures to measure immune responses. About two weeks after the final immunisation, mice are humanely killed, and the experiment ended. Tissues may be collected post mortem for further analysis in the laboratory as these are where immune responses are generated.

2) Some mice will be given an intestinal challenge of bacteria. Mice might be given treatments in their drinking water for up to one week. They will then be given a treatment to make them more susceptible to infection. After this, they will be administered with bacteria such as Shigella on up to four occasions by oral gavage. Mice may then be given treatments such as anti-inflammatory drugs to test their effect on bacterial infection. Blood sampling may be carried out to assess the level of infection in the blood stream, and faeces will be collected regularly. Mice are humanely killed no later than three months after the last dose of bacteria but typically after 10 days. Tissues might be harvested post-mortem to determine the impact of infection on relevant body sites.

3) Using the models of gastrointestinal disease, we will test the impact of vaccines on bacterial infection. In these experiments, mice will be immunised as in section 1),

but subsequently be analysed for protection against bacterial infection as described in section 2).

4) Genetically altered (GA) mice will be bred by conventional methods. The mutations these mice carry are not expected to cause the animals to deviate from their normal behaviour or wellbeing. The response GA mice to immunisation or gastrointestinal infection (see sections 1, 2 and 3) will be assessed in a few instances, and compared wth responses in wild-type animals.

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

Immunisation with vaccines is not expected to have any adverse effects other than a slight tenderness at the injection site which improves rapidly.

Mice that are given a bacterial challenge via the oral route may experience transient weight loss of up to 15% for up to 4 days post infection following which they will regain the weight loss. They will also develop loose stools and/or diarrhoea immediately following infection, but this improves over time. In some cases the mice might develop a rectal prolapse.

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

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

Mice 50% Subthreshold, 30% Mild, 20% Moderate

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

- Killed
- Used in other projects

Replacement

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

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

To test vaccines requires mammals that have a fully functional immune system to see the effects of the administered antigens. This is because the immune system cannot be replicated in vitro, and we are unable to study this in humans without major costs in terms of vaccine manufacture and the expense of clinical trials.

The immune system is composed of different cell types and body sites organised in a complex, interacting network. For example, immune responses can be generated in the spleen which has multiple distinct areas, with their own cell types and blood supply. This cannot be reliably mimicked by any cell culture or organoid models.

Similar considerations apply for models of intestinal disease as the mammalian intestinal tract contains many distinct regions from the stomach, to the small and large intestine. These sites are lined by their own specialised cells, and contain other bacteria as members of the flora that also shape the niche.

Each site presents bacteria with different environments and challenges (e.g. acid in the stomach, low oxygen in the intestine). Therefore, the ability of bacteria to survive and cause disease in the intestinal tract cannot be replicated by models in the laboratory.

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

We can assess bacteria in tissue culture to give us information on their ability to invade certain cell types. We are also able to investigate how bacteria can grow in certain specific environments found in the intestinal tract such as under low oxygen.

Why were they not suitable?

Whilst the answers we receive from our tissue culture studies allow us to answer some of our research questions, they do not accurately reflect the impact of the bacteria on the immune system as a whole, or their ability to survive in the complex habitats they occupy in the intestinal tract and to elicit inflammation in these sites.

Reduction

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

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

We have used our previous experimental data to allow us to estimate our numbers for this project. We may use pilot studies with a small number of mice to assess the minimum number of animals required to achieve statistical relevance. These groups are typically groups of three animals.

Group sizes of five are usually sufficient in our studies of immune responses.

We have 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?

We employ the NC3Rs' experimental design guidance and experimental design assistant (EDA) to help with experimental design, practical steps, and statistical analysis utilising the advice and support for randomisation and blinding, sample size

calculations and appropriate statistical analysis methods. We will use the EDA diagram and report outputs to support experimental planning with animal users.

We typically use wild-type mice in our project which are sourced from commercial suppliers or bred in our facility with known health status to eliminate any genetic or co-morbidities/co-infections which could affect the outcome of our experiments. This removes the need to repeat experiments due to eliminating this variation, and therefore reduces the number of animals we need to use.

Training in statistics is undertaken by the group as whole so that when we are planning experiments, we apply broad relevant experience thus making sure we keep group numbers to a minimum but with the required statistical relevance.

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

We undertake pilot studies with small group sizes (usually n=3) when modifying and optimising protocols (e.g. when trying different adjuvants). We also include as many experimental groups as possible on a single occasion to minimise the number of animals in either negative or positive control groups.

The gender of animals can be an important variable in experiments. We will generally use female mice in our experiments to reduce variation and hence reduce the number of mice needed to obtain statistically significant results. Female animals are easier to handle, and wild-type mice will be purchased from registered suppliers or bred in in-house facilities, so we always use animals with known health status to reduce variation and confounding effects of co-infections/diseases.

We compare the virulence of strains by mixed inoculum experiments and see which strain outcompetes the other. This reduces the number of animals needed for experiments as it eliminates between animal variation.

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. Cryopreservation of colonies not required in the short term will be considered.

Refinement

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

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

Rodents are the lowest form of mammal that can be used to study immune responses. There are extensive reagents available for studying immune responses in mice.

We use adjuvants to stimulate immune responses that are licensed for use in humans or those which are well tolerated in animals so their safety has been extensively tested. We monitor animals carefully for signs of distress and have procedures in place to minimise pain or suffering.

During the previous project most handling was performed by experienced technical staff employed full time to care for animals. We will continue use this approach to ensure that animals are handled by experts whenever possible.

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

We cannot use less sentient animals such as fish, worms, or flies as they do not have a fully functional immune system that replicates that of humans.

Terminally anaesthetised animals are not suitable as we need to assess the response of the immune system to vaccines and infection over an extended period of time (i.e. often > 1 week).

We use adult mice as they have a mature immune system unlike embryos or young animals.

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

We refine our monitoring of animals during small pilot experiments, so that they are checked at appropriate, regular times depending on procedure. The duration of experiment is kept to the shortest period possible that achieves our scientific objectives.

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

Our advice on best practice is obtain by regular interactions with our NVS at welfare and AWERB meetings, and discussing protocols with experienced NACWOs. We also refer to www.nc3rs.org.uk which provides information about latest developments. We will use the LASA guidance for the administration of substances and for advice on aseptic techniques.

The NC3Rs will be consulted where we are looking for best practice in minor procedures and ensuring we follow best practice in terms of the welfare of the animals.

We will follow the ARRIVE and PREPARE guidelines in ensuring our experiments are carried out in a manner that will allow reproducibility.

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

The current licence holder attends termly animal welfare meetings and has been a member of the Department's AWREB committee for over 10 years. Advances and best practice about the 3Rs are discussed at these meetings. Additionally, we refer to latest advice provided at www.nc3rs.org.uk.

18. Systemic effects of liver disease

Project duration

5 years 0 months

Project purpose

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

Key words

liver disease, cirrhosis, inflammation, microbiome, obesity

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

When liver disease develops there are a series of immune system mediated effects that cause dysfunction in other organs, including the kidneys, the brain and the immune system. The aim of this project is to understand how the problems occur in order to develop novel treatments.

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

Why is it important to undertake this work?

Liver disease is the only major cause of mortality that is still increasing in the UK (British Liver Trust, 2021). In part this is due to the ongoing obesity crisis that leads

to progressive liver damage that can result in organ failure. As such, there remains a clear need to understand the nature of disease progression in order to develop new treatments for the liver and other organs affected.

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

The research team has a strong track record in publishing scientific research, which is expected to continue. The new discoveries will be made available via scientific journals, talks at scientific meetings and public facing events.

Previously we have also made discoveries that have been patented and developed into new clinical treatments. Hopefully, this will continue and there will be further discoveries leading to additional novel therapies.

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

The scientific community benefits from the increased knowledge base resulting from our work. In the longer term, patients will benefit from our discoveries and the new treatments we develop.

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

We have been successful in working with a wide range of academic and industrial partners both in the UK, across Europe and the rest of the world. Work has been shared at conferences, through scientific papers and in mainstream media, all of which we plan to continue.

Species and numbers of animals expected to be used

- Mice: 4000
- Rats: 3500

Predicted harms

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

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

We will use a series of rodent and rabbit models that have been very well defined in terms of the development of liver injury. In this way we are able to examine the impact on other organ systems and relate this to the nature and stage of the liver disease.

For the most part we will use adult animals for the studies, though in some cases the models may be started slightly earlier (as juveniles) in order to observe a particular disease characteristic (e.g. early onset obesity).

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

A large proportion of models will be initiated by altering the animals diet as we know that using high fat/high cholesterol over several months leads to the development of liver disease that shares many common features with human disease. Other models will use injections of materials to cause liver disease over a three month period, or the use of minor surgery to induce the disease process over the following 4-6 weeks.

In some studies we will collect samples (blood, urine) at specific time points to monitor disease development or administer treatments to test their effectiveness. Some experiments will involve testing the brain function of animals using simple maze studies or memory tests to understand the effects of liver disease.

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

In most cases there is little outward indication that liver disease is developing. In humans, the majority of people are unaware that they have liver disease until the latter stages and this is similar for the animals. Animals receiving a high fat diet will gain some additional weight, and animals receiving surgery may temporarily lose weight over a few days before recovering. For the most part, it is difficult to identify symptoms without undertaking specific tests. In the latter stages (the final 1-2 weeks), animals may become lethargic and show evidence of jaundice though this is not associated with pain or discomfort.

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

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

The majority of models in these studies (80%) would typically be classified as moderate severity. This is due to our requirements to undertake measurements, administer treatments, conduct surgical procedures, administer substances to induce organ dysfunction or collect samples.

In the majority of cases final sample collection will be undertaken under anaesthesia.

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

- Killed

Replacement

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

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

The nature of these studies is to understand the complex relationship between the liver and other organs/body systems. As such, it is necessary to use the whole animal rather than isolated tissues. As an example, in order to study the interaction between the liver and brain it is also necessary to utilise the circulatory and immune

systems. Currently we do not have alternative technologies that can fully replace an animal for these types of studies.

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

We extensively use cell culture systems as part of our research and have investigated the use of culturing different cell/tissue types in combination to examine signalling mechanisms. However, these are limited in what can be achieved.

We also have developed complex computer models to examine the processes that occur in the body, though again these still lack sufficient sophistication to act as replacements at this time.

Why were they not suitable?

The interactions between different tissues within the body is complex. There are numerous different cell types and body systems that are involved and currently the culture / computer models cannot effectively replicate what we see in the body.

Reduction

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

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

Based on previous studies and the level of funding we would expect to receive, we have estimated animal usage over the next 5 years.

Historically, we have defined the types of models and can predict with a high level of accuracy the minimum group sizes we need to study in order to generate a statistically meaningful outcome.

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

We have used the NC3R's Experimental Design Assistant in the planning of studies, which allows us to base our work on relevant study outcomes. As such, we are able to use the minimum number of animals necessary to achieve the experimental objectives.

By also planning to use accurate and sensitive measurements during the studies we can limit the number of animals whilst maximising the information obtained. In some cases we will also be able to use non-invasive imaging technology to conduct measurements that previously would have required an animal to be culled.

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

Where possible we will use animals supplied by authorised breeders in order to avoid the maintenance of breeding colonies. It has been established practice in the research team to use pilot studies to better inform the planning and design of experiments, which will continue. Our institution has an effective tissue sharing system in place, which will be used as appropriate.

Refinement

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

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

This project will use small rodents (mice and rats) for the studies. Mice are utilised due to the options for genetic modification to examine the role of specific genes/proteins. As mouse models are limited in terms of sample collection volumes in many cases it is advantageous to use rat models due to their greater size. As an example, this is particularly important when dissecting specific regions of the brain for analysis of hepatic encephalopathy as it is untenable to differentiate the structures sufficiently to isolate tissue samples in the mouse brain. There are also advantages in conducting biliary ligation models in rat as they lack a gall bladder. In some studies where extra-corporeal filters are used, mice do not have sufficient minimum blood volume to safely maintain the circuit volume, though this is possible in a rat.

In most cases liver disease will be induced by variations in the diet (e.g. high fat) which causes very little distress to the animals. In others we will use a short surgical procedure to cause liver disease, though this will be conducted using general and local anaesthetic to minimise any discomfort.

In most cases, the development of liver disease does not result in obvious symptoms (which is why most people are unaware they have the condition). Animals may become tired more easily and rest more, but do not show any evidence of discomfort. In some studies we will test the brain function of animals by use of simple maze studies, though again this is not associated with evidence of major stress.

All end point studies will be conducted under general anaesthetic to ensure that the animals do not suffer.

In some studies we will look to see which proteins or signalling mechanisms are involved as the effects of liver disease impact on other organs. To do this we will use animals in which the specific proteins have been knocked out, or upregulated in

order to determine its role in the process. These genetic modifications are benign under normal circumstances, but can show protection or susceptibility to disease.

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

As it is necessary to have functioning body systems it is necessary to use mammals as they have similar physiology to humans which allows us to better in order to relate the findings to human health and medical advancement. Where possible we will conduct the studies on terminally anaesthetised animals, although it will be necessary to develop liver disease first in many cases.

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

We have steadily increased the amount of monitoring we have conducted over recent years and will continue to do this. Post-operative care has advanced in terms of the local anaesthetics available, in addition we can utilise our improved understanding of how best to monitor the recovery of our animals and prevent discomfort. Where appropriate we will use systemic analgesia (e.g. in surgical models where their hepatic metabolism and biliary excretion of metabolites are not contraindicated).

When conducting brain function or behavioural testing we will employ best practice in terms of number of studies and the timing of studies to limit the stress to the animals.

We also strive to use the most informative and accurate methods to limit the number of samples required and restrict the number of animals used. As an example, it is now possible to conduct a series of measures on a single drop of blood that would have required a much larger sample to be collected only a few years ago.

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

We will use the most recent examples from the scientific literature in order to ensure that all of our studies are conducted in an appropriate manner and that our findings will have demonstratable impact in the field.

We will use the published resources by NC3R's to ensure that our studies are fit for purpose, including use of the Experimental Design Assistant to plan studies, practical guidance for handling and assessing the responses of animals and ensuring that we use the ARRIVE guidelines for publication of our work.

LASA principles will be followed in relation to aseptic techniques and best practice for surgical procedures, and We also use the RSPCA guidance in relation to refinement, reduction and best practice (https://www.rspca.org.uk/adviceandwelfare/laboratory).

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

Members of the research group are very active in terms of 3R's engagement. Staff are members of 3R's groups at our institution and regularly attend meetings, workshops and seminars. We also regularly view the NC3R's, Understanding animal research (UAR) and RSPCA websites to keep updated on the latest information and also receive bulletins from our local named information officer (NIO).

19. The genetic and functional basis of proteinuria and kidney disease

Project duration

5 years 0 months

Project purpose

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

Key words

Kidney disease, autoimmunity, genetics, Glomerulonephritis, Proteinuria

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This project aims to understand the changes in gene expression and pathways that drive kidney diseases characterised by protein leak in to the urine, in order to better classify kidney disease, and identify and test new targets for treatment.

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

Why is it important to undertake this work?

Current treatments for most kidney disease are very limited and have serious side effects. By studying renal disease, at the level of changes in individual cells within the kidney, we can identify candidate genes or proteins that can be tested as targets

for new more effective and personalised treatments for kidney disease. Our work will also help us to better classify and diagnose renal disease.

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

- New insights into the cellular basis of glomerular kidney diseases
- Exploration and validation of candidate novel therapeutic targets in vivo and in vitro
- Evidence for in vivo effects of novel therapeutics, which may include drugs, antibodies or cell therapies
- Publications in peer reviewed journals sharing our findings

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

- Academic colleagues, locally and internationally, researching kidney disease or autoimmunity, through dissemination of our discoveries and data
- Clinicians, including nephrologists and renal pathologists, who may benefit from new approaches to human renal pathology building on our work
- Clinical trialists in kidney disease. Our work may lead in the longer term to new ways to classify renal disease and treatment responses, improving stratification and outcomes for clinical trials
- People living with, or at risk of kidney disease, who in the longer term can benefit from new more targeted, personalised treatments, more effective and with less adverse effects, based on our cellular insights.

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

- We collaborate with other research teams locally, and across Europe, to maximise the impact of our work
- New findings are shared at conferences and meetings, in the UK and internationally
- We publish our results in peer reviewed journals, including sharing raw data so that results can be replicated or new findings made.
- We publish negative or unexpected findings from animal models to share knowledge so that work is not duplicated, and assumptions can be challenged.

Species and numbers of animals expected to be used

- Mice: 6600

Predicted harms

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

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

The laboratory mouse is the species of choice for studying renal disease, and the ideal mammal for genetic studies where animals need to be generated rapidly. It has a kidney of similar structure and anatomical complexity to human organs.

Using mice provides us with an opportunity to directly study the role of genes in the maintenance of the glomerular filtration barrier, which is not possible in higher organisms, or sufficiently close to the human kidney in lower organisms. The similarity of human and mouse renal systems is reflected at a genetic and protein level. This means that human genes identified by sequencing projects almost invariably have mouse counterparts.

Mice can be genetically altered or treated to replicate chronic kidney diseases found in humans. Mouse strains with susceptibility to kidney disease are established, and these animals provide the least sentient system that recapitulates human immune and renal function. We can't use embryos or very young animals as their immune and renal system is too immature. Chronic renal damage takes time to accumulate so we study adult mice.

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

Animals will be bred, and after humanely killing, the kidneys and other tissues are harvested for experiments.

In some cases we will take blood from a tail vein to measure parameters such as kidney function and immune populations.

Urine provides a useful marker of kidney changes, including protein leak, and we will collect urine samples, in some cases by isolating individual animals for up to 3 hours in a cage with hydrophobic sand as substrate, to collect all urine produced.

Some animals are treated with a topical substance (e.g. a cream applied to the ears) to induce an immune response. This may be applied 3 times a week for 8 weeks.

Substances may be also be given intravenously (injection into a blood vessel), subcutaneously (injection under the skin), or orally, in order to induce, study or treat renal changes. In some cases, for example if multiple injections are required or to place a temporary cannula into a vein, injections may be performed under anaesthesia.

To perfuse the kidneys, for example with magnetic beads to enable harvesting of specific kidney tissues, or to measure renal filtration, we may inject with a perfusion fluid under deep terminal anaesthesia, before humanely killing and collecting tissue.

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

Renal failure is typically not symptomatic until the late stages, and so most animals will not experience adverse effects. However some animals with genetic or induced renal disease may develop symptoms of renal impairment. This can include mild fluid retention (oedema) due to urinary protein leak. Renal impairment, immune activity or toxicity from drugs administered, can also lead to weight loss, therefore we will monitor all at risk mice closely to ensure animals are humanely killed at a time point prior to the development of symptomatic renal failure.

Expected severity categories and the 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 Subthreshold 70%
Mild 5%
Moderate 25%

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

- Killed

Replacement

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

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

Human kidneys are highly complex with more than 30 different cell types, interacting with each other to perform the functions of filtration, maintenance of fluid and salt balance, and contributions to bone health and hormonal activity. Access to human kidney tissue is limited, and so mice provide a crucial adjunct to our study of human tissue, to enable study of the in vivo renal system, and to manipulate pathways to explore disease mechanisms. The insights we gain from animal work are essential to enable the development of targeted treatments for human kidney disease.

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

CRISPR/Cas9 gene edited human kidney cells Kidney organoids

Kidney on a chip systems (engineered or natural minature tissues grown inside microfluidic chips)

Why were they not suitable?

The use of in vitro CRISPR/Cas9 gene edited human podocytes and mesangial cells will complement the mouse work, however immortalised cells fail to fully profile in vivo cells, and cannot model their complex interactions in a living organism.

Complex new models of kidney structure such as kidney organoids, whilst offering huge potential, do not yet adequately mimic in vivo physiology. For example kidney organoids derived from induced pluripotent stem cells represent an immature stage in development akin to first trimester (embryonic) kidney, with substantial contribution from off-target cell populations such as neural cells. Nevertheless kidney organoid technology is used in our lab to study genetic developmental defects and environmental injury and will complement the animal work.

Kidney on a chip systems combine cell culture or co-culture with microfluidic devices to mimic stimuli such as blood flow. These systems may offer a powerful approach for screening compounds, for example for kidney toxicity. However architectural and mechanical limitations persist, and these engineered models fail to fully mimic the complex structure and cell interactions of the glomerulus.

Reduction

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

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

Sample sizes for our experiments are based on past experience, and a typical experiment could compare 6 treated or genetically engineered mice with 6 matched controls. Annual returns of procedures data, combined with planned experiments and work programmes, have been considered in estimating the numbers of animals required. Experimental procedures are reviewed within our group, and at Animal Welfare Meetings.

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

The emphasis in this programme is on using techniques that can reduce the number of animals. Our experiments are performed on co-housed inbred mice to reduce biological variation.

Use of statistics. Data in the form of various quantitative parameters (e.g. creatinine, cell proliferation, cell markers) are assessed by simple statistics or by ANOVA (analysis of variance) to allow for variable numbers of controls and mice of different age and sex. We are not normally in pursuit of low penetrance / subtle phenotypes and therefore do not normally need to examine large numbers of animals.

Experimental design, including efforts to increase uniformity and reduce random variation, is necessary to detect significant differences with low numbers of animals. Therefore our approach is to use factorial designs and blocking to remove nuisance variables, if numbers allow. The majority of our experiments produce quantitative data that can be analysed by parametric methods. Chi squared may be used if non parametric qualitative observations are made (e.g. histological classifications). The

NC3Rs experimental design assistant (https://www.nc3rs.org.uk/our-portfolio/experimental-design- assistant-eda) can support good design to ensure that the minimal sufficient animals are used to adequately answer the scientific question.

Avoidance of bias: Randomisation will be used with appropriate controls, and where appropriate, use of blocking to control for sources of variability not of primary interest (for example sex). Where possible samples will be labelled with anonymised codes by one investigator and assayed by another but given the small team, full blinding will often not be possible. Where appropriate quantitative read outs and automated image analysis systems will be used to minimise subjectivity.

So that others can scrutinise and replicate our findings, we will use the ARRIVE guidelines to improve the quality of animal reporting in our published research.

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

1. In vitro experiments. Where in vitro experiments can address the hypothesis they will be used first. Our ability to target cells with CRISPR/Cas9, is increasing the usefulness of renal cell lines, which were previously difficult to manipulate genetically due to resistance to transfection and genomic instability. We have established a programme for genetically manipulated kidney organoids, the first in our institution, which also provides an alternative and sometimes complementary way to interrogate the genetics of renal disease.

2. Maximising output for each experiment. Where possible the data from each individual animal will be maximised through the collection of multiple samples and data at endpoints that would otherwise have to be assessed in separate experiments.

3. Reducing breeding. 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. We previously showed that whole genome analysis in random mutagenesis studies reduces the need for additional breeding or outcrossing to another strain background. By using gene editing to directly induce mutations in renal susceptible strains we can reduce the need for backcrossing and additional breeding. When strains are no longer needed they will be archived. Where suitable lines already exist, animals will be obtained from the relevant supplier. Male and female transgenic mice will be used where possible to reduce the number of animals. For some renal phenotypes, e.g. lupus nephritis, the disease is more pronounced in females and in these cases only female mice will be compared to reduce variability.

Refinement

Give examples of the specific measures (e.g., increased monitoring,

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

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

We will study genetic mouse strains susceptible to renal disease and inducible models, where kidney disease develops due to administration of a drug or compound.

For example we apply a cream to the ears 3 times a week for 8 weeks to induce autoimmunity with renal involvement similar to human lupus, or give a single intravenous injection of a chemotherapeutic agent which causes the development of injury and inflammation in the kidneys and urine protein leak over a period of 1-2 weeks.

By studying early kidney damage, in models with mild disease, or at an age prior to the onset of renal failure, we can minimise the animal suffering, while focussing our research on early disease specific pathways more likely to be amenable to treatment.

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

We can't use embryos or very young animals as their immune and renal system is too immature. Chronic renal damage takes time to accumulate so our experiments need to be performed in living animals, rather than only under terminal anaesthesia.

Drosophila nephrocytes have some features in common with podocytes and can provide a less sentient model to study renal disease, however the nephrocyte differs significantly from podocytes in both cell architecture and the nature of the excretory system.

The nematode C elegans does not have an excretory system comparable with the mammalian kidney, and while it has provided a very useful model for cystic kidney disease, the closest equivalent to the podocyte is a neuronal cell, which shares some features of the slit diaphragm, but lacks a true glomerular structure or excretory function.

Zebrafish (Danio rerio) can provide a more complex renal architecture, the zebrafish pronephros has informed our understanding of renal development, and zebrafish have been used to model acute kidney injury and genetic kidney disease. However in contrast to the mouse, where urine and blood parameters can be monitored, a key limitation of the zebrafish is the glomerular injury read out.

Oedema typically occurs, easily visible by light microscopy, but is a non-specific finding not always linked to renal impairment. More specific measures such as visualisation of the glomerular filtration barrier by electron microscopy, or

microinjection of fluorescently labelled dextrans, are time consuming, technically complex, and can be very variable.

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

1. Sampling time points will be selected to maximise the value of the data while minimising the number of time points. For example tail bleeding will be done only at the most informative times following administration of renal toxins.

2. Some mice will be more susceptible to weight loss or malaise due to renal insufficiency or due to induced autoimmunity. Where this is predictable from our previous experience or published results, we will assess animals at suitable time-points to ensure severity limits are not exceeded; in other cases, we will use small pilot experiments and liaise with animal staff to highlight the need for added vigilance. We will closely monitor new strains, including where appropriate litters prior to weaning, in order to define the age of onset of renal impairment. Mice with isolated urine protein leak are expected to exhibit mild or no adverse effects. In some cases proteinuria may progress to low blood protein levels and occasionally but not typically fluid retention. Animals with weight loss > 10% from baseline would be given palatable food on the cage floor and have increased monitoring, including weighing at a minimum every 2 days. All animals given the renal toxic drug Doxorubicin will be weighed daily for the first 14 days post dosing since this drug is associated with weight loss in some animals.

3. We will use urine collection by brief handling to collect urine in small volumes for protein dipstick testing. For more accurate quantification, timed urine collection will be used to obtain larger volumes. The use of 3 hour collections, rather than 24 hour single housing, reduces isolation. We have established a method using hydrophobic sand, rather than a gridded floor, providing a more natural substrate within the cage environment during urine collection, and facilitating more complete collection. Animals will be provided with food and water throughout this period.

4. Toxins, inhibitors, agonists and other substances used to modify renal function will be reviewed carefully before use to ensure that limits are not exceeded. Agents that have not previously been used in mice will only be used when there is good reason to suppose they will be well tolerated and at the minimum dose. Route of administration will be selected to minimise adverse effects.

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

Guidance disseminated via the Named Training and Competancy Officer, and organisations including NC3Rs, Norway's norecopa and the Laboratory Animal Science Association.

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

Through attendance and presentation of protocols and work at regular internal 3Rs meetings to share good practice and learning.

By keeping up to date with advances through these meetings, information disseminated by the local NC3Rs regional manager, and on the NC3Rs website (www.nc3rs.org.uk).

20. Therapeutic monoclonal antibodies against difficult proteins

Project duration

5 years 0 months

Project purpose

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

Key words

Antibody, Disease, Medicine, Discovery

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overarching goal of this work is to use our immunisation technology to discover antibodies which bind to multi-pass membrane proteins such as G protein coupled receptors (GPCRs) and which modulate their function. The antibodies can then be developed into new medicines to treat severe diseases.

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

Why is it important to undertake this work?

There are many diseases for which we have an understanding of the underlying

problem, and where we know how we could intervene to improve the patient's condition, but we don't have a medicine whose specific mechanism of action can be used to address that underlying problem. In many cases the disease-causing proteins – the 'targets' - sit in the membranes of our cells. This kind of protein is extremely difficult to work with and to discover new biologic drugs against, because the proteins are generally unstable when removed from this cell membrane milieu. This project will address this problem, by allowing us to use a technology we have developed that lets us discover a class of drug called 'monoclonal antibodies' (mAbs). A critical part of the technology is injecting animals such as mice and rats, and then harnessing part of their immune response as raw materials for discovering the monoclonal antibodies.

Our work aims to discover treatments for severe diseases. We currently have programs in two different areas.

- Inflammatory bowel disease
- Rheumatoid arthritis

Over the course of the project we expect to expand this list of diseases and we are currently considering a range of disease areas including dis-regulation of the immune system, dermatological conditions and immuno-oncology. As well as making good therapeutic drugs, monoclonal antibodies are also useful for detecting chemical markers of health and disease called biomarkers.

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

This project will generate monoclonal antibodies derived from immunised animals.

The antibodies will be useful tools in research and can help improve our understanding the mechanisms behind human disease.

In the long term, the antibodies discovered from this project will be developed into drugs and tested as novel therapies for human disease.

The sequences of the identified antibodies will be published as part of a patent filing.

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

In the short term, antibodies which could be used as tools in research will benefit the scientific community. Antibodies to specific proteins can help identify that protein's role in health and disease. This would help establish if the particular protein is a good target to develop a drug against.

Antibodies can also help clinicians and patients in diagnoses. Monoclonal antibodies (mAbs) which could detect biomarkers could be further developed as tools for biomarker detection devices which are an important part of the personalised health revolution.

In the long term, functional antibodies from this project will be developed as potential drugs. The goal is to create new treatments and cures for inflammatory diseases and test these in human clinical trials. If successful, this would be an enormous benefit to

patients who are currently suffering from diseases without adequate treatment options.

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

We regularly give talks at conferences, teaching our findings. MAbs will eventually be patented and published, to provide protection to allow their development. Antibodies will be developed as drug candidates, which will involve testing in human clinical trials.

Species and numbers of animals expected to be used

- Mice: 5375
- Rats: 500

Predicted harms

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

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

The majority of work under this PPL will be using adult mice. Animals with a fully functioning active immune system are required to generate high quality monoclonal antibodies. The development of monoclonal antibodies as drugs suitable for human use is well-established from mice. Indeed, we have discovered several antibodies which are now being progressed towards clinical testing in our laboratories using this approach. In addition, we have an established protocol in place for the generation of antibodies to difficult targets that works well in mice.

In addition to mice, a smaller number of adult rats will be used. These animals will be used to investigate if our immunisation technologies will give diverse,high-quality monoclonal antibodies in additional species. An advantage of using rats is the ability to generate a mouse reactive antibody, which is very difficult in mice. Having a mouse cross-reactive antibody means it can be tested for efficacy and pharmacodynamics in mouse models, which enables therapeutic development of the discovered antibody. Another advantage of using these larger animals is that more immune material will be generated from each animal, increasing the chance of finding rare antibodies. Rats are routinely used in other published immunisation protocols for generation of monoclonal antibodies.

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

During the immunisation experiments, the animals will be injected with preparations designed to stimulate an immune response, similar to the administration of a vaccine to a human patient. Blood samples will be taken at intervals, typically once a month, to allow us to assess the level of antibody production. At the end of the experiment, under terminal anaesthesia a larger blood sample will be taken before the animal is humanely killed.

Genetically altered mice will be bred which is not expected to cause any adverse effects. The genetic modification of these strains is not expected to cause any adverse effects that are more than mild and transient. Typically, animals will spend 4 months on the immunisation protocol.

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

The adverse events of the immunisation are expected to be similar to those expected after human vaccination, as a result of the immune response: some local soreness and some systemic effects which cause temporary discomfort. The animals may show adverse effects by having hunched posture or ruffled fur. Typically, these signs of ill health will start to resolve in 24 hours and fully resolve in 48 hours, however if animals are still showing symptoms 72 hours after immunisation they will be humanely killed.

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

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

Severity	Mouse	Rat
Moderate	10%	10%
Mild	70%	90%
Sub-threshold	20%	0%

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

- Killed

Replacement

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

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

We need to use animal models to generate antibodies in response to immunisation. This process happens in lymphoid tissue that are very complex structures with many different cell types interacting and communicating with each other. In addition, these immune cells are in constant movement that allows them to interact with different partners at different stages of their development. These processes are so complex that currently no in vitro system is able to replicate this.

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

There are some methods that do not involve live animal injection for discovering monoclonal antibodies, such as synthetic libraries. These libraries consist of 10s of billions of antibodies which could potentially bind to the target of interest. We work

with colleagues who are working on the challenge of using these libraries for discovery of antibodies which target proteins that sit in cell membranes.

Characterisation of function of antibodies will primarily be performed in vitro. We have a plethora of cell-based assays which can be performed which inform the characteristics of the antibodies produced from this work. We are able to test the binding affinities, the functional interaction with the drug target and even replicate some biological process using human blood.

Why were they not suitable?

These alternative methods have not been successful for working with target proteins that sit in cell membranes and are challenging to work with. We have worked with well-characterised and validated libraries of antibodies, however they have not generated any antibodies which have 'drug-like properties' and can be developed as therapeutics.

We have shown that our technology is superior to all alternative methods in benchmarked studies, particularly when working with membrane proteins.

Reduction

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

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

We have estimated the number of animals to be used based on previous work and records as well as published data. We will typically use groups of 10 animals.

We will start by performing an exploratory study for monitoring of the immune response. This analysis will inform which regimens and doses are effective, but also inform group sizes that will be sufficient for discovery of 100s of mAbs. Typically, we can isolate 6-8 distinct antibodies from each mouse, therefore around 150 mice are required in total to generate enough antibodies to produce a therapeutic candidate.

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

We are continuously refining/improving our antibody isolation methods, meaning fewer animals are needed to isolate rare/potent antibody clones of interest. For example, we now take more immune tissues from animals and cryopreserve them in smaller volumes and more vials, which allows more downstream in vitro experiments per animal. We are also deploying next-generation sequencing technologies to extract the maximum immune diversity from each animal.

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

When working with any new target protein we perform in vitro characterization to confirm proper target behaviour prior to commencing any in vivo work. We always perform pilot studies to ensure that the immunisation method we are using is optimised for the specific target protein. This means that when we start a large-scale immunisation campaign, we are confident that our immunisation method will work well. We are also aiming to develop our technology which already works with mice to work with rats. This will enable us to discover antibodies that recognise the mouse version of the target. Because this species is larger, we will be able to use fewer of them to discover the same number of lead monoclonal antibody medicines, without increasing the severity. Using rats will also allow us to discover rarer antibodies that are not present in mice.

We will perform multiple techniques to isolate antibodies from our animal-derived immune material. We cryopreserve immune material from each individual animal, which supports a range of different discovery techniques. This will increase the number of antibodies that we can discover from a single animal, and therefore reduce the number of animals required to isolate antibodies for therapeutic development.

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.

Refinement

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

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

Mice and rats are the least sentient mammalian model that mimics the human immune system. Most of the stages in monoclonal antibody medicine discovery that come downstream of the animal immunisation step are best optimised for mouse antibodies, which means that there is a higher likelihood of successfully developing a new treatment. There is also a variety of genetically altered mice that potentially deliver an increased rate of antibody discovery, reducing the number of individual animals required.

In addition to mice, we will use a smaller number of rats. Rats are the most refined choice of animal model for using as an alternative to mice. Both are well-

documented model species for antibody discovery, with well-established methodologies for antibody isolation which maximises the chance of success with working with these species.

The vast majority of animals will only experience mild severity levels from the experiments. However a minority may experience moderate severity due to the nature of the immunisation material.

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

Non-mammalian animals are limited in their use because they either do not have the right type of immune cell or their immune system is too different from the human immune system to provide relevant results. We can't use embryos or very young animals as their immune system is immature and doesn't respond to antigenic stimulation in the way mature animals do. We will use mice and rats as these are the least sentient species that will be able to meet the scientific aims.

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

Some animals experience moderate adverse events after the injections. We have an increased monitoring schedule after injections to ensure that we spot any adverse events as soon as possible and will humanely kill the animals where the side effects are not improving in the first 24 hours after immunisation.

If weight loss in some animals becomes apparent the animals will be weighed regularly and monitoring of physical conditions will increase. If animals reach 15% weight loss they are humanely killed.

When injecting substances, we use refined routes of injections and where appropriate to reduce distress, we use anaesthetics. We will follow the LASA guidelines for dose volumes and frequency of administrations.

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

All researchers will regularly review websites with 3Rs information to monitor for potential refinements including:

www.nc3rs.org.uk https://norecopa.no https://www.lasa.co.uk

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

We will use local and national resources:

- All researchers will attend regular establishment/internal meetings where best practice on the 3Rs is shared.

- All researchers will regularly review websites with 3Rs information, including www.nc3rs.org.uk and https://science.rspca.org.uk.

21. Understanding and modulating tissue inflammation, repair and regeneration

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Inflammation, Regeneration, Repair, Lung, Tissue Injury

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to understand the factors that control inflammation, tissue repair and regeneration during homeostasis and after organ and tissue injury. We know that aberrant inflammation as well as defective tissue repair and regeneration is a frequent outcome in human disease states, but the factors controlling inflammation, repair and regeneration remain poorly understood with no current treatments available for humans that target tissue repair/regeneration pathways.

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

Why is it important to undertake this work?

Our primary focus will be the lung, where we know that inflammation impacts upon both lung health and disease states, and where defective tissue regeneration and repair (including excessive fibrosis) are frequent outcomes in disease states in humans. Lung disease remains an urgent health challenge and the third leading

cause of death; last year over 12 million people died from lung disease. Lungs are continually exposed to infections and toxins, which frequently cause tissue damage. This tissue damage compromises lung function and occurs in fatal lung injury. For humans that develop the most serious form of acute lung injury (known as acute respiratory distress syndrome) this carries a 50% mortality, and no effective treatments exist beyond supportive care.

In response to tissue damage lung inflammation is triggered, which needs to be tightly coordinated to protect the host from potential invading pathogens, but also limit any exacerbation of tissue damage. In tandem, it is vital that the lung can efficiently regenerate and repair itself to ensure optimum functioning and protect against morbidity and mortality. But uncontrolled unresolved inflammation and defective tissue repair and regeneration are frequently seen in human lung diseases and no existing treatments promote lung regeneration. Understanding the processes of how inflammation is coordinated and regulated and how the lung naturally regenerates after injury is critical to help develop new treatment approaches that promote lung regeneration and repair after serious infection or damage.

Excitingly, we have shown that inflammation and tissue repair and regeneration are not separate independent processes, but that the inflammatory response is critical for successful tissue repair and regeneration and that timely repair and regeneration is key to preventing excessive inflammatory processes. Tissue inflammatory cells frequently increase in numbers in response to tissue injury and we have recently demonstrated that increased inflammatory cell numbers (especially macrophages) are critical to promote successful regeneration of lung epithelium after injury. But the mechanisms of how inflammation, including changes to inflammatory cell numbers, are controlled in response to injury, and how inflammation and tissue repair and regeneration pathways are coordinated remain extremely poorly understood. This work will identify new mechanisms of inflammation, tissue repair and regeneration which together will help to identify new molecules and processes that promote tissue recovery and successful regeneration after injury.

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

Outputs from this programme of work include:

1. New datasets, which will be available to other researchers in both academic and industry settings, investigating inflammatory and tissue repair and regeneration processes. This includes not just lung focused researchers but a myriad of other researchers investigation tissue injury in numerous other organs and contexts.

2. New understanding of the mechanisms that govern inflammation and tissue repair and regeneration to allow tissue/organ recovery after injury. This includes the identification of methods and molecules to modulate tissue inflammation, repair and regeneration which will be essential to identify potential therapeutic approaches to treat some of the most serious human diseases.

3. Publications arising from this work that detail our novel findings, techniques and methodology.

4. Using our new knowledge to drive patient and public involvement in research.

5. Additional research funding based upon our findings.

6. Presentations in national and international congresses.

7. Supporting student research projects.

8. Extend and develop national and international collaborations.

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

As lung disease accounts for 1 in 5 deaths globally and severe lung injury has been the leading global health emergency of this decade, this programme of discovery science has real potential to advance knowledge in an area of unmet human need. Our ultimate goal is to identify potential therapeutic targets to modulate inflammation and promote lung regeneration and successful repair. Therefore, there is potential for broad academic and industry benefit and alignment across several sectors.

1. Research scientists: The main academic beneficiaries will be those working in the fields of inflammation biology and tissue repair and regeneration. Knowledge on inflammatory processes and inflammatory cell functions and the recovery from tissue injury is relevant to a myriad of researchers in inflammatory and traumatic diseases. Immunologists and inflammatory cell researchers will be able to access our datasets for further analysis, looking to identify new and conserved molecules involved in inflammation, tissue injury and regeneration. Researchers in translational chemistry and biomedical imaging will be interested in the targets and pathways we will identify, which have potential to aid early detection and impact upon precision medicine in organ injury and recovery. Our fundamental discovery science programme will be translated into respiratory research as well as allied fields, further impacting on ways to treat and prevent disease and to advance the health of people worldwide.

2: Patients and clinicians: Understanding mechanisms of successful or failed organ regeneration and inflammation may identify biomarkers which allow prevention and early detection of disease states.

Ultimately our goal is to use our mechanistic knowledge to develop advanced therapies in organ/tissue injury, a current unmet need in human disease states. These therapeutic targets for clinical use will also have utility in a number of other human diseases characterised by severe inflammation, organ injury or failed tissue regeneration.

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

1. Publish our findings in open access journals to ensure those working in academic, clinical, charity-based and pharmaceutical organisations can access my work.

2. Continue to present our findings at scientific meetings and conferences (local, national and international), to allow early dissemination of our findings as well as engagement with our research community.

3. Continue to be supported by the University's Press and Public Relations office to promote and procure media coverage and press releases to ensure maximum dissemination of our research activities and new findings.

4. Continue with our public and patient engagement activities to communicate our research and experiences to a broad audience.

Species and numbers of animals expected to be used

- Mice: 7625

Predicted harms

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

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

Use of tissue from, and experiments performed on, genetically modified mice will allow investigation of mechanisms of inflammation and tissue repair and regeneration. These experiments cannot be performed on living humans. Existing knockout and transgenic mouse lines will allow testing of specific mechanistic hypotheses, and as the major organ of interest is the lung, a species with lungs is required for many of our experiments. Pre-existing expertise and refinement of our models of mouse lung injury and infection will be used to study inflammation, tissue repair and regeneration. Adult mice are therefore the relevant species to be used for most of our in vivo work. As inflammation and tissue repair/regeneration requires the coordinated input of multiple cell types, this cannot be accurately recapitulated in vitro, and in vivo experimentation is necessary to meet our aims.

Human tissue (including blood derived inflammatory cells, and lung cells collected during procedures and following surgical resection of the lung) will continue to be utilised to replace animal experiments wherever possible.

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

Mice:

1. Mice will be used for breeding and colony maintenance

2. Mice will often receive treatment(s) to alter a gene or cell type. This may result in impacts on inflammation, tissue repair and/or regeneration.

3. Mice will usually receive a treatment to modify the inflammatory response or to impact tissue repair or regeneration.

4. Mice will occasionally have modulation of inflammatory cells by irradiation and adoptive transfer of bone marrow, or by adoptive transfer of cells (for example administered intraperitoneally, intravenously or into the lungs) with/without prior cell depletion.

5. Mice will usually receive a treatment to induce lung injury. In some mice this will be by administration of an infection.

6. Prior to the end of the study, some mice will receive treatment(s) to allow us to assess inflammation, tissue repair and/or regeneration.

7. Animals will be humanely killed at the end of an experiment, or if a humane endpoint is reached.

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

No adverse or harmful phenotypes are expected from breeding and maintenance of genetically modified mice. Mice will be monitored for ill health and will be humanely killed if a humane endpoint is reached.

We expect that most treatments to alter a gene or cell type, or to modify the inflammatory response, will result in minimal and transient discomfort only.

Mice will take a little time to recover from anaesthesia; mice will be monitored for signs of distress or ill health until recovered from anaesthesia. Mice will be humanely killed if a humane endpoint is reached.

For mice receiving whole body irradiation and adoptive transfer of bone marrow, damage is mainly restricted to the hematopoietic system and intestinal mucosa. Radiation sickness could develop but in our experience this occurs in less than 2% of mice. Mice receiving whole body irradiation are also at transient risk of opportunistic infection. With chemotherapeutic agents such as busulfan, the risk of mucosal damage and infection is reported to be lower than irradiation, although mice may experience transient weight loss of 5-10%. Mice that receive a sufficient number of haematopoietic cells to effect complete engraftment are not expected to show anything more than mild symptoms. Mice that receive partial body irradiation are not expected to experience adverse effects from irradiation, but will experience mild discomfort from the injectable anaesthesia.

Following induction of lung injury/inflammation, weight loss and a transient reduction in animal activity is common. From experience, persisting respiratory distress is infrequent in these models. Mice will be monitored for ill health (standardised scoring and humane end pointe detailed in protocol details) and will be humanely killed if a humane endpoint is reached.

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

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

All animals used for breeding and maintenance of genetically modified animals are expected to be of a mild severity.

The majority of animals (90%) undergoing lung injury/inflammation are expected to be of moderate severity; the remaining 10% are expected to be of a mild severity.

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

- Killed
- Used in other projects

Replacement

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

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

Our primary aim is to understand the mechanisms controlling tissue inflammation and how tissues can regenerate and repair after injury. As these processes rely on many interacting cell types, in vitro systems do not yet allow reproduction of the complex in vivo environment of the lung which is in intimate communication with the systemic circulation. Animals are therefore necessary to achieve our aims.

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

1. Ex vivo systems, including organoids and living airways

2. Experiments on human tissues and cells

Why were they not suitable?

1. Organoids, which are soaked in culture media, do not allow us to accurately replicate lung epithelium, which have polarity with air on one side. The ability to reliably and accurately introduce and incorporate inflammatory cells to study cellular interactions is not possible. Living airways allow the reductionist study of epithelium and one immune cell type, and this is a biological system that we have established within our laboratory to allow replacement of animals to answer very specific questions. However, tissue injury/inflammation and the reparative and regenerative response in a whole organ involves a multitude of cell types interacting with each other. This cannot be accurately recapitulated in this system.

2. Human tissue samples are routinely used within our group, but only allow a ‘snapshot’ of tissues/cells at a particular timepoint. Humans with lung injury are extremely sick and therefore sampling of lung tissue is exceptionally dangerous and not ethical.

Reduction

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

These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

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

Numbers have been calculated based on our work over the previous 5 years, and based upon our currently planned experiments over the next 5 years.

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

Experimental planning will be undertaken such as the use of the NC3Rs EDA (experimental design assistant) to ensure appropriate numbers of animals are used. This includes use of power calculations.

We have a number of technical approaches, such as separating the lung lobes for different types of tissue analysis, that allow us to maximise the use of tissue from each animal and limit the numbers of mice undergoing regulated procedures.

Group members will undergo research training, to ensure numbers of animals used are minimised.

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

Efficient breeding will be undertaken, assisted by use of our in house animal colony management software (tick@lab). Cryopreservation of lines will be undertaken after the use of a line is finished.

Where appropriate we will undertake pilot studies when investigating a new intervention in order to determine the most appropriate and meaningful parameters (for example dosing of drugs/compounds, selection of optimal timepoints for analysis etc.)

Tissue and data that we generate will be shared with the scientific community to maximise this resource. We will also make use of archived tissue wherever possible to reduce the need for repeat or multiple experiments.

Refinement

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

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

Use of tissue from, and experiments performed on, genetically modified mice will allow investigation of mechanisms of tissue inflammation, repair and regeneration; these experiments cannot be performed on living humans nor recapitulated in in vitro systems. As the primary tissue of interest is the lung epithelium, a species with lungs is required. Existing knockout and transgenic mouse lines (e.g.

Cre/LoxP lines and fluorescent reporter lines) will allow testing of specific mechanistic hypotheses. Pre-existing expertise in models of mouse lung injury will be used to model tissue inflammation, repair and regeneration in a meaningful way with relevance to human lung injury, but while minimising pain, suffering, distress, or lasting harm to the animals.

For drug/substance administration we will use the most appropriate route which causes the least animal distress/suffering.

All experiments necessitating direct intratracheal administration will be performed using a blunted catheter passed between the vocal cords via the mouth under direct transillumination of the thorax, thus obviating the requirement for the traditional approach of surgical exposure of the trachea. Where appropriate, substances will be administered into the lungs via indirect intratracheal administration (i.e. ‘oropharyngeal aspiration’) under transient isoflurane anaesthesia; this approach avoids both surgical exposure of the trachea and injectable anaesthesia. Recovery from transient isoflurane anaesthesia is extremely rapid (induction of anaesthesia, indirect intratracheal administration via oropharyngeal aspiration and recovery from anaesthesia happens in less than 10 minutes), and is much quicker than injectable anaesthesia approaches.

In some studies we will induce lung injury in mice to study, understand and modulate tissue inflammation, repair and regeneration. While any injury to the lung can have systemic consequences (as is observed in humans as well as mice), such as weight loss and illness behaviour with reduced activity, many of our models have already been refined to minimise adverse effects and cause the least pain, suffering, distress or lasting harm while still generating meaningful biological readouts of relevance to understand human lung injury.

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

To allow us to fully understand tissue inflammation, repair and regeneration in the lung this requires use of adult mice with fully developed lungs. Inflammation, tissue repair and regeneration is known to be altered in earlier life stages. The inflammatory response and tissue repair and regeneration often occurs over days-weeks; therefore studying terminally anaesthetised animals for a few hours will not allow us to answer our aims.

Sometimes specific mechanistic questions on inflammatory cells and tissue repair and regeneration can be answered in models of tissue repair and regeneration.

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

Animals will be routinely checked while on a study to assess for pain or discomfort. Animals will be humanely killed if any health issues arise that cannot be immediately treated. Our monitoring strategy includes standardised scoring sheets (detailed in protocols).

Where possible tissue specific gene or cell manipulations will be undertaken, to minimise the possibility of whole animal genetic/cellular manipulations that could have unexpected harmful effects.

For drug/substance administration we will use the most appropriate route which causes the least animal distress/suffering.

All experiments necessitating direct intratracheal administration will be performed using a blunted catheter passed between the vocal cords via the mouth under direct transillumination of the thorax, thus obviating the requirement for the traditional approach of surgical exposure of the trachea. Where appropriate, substances will be administered into the lungs via indirect intratracheal administration (i.e. 'oropharyngeal aspiration') under transient general anaesthesia; recovery is extremely rapid (induction of anaesthesia, intratracheal administration and recovery from anaesthesia happens in less than 10 minutes) and is well tolerated.

For animals undergoing lung injury protocols, we will administer supplemental fluids during periods of anorexia and weight loss to minimise dehydration and maximise well-being (for example, naphthalene lung injury causes anorexia and weight loss over the first 2-3 days before rapid recovery; supplemental fluids will therefore be administered e.g. for the first 2 days).

All animals will have environmental enrichment and will be co-housed (unless e.g. fighting/aggression prevent this) to allow normal behaviours.

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

Wherever possible we will use published best practice guidelines in our experiments. Standard approaches (including standard operating procedures - SOPs) and rigorous training of team members will ensure experiments are conducted in the most refined way.

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

We will continue to receive and engage with the 3Rs newsletter as well as our Institute's communications and roadshows. We will continue to adopt newer techniques and/or approaches that allow us to minimise pain, suffering, distress, or lasting harm to the animals while still allowing delivery of our project aims.

22. Creation, production, maintenance and supply of genetically altered mice

Project duration

5 years 0 months

Project purpose

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

Key words

Production, creation, breeding, rederive

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This project aims to provide a service to create, breed, maintain and supply GA mice for research teams primarily within our establishment but also researchers in other establishments.

We plan to rederive animals using either surgical or non surgical methods into Biological services facilities.

This includes breeding of GA lines to support rederivation work and the holding of transgenic colonies required within the establishment.

Potential benefits likely to derive from the project, for example how science

might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

To enable researchers to have a service that can create new transgenic lines which assist in their research or transfer animals from a lower health status to a higher health status or to enable embryos to be imported and implanted rather than live animals being shipped from abroad. Which protects our existing colonies from outside pathogens being introduced to the BSU facilities.

We also plan to provide a breeding service for GA animals for production and/or supply eg. NSG, Prm1,Cre and many other current and future GA lines.

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

To provide researchers the facility to generate/establish/develop new GA lines that mimic human conditions and also to enable researchers to improve the health status of existing lines.

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

Providing our researchers with a centralised service license which will be able to perform the protocols described, ensures the use of skilled, highly trained staff and facilities for a local dedicated and standardised creation, breeding and supply of animals that are healthy. This reduces the number of animals used and reduces the duplication of lines.

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

This project will reduce the number of animals used and reduces the duplication of lines by the sharing of colonies within UCL and the wider research community.

Species and numbers of animals expected to be used.

- Mice: 101,000

Predicted harms

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

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

GA mice have made significant contributions to biomedical research. However the function of many genes is still not known or is not fully understood, either individually or in the ways they interact to produce their intended effects, or how they go wrong in

disease processes. The use of animal models is necessary to determine these processes and to find new treatments for human diseases which are too complex to be modelled in lab or computer based systems.

All stages of life will be required to create and maintain breeding colonies.

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

We will be breeding genetically sterile males, these will then to be used to mate with wild type females to supply pseudo pregnant females for embryos imported from other locations or embryos that have been genetically altered to be implanted into by surgical or non surgical routes and allowed to develop and result in live births of genetically modified animals to create new genetically modified lines at our establishment.

We will also use wild type female mice, which will undergo Hormone injections to provide eggs to be microinjected with sperm that has been imported or to be used to create embryos that have been genetically altered these will then be implanted into pseudo pregnant wild type females and allowed to develop and result in live births to create new genetically modified lines at our establishment.

All offspring resulting from above methods will be biopsied to allow for genotyping of the tissue to determine genotype to animals, those required genetically modified animals will then be bred and/or held under this project as required.

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

Transient pain or discomfort when receiving Intraperitoneal hormone injections.

Mild or moderate pain or discomfort post surgical implantation Transient Pain or discomfort associated with ear biopsy.

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

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

98% of these animals would be expected to experience mild severity.
2% of these animals would be expected to experience moderate severity.

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

- Killed
- Kept alive
- Used in other projects

Replacement

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

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

This service PPL by its nature requires the use of animals, and will result in GA animals being made available for use in most of the PPLs used across the establishment, for which the benefits are clearly described within each PPL and will be published via the scientific groups holding these PPLs.

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

This project cannot be achieved without animal use.

Why were they not suitable?

This project cannot be achieved without animal use.

Reduction

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

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

This number has been estimated by looking at numbers used in the previous service licence.

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

As this is a service licence we cannot use statistics to plan/reduce numbers, but we strive to use the minimal amount of animals necessary to provide sufficient offspring to supply researchers. Colonies.

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

The generation, breeding and supply of mice is unquestionably appropriate. Animals will only be bred once a user requirement has been established and agreed. The breeding programme will be subject to regular review to optimally meet anticipated demand.

Numbers will be kept to a minimum by use of in house colony management protocols and training staff to high standards.

Database tools to allow the tracking of animals and any adverse effects accurately, which allows us to tailor demand and reduce overproduction of animals and monitor potential harmful phenotypes.

Through centralising the supply of animals, over production and duplication can be reduced. The employment of specialist staff skilled in the breeding and maintenance of GA animals and transgenic technologies, yields a high rate of management success.

Cryopreservation is used as a method to reduce the number of live animals maintained by archiving lines.

Refinement

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

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

Mice models will be used and subjected to the following:

Superovulation (hormone injections IP)
Surgical and non surgical implantation of embryos Breeding
Ear biopsy

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

This service PPL by its nature requires the use of animals, and will result in GA animals being made produced for research under other projects.

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

To remove the need to perform vasectomy of males we have sourced a genetically sterile male strain which we breed internally to supply males for this project.

When applicable we use non surgical implantation of embryos
We also continue to monitor above sources for ways to implement the 3R`s Aseptic technique will always be used when performing surgery.

Pain relief given as advised by NVS.

Use of importation mouse passport with relevant information of strains phenotype

and specialist breeding needs.

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

Generation and breeding of genetically altered mice (NC3R`s) https://nc3rs.org.uk/3rs-resources/breeding-and-colony-management/colony-management-best- practice.

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

We will stay informed of advances in 3R`s initiatives by interaction with the named information officer, AWERB and 3R`s committee and by attendance at relevant seminars and courses in addition reading up to date literature regarding this field of work.

Regular interaction with NVS, local BSU NACWO along with BSU technicians will help us by advising on the care of our animals and the implementation any of improvements that can be made.

23. Evaluation of innovative, miniature medical devices for improving current diagnostic and interventional medical procedures

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Gastrointestinal endoscopy, Abdominal surgery, Bronchoscopy, Robotic endoscopy, Robotic surgery

Animal types	Life stages
Pigs	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to evaluate feasibility (that is the quality of being doable) and safety of innovative small medical robotic devices for inspection of the digestive tract (gastrointestinal endoscopy) or the bronchial tree (that is the airway providing passage of air to the lungs), or for abdominal surgery (that is surgery in the area of the belly). The following will be answered: Is the concept feasible? What is the most effective design? Is the concept safe and suitable for transitioning to redesign towards clinical use?

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

Why is it important to undertake this work?

The novel devices developed in this project will allow diagnostic and therapeutic functionalities (such as visualization, disease detection, tissue collection, tissue removal) in areas that are difficult or impossible to reach with current medical instruments (such as the peripheral areas of the lungs, the pancreas or the small bowel). These novel devices are related to high impact, major clinical needs with millions of individuals potentially benefiting from the successful implementation of the technologies. For example, bowel cancer survival rates could be drastically improved with the adoption of our robotic system which could provide an efficient, easy-to-use, painless alternative to the more invasive conventional procedure. Wider groups (e.g., the NHS and the UK as a whole) could also benefit from significant economic savings, particularly with the successful adoption of low-cost concepts.

The work will be disseminated in peer reviewed journals and conferences, with the publications being relevant to actively researched areas in medicine and robotics.

This will provide valuable knowledge to the wider research community.

The work focuses on high impact, clinically relevant areas where there is still significant scope for innovation. Therefore, the technologies developed have a tangible and significant need, ensuring that support is readily available to push the innovations to clinical use. The extensive facilities, technical expertise and previous research track record of the group will ensure maximum success rate.

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

The intended outputs from this work are novel designs of medical technologies, as well as methods of operating and testing them. These will be disseminated primarily in peer reviewed journals, conferences and in filed patents. Publications will be relevant to actively researched areas in medicine and robotics, providing valuable knowledge to the wider research community.

Other potential outputs will be test data required for pre-clinical assessment of these devices, paving the way for future studies to pursue regulatory approval and spin-out companies to translate the technology to market. Regulatory approval is beyond the scope of this project.

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

The innovations developed in this project are related to high impact, significant clinical needs (e.g. early cancer diagnosis and treatment) with millions of individuals potentially benefiting from the successful implementation of the technologies. Wider groups (e.g. the NHS and the UK as a whole) could benefit from major economic savings, particularly with the successful adoption of the low-cost concepts being studied.

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

We will maximise the output of this work via publications that will benefit the research

community throughout the lifespan of this project.

We also plan to patent and translate medical technologies showing promising results in pre-clinical trials. If successful, our technologies may have an impact on patients within the next 5 – 10 years (i.e. 5 for the already established technologies and 10 for novel concepts generated).

Species and numbers of animals expected to be used.

- Pigs: 120

Predicted harms

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

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

The adult pig model is widely accepted as a suitable replacement for the in-vivo adult human model for abdominal surgery, bronchoscopy (that is the inspection of the lungs via a flexible probe with a camera on its tip introduced by the mouth of the patient) and gastrointestinal endoscopy (that is the inspection of the stomach or the bowel via a flexible probe with a camera on its tip introduced either by the mouth or the anus of the patient) because of its comparable size, tissue properties and, with the exception of the large intestine (spiral part of the colon), anatomy.

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

Animals undergoing gastrointestinal endoscopy (that is the inspection of the stomach or the bowel via a flexible probe with a camera on its tip introduced either by the mouth or the anus of the animal) will be prepared according to existing literature on feeding management before studies performed in the gastrointestinal tract. In the seven days preceding planned procedures, animals will be socially housed without straw or bedding, but will be provided with supplemental heat and additional enrichment items. Social housing will be adopted whenever possible. High-energy liquid diet will be introduced during this period and in the 36-48hrs prior to procedures, animals will be maintained on liquid diet only.

On the day of the procedure, anaesthesia will be induced and maintained by qualified staff. Any animal showing anaesthetic complications that jeopardise animal welfare or scientific aims will be killed immediately by schedule 1 method (anaesthetic overdose).

Animals undergoing colonoscopy (that is the inspection of the colon via a flexible probe with a camera on its tip introduced by the anus of the animal) may be administered an enema (that is an injection of a liquid through the anus to stimulate evacuation) if prior feeding management has not sufficiently cleared the lower bowel.

When testing small medical devices in the gastrointestinal tract, the lungs and the

abdominal cavity, we will use non-invasive or minimal invasive techniques. When performing surgical procedures, they will be carried out under aseptic conditions. During laparotomy (that is a cut into the abdominal cavity), haemostasis (that is the mechanism that leads to cessation of bleeding from a blood vessel) will be achieved with electrocautery (that is a method of using electricity to apply heat to tissue), ligation (that is the surgical process of tying up a blood vessel) and/or pressure.

During procedures in the lungs or the gastrointestinal tract, haemostasis will be achieved with electrocautery.

Animals may be used to test more than one medical device in one session, although time under general anesthesia will be limited to 6 hours. On conclusion of the procedure, an aesthetised animals will be killed by a Schedule 1 method (anesthetic overdose).

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

No adverse effects were identified in literature in relation to the high-energy liquid diet prior the procedure.

Animals will be at risk of the usual complications of general an esthesia, namely cardiorespiratory depression (that is the slowing of breathing and heart rate) and malignant hyperthermia (that is a condition where the animal develops high body temperature that cannot be controlled). The entire procedure may take up to 6 hours during which intravenous fluids (fluids injected into the veins) will be administered routinely. Potential risk of hypothermia (that is low body temperature) will be reduced by providing supplementary heat during the period of anesthesia. Any animal showing anesthetic complications that jeopardise animal welfare or scientific aims will be killed immediately by schedule 1 method (anesthetic overdose).

In our experience, enema to clean the colon in an aesthetised animal does not cause any adverse effects.

As all procedures will be carried out under terminal anesthesia (that is anesthesia from which the animal does not recover consciousness before death), we do not expect any adverse effects during manipulation of small medical devices by non-invasive or minimal invasive techniques.

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

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

Non-recovery (100%)

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

- Killed

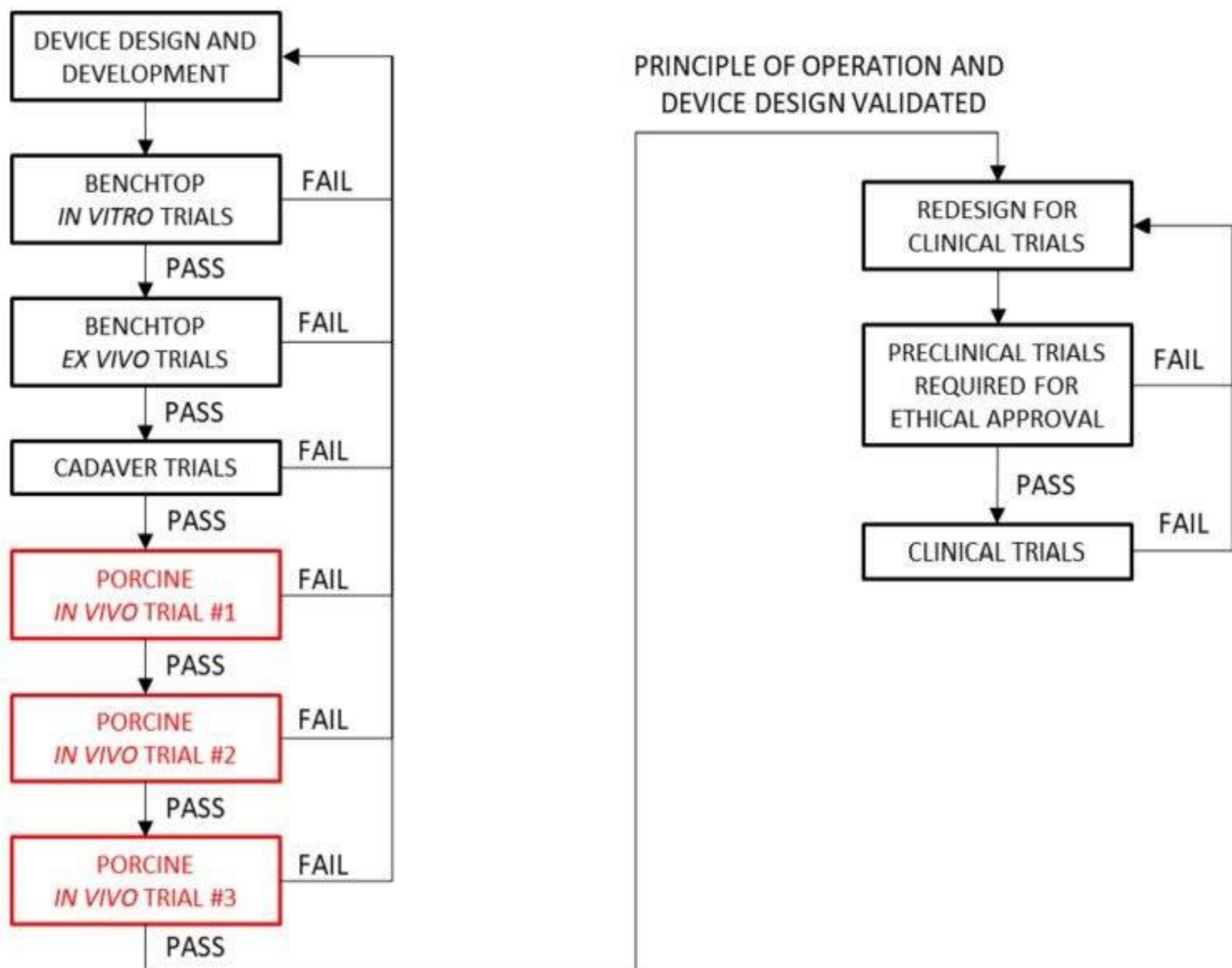
Replacement

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

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

In order to design appropriate medical technologies and evaluate their safety, an environment as close to that of the in-vivo (that means living) human is required. In-vivo conditions are necessary to fully assess safety and functionality of the device, including those impossible to recreate with simulators on the bench-top. Testing in an animal model facilitates the refinement of the device design, and specifically the reduction/mitigation of risks associated with its in-vivo use.

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



Flow diagram of the design and validation. The current project license is intended to cover the blocks in red.

To completely evaluate a medical device without testing in-human, the anatomy and the physiological properties of the environment in use should be realistic. Referring to

the flow diagram reported above, the devices/technologies in this project will be developed first in the laboratory using synthetic environments (e.g., silicone) and where possible, in simulation.

Next, ex-vivo animal tissue (that is taken from a dead animal) will be used in the laboratory (benchtop trial) to explore the effect the device had on tissue.

The subsequent increment in testing requires a more realistic environment. Human embalmed cadavers are available and are utilised where necessary as they provide a realistic, human anatomy. However, the tissue properties are fundamentally different than the in-vivo model.

The final and most realistic model to assess feasibility – especially to validate safety and functionality in a physiologically relevant environment – is a large, in-vivo animal model. Pigs are often used because of their similar size and tissue properties to humans.

Why were they not suitable?

The devices in this project are designed to interact with the living soft tissues and hence their properties (i.e., temperature, humidity, tissue compliance/stiffness, motions due to respiration, potential for bleeding, physiological response) are crucial. To-date, no synthetic alternatives are available that can completely recreate the in-vivo conditions found in a human because of the immense complexity.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 this document is being written, we already have more than five different technologies that are in need of feasibility testing in animals. Looking forward, we envisage that, in the next five years, we will be developing more devices that will reach the animal testing stage. This is based on our previous experience (i.e. in the last five years, I have used one non-survival experimental session with one animal every month and I expect that the volume will be the same) and takes into account the fact that this project is aimed at pilot feasibility trials. Therefore, data analysis will be descriptive and graphical. No formal statistical comparison will be made.

At this stage and based on previous experience and on the fact that each device/technology needs to show success in three consecutive animal trials, we plan about one non-survival animal experiment per month. To guarantee social housing, we plan two animals per experiment, leading to a total number of 120 animals for the five year duration of this project license. This is the maximum number required to

ensure the effective, safe and ethical development of our technologies.
What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Synthetic test environments will always be used extensively first and, where possible, in-vivo work will be avoided completely. This benchtop work will be used to answer fundamental questions and to refine the concepts as much as possible. Once operating with satisfactory performance and achieving full functionality, the devices will be tested in ex-vivo environments (e.g., excised porcine tissue). This will provide further insight and time for concept refinement. Once the concept development has passed these stages, in-vivo animal models will be used only where is completely necessary for the assessment of device safety and efficacy.

Typical quantitative outcomes that will be used to assess feasibility and safety during in vivo trials include workspace of motion, time to complete the procedure, ability to complete the diagnostic/interventional task, safety of operation as demonstrated by post-mortem visual inspection and histological analysis. All these quantitative outcomes will be benchmarked against conventional techniques. Additional success criteria will be defined in relation to the specific task that the device/technology is set to achieve. As highlighted in the flow diagram attached earlier, each design/technology will successfully clear this stage if positive results are obtained in at least three consecutive in vivo porcine experiments.

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

Whenever possible, multiple devices will be tested on the same, single animal and thus make most efficient use of the available resources. If the testing required is minor, i.e. requiring only a short duration and/or very basic data collection, where possible the testing will be postponed until additional work can be added to the tests (e.g. more thorough tests and/or the testing of an additional device).

In case we do not have at least 2 different devices/technologies to try on a single animal, the experiment will be postponed to next month in order to reduce the number of animal used.

Unused tissues will be offered to colleagues for use in other projects.

Refinement

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

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

The pig model is widely accepted as a suitable replacement for the in-vivo human model for abdominal surgery, bronchoscopy, and gastrointestinal endoscopy because of its comparable size, tissue properties and, with the exception of the large intestine (spiral part of the colon), anatomy.

All devices/technologies will be tested on benchtop prior to animal work. This will ensure the in vivo work in terminally anesthetised animals is done with minimal risk/harm to both the operators and animals.

Planned pre-endoscopy feeding management requires the removal of usual husbandry items (eg. straw/bedding/pelleted diet) for a limited time period. This is required to reduce the risk of debris affecting visibility within the gastrointestinal tract that could impact success of device testing. In order to mitigate any distress that this may cause, animals will be provided with additional enrichment items to ensure comfort and reduce boredom (e.g. Heat lamp, dog toys, traffic cones). Rubber matting will remain where possible to improve comfort for the animals.

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

All testing will be performed under terminal anaesthesia. The pig model is widely accepted as the most suitable replacement for the living human model for abdominal surgery and interventions relating to the digestive tract and the respiratory system (Ortiz-Fernández-Sordo J, et al 2011; Adolfo Parra-Blanco, et al 2012). This is because of its comparable size, tissue properties and - with the exception of the large bowel - anatomy.

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

With experience coming from in vivo animal trials, the device and the procedure will be continuously refined. All testing will be performed under terminal anesthesia.

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

I will use the resources available in the NC3Rs website (Resource Library section) and monitor relevant peer-reviewed journals on the topic, including Laboratory Animals Limited.

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

I will be monitoring the researcher newsletter and the establishment intranet for updates in advances in 3Rs. I will also monitor the NC3Rs website and relevant scientific literature as I have been doing for my previous Project License.

24. Gene function in musculoskeletal system formation, homeostasis and disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Limb birth defects, diseases of the musculoskeletal system, muscle repair, ageing of the musculoskeletal system

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to understand the normal processes that control formation of the muscles and bones of the limbs and a healthy musculoskeletal system throughout life and how disruption of these processes can lead to limb birth defect and diseases affecting the limb musculoskeletal system.

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

Why is it important to undertake this work?

Limb birth defects are common and diseases affecting the limbs are a common feature of trauma injuries and aged-related disease. Properly functioning limbs (arms and legs) are critical for everyday life and the independence of an individual. Diseases that affect either formation of arms and legs or the normal functioning of

arms and legs during the life course have a significant clinical impact on affected individuals and their carers and represent a significant burden on healthcare budgets.

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

WE expect to make advances in our understanding of how limb tissues are generated during embryonic development and which events of embryonic limb development have been disrupted to cause certain limb birth defects. We will disseminate our results by publishing our work in open access scientific journals.

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

IN the short term the scientific community studying limb development and the origins of limb birth defects will benefit from the new knowledge will generate. In the longer term our work and the worker of the broader scientific community will lead to progress in treatments to better diagnose limb birth defects, improve strategies for prognosis, management and treatment of diseases affecting the limbs.

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

We will disseminate of results through open access publications and presentations at conferences.

We actively collaborate with surgeons who treat individuals affected by limb birth defects and together we look for ways we can use the knowledge we generate to best impact patient care.

Species and numbers of animals expected to be used.

- Mice: 5000

Predicted harms

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

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

We are using whole animal models to study diseases affecting the musculoskeletal (bone, muscle and tendon) system.

Much of our work focusses on embryonic mouse stages as we want to study, and subsequently understand, the processes and mechanisms that govern normal formation of musculoskeletal tissues and the origins of birth defects and other diseases affecting the formation of these structures. We study juvenile and adult stage mice to study the processes that regulate the maintenance of healthy tissue (tissue homeostasis), tissue repair and the effects of ageing on musculoskeletal

tissues. While some aspects of these biological events can be modelled to some extent using cell lines, there is still no ideal alternative to using the whole animal model to study musculoskeletal tissues and the effects of disease and ageing. We complement our whole animal model work with in vitro alternatives when this is possible and appropriate for our experimental objectives.

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

The majority of animals will be used for breeding and maintenance of lines. Some animals will be used in terminal procedures to harvest embryonic material or tissue samples. Some animals will receive injections or undergo procedures to deliver substances that can induce markers of particular cell types or tissues (eg fascia or the periosteal cells that cover bones) or disrupt the production of the protein products of specific genes of interest.

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

In the majority of cases, we do not expect an adverse effects on the adult mice we will maintain. In a minority of cases, we will be inducing effects on gestating embryos which will be harvested in a terminal procedure and the tissues subsequently analysed.

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

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

For the vast majority of animals, we will use the severity will be mild (we expect no adverse effects). In a minority of cases (<5%) of total animals, we will use injections or oral gavage to administer substances that are classed as moderate severity.

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

- Killed

Replacement

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

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

The whole animal model is the only alternative to study the formation, repair, homeostasis and ageing of the interconnected array of musculoskeletal.

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

We complement our whole animal work by using cells in 2D and 3D tissue culture experiment. We are also collaborating with mathematical modelers to build agent based modelling simulations of the biological phenomena we are studying and using the "real world" values we can measure from whole animal and tissue culture wet lab work to paramterise these models.

Why were they not suitable?

In vitro and in silico models are used to complement our whole animal model work but they are currently not suitable to fully replace whole animal work as we do not know enough about the complex processes the control tissue formation/repair/homeostasis and ageing and the interactions between tissues.

Reduction

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

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

The estimate is an upper limit based on experience of our animal usage and taking in to account the experiments we have planned for the next 5 years.

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

We have designed our experiments to use a sufficient number of animals to generate data that has the required statistical power to be scientifically robust.

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

We maintain stocks as compound homozygous stocks when possible and appropriate for our studies.

We carry out pilot studies prior to starting a larger full experiment and refine our experimental design accordingly.

We maximise the use of harvested embryonic material so that as much material is used is possible and practical.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe

the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

We will use the mouse model at embryonic, neonatal, juvenile and adult stages. The vast majority of our procedures will have no adverse effects on the animals. We harvest tissues only after sacrificing the animals following approved procedures. We will administer substances at doses that in the vast majority of cases has not adverse effects on the adult animal.

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

The bulk of the material we will harvest of analysis will be at embryonic stages.

We harvest tissues only after sacrificing the animals following approved procedures.

We are using the mouse model as it has a musculoskeletal system that is very similar to humans and there are powerful experimental tools available in this model system that enable us to refine our experimental procedures to target cells and tissue types of interest and thereby learn as much as possible that could be relevant to human health.

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

The bulk of our procedures are breeding and maintenance.

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

We regularly seek guidance from local BSU on best practice guidance and liaise with vets, if necessary

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

The local BSU keeps labs informed of relevant events though its monthly newsletter, including information about NC3Rs workshops etc.

We also visit the NC3Rs website.

Local BSU helps with implementation of any advances.

25. Improving oral iron supplementation in early life

Project duration

5 years 0 months

Project purpose

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

Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Iron supplementation, inflammation, iron uptake mechanisms, Piglet model for human infants, Microbiota

Animal types	Life stages
Pigs	neonate, juvenile

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The primary aim is to explore the efficacy of standard and novel oral iron supplements in piglets as models for human infants. This will be done under both healthy and inflammatory conditions, and sometimes in association with supplements where there is some evidence that they can improve iron absorption from the gut.

The project also aims to improve understanding of the mechanisms underlying supplementary iron absorption, which is currently lacking. This is to inform the development of novel iron supplements.

A further aim is to explore the longer-term impact of oral iron supplementation on gut microbiota, immune and metabolic development.

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

Why is it important to undertake this work?

Iron is an essential nutrient since it has vital roles in a wide range of key biochemical processes, such as oxygen transport. However, Iron deficiency is the most prevalent human micronutrient deficiency, disrupting the physiological development of millions of infants and children, and the detrimental effects of iron deficiency can last a lifetime.

Although iron supplementation is often shown to prevent and cure anaemia, there are many problems with different forms of supplementation ranging from diarrhoea, low compliance due to palatability issues, presence of digestive inhibitors in the diet and infection/inflammation which block the natural pathways for iron absorption, which is already highly inefficient. The more bioavailable forms of iron tend to have palatability issues, and those with better sensory profiles, are less soluble and therefore poorly absorbed. Ideally, the most bioavailable and safe iron compounds are selected for food fortification. But in practice, the selection is based on compounds that provoke the least or no sensory changes to the food they are added, thus jeopardizing absorption potential. There is no compound available today that combines the needed sensory and safety profile that is also highly absorbed in the presence of stressors from both the diet and host - and all for a cost that food manufacturers can easily pass on to consumers in low and middle-income countries (LMICs).

The program of work in this application will test novel iron supplements in the piglet, a highly translatable model species, in which we can generate inflammatory conditions very similar to those seen in many infants in LMICs. This is important since inflammation in children is common in (LMICs), where iron deficiency is prevenient, and is known to impede iron uptake from the gut.

There is some evidence that iron absorption can be significantly improved if given in combination with some pre- and/or probiotics which could result in changing iron to a more absorbable form. Some pre- and probiotics can also reduce inflammation, which could, in itself, increase iron uptake. The proposed work is important because it could provide further evidence for this, which is currently promising, but rather sparce. Improving iron uptake could mean reduced amounts of oral iron are used in supplements, which could improve the negative side effects associated with iron supplementation.

This work is important because it could offer solutions to the 'iron supplementation problem' and has huge potential for positive impact across LMICs. It is also highly relevant to the pig industry where iron deficiency can be problematic, but excess luminal iron could be contributing to post-weaning diarrhea, the primary welfare and

productivity issue in pig farming.

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

Increase understanding of iron up-take mechanisms in a piglet model of human infants.

Determine whether the novel plant-based heme iron is absorbed as well as currently used alternatives under normal and inflammatory conditions.

Determined the impact of the novel iron supplement on the composition and metabolic activity of the gut microbiota compared to other forms of iron supplementation.

4, Determination of how that immune and metabolic development is affected by the developing gut bacteria in piglets fed both standard and novel iron supplements.

Ultimately, demonstrate in a highly translatable *in vivo* model that the novel iron supplement is superior to currently available supplements in terms of absorption whilst reducing the detrimental effects on other parameters.

Due to our systems approach, this work is highly likely to generate novel information and lead to peer- reviewed publications. It will also generate data to inform the development of further grant applications. Furthermore, considerable physiological similarities between pigs and humans means that findings are more likely to translate into human healthcare than other non-primate models. We are working with the Bill and Melinda Gates Foundation and their partners to eventually use this novel plant-based heme product across LMIC. For this reason, the project has very high potential for significant impact.

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

Pigs and humans are physiologically very similar, so the results we obtain from these studies will benefit humans. Far more so than if this program of work was carried out using mice or rats. The major benefit will be seeing if our safe, more palatable, cost effective and novel plant-based heme iron supplement can reduce anemia more effectively than currently available alternatives. This will be relevant and beneficial for clinicians (especially pediatricians) and infant food development industries. Many human immunological diseases (autoimmunity, allergy) are being linked to problems with immunological development in early life, but our understanding is limited. Since immune development is driven largely by the gut microbiota, and due to its accessibility for modification, altering the gut microbiota in early life has the potential to reduce the risk of disease development. This program of work will inform the development of nutritional interventions to reduce the risk of immune-associated diseases in later life. Although this is long term, the benefits of this work could be societal as incidences of immunological-based diseases are increasing rapidly in modern society. There is no cure for many such conditions and so prevention is a valuable approach from which many 'at risk' individuals could benefit in the future.

Environmental enteric dysfunction (EED) is a considerable, but little understood,

condition which ultimately results in stunting and life-long health implications. There are currently no rodent models of EED, which has hampered the generation of knowledge of disease development and progression.

However, our EED pig model will start to address this issue, which will benefit infants and children in LMICs.

Academics will benefit from this work as it will help to unravel the complex interactions which occur between the gut microbiota, metabolism and immunity under normal conditions in our control animals. Gut microbiota research is expanding rapidly as it appears to be involved in many aspects of normal development. This will benefit human and animal medical and nutritional professionals.

Animal feed industries will also benefit from this work. We have already been asked to present finding from these studies to a large international animal feed producer (which manufactures our bespoke feed for our trials). We have also received materials in kind from several other animal feed producers and manufacturers which demonstrates their interest in our work and how they may benefit. Of major concern is the effects of abruptly weaned piglets being exposed to very high levels of iron in weaner feed at a time where they are highly vulnerable. This is because luminal iron impacts the gut microbiota and generates a hospitable environment for enteric pathogens.

In the pig industry, it is essential that piglets 'get off to a good start'. It is likely that information generated by our studies will benefit pig producers. Nutritional intervention in early-life could lead to increases in productivity and welfare within the industry, although this is a longer-term benefit as here we are proposing fundamental research.

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

In addition to serial blood and stool collections, once piglets have been killed, we will collect a variety of tissues and body fluids, for example duodenum, proximal and distal jejunum, colon, caecum, liver, mesenteric lymph nodes and urine, which will be processed and stored for later analysis. This will most likely be by 4-colour fluorescence immunohistology (for quantification of protein expression) and/or gas chromatography / mass spectroscopy (metabolic profiling) and/or gas chromatography (bacterial metabolic end-product analysis) and more. This is to facilitate a systems approach to characterise the underlying mechanisms of different forms of iron absorption and deviances induced by anaemia. We will link gut microbiota composition to microbial metabolic outputs and identify how the gut microbiota interacts with the host under different iron statuses.

The cryopreserved tissue banks are maintained for years to enable revisiting the samples at later dates when additional technologies are developed or further research questions arise. Our pig tissue banks have also been used to provide samples to other researchers. This is aligned with the 3Rs since it reduces the use of animals in research, and also facilitates maximum output from each pig trial.
The work proposed is part of a large-scale collaboration involving several world-leading researchers from prestigious universities. The applicant and collaborators all

have strong track records of peer- reviewed publications (>200) in a range of journals, and of giving invited talks at international conferences. Taken together, these provide considerable opportunities to disseminate the findings from this work. In addition, the applicant has a co-supervised PDRA working on a research project in collaboration with medics. As a result, the applicant is often asked to talk at medical conferences, and directly to surgeons and doctors, which also provides opportunity for dissemination of results directly to interested parties.

A further collaboration is being developed with academics at an additional University with whom this program of work has been developed. These academics are renowned researchers in their field and also provide dissemination opportunities. We have already started *in vitro* work together to inform the development of our pig trials proposed here.

The applicant has up-to-date Loop and ResearchGate profiles (>25k views) and research networks built up over 18 years of working in the field.

Our university has firm commitments to the aims and principles of Open Research and has recently updated its Open Research Action Plan. This promotes the publication of unsuccessful research approaches in appropriate, publicly available databases.

Species and numbers of animals expected to be used.

- Pigs: 200 pigs

Predicted harms

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

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

Iron deficiency anaemia (IDA) is an important global problem, especially in infants, and is often addressed by oral iron supplementation. However, there are many negative side effects of using such treatments, including irritated and inflamed guts, increased risk of infection and diarrhoea. In addition, current iron supplements are often poorly absorbed or have low sensory appeal.

Firstly, without iron supplementation, all piglets rapidly develop IDA without requiring iron-free diets overextended periods. In addition, they share many physiological characteristics and dietary requirements with humans which means that results have high potential for translation into human healthcare. Moreover, piglets are born far more developed than most other mammals which means that they can be removed from their mothers at birth and thus their nutritional intake can be tightly controlled.

Finally, iron absorption is impaired under inflammatory conditions, which we can easily generate in piglets by weaning them early and abruptly onto subsistence-type

diets. For these reasons, this project will use young piglets as models for human infants to explore the effects of IDA, and various forms iron supplements, under normal and inflammatory conditions.

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

Depending on the trial, piglets may be removed from their mothers during their first day of life (always following colostrum consumption) and transported to our facilities where they will be housed in individual pens in warm, light-controlled conditions.

They will most likely be weighed weekly, or twice weekly, and blood samples are likely to be taken no more frequently than on a weekly basis. In other trials, piglets will remain with their sows until weaning (between 3-4 weeks) before being transported to our facilities. The duration of all trials will be no more than 12 weeks.

Some piglets will receive a form of non-radioactive iron (Fe^{57}) supplementation from 1 day old. Naturally, Fe^{57} makes up about 3% of heme-associated iron, but will be increased to around 60-80% if it is the sole source of iron. This has been done in rats before with no ill effects, as our partners have previously demonstrated (Shen *et al*, Nature Nanotechnology, 2017). The Fe^{57} enriched blood will then be harvested and used in a human trial by our partners as it can be easily traced and differentiated from host blood which will generate further knowledge on iron uptake mechanisms, which is currently lacking. In other trials, piglets will receive different forms of oral iron and/or 1-2 injections of iron into their thigh.

In order to generate inflammation, piglets will sometimes be abruptly weaned at 21 days onto subsistence diets consisting of 50% of the amount of feed required for optimal growth. This will not cause weight loss, but will reduce growth-rate and is associated with characteristics such as gut inflammation and decreased gut barrier function. Other piglets will be injected with mini-hepcidin. This is because hepcidin reduces iron absorption and levels are elevated when there is inflammation. No piglets will receive hepcidin and be put on a subsistence diet.

Some prebiotics and probiotics have been shown to enhance oral iron absorption and some piglets will receive iron supplementation along with specific prebiotics and probiotics. This is unlikely to cause any harms.

In some cases, control animals will not be provided with iron supplements and will develop anaemia. In our experience this results in some lethargy and reductions in weight-gain, but doesn't impact on appetite or other behavioural parameters. The period of anaemia will be restricted to no more than 6 weeks.

Since we are particularly interested in the sustained effects of early-life nutritional interventions on physiological development, we will sometimes mix piglets following the intervention so we can compare control and treatment groups after they have all been treated the same for a period of time.

At the end of each trial, piglets will be euthanised using an overdose of barbiturate.

Larger piglets will receive an injection of a sedative prior to being killed, to facilitate handling and thus reduce stress and risk of injury. In a limited number of cases, piglets may be transported to an abattoir at the end of a trial.

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

Early removal from sows and transportation is stressful for piglets. Journey times are estimated to be around 2 hours long. Due to their social nature, individual housing is stressful for piglets, but they will be in physical contact with each other through large-opening wire fencing. Piglets will not be individually housed for more than 12 weeks since all of our trials will be completed by this point. Some piglets will be mixed for a time following individual housing, which may lead to a limited amount of fighting. This usually settles down quickly as the hierarchy is established. Piglets which persist will be given a mild sedative to prevent them from fighting while they get used to each other.

Piglets on subsistence diets will feel hungry, but they will be fed at regular intervals and feed will be increased according to the weight of their siblings on normal diets. In our experience, such piglets do not perform unusual behaviours.

Blood-sampling is a stressful and uncomfortable experience for piglets. This will be performed by skilled staff and the process is completed rapidly. After blood-sampling, piglets are fed in an attempt to neutralise the expectance.

Some piglets will receive injections which will be painful. Injections will be administered slowly to limit their discomfort.

Sow welfare must also be considered and it would be extremely stressful were the sow to have an entire litter removed. However, we will only ever remove a maximum of 6 piglets from each sow to split between treatment groups. Typical litter sizes are over 14 piglets, so removal of some will probably benefit the sow and enhance the welfare of the remainder of the litter by reducing competition for food. In our experience, sows do not appear to even notice when piglets are removed.

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

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

The expected severities are mild to moderate, largely due to the accumulative effect. This will be as a result of restricted diet, individual housing, early weaning, early removal from their sow, injections, mixing, blood-sampling and/or anaemia.

However, most piglets will be on different forms of iron supplements with some also receiving prebiotics and/or probiotics and will experience only mild severities.

Following initial group housing for 1-2 days to allow piglets to learn to drink from bowls, most of the piglets will be individually housed for the duration of each trial.

Most piglets will also undergo early removal from their sows OR early weaning (but not both) and blood-sampling. About 1/3 will also undergo some form of diet restriction, including reduced feed intake, and restricted iron.

35% of the pigs will experience moderate severity, while 65% will experience mild severity.

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

- Killed
- Rehomed

Replacement

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

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

So far, it has not proved possible to model the interaction between gut bacteria and the complex immune, metabolic, nervous and hormone systems of humans without using experimental animals. Less advanced, non-protected, animals do not show the same complex interactions and do not provide reliable models.

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

We have considered the use of verified fermentation and continuous *in vitro* gut model systems. We use these often as a pre-animal trial screening step, or where we want to assess the effect of, for example, a nutrient (or lack of) on the gut microbiota and/or pathogenic gut bacteria growth and survival. For example, we can screen probiotics/prebiotics for their ability to produce a beneficial change to the gut microbiota and/or its metabolic output prior to selecting specific pre- and probiotics to use in pig trials. This significantly reduces the number of animals we use.

We have also considered using microphysiological systems such as 3D cell cultures, along with computer modelling and gut-on-a-chip approaches.

Why were they not suitable?

Gut models are not suitable for this work since a key aim is to demonstrate that nutritional intervention has an impact on biological systems, which can not be reflected *in vitro*. For example, we aim to demonstrate that plant-based heme iron can be absorbed more effectively than non-heme iron. We will also explore whether prebiotics/probiotics can enhance iron absorption. A further key objective is to determine whether plant-based heme iron can be readily absorbed under inflammatory conditions.

None of these objectives can be explored using gut model systema.
following exploration of the literature and discussions with experts in the field, it is

clear that 3D cell models and computer modelling methods use reductionist approaches and, although helpful, are not yet at a point where they accurate reflect full biological systems, which will impact on translation potential.

The gut-on-a-chip approach is largely used for pharmaceutical purposes (assessing drug delivery and potential for inflammatory responses) and again, is a reductionist approach - for example, the gut microbiota is not present. This proposal is focused on exploring microbe-host interactions and so this is not an appropriate method.

Reduction

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

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

We have published widely in the area of neonatal nutritional interventions using piglet models and have demonstrated that the numbers proposed are sufficient, but not excessive, to observe statistical differences in immune and metabolic development in the host. We also have data specifically regarding significant effects of iron absorption in young piglets and will use these data to inform trial design for the proposed work.

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

Professional statisticians were consulted to ensure that experiments in this project were designed using only the number of animals needed for the research questions to be answered.

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

Litter-matching will be deployed wherever possible. This is where members of the same litter are split between treatment groups to reduce genetic variability between these groups (effectively ‘twin’ studies). Coupled with housing animals in individual units, this strategy means far fewer animals are required than if the treatments were applied to group-housed piglets which had not been litter-matched into treatment groups.

Refinement

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

the lifetime of the project.

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

Pigs are valuable comparative models for human studies1 since they share many features of physiology, immunology, metabolism, microbiology, diet and genetics. As in human infants, heterogeneity in pigs can lead to large ranges in biological responses, something difficult to emulate in inbred animals. With regard to nutritional studies, the gastrointestinal system of the omnivorous pig is similar to that of humans, and pigs metabolise dietary carbohydrates and lipids in a similar way.

Moreover, predicted protein sequences (identified from the Porcine Genome Project) were compared with their human orthologues and 112 positions had the same amino acid that is implicated in human multifactorial traits such as obesity (ADRB3, SDC3) and diabetes (PPP1RA, SLC30A8, ZNF615). Pigs are particularly valuable when assessing host-microbe interactions since the gut microbiota is substantially more stable over time in both pigs and humans compared to rodent models. Furthermore, inter-individual variability is more similar between humans and pigs than between humans and mice, thus in these respects the human population is better reflected in this outbred animal species. In terms of early-life studies, piglets are precocial; they can be removed from maternal influences at birth, allowing individual identification and manipulation from a very early age. Whereas the earliest pup-in-a- cup rodent model starts at 5 days old and new born rodents are less mature at birth and better represent premature infants. In addition, the small size of rodent does not permit analysis of the same tissue using the multi-platform approach outlined in this work.

There is a paucity of knowledge regarding how both iron deficiency and luminal iron availability affect gut microbiota development, or the subsequent impact on immunity, which are likely to be contributors to the increased risk of infection. Piglets are naturally iron deficient. This is largely due to their low iron endowments at birth (primarily due to large litter sizes), and their rapid growth combined with the low iron levels in sow milk. Thus, piglets consistently become iron deficient within days of birth which rapidly progresses to anaemia in the absence of iron supplementation.

This means that unlike other species, they do not require long periods of iron deficient diets to become anaemic. They also have similar iron up-take mechanisms to those in humans.

Piglets are very similar to humans in terms of their gut physiology, immunity, metabolism and gut microbiota and therefore are excellent translatable models. The findings from this program of work are more likely to translate into human healthcare than the use of other species might. The above reasons are why piglets are by far the best translatable model for these specific experimental protocols.

Most of the proposed studies compare the effects of different sorts of iron supplementation, which means that the majority will not be anaemic. However, anaemic controls will be required and these control piglets will have systemic haemoglobin levels monitored weekly and the period of anaemia will be kept to a

minimum. Our previous work in this area demonstrated that young anaemic piglets behave similarly to their iron sufficient counterparts and do not show signs of distress, although they are a little smaller. The time frame that piglets remain anaemic will be kept to a minimum and piglets will be culled when still young, so there will not be lasting effects.
The environmental enteric dysfunction model piglets will be maintained on half the amount of food required for optimal growth to reflect a subsistence diet in humans. We have used this model before and our data shows that these piglets grow more slowly than counterparts which receive adequate diets, but they do not lose weight. Long-lasting harm is avoided by culling piglets while still quite young and thus avoiding significant health implications in later-life.

For all piglets, there will be some discomfort during blood-sampling, but this will be very quick and carried out by staff with a lot of experience blood-sampling piglets. To minimise the replicates required, piglets will be individually housed so that each piglet is a unit. However, wire mesh, as opposed to a solid wall, will be used to separate pens so that social piglets will be in contact with each other and thus reduce their suffering. In addition, piglets which are younger than 28 days are provided with heat- lamps across the corner of 4 adjacent pens, thus permitting them to keep warm at the same time as being in close proximity to each other. If heat lamps were to be placed in other locations in the pen, piglets would have to chose between the comfort of each other or comfort from their heat lamp. Where individual housing is not necessary (e.g. when generating Fe^{57} heme), piglets will be housed together.

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

Less advanced, non-protected, animals do not show the same complex mammalian host-microbe interactions, or iron uptake mechanisms, and do not provide reliable models for this sort of work. Data generated using such models is far less likely to translate into human healthcare. The interventions are over several weeks and so using terminally anaesthetized animals is not appropriate.

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

Trial design will be amended where possible as the program of work progresses. For example where earlier studies in the program of work inform the design of later trials to minimise the welfare costs.

Following each trial, all personnel involved with the project attend a follow up meeting to discuss how the trial progressed, and whether modification could be made to improve welfare during the next trial. For example, following an earlier trial (under a different licence) we concluded that different bedding would be more preferable for the piglets and we have adopted this for subsequent trials. Piglets learn rapidly and we feed them immediately after blood-sampling with the aim of generating a positive association between feeding and being bled. This may go some way to reduce stress for piglets. We also remove the pentobarbital solution from the fridge an hour or so before using it on the piglets so that it is at room

temperature, rather than 4°C, when it is injected into the pigs.

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

Over the years the applicant has developed a network of researchers working in similar areas and we share ideas to promote the refinement of experiments. The applicant has been carrying out piglet trials of a similar nature for over 18 years and has a track record of publishing the results. The applicant was initially trained by experienced pig researchers at a different university who had been working successfully with piglets for several decades under a variety of project licences. Best practices have been shared and this project will follow these best practices and adapt where appropriate to meet specific needs.

Animal technicians at our establishment have track records of working with pigs and have completed successful trials in neonatal piglets under the applicant's other project licences (and others). They also regularly complete pig work for other institutes and we often share best practices. For example using a purpose designed 'cradle' for the restraint of larger piglets to make the process easier and less stressful. Specific plans and processes are also discussed with the named vet beforehand and there are several discussions with animal care staff and members of AWERB prior to each pig trial so that the most refined trial can be designed.

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

The applicant reads the monthly NC3Rs newsletter sent by the institute's animal ethics staff. And attend 3R workshops at both their own institution and elsewhere.

Amendments will be made to the program's animal trials where relevant and appropriate. The PI is also in contact with others in the UK working with young piglets and advances in techniques and practices are shared. We have advised other research groups who have since adopted and modified our practices to suit their specific purposes. In this area, we benefit from other pig researchers using our site and staff. The applicant also attends licence holder events at other institutes. Aligning with the 3Rs is an on-going process.

The applicant also keeps up-to-date with current literature in the field and networks at conferences where new techniques which align with the 3Rs are discussed. For example, the applicant has a PhD co-supervisor at another institute which is currently developing 3D gut modelling, but this is not yet fully developed for use. However, the system could be finalised and verified during this licence and could be adopted where appropriate.

26. Mechanisms of Inflammation and New Treatments for Respiratory Diseases

Project duration

5 years 0 months

Project purpose

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

Key words

Lung Disease, Infection, Cough, Airway irritability, Treatments

Animal types	Life stages
Rabbits	adult
Guinea pigs	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall objective is to understand specific mechanisms that contribute to impaired breathing capabilities in lung diseases such as asthma and COPD (Smoker's Disease) to help develop new treatments for patients. We are also looking to develop new treatments for the hard-to-treat lung infections.

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

Why is it important to undertake this work?

Currently, smoker’s disease (COPD) and asthma are wide reaching lung diseases with recent numbers from the World Health Organisation highlighting that 174 and 339 million people were impacted globally by COPD and asthma respectively.

Furthermore, recently numbers have implicated COPD as the third leading cause of death in 2019 and was attributed to 3.23 million deaths, whilst asthma was associated with approximately 0.5 million deaths contributing to a combine cost of nearly £5 billion to the NHS every year. Whilst current treatments demonstrate an ability to improve several symptoms associated with both COPD and asthma, there remains a portion of this patient population that are unresponsive to current therapies, furthermore, no therapies currently exist for the effective reversal of the increased sensitivity of the airways associated with these diseases. There is therefore a need to find better drugs to treat the underlying causes of these diseases.

These diseases are also often associated with lung infections that can lead to rapid and severe deterioration of the patient. Increasingly many of these infections are attributed to antimicrobial resistant pathogens (bacteria that do not respond effectively to current antibiotic drugs), a process that has been made worse by a significant decline in the approval of new antibiotics. It is predicted that that by 2050 over 10 million deaths may be attributed to infections with antimicrobial resistant bacteria, contributing to a global economic burden of £66 trillion. Our research will therefore continue to provide valuable information on the mechanisms underlying asthma, smokers’ disease and persistent lung infections that is required to help both our research group and the wider research community other researchers to better understand these diseases and develop new and better strategies for their treatment.

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

We anticipate that the outputs of this project will be identification of mechanisms underpinning the development of airway hyperresponsiveness, chronic cough, and lung infections in patients with respiratory diseases to aid in the identification of novel therapeutic strategies for patients. These findings will be published in peer reviewed journals, presented at both national and international scientific meetings, whilst also being used to secure future research funding. The Identification of approaches will therefore facilitate the development of new drugs for the treatment of both infectious and inflammatory respiratory diseases.

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

In the short to medium term, we anticipate that our work we will gain a greater understanding of the broad inflammatory mechanisms associated with lung diseases such as asthma, COPD or difficult to treat lung infections that lead to pneumonia and hard-to-treat persistent lung infections. This will be of benefit to pre-clinical scientists and clinicians that are interested in the mechanisms and treatment of airway diseases. We also anticipate that this work will be of interest to both the biotech and pharmaceutical industry seeking to find novel treatment strategies for these diseases.

However, in the longer term, our work will contribute to the discovery and subsequent

development of improved medicines for the treatment of patients with lung diseases such as asthma, COPD, chronic cough, and lung infections where a significant unmet clinical need remains, thereby accelerating their progression for clinical use.

To ensure the maximal benefit of our research, we will ensure that our work is as widely disseminated as possible through publication in internationally recognised peer review publications, through presentations at appropriate scientific and medical meetings and through patient and public involvement.

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

We will seek to maximise the output of this work by collaborating with both academic colleagues and industrial partners in the respiratory field. We will disseminate our findings in accordance with ARRIVE guidelines to international audiences through open-access publications in high impact peer-reviewed journals alongside presenting our work at both national and international symposiums such as the European Respiratory Society and the American Thoracic Society. Sharing our findings with an international audience will help the wider scientific community continue the development of novel therapeutic strategies for patients with inflammatory lung diseases or persistent lung infections.

Species and numbers of animals expected to be used.

- Guinea pigs: 1500
- Rabbits: 500

Predicted harms

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

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

Adult Guinea pigs have been widely used in the development of drugs for the treatment of airways diseases. They have many similarities with humans with respect to their lung physiology. They are also large enough to measure lung function robustly and unlike lower species such as mice and rats are able to cough to the same stimuli as people. We will also use adult rabbits in longitudinal studies such as lung function and cough as unlike guinea pigs, the size of rabbits allows for these measurements to be taken without the need for surgery under recovery anaesthesia.

This will allow for studies on longer term effects of drug regimens and/or disease progression. Rabbits have been widely used to provide models of lung infection and inflammation and much of our work on understanding the neuronal control of lung physiology has derived from work in rabbits and guinea pigs.

Typically, what will be done to an animal used in your project?
Example of Experimental Design for Cough in Rabbits and Guinea-Pigs:

For the measurement of cough, rabbits or guinea pigs are exposed to stimuli such as ozone and then transferred to a purpose-built cough chamber. The animals are exposed to a substance to cause cough and the number of coughs and sneezes counted over a defined period of time. This procedure may be repeated once per week. Animals may receive drug treatment throughout the duration of the study. Seven days after the last cough experiments are completed, studies will be performed to evaluate lung function. Following this, we will perform a Bronchoalveolar lavage, a saline wash of the lungs which allows sampling of inflammatory cells within the lungs. At the completion of the experiment, animals will be humanely killed with an overdose of appropriate terminal anaesthesia.

Example of Experimental Design for Lung Function Challenge in Rabbits:
Lung function in rabbits will be measured under anaesthesia under normal breathing without surgery. This will allow us to measure lung function in the same animal over a longer period of time. Animals may receive drug treatment prior to or during the recording of lung function.

Example of Experimental Design for Lung Function Challenge in Guinea-Pigs:
Lung function in guinea-pigs will be measured and recorded using a FlexiVent machine under non- recovery anaesthesia (animals will be killed whilst remaining under anaesthesia).. Drugs may be given by routes including injections or inhalations prior to or during lung function measurements. At the conclusion of each lung function experiment, animals will be humanely killed with an overdose of appropriate terminal anaesthesia, this will be followed by a Bronchoalveolar lavage (a saline wash of the lungs to capture fluid samples to allow sampling of inflammatory cells within the lungs). Finally, the lungs will be removed to allow us to measure any disease induced structural changes to the lungs in these animals.

Example of Experimental Design for Lung Infection:
Guinea pigs will be infected with different bacteria that commonly infect human patients. Animals may receive drug treatment throughout the duration of the study which may run up to 7 days post infection. On the last day of the study, lung function experiments may be performed as described above. At the conclusion of each lung function experiment animals will be humanely killed with an overdose of appropriate anaesthesia, this will be followed by a Bronchoalveolar lavage (a saline wash of the lungs to capture fluid samples to allow sampling of inflammatory cells within the lungs). Finally, the lungs will be removed to measure bacterial numbers and allow us to measure any disease induced structural changes to the lungs in these animals.

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

The majority of animals used in this project will generally only experience mild adverse effects or will be under terminal anaesthesia. However, some animals may experience transient moderate symptoms of respiratory distress in the first 1-2 hours of the study, discomfort at sites of injection, weight loss (5- 10% of starting weight), hunching, reduced movement or abnormal walking in the cages, and piloerection/scruffy fur from certain types of drugs such as capsaicin or through local pulmonary bacterial infection. Furthermore, some animals may experience transient moderate symptoms such as post-surgical discomfort at the site of the incision

following surgical procedures such as the implantation of devices for the remote monitoring of animal welfare, or devices for the slow release of drugs. At the end of an experimental procedure or if it becomes necessary to alleviate unacceptable suffering at any time under any protocol then animals will be humanely killed via an appropriate method.

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

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

Rabbits
It is expected that no rabbits will experience more than moderate severity across protocols 1-3. Specifically, only 10% of rabbits (50 animals) treated with agents such as Capsaicin would be expected to experience moderate severity with the remaining 90% (450 animals) only expected to experience mild severity.

Guinea-Pigs
It is expected that no Guinea pigs will experience more than moderate severity. Specifically, only 10% of guinea pigs treated with agents such as Capsaicin on protocols 2 and 3 would be expected to experience moderate severity (100) with the remaining 90% (900 animals) only expected to experience mild severity. However, it is expected that Guinea pigs used in Protocol 4 will experience moderate severity as a result of pulmonary bacterial infection (500 animals).

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

- Killed

Replacement

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

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

We are interested in understanding the mechanisms that give rise to the physiological changes to the lung during inflammation and infection that contribute to airway irritability such as increased airway sensitivity and chronic cough. Whilst we can study individual cell types in culture there is currently no test-tube based technique that can robustly replicate the changes associated with airway irritability or function, as they are known to involve airway reflexes from the lung to the brain and back.

It is increasingly recognised that diseases of the lung such as asthma, COPD (Smoker's disease) and infection are complex that cannot be replicated using test-tube experiments alone. Therefore, we must use experiments on living animals alongside the study of cells obtained from patients to fully understand the basis of

these conditions and to help us identify improved treatments for these common disorders that still have significant unmet medical need.

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

Where possible we do undertake experiments using human cells in culture derived from both immortal cell lines and patient samples, or cells derived from the blood of human donors. We employ a number of techniques to investigate airway smooth muscle functionality *in vitro* following electrical field stimulation of isolated airways, including the measurement of smooth muscle contraction on neurotransmitter release. These techniques can also be used to elucidate the effect of agents on an isolated nerve/airway and skin preparations. Other techniques that we employ include the use of *in vitro* cell culture assays to examine proliferation of airway smooth muscle cells, inflammatory cell adhesion to endothelial cells and the function of individual inflammatory cells. However, for more complex situations such as cough and airway irritability, there is no simple *in vitro* model that can be used. Whilst we can investigate isolated inflammatory cells and even co-cultures involving more than one cell type, alongside using complex *in vitro* models such as the Hollow Fibre model of infection it is not yet possible to mimic the complex nature of a chronic lung infection and the bodies response to injury *in vitro* therefore we continue to require *in vivo* models of pulmonary bacterial infection.

Why were they not suitable?

Whilst we have considered the use of the *in vitro* cell culture assays, the clinical observation of airway irritability that may be a function of the interaction of all of these individual components can only be demonstrated and studied in the whole animal. Indeed, the phenomenon of airway irritability can only be observed in an intact mammal. Previous studies have also shown that airway irritability cannot be measured even in isolated human isolated airway preparations from individual who dies of their asthma.

We also measure cough which is a physiological response that can only be studied with certainty in particular mammals (e.g. guinea-pigs and rabbits as cough cannot be elicited in mice or rats). Whilst restricted aspects of neuronal function can be studied in isolation using cell culture techniques of neuronal cell which innervate the lung, there is no *in vitro* functional correlate of cough and *in vivo* experiments are required to study the nerve reflexes involved in cough.

Reduction

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

How have you estimated the numbers of animals you will use?
All experiments will be designed to achieve the scientific objectives with the

minimum number of animals. In most of our experiments we have the power to detect at least a 50% difference in the magnitude of a response using factorial design with at most 4 treatment groups. Consequently, our standardised differential is approximately 1.5 necessitating the use of 6-8 animals per treatment group. For studies that are less well established and for which historical data with the department is not available, the literature will be consulted to help establish group size.

For less well-established study types, a preliminary pilot study may be conducted where smaller numbers of animals may be used to generate data in order to ensure that the experiment operates to expectations and to generate some data which be used to optimise the study design for future experiments. From such studies, the inter variability of the biomarker output is evaluated and used in calculations to determine the number of animals per group required to identify the effect we are looking to measure.

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

We are committed to maximising the amount of experimental information we obtain from each individual experimental animal, enabling us to hopefully minimise usage. Using careful experimental design, we will ensure the minimum number of animals are used per experiment consistent with the scientific objectives. An example of this is the use of advanced measurements of lung function in conscious animals and the use of advanced imaging techniques whereby animals can be used as their own controls, thereby minimising the overall number of animals required in any given experiment.

Other examples include comparing multiple drugs of interest/multiple doses of drug in the same study against a single control to reduce the required number of control animals for the study.

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

Where possible and consistent with the scientific objects of the wider research group we will use single strains of animals in multiple research areas to reduce variability. This allows us to use multiple organs collected from individual animals and share tissue amongst the wider research group in order minimise the number of animals required.

Refinement

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

why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Guinea pigs are under terminal anaesthesia during lung function experiments to minimise any potential pain and stress during the experiment. Rabbits are anaesthetised during lung function experiments to minimise stress. Guinea pigs will be the most common animal used in these studies as they are the species on the lowest level of the phylogenic scale compatible with the scientific objectives of the project. However, on occasions where we seek to measure lung function longitudinally under recovery anaesthesia rabbits may be utilized as they are able to serve as their own controls in experiments requiring repeated measurements.

Cough experiments using both rabbits and guinea pigs (in conscious animals) are monitored closely to minimise suffering to the animal and protocols will be adjusted if exposure is causing an increased level of distress by reducing the doses of tussive-agents used. Cough has to be recorded in a conscious animal and is a mildly stressful experience, however all measures will be put in place to minimise harm to the animals.

Lung infection model studies will be performed in guinea pigs for the assessment of changes in lung function due to infection and efficacy evaluation of novel therapeutic interventions. Lung function experiments will be performed under terminal anaesthesia to minimize any pain and stress during the experiment.

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

Studies evaluating cough: Unlike guinea pigs, rodents including mice and rats do not have a robust cough reflex. Guinea pigs and rabbits like humans' cough in response to cough inducing agents like citric acid and therefore are a more relevant species to investigate cough and to better translate our findings to future treatments for humans.

Studies evaluating respiratory lung mechanics: Many groups including our own have used the rabbit and guinea pig to model various aspects of airway disease. The advantage of this model is that the size permits the recording of changes in lung mechanics repeatedly in the same animal, thereby increasing the statistical power of the experiments and allowing animals to be used as their own controls as would be the case in clinical studies. This is of particular relevance when monitoring changes in lung function over time in models of asthma, chronic obstructive pulmonary disease and chronic bacterial lung infections.

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

Where possible we will seek to employ non-adversive handling, provide habituation to handling and provide appropriate environmental enrichment to reduce stresses, improve animal welfare, and thereby decreasing variability and improving experimental reliability. We will continuously monitor the animal welfare following recovery procedures and adjust protocols to ensure the minimum exposure to harmful stimuli is used to achieve a significant result (eg. Through the use of Body

Conditioning Scoring). This will be achieved through the use of pilot studies utilizing the most refined methods available to us when appropriate during a model characterization phase. We will also use both recovery and terminal anaesthesia where appropriate (eg. Use of terminal anaesthesia in lung function studies) to minimize animals experience of pain and distress throughout the studies.

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

We will follow the published ARRIVE guidelines alongside prior established protocols and codes of practice including but not limited to the code of practice for housing and care of animals bred, supplied or use for scientific purposes, LASA guidelines, Universities Federation for Animal Welfare (UFAW) guidelines and publications, NC3Rs procedures with care, and local AWERB standards to ensure all recent refinements are adopted to establish a clear scientific response allowing us to minimise the number of animals used and also improving the welfare of animals used in this application.

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

We will constantly review the literature for any new developments in experimental protocols, whilst staying up to date with other research and guidelines in good laboratory practice and animal welfare through attending relevant seminars advertised by the NC3Rs, access material available via the NC3Rs website and through continued discussions with colleagues including but not limited to the staff within institutes biological services unit and the NVS. Furthermore, we will engage and participate in any relevant training sessions and seminars available to us through the sources such as the NC3Rs.

Any advances in the 3Rs identified by these approaches will be discussed as part of both lab and wider departmental meetings alongside discussions with external colleagues to enable their effective implementation.

27. Regulation of the immune and matrix environment 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

Allergy, Inflammation, Extracellular-matrix, Immunity, Stromal cells

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We want to understand how immune cells, tissue structural cells and the extracellular matrix interact to shape how tissue functions. The extracellular matrix (made up of components such as collagen and elastin) serves as a scaffold for tissues and organs throughout the body and plays an essential role in their structural and functional integrity. By extension, we aim to investigate the cell and tissue derived signals that influence these cell-cell and cell-matrix interactions and how disruption of these networks leads to chronic allergic diseases in the lung.

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

term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Aeroallergens and other airborne irritants (e.g. pollutants, infectious agents) are abundant in the environment but usually harmless and rapidly eliminated from the airways by our immune system. However, in some individuals the immune system overreacts to these substances triggering localised inflammation which may develop into severe chronic inflammatory diseases like asthma, a respiratory disorder that affects over 300 million people worldwide. Aside from chronic inflammation, the lungs of people with asthma also undergo structural changes whereby the extracellular matrix (ECM) is altered. The ECM is a network of molecules that not only supports the structure and mechanical properties of a tissue, but also widely influences where cells localise to within a tissue and how cells function. These cell-matrix interactions help to keep the lungs healthy and can facilitate tissue repair following injury.

However, in people with asthma, pathogenic remodelling of the matrix and disruption of the cell-matrix networks ultimately contributes to a loss of lung function. Whilst it is known that immune cells and mediators can influence the ECM (and vice-versa), targeting chronic inflammation alone is not enough to reverse pathological matrix changes. Moreover, little is known about the mechanisms and consequences of cell-matrix interactions within the lung environment during the switch from health to pathology. Therefore, this fundamental research into crosstalk between immune cells, tissue resident cells and the matrix will not only lead to improved understanding of how chronic lung pathologies such as asthma and other fibrotic diseases occur, but should also provide insight into targets that can rejuvenate a dysfunctional cell-matrix environment in the lungs.

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

A major goal of this project is to identify the fundamental communication pathways between our immune system and the extracellular matrix that underpin the development of chronic diseases.

One of the primary outputs of this project will be the generation of vital data and knowledge to help understand the precise immune signals that change the extracellular matrix and vice versa leading to inflammation and remodelling of the tissue environment, with a large focus on lung pathologies. Such outputs will be disseminated through publication in open access, peer-reviewed journals and presentations at conferences, seminars and workshops. Appropriate data outputs including methods (e.g. large datasets) will also be published in open-access repositories. The lab will continue to actively participate in public engagement events to increase public awareness of our work, its implications for health and disease and importance of biological research. Furthermore, certain aspects of our work will identify druggable pathways and in the longer term may be used to generate commercially exploitable therapies to treat inflammation and/or matrix remodelling in chronic disease pathology.

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

This project will aim to discover new knowledge and mechanisms centred around immune-mediated pathology relevant not only to human and animal disorders but fundamental for understanding how to maintain health.

Although hypothesis driven, the discovery nature of our work means that in the shorter term (over the first 5 years of the project) the primary beneficiary of this work will be the academic community and clinical scientists. Our research is largely focussed on lung diseases models. However, communication between the immune system and extracellular matrix is fundamental for the function of any tissue. Therefore, the concepts discovered during this project will benefit a broad range of research and clinical scientists that specialise in chronic diseases and ageing.

Importantly, we will also screen immunotherapies and anti-fibrotic agents to determine their impact on the extracellular matrix and/or immune system using animal models of pathology. Within this project, we will also be collecting and utilising samples from human patients to translate findings from animal models to determine features of cell-matrix communication that is reflective of human disease.

In the longer term, after completion of this 5 year project, our goal is to steer future research towards new therapeutic strategies that influence interactions between immune cells and the extracellular matrix that are important for driving disease. We would aim to develop novel drug candidates which would be directed at treating chronic diseases either individually or combined with current established treatment regimens to improve patient health.

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

As this project is multidisciplinary, we will communicate findings by publishing in journals and presenting at local, national and international conferences and seminars that reach across a broad spectrum of biological and medical disciplines. To ensure maximum dissemination we will only publish in journals with available open access policies. Furthermore, to speed up access of data and knowledge, findings will be published on open access preprint server such as BioRxiv. We will also aim to publish all large data sets generated (including negative results) and methods used for analysis on repositories such as Github, allowing researchers to rapidly access and utilise data and avoid unnecessary repetition of experiments.

To enable rapid translation of findings to the clinic, we will exploit new and existing collaborators and utilise local clinicians for access to clinical samples. In this respect, our local scientific research environment associated with human tissue biobanks is ideal for developing collaborations and translating findings from animal models to a clinical setting.

Species and numbers of animals expected to be used.

- Mice: 8000

Predicted harms

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

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

We study adult mice because the immune system, tissue organisation, ECM environment and development of all mammals are similar. Therefore, the use of mice allows us to model human systems and other animals. Over the years, researchers have created many genetically altered mouse lines and tools specific for mice, allowing us to mechanistically analyse what happens during an immune response or during ECM remodelling. For example, we can study in fine detail how specific cells and molecules within a tissue can interact with each other and the ECM environment to repair tissues and respond to environmental challenges such as allergens.

Manipulation of such cells/molecules can help us understand how cell-cell and cell-matrix processes go wrong in chronic inflammatory disease like asthma and may enable us to design new future therapies that keep our tissues healthy.

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

Typically, animals will receive repeat doses of allergens through the nose following brief exposure to inhalation anaesthetics (either singularly or as an allergen mixture) to mediate allergic inflammation and remodelling of the ECM. Experiments might look at the immediate immune responses in the first few days, but experiments will also last as long as 8 weeks to allow full development of a diseased tissue with a remodelled ECM. Mice may be administered allergens up to 5 doses a week, but typically will only be exposed to allergens twice a week (for between 1 to 8 weeks).

n some experiments, mice may be given allergens to induce chronic inflammation and remodelling, then rested without further treatment (typically 4 weeks but may rest period may last 8 weeks) to determine the reversibility of disease features.
In many cases, mice will also receive a single or multiple injections containing an immunomodulatory substance (e.g. antibodies to neutralise a molecule or deplete a specific cell type) either alone or before, during or following exposure to allergens. In addition, small volumes of blood may be taken from a vein to screen for changes in blood cells or molecules. Some genetically modified animals will receive a drug (e.g. tamoxifen) to induce expression of a gene either alone, or before, during or following exposure to allergens or immunomodulators. Gene inducing agents may be given as a single or multiple injection, but for repeat administration we will use the route that provides the least trauma.

Some mice may receive cells (either pre-exposed to allergens or not) prior to, or after treatment with an immunomodulatory substance. These mice may then be further challenged with allergens. Typically, these experiments will last 1-21 days, but longer experiments (8-12 weeks) may be required. In order to trace cells, some mice will be administered a labelling agent (e.g. BrdU). These agents will typically be administered on one occasion following exposure to allergens. However, on some occasions, a cell labelling agent will be administered to before, during of following exposure to allergens or immuno- modulators to track cells.

Overall, the cumulative experience of a typical mouse will include exposure to between 2 to 3 procedures (if repeat intranasal dosing is regarded as a single procedure) that may each cause short but usually separated periods of typically mild or potentially moderate degrees of suffering. Repeated administration of inhalation anaesthesia may also lead to short, separated periods of mild or potentially moderate degrees of suffering due to 1) developing an aversion to the smell of the anaesthetic and/or 2) shorter recovery periods if allergens are administered 5 times a week as opposed to twice weekly.

Experiments will end with animals being killed humanely. For the majority of experiments, this will involve exposing mice to rising carbon dioxide levels. This schedule 1 approved approach is the best approach for our research as it enables us to isolate samples from the airway. Other methods such as cervical dislocation cause damage to the trachea preventing us taking samples from the airway. In some instances we need to euthanise the animal under terminal anaesthesia due to potential damage to the lung tissue by being exposed to high levels of carbon dioxide (e.g. when studying epithelial cells that line the lung airway).

Separate from the above experiments, some genetically altered animals will be used only to breed and maintain animal lines.

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

The vast majority of animals will experience no adverse effects or only mild adverse effects.

Our models of repeated allergen exposure are generally very well tolerated even when allergen is given over long periods of time. However, in rare cases allergens can cause temporary breathing problems ie laboured breathing, increased/decreased respiration. The ECM remodelling allergy experiments have been specifically designed to assess structural changes to the tissues without major overt clinical symptoms.

Most immunomodulators or labelling/tracing substances are not expected to have adverse effects in their own right. However, administration of some substances (e.g., LPS) may have systemic immune or inflammatory effects causing some signs of moderate effects e.g. appear hunched with ruffled fur (<30%) with their eyes partially closed and weight loss (up to 15%). Mice may develop soft faeces and/or diarrhoea.

Inflammation (redness or swelling) could occur at the site of injection but should resolve within 2-3 days.

In all experiments, animals will be carefully monitored and humanely killed before they exceed moderate severity limits. Guidance will be sought from the NVS and NACWO should any animal display signs of abnormal behaviour or any unexpected change in physical appearance.

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

per species.

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

We expect approximately 90% of mice to experience mild severity and 10% of mice to experience moderate severity.

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

- Killed
- Used in other projects

Replacement

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

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

The extracellular matrix environment, which is made up of hundreds of molecules, undergoes dynamic changes in a tissue and provides essential biological and physical cues that dictate how immune cells and tissue structural cells function and migrate. Furthermore, the mammalian immune system is extremely complex and further requires co-ordinated interactions between different cell types, the extracellular matrix and tissue molecules, and also input from the circulatory system.

Reviews of research databases (E.g. PubMed, Preprint Archives, Web of science) demonstrates published data that examines immune responses or matrix changes in isolation or alternatively may focus on an individual cell type or matrix molecule, rather than examining complexity of cell and matrix networks within the tissue environment. In vivo systems are required to shed light on the key mechanistic cell-cell and cell-matrix interactions that influence tissue inflammation, physiology and pathology in response to pulmonary challenges. Unfortunately, in vitro assays currently cannot fully emulate the complexity of the lung tissue cellular and matrix environment. As such, in vivo models are essential to get an accurate picture of how whole tissue processes change dynamically and contribute to lung pathology.

Current alternatives include ex vivo culture of human tissue, in vitro cell culture systems or engineered tissues. We will be utilising lung tissue resections from humans in order to study cell-matrix interactions using co-culture systems and tissue slices to inform murine studies to ensure only pathways valid in human health and disease settings will be investigated in mice. However, such alternative approaches are limited in their use for addressing cell-cell and cell-matrix interactions because they don’t contain input from the circulation or lymphoid tissues and therefore cannot completely replace in vivo models.

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

Utilising NC3Rs and FRAME, alongside relevant literature searches we have investigated the possibility of using non-animal alternatives for this project that encompass in vitro tissue models, engineered tissue scaffolds, organoid cultures and microfluidic devices. From this research, the following alternatives have been considered:

In vitro cell culture assays
Organoids, organ-on-chip
Human tissue and data

Why were they not suitable?

Cells can be isolated from a tissue and put into artificial culture vessels. However, a lot of immune and tissue resident cells will change their physical and physiological phenotype and functions once out of their microenvironment. Cells can be grown in culture vessels coated in matrix components or artificial gel matrices, but such approaches cannot replicate the complexity and dynamics of the tissue matrix in vivo. Furthermore, even the ability to co-culture several different cell types together to understand cell- cell interactions is not reflective how the immune system works in vivo.

More sophisticated culture techniques such as human 3D lung slices can be used whereby tissue resident cells, including resident immune cells can be studied 3D within the natural lung architecture and ECM environment. Whilst tissue slice cultures represent a significant advantage over traditional in vitro assays, they still lack input from the circulatory system and therefore cannot be used to study cell recruitment. Organoid cultures are another technique that would allow us to study some limited aspects of cell-cell interaction and function, but they cannot replicate tissue matrix changes and cell migration during tissue remodelling responses. Additionally, it is not currently feasible to manipulate the matrix environment ex vivo.

The experiments that require dynamic tracking of cells and how they function in vivo is not possible with human tissue biopsies. Additionally, mechanistic studies require manipulation of the tissue environment or cells in vivo and this cannot be done experimentally in humans.

Overall, none of the alternatives investigated will fully replicate the lung tissue environment and ECM network and the way in which circulating immune cells interact with resident cells and the matrix.

However, we will regularly investigate alternatives throughout the duration of the project from resources such as NC3Rs, FRAME and Norecopa databases to ensure that any suitable techniques and advances to replace animals are put in place in our programme of work. Regardless, the alternative techniques mentioned above will be used throughout the project to guide, complement and translate our in vivo experiments, thereby reducing the numbers of animals required for in vivo studies.

Reduction

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

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

Numbers of mice are based on extensive previous experience using these experiment techniques during my career working on several Home Office licenses. Numbers take into account breeding strategies for genetically altered mice and the numbers of animals we anticipate using in the planned experiments across the course of this project.

In line with previous activity for the lab, we expect to use 5000 transgenic mice which takes into account important littermate breeding strategies for experiments (enabling use of wild-type (+/+) controls and GA mice within an experiment) and maintenance of transgenic colonies; with an estimated 3000 mice transferred to experimental protocols. Additionally, we anticipate using 3000 wild-type mice (in house breeding or commercial supplier) in experimental protocols when transgenic mice are not required.

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

My lab has and will continue to use the Experimental Design Assistant (EDA) on the NC3R's website. Any new staff members or students joining the group will be trained in using the EDA and will be encouraged to continue using it throughout the course of their individual projects and update their training with online courses offered by NC3Rs. The EDA allows us to review and discuss practical steps (e.g. randomisation, blinding) and data analysis (e.g. sample size and appropriate analysis tests). Additionally, all new lab members are trained in statistical analysis methods to ensure statistical significance with minimum number of mice, and consultation with statisticians whenever necessary.

Plans for experiments, including approaches for data analysis are discussed routinely in lab meetings so there is agreement that best practices and methods are being followed.

For all experiments we use inbred mice to reduce inter-animal variability. Most experiments also utilise littermate animals for both wild-type and genetically altered mice ensuring variation caused by differences in microbiota between mice is significantly reduced. Other variables such as age and weight are also taken into consideration for all studies. Overall, these strategies to limit variations allow us to reduce the numbers of control animals/groups required in experiments. We also routinely use both sexes (control matched) allowing for a larger percentage of mice produced during breeding to be used in experimental protocols.

In an increasing number of experiments where appropriate, we use inhibitors, neutralising antibodies, or cell depletion strategies in wild-type mice rather than using

transgenic mice, reducing breeding numbers. Additionally, we have refined our techniques to enable multiple parameters to be measured from mice (e.g. one lung is routinely divided to provide airway lavage, cells for phenotyping, tissue for protein assessment, tissue for RNA, tissue for histology and imaging) which reduces the number of animals required to answer our objectives.

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

Breeding of genetically altered animals is a large portion of our overall animal use throughout the project. However, when mouse lines are not being used experimentally, breeding goes onto a tick-over protocol to ensure only the minimum number of animals are bred to maintain the line. Our littermate breeding strategy also allows for littermate animals to be used as controls in experiments involving GA mice reducing the numbers of out-sourced wild-type mice required. Discussions about breeding lines and numbers of animals are done monthly in lab meetings to make sure we plan accurate for future experiments and do not overbreed any mouse lines. Importantly, we work closely with experienced animal facility technicians to optimise breeding strategies to reduce animal numbers.

During our routine meetings with the lab and other groups, we discuss all animal experiments from design through to analysis and results. Such discussions ensure all animals are used effectively and allow for experiments to be combined or tissue shared across multiple researchers. I have extensive experience successfully implementing this approach over my career whilst working in other labs, and it is an important tool we will use to reduce animal usage. In addition, we routinely harvest and fix or freeze as many relevant tissues as possible post-mortem for later use.

This approach has been used effectively to assess immune responses in other organs (e.g. pleural cavity, liver, heart) and has highlighted potential avenues for inter-tissue communication during allergic responses.

Pilot experiments are routinely used to determine the appropriate numbers of animals required to achieve statistical power and to ensure feasibility. To ensure reproducibility and reveal less pronounced effects, experiments are performed on a minimum of two separate occasions and data pooled for statistical analysis. For some experiments, we will use lung slice cultures or in vitro assays using primary lung cells to test new hypotheses prior to performing in vivo experiments. Such approaches require very few animals (e.g. >15 slices of lung can be obtained from one animal) and ultimately reduce the numbers of experiments and hence animals required for subsequent in vivo studies.

Importantly, in some cases these tissues can also be obtained from other research groups within the Institute who are performing experiments where the lungs are not required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-

operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

This project will use mouse models of inflammation and tissue remodelling to study how altered communication between immune cells, tissue stromal cells and the extracellular matrix contributes to changes in the lung. Mice are the most appropriate model to use in this in vivo setting as the immune system and matrix in mice have been extensively described and continue to provide insight into the importance of these cells and molecules in the physiology and pathology of human respiratory disorders.

Delivery of allergens or immunomodulators is performed to assess immune responses and changes to the extracellular matrix environment. Over the years our allergy experiments have been refined to study immune responses and matrix remodelling with the minimal number of times allergens are delivered to mice. Whilst chronic inflammation and matrix remodelling can be observed in the tissue and mimic human disease, long term allergen administration to mice does not cause long lasting harm to animals and are terminated before the animals exhibit serious breathing problems. We will continue to assess our methods, consult with collaborators, and review the literature to continually refine our approaches so as to cause minimal pain, suffering and distress whilst still being the best possible scientific approach.

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

To our knowledge, there are no other species of lesser sentience that can fulfil the requirements of this project to the same extent as the laboratory mouse. We are investigating complex and often chronic processes in relation to an adult immune system and extracellular matrix environment. Neonatal mice have a fundamentally different and changing immune system and extracellular matrix and therefore would not provide meaningful results as to how the adult system behaves.
As we wish to study the impact of modulating the immune system and extracellular matrix environment and how that impacts the lungs over time periods of weeks, procedures cannot be carried out on terminally anaesthetised mice.

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

Procedures will be performed by trained and skilled personal license holders, who will handle animals with care. All animals are monitored for adverse effects using objective measurements of clinical signs associated with adverse effects. Increased monitoring will occur if animals show adverse effects prior to humane endpoints being reached.

We will continue to use the most refined techniques in animal handling (e.g. tunnel handling) in order to reduce the stress associated with any procedures on mice.

Wherever possible, the least invasive methods for dosing and sampling will be used throughout the project. For some procedures, we use anaesthesia where appropriate and suitable (e.g. for humane restraint during intranasal administration of substances). Such procedures will be discussed with the NVS and experienced animal technicians to ensure they continue to be the most appropriate technique.

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

We follow the LASA guidelines (https://www.lasa.co.uk/current_publications) for best practice and follow PREPARE guidelines (https://norecopa.no/prepare) for experimental planning. Additionally, we routinely consult NC3R's resource library (https://www.nc3rs.org.uk/3rs-resources) including online training courses and videos for maintaining and improving best practices. The lab uses the NC3Rs Experimental Design Assistant to ensure all experiments are designed in a way that will allow us to achieve statistically significant results with the minimum number of animals required.

The lab routinely discusses 3Rs during lab meetings to ensure everyone actively discusses and considers all aspects of the 3Rs. Additionally, we read, share and discuss publications from other groups doing similar experiments to ensure the most refined procedures are being used for the topic being investigated. Collaborators and other researchers working on similar models will also be consulted on a regular basis.

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

We will hold frequent discussions with NACWOs, NIO and NTCO and dedicated veterinarians to seek guidance to make sure we maintain the highest level of animal welfare and constantly refine our animal use. We also will continue to present our research and studies to facility staff and get feedback from expert technicians to ensure we are following current best practices.

The lab will keep track of the latest advances in improving animal welfare via discussions with colleagues, attending conferences and assessing published literature. We routinely consult the NC3Rs resources page and FRAME website to ensure we are informed about all the latest advances in 3Rs and new approaches to using alternatives for animal research. The lab will attend any appropriate online seminars and training offered by NC3Rs or in-house and events highlighted to my research group. In line with the our local commitment to improving reporting of animal research, the lab will continue to publish all research outputs in line with the ARRIVE guidelines.

28. State-dependent Neural Processing

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neural circuits, Information processing, Physiological states, Behaviour, Hormones

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Our research aims to understand how the state of the body (e.g., hormonal or reproductive state, sleep, stress, hunger) affects information processing in the brain, and how this in turn affects behaviour. To achieve this, we will characterise state-dependent changes in networks (circuits) of brain cells (neurons) which control behaviours that are affected by such states (e.g., parenting circuits, feeding circuits).

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

Why is it important to undertake this work?

It has become increasingly clear that the brain does not operate in isolation. Rather, its form and function are profoundly shaped by the state of the rest of the body (e.g., hormones, inflammation, gut microbiome etc.). We will be studying these processes in brain networks and single neurons in the mouse brain, where exquisite genetic tools allow us access to well-defined populations of neurons. By studying networks

of neurons (neural circuits) in a mammalian model system, we hope to gain a better understanding of the causal and mechanistic links between neural networks and behaviour. Psychiatric and neurological disorders are increasingly thought of as network disorders, and it has become clear that the function of such networks is extremely sensitive to physiological parameters. Examining how physiological states such as pregnancy, stress or sleep, or metabolic changes impact the brain will allow us to unravel crucial mechanisms underlying both normal cognitive processes as well as neurological disorders. One example stems from our recent work into the neural effects of pregnancy: knowledge of where and how ovarian hormones act to change circuit function in an adaptive manner during pregnancy now puts us in a position to investigate what might underlie the maladaptive changes occurring during e.g., postpartum depression. Our work will thus advance a more integrative view of brain physiology in health and disease.

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

This project will generate new scientific knowledge about how information processing in the brain is influenced by internal states. Understanding how pregnancy hormones, among other factors, impact brain information processing could potentially advance the development of effective strategies for managing prevalent yet under-researched postpartum mood disorders, including postpartum depression and anxiety. Furthermore, this investigation might lead to insights contributing to the creation of highly targeted categories of contraceptives that operate peripherally, thus avoiding any potential adverse effects on the brain. In a broader context, this programme can uncover how neural and molecular signals from the periphery – such as those originating from the gut microbiome, inflammatory processes, and hormones – affect brain function in health and disease.

We will publish our findings in high-quality, peer-reviewed journals and we will make our data (e.g., neural recordings, behavioural videos, imaging datasets) available in accessible, curated and well- annotated repositories. This will allow other researchers to access our data for replication or complementary analysis, thereby maximising the impact of our work.

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

This is a basic research programme which will provide fundamental insights into how bodily states affect brain form and function. Its short- and medium-term benefits are to uncover how specific neurons and neural networks in the brain are affected by processes in the rest of the body, via either wired (peripheral nervous system) or wireless (hormones, circulating molecules) communication. We hope to discover new principles by which bodily states can influence the brain to ensure state-appropriate behaviour, and to provide mechanistic insights into how maladaptive states affect brain function.

Obtaining such mechanisms has the potential to further our understanding of conditions such as postpartum depression and other disorders which are caused by dysregulated physiological states.

We will aim to present and disseminate our work at national and international

conferences and communicate the gained knowledge and understanding through publishing our work in high-impact, peer reviewed journals as well as via preprint servers (bioRxiv) for more rapid dissemination. New tools (e.g., hormone reporters, tracing reagents, mouse lines etc.) developed in this project will be valuable to other scientists investigating state-dependent neural processing. We will make such technology, animals, protocols, insights, and reagents available to others via repositories such as Addgene, JAX, GitHub, Figshare or other open access databases.

In the long term, elucidating the mechanisms by which e.g., pregnancy hormones influence information processing in the brain might contribute to the development of strategies for managing postnatal mood disorders, such as postnatal depression and -anxiety. These disorders are highly prevalent, yet dramatically under-researched.

Another possible outcome would be novel insights contributing to the design of highly selective classes of contraceptives that act peripherally, without side effects on the brain. More generally, this programme will contribute towards overcoming the Cartesian divide between the body and the 'mind'. It has become increasingly clear that the brain is profoundly sensitive to both neural as well as molecular signals from the periphery (e.g., gut microbiome, inflammatory processes, hormones etc.). Such peripheral signals are suspected to be directly involved in mental disorders. By investigating the mechanisms by which they influence the brain, our work will advance a more integrative view of brain physiology in health and disease.

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

During this project, we will actively collaborate with colleagues from fields such as neuroendocrinology (to gain a better understanding of hormonal effects on the brain), systems and theoretical neuroscience (to model state-dependent effects on brain network function), and immunology / cancer biology (to explore how disease states affect brain form and function). Apart from advancing our core projects, these collaborations will aim to provide a broader, more generalisable context for our findings.

In general, we will maximise outputs through rapid publication of results on preprint servers (e.g., bioRxiv) as well as through publishing raw data on suitable platforms (e.g., Figshare), algorithms and protocols on appropriate outlets such as GitHub and negative results on Wellcome Open Research.

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.

Mice are the species with the lowest sentience that are still suited for studying mammalian brain function. Therefore, mice have been used in neurophysiological and systems neuroscience research for many decades. As a result, many transgenic mouse lines and viral reagents are available to label and interrogate specific, genetically defined populations of neurons in the mouse brain. In addition, the neuroanatomy of the mouse brain has been extensively studied, and high-resolution brain atlases are available, permitting targeted recordings and manipulations, and greatly facilitating the generation of hypotheses. As we mainly aim to unravel the effects of physiological states on neural information processing in the mature brain, most experiments will focus on adult mice. However, in some cases the development of circuits for specific behaviours or influence of hormones on circuit remodelling and maturation will be particularly insightful (e.g., during puberty), necessitating some experiments to be performed at earlier developmental stages. Neonates (P1-4) are used in observational experiments in which parental behaviour of adult test animals is characterised. In rare cases, surgeries (such as intracerebral virus injections) have to be performed at the neonate stage in order to subsequently investigate the function and development of neural circuits.

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

Part of the project will involve the raising of genetically altered mice to allow us to study the functions of particular hormone receptors, molecules or genetically defined neurons in state-dependent neural processing. These animals are expected to be no different in the way they behave from wild-type controls. In some cases, the animal's hormonal states may be altered by surgical (e.g., ovariectomy) or chemical (e.g., injection with hormones) means. These experiments will be performed by, or under supervision of, highly trained animal technicians at the host institute and will only cause temporary discomfort due to the use of general anaesthesia and appropriate pain management. The behavioural testing procedures we will use to measure state-dependent behavioural changes in freely moving or head fixed animals are painless.

During these experiments, mice will be exposed to pups or adults of the same species, or sensory stimuli. All the stimuli are below the pain threshold, and animals will be habituated to them. In some behavioural experiments, animals will be unable to move their heads as they will be mechanically restrained. This is necessary to allow for measurements of brain function that require a still head.

Some mice will undergo surgical procedures. A portion of the experiments will involve the insertion or implantation of a device such as a silicon recording probe, glass fibre, recording window, or cannula into the brain. Most of these approaches will cause little to no discomfort or pain as the brain itself does not feel pain (e.g., patients in neurosurgical settings are often awake). Some experiments will involve functional imaging from the brain where the brain surface is illuminated or scanned with a light source to measure activity in the brain. Some experiments will also involve the injection of a gene-delivery agent (such as a virus used as a gene ferry) that allows to label specific cells. These labels will allow us to monitor activity from cells or even control activity (e.g., exciting or inhibiting cells to test predictions of computational models). Adverse effects may occur, but the incidence is likely to be

low and methods of control (e.g., pain relief, antiseptic conditions) and the most refined experimental techniques will always be used to mitigate them. Postoperative pain and inflammation will be closely monitored, and animals will receive preventive pain killers during and after the surgery. Animals will be left one week to recover before undergoing any other procedures. Animals will be killed humanely at the end of the experiment. The duration of experiments varies between 2-3 months, but longer experimental timescales (up to 18 months) might be necessary e.g., in rare cases where long-term pregnancy-mediated behavioural and/or physiological changes will be characterised. Typical protocols include one surgical procedure over the course of the experiment, followed by repeated combined behavioural testing and neural recordings. In certain experiments (e.g., for the stepwise tracing of neuronal connections in complex brain circuits) the number could reach a maximum of four surgical procedures over the course of the experiment.

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

Surgical procedures will be carried out aseptically. Peri- and post-operative pain relief will be provided; agents will be administered as agreed in advance with the Named Veterinary Surgeon. Adverse effects from surgical procedures such as weight loss are infrequent, and animals typically make a rapid and unremarkable recovery within 2 days. Animals may experience pain during injection of substances which typically lasts for minutes. Mild and transient agitation or stress might occur as a result from head restraint. To mitigate such effects, animals will be habituated to the recording setup in incremental steps starting with short durations on the order of several minutes. No adverse effects are anticipated from behavioural tests, but animals will be closely monitored. During habituation to the setup and testing arenas mice will be taken through a habituation phase. To minimise stress animals are kept in a dark and noise-free environment during habituation periods of typically 10–30 minutes.

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

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

Mild - 50%
Moderate - 50%
Severe - 0%

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

- Killed
- Kept alive
- Used in other projects

Replacement

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

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

The experiments in this proposal aim to improve our understanding of the mechanisms of state- dependent neural processing. Our goal is to identify the cellular and neural network mechanisms by which bodily states (e.g., pregnancy hormones) can lead to changes in behaviour (such as onset of parental behaviour or changes to feeding routines). This requires studying the intact brain-body system and being able to assess the animal's behaviour as a readout. As we learn more about the neural networks under investigation, we will be able to computationally model them, but for such approaches to be useful, they will need to be tightly constrained by biological measurements.

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

Following the PREPARE guidelines and RSPCA suggestions, we considered primary cell cultures, simpler non-vertebrate organisms, and mathematical and computer simulations. We routinely explore such options by searching biomedical databases such as PubMed, Google Scholar or Web of Science, and by attending conferences in theoretical and computational neuroscience (e.g., COSYNE). We are also in constant exchange with colleagues working with non-vertebrate animal models.

Why were they not suitable?

We will supplement our research with the above-mentioned non-animal alternatives whenever possible, but our objectives cannot be achieved by non-animal alternatives alone. While we have considered other techniques such as cultured neurons, these are unfortunately inappropriate, since the culturing procedure destroys the organisation of the network and, crucially, precludes behavioural assessment.

Similarly, brain organoids – while occasionally exhibiting connectivity patterns vaguely resembling brain connectivity – are not suitable for our research since they are not part of an intact organism with physiological states. At a very fundamental level, cells in the living system behave very differently from cells in a dish (e.g., see Meister M, Neuron, 2017). Nevertheless, several crucial experiments, such as single-cell expression profiling and biophysical characterisation of neurons at different stages of pregnancy will be performed in isolated neurons and brain slices, respectively.

Similarly, simpler non-vertebrate organisms do not have the behavioural and neural complexity of mammalian brains. Since the biological basis of many brain functions remains unknown, they cannot yet be accurately modelled or simulated. The same applies to neurological disease states. We will attempt, where possible, to build computational models of cell and circuit function during different physiological states, but for such approaches to be useful they will need to be tightly constrained by biological measurements.

Reduction

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

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

Since an integral part of this research is to study the role of different cell types and neuronal and molecular pathways, we require different mouse lines in which such specific cell types and pathways can be targeted. Where physiological or other interventions are required, we expect that 5-6 animals per treatment group will usually be enough to obtain robust results. This is based on effect sizes from existing studies in our lab and the related literature. For most of the quantitative experiments, design will be based on PREPARE guidelines. Otherwise, we will use the minimum number of animals to provide an adequate description, generally on the basis of previous experience (our own and from the literature). We have statistical expertise within our group (and a resident biostatistician at the establishment) to adequately determine sample size based on power analysis. We will also use the NC3R's experimental design assistant to help us determine the appropriate animal numbers.

Finally, we will use the ARRIVE guidelines when we publish our work to assure that our work can be reproduced.

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

The overall experimental design relies on using the same animal for performing experiments and controls (e.g., virgin state vs postpartum state), as well as for gathering in vitro and in vivo data. This approach reduces the number of required animals since it (a) prevents duplication of experimental procedures and (b) reduces variability, thereby increasing sensitivity. By recording repeatedly from the same animals before, throughout and after changes in physiological state (e.g., pregnancy), we will obtain more valuable data and increase the statistical power of each experiment, as compared to single time-point experiments that require larger numbers of experimental subjects. Moreover, the use of viral vectors for visualising and manipulating specific neuronal populations will reduce the need for making transgenic mouse lines. My group has been using such approaches routinely in the past 5 years. Experiments will typically start with pilot studies involving small numbers of animals in order to recognise unexpected adverse effects and difficulties with experimental assessment early on. These pilot studies will also allow us to assess the variability in outcomes of our experiments, and allow us to devise strategies for further minimising such variability. In general, lines will be maintained in a homozygous state (i.e., having two identical copies of a given gene or genetic marker), to limit the number of offspring that do not have the gene / genetic marker. If it is impossible to generate homozygous animals, any offspring without the gene / genetic marker will be used as age and sex matched controls.

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

Together with our local biological research facility, we will identify the most efficient breeding strategies for transgenic mouse lines. This will involve e.g., using breeders that have two identical copies of a given gene or genetic marker (homozygous) to ensure that all offspring have a suitable genotype, as well as cryopreservation of embryos to enable breeding only when animals are needed. Over the last 5 years, we have been crossing all of our transgenic mouse lines to C57BL/6J mice, a common inbred mouse strain. Since all of our mice are now genetically very similar, this reduces inter-individual variability in our experiments. Wherever possible, we will coordinate experiments so that several orthogonal types of data can be obtained from the same animal (e.g., obtaining behavioural data, neural tracing data and brain slice electrophysiology data from the same individual). Such datasets have the additional benefit of allowing for correlations between different experimental readouts to be established. All experiments will be conducted in animals of both sexes, unless a scientific reason suggests the use of one sex. Finally, my group has established very efficient communication and data sharing protocols between members, thus reducing the chance of unnecessary duplication of experimental efforts.

Refinement

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

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

We will use mice for this project. Mice are sufficiently close to humans to reveal principles of information processing in the brain. Furthermore, rodents have been a long-standing useful model for behavioural studies of instinctive behaviours, enabling us to build upon a large body of existing research already, and to relate our findings to previous results. Our primary experimental approaches will include a) stimulation and recording from brain slice preparations, and from different regions of mammalian brain in the living, behaving animal, b) monitoring and manipulating the animal's physiological state and c) behavioural observations during social interactions of test animals with mice of either sex and of different ages (pups, juveniles, adults).

These are state-of-the-art approaches for studying how specific neural networks are connected and how they process information. They can be combined with observations of animal behaviour, which allows us to test the contributions of specific networks to the control of behaviour.

We have continuously refined the surgical procedures relevant to this project to minimise pain and distress to the animals. For instance, all surgeries will use light-

curable dental cement, reducing surgery time by about 20 minutes, and implanted optic fibres have magnetic connectors that can be attached without restraining the animal. In addition, since we have previously targeted many of the brain areas relevant here, there is typically no need to verify stereotaxic coordinates before surgery.

After surgery, animals are directly monitored in a recovery chamber and then returned to their home cage. Typically, once animals are returned to their home cage they are observed at least twice daily. Experiments will be done in awake animals where existing data indicate that brain function in anaesthetised animals significantly differs from brain function in the awake, healthy brain, and where behavioural readout is required. Furthermore, we typically group-house mice and provide environmental enrichment, which reduces stress levels significantly compared to isolated housing.

When recording neural activity under anaesthesia, we will ensure that the animals are sufficiently deeply anaesthetised using standard procedures (e.g., pedal withdrawal, tail and ear pinch reflexes, rate, depth and pattern of respiration, colour of mucous membranes or capillary refill time), and by monitoring vital parameters such as ECG, EEG and body temperature.

Genetic perturbations will be mostly limited to validated genetically altered mouse models, and in most cases, we will acutely induce genetic changes in specific neurons using viruses to deliver gene-editing payloads. This maximises the specificity of the phenotype and reduce the likelihood of generating severe phenotypes. When using pharmacological agents, dose-response curves will be generated in cultured cells or brain slices, to guide applications in the living animal and to minimise side effects.

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

This project aims to unravel how information is and processed in the mammalian brain. We want to unravel mechanisms that allow us to further our understanding of how the healthy brain works – which is essential for our understanding and treatment of complex neurological diseases such as schizophrenia or autism. While species with less complex brains provide very interesting model systems for fundamental aspects of nervous system function (and certainly inform our experiments), e.g., insect brains are too dissimilar from mammalian brains (in size, complexity, wiring, as well as in the properties of individual neurons) to draw direct conclusions to the human brain. For such work to be translatable to human patients, mammalian species are needed. Mice are well established in neuroscience, provide key tools such as genetic access and are to the best of our knowledge the species with the "lowest sentience” that still replicates key aspects of the human brain. Transgenic animals offer access to specific cell populations as well as providing certain disease models. For recordings from, and manipulations of, specific cell populations, viral gene delivery is crucial as this allows precise spatiotemporal control (this is critical for establishing causality). As viruses need several days to weeks for stable expression, surgical procedures under anaesthesia have to be followed by recovery time and subsequent neural recording or manipulation procedures. Moreover, it is now well established that brain function is profoundly influenced by anaesthesia.

Therefore, some recordings need to be performed in awake animals.

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

To reduce the stress associated with behavioural experiments, delivered stimuli are below the pain threshold, and animals will be frequently handled and habituated to the experimental setup over the course of days. Whenever possible, neural recordings will be done in freely moving animals using lightweight implants that are easily supported by the animal, but, in some cases, head-restraint may be needed to enable valid results. In this case, animals will be habituated to the recording setup in a stepwise manner, starting with short durations on the order of several minutes.

Surgical procedures will be conducted under anaesthesia and aseptic conditions to alleviate pain and reduce the risk of infection. Over the past 5 years, we have been continuously refining our surgical practices for e.g., implantation of imaging lenses into the brain, shortening the overall duration of surgery while increasing success rates. Animals will be left one week to recover before undergoing behavioural experiments. We will typically group-house animals and provide enrichment including nesting material to increase animal welfare.

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

Whenever applicable, we will follow the best practice guidelines provided by the NC3Rs (e.g., for blood draws or for husbandry). For surgical and non-surgical procedures, we will follow the recommendations of the Laboratory Animal Science Association (https://www.lasa.co.uk/wp- content/uploads/2018/05/Aseptic-Surgery.pdf) and of the Procedures with Care website (https://researchanimaltraining.com/article-categories/procedures-with-care/).

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

We constantly monitor best practices and new information available in the international literature and on the NC3RS and RSPCA websites (www.nc3rs.org.uk/our-resources www.rspca.org.uk/adviceandwelfare/laboratory). We also subscribe to the newsletter of the NC3RS (www.nc3rs.org.uk) and receive regular newsletter updates on best practices by our institution's biological research facility team.

29. Testing the Consequences of Haploid Selection in Animals

Project duration

5 years 0 months

Project purpose

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

Key words

Genetics, Reproduction, Fertility, Parental effects

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Sexual reproduction is a key biological process but we still know very little about the genetic mechanisms underlying it. Our project aims to understand how sperm are selected for successful fertilisation and how they interact with the female egg.

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

Why is it important to undertake this work?

Understanding the genetic mechanisms involved in reproduction helps us to improve artificial technologies in fertility to be applied in animal breeding and human reproduction.

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

The results from this project will be published in scientific journals with open access to make them available to a wide audience. They will lead to patents that have direct application in animal breeding and human reproduction and fertility. The results will also be presented at scientific meetings and conferences and in outreach events to communicate them to the general public.

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

The outcomes will benefit the scientific community in a wider sense, the medical community and the general public. The publication of scientific articles will occur over the duration of this project, and so will the filing of the patents. The application of the patents will reach well beyond the duration of this project and feed into improving the protocols and standards currently in use for Artificial Reproduction Technologies.

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

The project involves collaborations with national and international colleagues in related and complementary fields. The dissemination of our knowledge will be achieved by oral and poster presentations at national and international meetings and conferences to colleagues and at outreach events at schools, science fairs and other events to the general public.

Species and numbers of animals expected to be used.

- Zebra fish (Danio rerio): 10550
- Mice: 0

Predicted harms

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

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

Zebrafish are externally fertilising which makes it an ideal study species to observe and manipulate any processes involved in reproduction, fertility and early embryo development in life animals with minimal impact on the animals welfare. Studying sexual reproduction requires the use of adult, sexually mature fish. Gametes can be collected from live male and female fish using a minimally invasive methods, in vitro fertilisations can be performed in a natural environment, and embryos observed in vivo. In addition, zebrafish produce large clutches of offspring during every spawning event, and have a relatively short generation time compared to most other vertebrate model species.

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

The protocols included in this project are:
Breeding and maintenance of GA fish via male/female pairing for natural spawning: GA lines, initially sourced from International Zebrafish Resource Centres, or obtained from other projects, and protocols within this project, with authority to breed and maintain genetically altered zebrafish of that type and to provide them for use on other projects. Where necessary, genotyping of fish will be carried out via tissue sampling and/or Fluorescent microscopy or other non-invasive imaging technique. Stocks will then be maintained via natural spawning. Fish will be kept to a maximum age of 24 months. Severity: Mild.

Breeding and maintenance of GA zebrafish via in vitro manipulation of gametes or zygote, blastulae, embryos and/or fertilisation (generation of founder stock) Fish will be generated by CRISPR:Cas-9 and reared to adulthood for breeding and maintenance of the strains (dnd-1 knockout strain, strains with Vasa:Cas9:GFP labelling in a tissue specific manner) under standard laboratory conditions. Additionally, GA lines, initially sourced from International Zebrafish Resource Centres, or obtained from other projects, and protocols within this project, with authority to breed and maintain genetically altered zebrafish of that type and to provide them for use on other projects. Where necessary, genotyping of fish will be carried out via tissue sampling and/or Fluorescent microscopy or other non-invasive imaging technique. Stocks will then be maintained via IVF. Fish will be kept to a maximum age of 24 months. Severity: Mild.

Gamete collection: Adult male and female fish will be exposed to a standard anaesthetic, blotted dry around the genital papilla and gently massaged in a cranio-caudal direction for release of male (sperm) and female (eggs) gametes. Following the procedure, fish will either be euthanised via a Schedule 1 method or transferred to a recovery tank for full recovery before putting back in to the system. Severity: mild.

Exposure to temperatures up to 35°C of embryos, juvenile and adult fish (still within natural range): embryos, juvenile and adult fish of both sexes, all developmental stages and ages will be exposed to temperatures above standard (28°C) temperature of up to 35°C which is still within the natural range of temperatures experienced by zebrafish for a maximum duration of 14 days. Fish may be exposed to more than one 2-week period of high temperature and at different stages of development. Typically, a fish will be exposed for up to five times throughout life and fish may be kept alive up to the age of 24 months. Severity: mild.

Exposure to sub-lethal dosages of gamma radiation: Embryos, juvenile and adult (GA and wild- type) fish will be exposed to one sub-lethal dosage (70Gy) per individual per lifetime for the induction of mutations in somatic tissues and the germ line. After exposure, fish return into the system tanks and kept alive for a maximum of one week. Severity: mild-moderate.

Transplant of germ cells into embryos/larvae and subsequent rearing to adulthood: Germ cells collected from dead embryo and adult fish (GA and wild-type fish) following dissection will be transplanted into early stage embryos/larvae (< 5 days post fertilisation) which will be reared to adulthood up to the age of 24 months. Severity: mild.

Feeding of folic acid and/or similar non-toxic supplements: Adult fish will be fed with normal food supplemented with folic acid or similar non-toxic supplements for up to 3 months. Severity: mild.

The role of the microbiome composition in personalised nutrition and its implications on dietary fibre interventions: Germ-free embryos will be created then, as adults, fed a diet supplemented with dietary fibres alongside regular wild type fish. Severity: mild

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

Most procedures will have no effects or mild effects and may lead to mild pain and abnormal behaviour. Fish will be carefully observed for any signs of pain or abnormal behaviour and any fish showing symptoms above threshold will be killed by a schedule 1 method.

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

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

Protocol 1: Breeding and maintenance of GA zebrafish via male/female pairing for natural spawning (Severity: Sub-threshold - Mild)

Protocol 2: Breeding and maintenance of GA zebrafish via in vitro manipulation of gametes or zygotes, blastulae, embryos and/or fertilisation (generation of founder stock) (Severity: Sub-threshold - Mild)

Protocol 3: Obtaining zebrafish gametes (Severity: Sub-threshold - Mild)

Protocol 4: Temperature variation (Severity: Sub-threshold - Mild)

Protocol 5: Irradiation of GA zebrafish (Severity: Sub-threshold - Mild)

Protocol 6: Germline Transplant in zebrafish (Severity: Sub-threshold - Mild)

Protocol 7: Feeding of Folic Acid and/or similar non-toxic supplements (Severity: Sub-threshold - Mild)

Protocol 8: The role of the microbiome composition in personalised nutrition and its implications on dietary fibre interventions (Severity: Sub-threshold – Mild).

Project summary:
Zebrafish: 80% sub-threshold, 20% mild

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

- Killed
- Kept alive

- Used in other projects

Replacement

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

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

Sexual reproduction is highly complex and we still understand very little about the molecular processes occurring in the germ line and the moments before, during and after fertilisation. These processes can only be observed in vivo and using an externally fertilising species like the zebrafish where gametes can be obtained in a non-invasive way and in large numbers and where offspring can be observed from the earliest developmental stages into late adulthood is ideal. Mice are used as a common model to study human health aspects. It is necessary to study some aspects opf reproduction in mice due to the differences in reproduction between externally fertilising zebrafish and internally fertilsing mammals such as mice and humans.

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

There are currently no non-animal alternatives available for the study of sexual reproduction in animals. Much of our research is done on gametes (sperm and eggs) and early-stage embryos and we try to reduce the use of animals older than 5 dpf as much as possible. We work with gametes (cells) wherever possible, but we need adult fish and mice to produce the gametes.

Why were they not suitable?

The biological processes of sexual reproduction and fertilisation in animals are fundamentally different from non-animals such as plants, fungi. Our aim is to understand the mechanisms that specifically apply to animals and humans and this cannot be done by using any non-animal alternatives.

Reduction

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

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

The number of animals needed for this project are based on the protocols and experiments performed under previous project licenses. Our lab has extensive experience of working with zebrafish and all procedures except for the germ cell transplants have been previously applied in our lab. The numbers estimated for each

protocol are based on the success of breeding the animals (the breeding cycles are not entirely predictable and 30-50% of fish breed each cycle). We continuously revise our feeding regimes and attend seminars to optimise the breeding success in our animals, but there are many uncontrollable factors that contribute to this.

Where relevant, factorial experimental designs will be used to maximise the information obtained from the minimum resource. For most of the quantitative experiments, sample sizes may be set using statistical analyses based on existing data and effects desired to be detected (power analyses, generally using a significance level of 5%, a power of 80%, and a least practicable difference between groups of 25%). Otherwise, we will use our previous experience to select sample sizes. In terms of the numbers of animals required, we expect that 60 animals per treatment group should be sufficient to obtain the required results. However, these numbers may change once preliminary data are collected and power analyses repeated in order to ensure that the balance is correct between sufficient power and minimal animal numbers.

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

The number of animals for this project are based on previously published experimental work from our lab.

We also have a number of completed projects with unpublished results in the pipeline that help us to inform on numbers of animals to use.

In addition, wherever possible we use tissues, gametes and organs from animals from other protocols and projects that have been bred in surplus and are being killed by Schedule 1 methods.

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

We are running careful breeding programs in our lab to optimise the number of animals needed to maintain GA strains in our lab and to run our experiments. Wherever possible we use cryopreserved gametes (eggs and sperm) to maintain lineages that are not directly needed for experiments. We will continuously monitor our data and results and adjust the samples sizes based on the effect sizes obtained in pilot data and reduce further wherever possible.

Refinement

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

Which animal models and methods will you use during this project? Explain

why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We take careful precautions to minimise the pain and stress and lasting harm in all animals used in this project:

We use an anaesthetic to sedate animals to undergo procedures requiring direct handling of the fish including fin clipping and the collection of gametes. The anaesthetic removes the stress of direct handling without any lasting harm.

We limit our treatments to previously tested levels with well-established thresholds (temperature, gamma irradiation).

The use of an externally fertilising animal reduces the need for invasive procedures as many aspects of the project can be done in vitro.

We collect gametes from freshly killed animals by Schedule 1 from other projects that were discarded from these projects in order to minimise the use of animals.

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

The characteristic of external fertilisation is a key advantage of the use of zebrafish and there are no other less sentient vertebrates available to perform the project with. The use of a vertebrate is important as our project has direct implications for animal stock breeding and human reproduction and fertility and any invertebrate animal with external fertilisation (e.g. molluscs, echinoderms etc.) are too different to fulfil this requirement. Many of these invertebrate model species are also very difficult to maintain in a lab environment for several generations and often have long generation times. Terrestrial invertebrate species are not useful for this project as they cannot be used for in vitro fertilisation due to their internal fertilisation mechanisms. In addition, their fertilisation mechanisms differ substantially from vertebrate systems and the results obtained would not be translatable to mmmals including humans.

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

All animals bred and maintained in our lab are under daily supervision and are checked upon at least twice per day. Most procedures to be used in this project are sub-threshold or mild. All animals that have undergone procedures are closely monitored and kept in separate tanks if need be until they show full recovery and are ready to be put back into the system tank. We keep ourselves informed about alternative ways to perform procedures that we use in our project and will adjust procedures to levels with further reduced severity.

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

We refer to the website published by the Zebrafish Husbandry Association (https://zhaonline.org/resources/zebrafish-basics/) and the guidelines and references published there. The website is continuously updated and represents the latest

knowledge in zebrafish husbandry.

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

Our lab keeps close ties to the best practice advice in zebrafish by participating regularly in online and in person workshops and meetings where the latest developments are discussed and experience shared. This helps us to keep our lab up to date with the latest developments around zebrafish. We also continuously are on the lookout for alternative models and use for example cryopreserved gametes where possible. In addition, we do use mathematical modelling and power calculations to optimise and reduce the use of animals wherever possible.

30. Ecology of fish: from river to sea

Project duration

5 years 0 months

Project purpose

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

Key words

Fish, Climate change, Biological invasion, Migration barrier, Spatial ecology

Animal types	Life stages
Brown Trout (Salmo Trutta)	adult, juvenile
Pike (Esox lucius)	juvenile, adult
Salmon (Salmo salar)	adult, juvenile
Rainbow Trout (Oncorhynchus mykiss)	juvenile

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To quantify the behaviour and ecology of fish across freshwater, estuarine and marine environments through the study of their interactions with other species and their environment, including their responses to environmental changes.

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

Why is it important to undertake this work?

This work is important to generate new understandings of how animals behave and interact with their environment, including how environmental changes (such as

climate change, biological invasions and habitat fragmentation) alter these behaviours and ecological interactions. These new understandings will then be used to influence policy and practice at local, regional, national and international levels in order to promote sustainability in the exploitation of aquatic resources. This sustainability is important given the substantial declines detected in aquatic biodiversity in recent decades, especially in freshwaters.

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

The data outputs of the programme of work are the quantified consequences of how human-mediated environmental changes are altering the ecology and behaviour of native fish communities, including species that are increasingly threatened in their range in Britain. It will produce publications that will influence public policy on the exploitation and management of aquatic resources. These outputs will be subjected to the rigour of peer-review prior to their publication in scientific journals, with the results also shared with the research community via presentations (oral and poster) at scientific symposia and seminars. Data will also be made publicly available through an institutional repository to promote its wider use.

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

The users of the data outputs will be researchers, academics, non-governmental organisations (NGOs) and government departments and agencies. Researchers and academics will use the outputs to refine their own work, NGOs will use the outputs to inform their practises (for example, UK River Trusts using outputs to improve their management of alien species) and government departments and agencies will use the outputs to improve and refine their policy and practice in marine and freshwater ecosystems.

The benefits of the project are thus three-fold: (1) Scientific advancement; (2) Development of policy and environmental management; and (3) Delivery of an improved natural environment with healthy fish populations that are not impacted by human stressors. These are explained below, including their timescales.

Scientific advancement (short- and medium-term)
Knowledge on the consequences of anthropogenic-induced challenges on aquatic ecosystems is a fundamentally important area of science in driving forward understandings of the ecological consequences of anthropogenic stressors. Its novelty in using integrative approaches across multiple levels of biological organisation to determine consequences from the individual to the ecosystem level will enable the project to deliver high quality science of high relevance to society.

Indeed, these are crucial components of how local and global changes have implications for biodiversity (including threatened species) and how these then have implications for society more generally. The outputs of the project would thus be published in peer-reviewed academic journals to demonstrate its originality, rigour and significance.

Development of policy and environmental management (medium- and long-term)
The outputs of the project will directly support the policy objectives of the UK

Government, in particular Defra's statutory responsibilities to protect the ecological status of GB wild fish communities (in both freshwater and marine environments), as it will provide an understanding of the threat of stressors to the ecological status of fish communities. This has high relevance to the UK responsibilities under the regulations replacing the EU Water Framework Directive (WFD) and Habitats Directive following the UK withdrawal from the European Union. In particular, the influence of climate warming, non-native species, unsustainable fishing, habitat loss and nutrient enrichment is likely to be having profound influences on the ecological status of fish communities and thus understanding how these influence river classifications under current and future legislation is extremely important. Where failures to meet good ecological status occur under this legislation, then management decisions have to be taken that seek to remediate this failure. In this regard, the project will be hugely beneficial in providing both empirical data and predictive models that will highlight which stressors impact fish communities and prevent good ecological status being achieved.

In terms of policy, the ability of the research to result in policy changes is high, with liaison with UK regulatory bodies already completed on a regular basis. Thus, the translation of science into policy makes this research highly important and thus very worthwhile. Consequently, a final, lasting benefit of the programme of work is: Delivery of an improved natural environment with healthy fish populations that are not impacted by human stressors (long-term).

In developing new scientific knowledge that drives public policy and process at national and international levels, the long-term benefits of the research will feed into the development of an environment which is less impacted by anthropogenic activities, in which wild fish populations (and other taxa) are relatively unaffected by stressors such as alien species and climate change. In doing so, this benefit should provide enhanced human well-being through an enhanced environment.

Also, it should be noted that the work being completed in marine environments under this licence involves shark species that, in their UK ranges, are considered as of high conservation importance and thus the work contributes strongly to the UK meeting its biodiversity and conservation targets and measures in these species. Also, note that the project licence is compliant with the conservation designations of these sharks (e.g. none are CITES List A species).

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

Open access will be used for all publications (gold open access where possible, green open access otherwise). Datasets will be made available post-publication (via open access, using a digital object identifier) and used as part of larger meta-analysis type studies on fish behaviours and ecological interactions. The project will enable the collaboration with researchers in the field across the world, enabling larger-scale studies to be developed based on data generated within the project.

Species and numbers of animals expected to be used.

- Other fish: No answer provided

- Salmon (Salmo salar): 1500
- Rainbow Trout (Oncorhynchus mykiss): 200
- Brown Trout (Salmo Trutta): 1700

Predicted harms

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

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

The animals used are fish, with the species dictated by the research question being asked/ hypothesis being tested. In the facility, all fish used are less than 15cm in length and less than 75g in mass, which for most species used are immature (juvenile). These sizes and life-stages are used as they are the most appropriate for measuring rapid responses to different environmental conditions and interactions with conspecifics and other species. They are also appropriate for being able to measure behavioural responses using video equipment and are suitable for holding in both groups and individually. The species used all have relevance to the sustainability of aquatic resources in the UK, with the freshwater and anadromous fishes used all being sensitive to issues including habitat loss and fragmentation, and biological invasions, with those used in estuarine and marine habitats being species that are highly responsive to environmental changes and disturbances, and/ or there remains substantial knowledge gaps in their spatial ecology and behaviours which is inhibiting the development of appropriate marine policy following the UK withdrawal from the European Union.

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

On their initial arrival into the facility, the fish will enter a quarantine stage where they are held in an isolated flow-through tank system for 10 days, with checking and feeding twice daily, where the end-point of an individual fish is one of (i) the loss of body equilibrium, (ii) persistent irregular operculum movement, and (iii) no feeding response in the individual across 72 hours. Following quarantine, the fish are typically held in groups in holding tanks, where the water quality is maintained using flow- through filtration systems, with feeding at least once per day. In this period, the fish are tagged individually, involving general anaesthesia (Tricaine methanesulfonate (MS222), dose appropriate to species and lifestage (e.g. 0.04 g l^{-1})), implanted with a 7 mm passive integrated transponder (PIT) tag and measured (length and/ or weight). When used in trials, the fish are used either individually for short periods (1 to 24 hours) and their behaviours measured, or used in groups for longer periods (15 to 50 days). Feeding is maintained daily at a minimum rate of maintenance feeding (e.g. 1% mean body weight per day) but with ad libitum also used on occasion (e.g. 2% mean body weight per day). No trial is designed or expected to cause additional stress to the animals, with the response variables being measured comprising of behaviour, foraging rates and/ or changes in body sizes (i.e. measurements of growth increments). At the conclusion of trials, the fish are

removed from tanks, with the final step being administration of non-recovery anaesthesia (Schedule 1 euthanasia by an approved person), after which the fish are remeasured and any tissue samples needed for further work taken.

At POLES, fish are typically captured for use by the most appropriate method for that waterbody and the species being targeted. This is likely to be electric fishing (all species), rod-and-line angling (common bream, European barbel, chub, Twaite and Allis shad, all shark species), fyke nets (eels), fish traps (topmouth gudgeon, sunbleak) and seine nets (all species in stillwater conditions). The captured fish are initially held in recovery tanks containing water collected from the POLE, with aeration provided in warmer temperatures (e.g. >15C, but appropriate to the species). After inspection of the fish to ensure it is of appropriate condition (e.g. no external wounds, the ability to hold body equilibrium, responds to external stimuli), it is placed into an anaesthetic bath (MS222, appropriate dose, e.g. 0.4g per 10 L for most species but higher for European eel) until equilibrium is lost and the fish no longer responds to external stimuli. It is then measured, scales and/ or a tissue biopsy taken, and a PIT tag inserted into the body cavity (12 or 23mm length, depending on the size of the fish). The fish is then placed into a recovery tank of clean, aerated water. In a small proportion of fish (<10%), a larger incision will be made in the body cavity and an acoustic transmitter implanted along with a PIT tag, where the transmitter is between 6 and 13 mm in diameter and with a mass that is <2% of the fish body mass (to minimise tag burden). This incision will be closed with at least one dissolvable suture.

The fish is then placed into the recovery tank. Once the fish has resumed normal body (shown by it maintaining body equilibrium and responding to external stimuli), it is taken to the river and held facing upstream in the river current, and released once it shows a willingness to swim away. For a fish that has failed to recover body equilibrium and does not respond to external stimuli after two hours post- procedure, this is the end-point of that individual and the most appropriate Schedule 1 method is used to euthanise (administered by an approved person).

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

All animals that have tissue biopsies taken and PIT tags inserted under general anaesthesia are expected to recover quickly and resume normal behaviours (e.g. willingness to feed within 24 hours, but often within 1 hour). Animals that have had an acoustic transmitter inserted and the wound closed with a suture are released into the river on resumption of normal behaviour (the ability to maintain body equilibrium and respond to external stimuli in holding tanks, and swimming away normally on release into the river). However, a short-term behavioural impairment (< 48 hours) could occur (e.g. increased use of refugia). However, all individuals are expected to resume their normal behaviour (i.e. those behaviours that were being expressed pre-procedure) after 48 hours.

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

What are the expected severities and the proportion of animals in each

category (per animal type)?

There are 4 protocols in the project.

In 2 of these protocols, 100% of animals will experience mild severity (Protocol 3 and 4).

In one of these protocols, 90% of animals will experience mild severity and the remaining 10% will experience moderate severity (Protocol 1).

In one of these protocols, 100% of animals will experience moderate severity (Protocol 2).

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

- Killed
- Set free

Replacement

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

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

Animals need to be used to achieve the project aim as there remains substantial gaps in knowledge on how animals in aquatic environments - from individual to ecosystem levels - respond to environmental changes, including through alterations in their behaviour and ecological traits. Fish have been selected as the organisms to use in the project. This is because fish are recognised as being adaptable and tractable experimental organisms that provide excellent model systems for studies on ecology and behaviour, and they lend themselves to both short- and longer term experimental approaches where a range of relevant metrics can be measured that represent their individual, population and community responses to different environmental and biological conditions.

The generation of empirical data in controlled, semi-controlled and wild conditions is required to fill the knowledge gaps identified in the project and to provide the basis of approaches that enable some subsequent replacement of animals through use of these empirical data within in-silico approaches (e.g. individual based models). The protocols and procedures used within the project have been refined whereby they enable the animals to be implanted with internal transmitters that enable their individual identification and the measurement of their movements while minimising adverse effects.

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

Systematic review and meta-analyses, and in-silico approaches using predictive models and simulations, were all considered as non-animal alternatives.

Why were they not suitable?

The scientific literature on the behavioural and ecological responses of fishes to environmental changes is incomplete, with systematic reviews being insufficient to answer contemporary questions relating to species' responses (note, systematic reviews will be used in the project to assist identifying knowledge gaps and refine questions and hypotheses). In-silico approaches provide opportunities to simulate scenarios that are beyond the scope of empirical approaches to complete, but still require empirical data to both develop and calibrate/ validate models. Accordingly, while such predictive approaches provide highly useful approaches, they cannot yet replace empirical data collection from live animals.

Reduction

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

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

For protocols 1 and 2, completed in POLES, the estimated number of animals to be used are based on our ability to capture, implant with an appropriate transmitter to measure movements, and then have sufficient animals detected making these movements in order to provide a sample size that is appropriate for statistical testing. This is because when measuring the movements to sea of anadromous fishes, such as Atlantic salmon, not all the tagged fish will move at the same time (within year migrations can occur across a six to eight week period), some individuals will delay migration until the following year, and some might be predated upon before they start their migration (or during it).

Thus, the tagging of relatively large numbers of juvenile anadromous fishes ensures sufficient numbers are detected during their migration to provide data of sufficient rigour to enable statistical based methods to test hypotheses.

For protocols 3 and 4, experimental designs are based on fully factorial, block designs and, in conjunction with extant infrastructure and our prior experience in running these types of experiments, we are able to be confident in using fish numbers in treatments that provide the appropriate statistical power to measure differences between the experimental units.

Accordingly, we have determined these estimated animal numbers from work that has been planned within the project (and, in most cases, already funded) coupled with the numbers of animals we estimated would be needed to complete this work.

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

To reduce the number of animals used in experiments, trials designed within the project will be approved by AWERB. The process will require experimental designs to be circulated to core AWERB members (n = 5) by the personal licence holder intending to utilise the designs under this project licence. This design will include information on the acclimation period of the fish, the number of treatments and replicates, and individual fish numbers, along with their justification/ rationale. These designs may refer to statistical power and the number of animals needing to be used to achieve this (at typically $\alpha = 0.05$), but can also be developed from best practice guidance for specific experiments where appropriate (e.g. PREPARE guidelines, https://norecopa.no/media/kctfpwb3/prepare_guidelines_norecopa.pdf), and including our experience of running similar experiments.

Where appropriate, control groups will be used within fully factorial designs, with variability reduced between controls and treatments by using fish from the same hatchery sources (not wild) and being size matched (as length or weight). Replicates are expected to be randomised. Approval for use of these trial designs will be from a minimum of 3 AWERB members, including the NVS and NACWO. All critical comments should also be responded to, including where a revised design is required, and approval granted before the trial can commence.

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

The measures will be that any tissues/ scales that have been collected but not used will be held in storage (e.g. freezing for tissue samples) and made available for other researchers working on similar questions. We will use individual based models to simulate scenarios based on the empirical data collected, where simulations can be run across multiple scenarios, so avoiding these scenarios being completed using live animals.

Refinement

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

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

For the experiments to produce meaningful data, the animals must be able to express their natural behaviours. As such, handling is minimised during data collection and any tissue biopsies or transmitter implantation is completed under general anaesthesia (A/B). Where temperature and environmental conditions are being varied within a trial then these will be within the range of those experienced by the animals in natural situations. Appropriate periods of acclimatisation to tank conditions (10 days) and the least invasive tagging methods will be used at all times.

All tagging will be completed by competent personal licensees and under general anaesthesia (A/B) (except where fish size and logistical constraints prevent this in Protocol 2). Release back into the wild will only be after recovery to natural behaviours and according to set criteria as outlined in the relevant protocol(s).

Where surgical implantation of larger tags has been completed then the personal licence holder completing the procedure has the option of applying a localised low dose of lidocaine to the wound site to aid perioperative analgesia to reduce the short-term effect of the procedure.

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

Less sentient animals (such as invertebrate models) cannot be used as a refinement as their behaviours, ecologies and life histories are quite different to the fish species being used here and would not provide adequate data on behaviour and ecological interactions. The lifestages used are all appropriate to the methods being used (earlier lifestages could not be used). The required data could not be collected from animals that have been terminally anaesthetised, as the spatial ecology and behaviours of the fish are being measured, which requires the animals to be alive and in good health.

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

In the facility where trials are completed within aquaria, the conditions (e.g. temperature, light/dark cycle) can be controlled and the animals viewed easily, with monitoring of the general behaviours of the animals completed at least daily. A scoring scheme is used to assess animal behaviours during monitoring. Water chemistry is maintained through filtration and checked weekly.

In the POLE, refinements to the implantation of transmitters into the animals have been made, where internal implantation rather than external attachment is used (as this removes the risk of water drag and biofouling), with all procedures completed under general anaesthesia using doses that have already been refined for the species in previous work.

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

We will use PREPARE guidelines where this is appropriate (e.g. https://norecopa.no/media/kctfpwb3/prepare_guidelines_norecopa.pdf)

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

We will regularly check the information provided on the NC3Rs website (https://nc3rs.org.uk/) and are signed up to the NC3Rs newsletter.

31. Evaluating genes and translational interventions on atherosclerosis

Project duration

5 years 0 months

Project purpose

Basic research

Key words

heart attack, atherosclerosis, brain, inflammation

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To determine the efficacy of therapeutic interventions for atherosclerosis using representative mouse models and thereby stratify candidate therapeutics for progression to validative studies within large animal models.

To assess the contribution of disease-related genes to the development of atherosclerosis.

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

Why is it important to undertake this work?

Despite the use of primary prevention medications (anti-hypertensives and lipid-lowering drugs) alongside interventional procedures, cardiovascular diseases, including atherosclerosis, cause more than a quarter of all deaths in the UK, with an average of 420 people losing their lives each day.

Worryingly, progress in mortality outcomes has halted, attributed to a combination of aspects including a rise in risk factors, and recognising the contribution of inflammation. As such, cardiovascular disease accounts for at least 1.18 million hospital admissions and reflects the enormous economic burden of cardiovascular disease, estimated at £19bn in the UK. Atherosclerosis is associated with an abnormality of the cells within the arterial wall and characterised by the development of distinctive lipid- rich atherosclerotic plaques which show a high tendency to rupture, leading to thrombo-embolic events and/or ischaemia. Consequently, myocardial infarction (heart attack) and stroke are almost invariably associated with coronary and carotid artery atherosclerosis respectively, underlying the high mortality rates associated with this disease. Despite medications to target elevated blood pressure and cholesterol levels, mortality rates have stalled, necessitating the identification of novel diagnostic and therapeutic interventions intended for use in a clinical setting. As such, the purpose of the outlined studies is to use representative mouse models to assess new biomarkers and interventions for the treatment of atherosclerosis. A new avenue of our studies is the contribution of other inflammatory diseases to atherosclerosis and vice versa. Examples include periodontitis accelerating atherosclerosis, and atherosclerosis driving inflammation within other organs, especially the brain. As such, evaluating the brains from atherosclerotic mice may also reveal novel therapies and biomarkers for neuroinflammatory diseases such as vascular dementia and Alzheimer's Disease.

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

The data generated from the studies within this project will demonstrate if targeting immune cells and their products protect against atherosclerosis. If successful, this project will highlight potential therapeutic targets alongside possible biomarkers of disease progression, and therefore support their advancement into trials or safety and effectiveness assessment in large animal models. The studies will also determine effective delivery strategies (such as nanoparticles, CAR T-cell, antisense oligonucleotides) and efficient administration routes for potential new medicines in future mouse and large animal studies. We therefore envisage beneficial outputs from this data to include new knowledge which will be delivered through presentation at scientific conferences and within peer- reviewed, open-access publications, alongside identification of candidate therapeutic targets/compounds and associated filing of patents.

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

The prime objective of the outlined work is to elucidate the most appropriate and effective therapeutic target to protect people from the clinical complications of atherosclerosis.

The expected short to medium term benefit is the generation of new knowledge, defining how select immune subpopulations behave during the progression of atherosclerosis, and how inhibiting their function or secreted molecules alters disease advancement. These data will be presented at local, national, and international scientific conferences/ public events respectively, and published in peer- reviewed academic journals and will provide a new appreciation of how distinct immune subsets can be assessed to predict disease susceptibility and

therapeutically targeted to limit cardiovascular disease. This will benefit our research group and collaborators, others within our establishment and researchers further afield, alongside the pharmaceutical industry and clinical colleagues.

The long-term potential benefits of these studies are that data generated may have far-reaching implications for the diagnosis and treatment of atherosclerosis, benefitting patients and clinicians by contributing to the development of predictive tools and effective therapies, which will ultimately reduce the economic and health burden caused by this pivotal cardiovascular disease.

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

All methods to regulate the expression of genes/non-coding RNAs/proteins in immune cells are well established and we have been successfully using these under my existing project licenses. Specific regulators of immune cell behaviour are expected to affect the progression of atherosclerosis. Thus, it is highly likely we will identify novel drug targets through our assessment and analysis of distinct immune subpopulations during the progression of atherosclerosis. Accordingly, If the outlined mouse studies prove successful, the therapeutic targets and most effective delivery approaches will be advanced to testing in appropriate large animal models for evaluating safety and efficacy. To this end, we already have an existing project license in place which contains large animal models of atherosclerosis and aneurysms. Additionally, we have an existing network of collaborators and contacts within the pharmaceutical industry to facilitate large animal studies and generation/provision of bulk in vivo-ready therapeutics. In due course, our findings will be published in peer-reviewed, open-access journals, and where appropriate, pre-print servers (such as Research Square or BioRxiv) which permits dissemination of new knowledge and publication of unsuccessful approaches in advance of the protracted peer-review process. Alongside presentation at scientific meetings, resources will be made available to researchers through provision of data, animals, and tissues/cells.

Species and numbers of animals expected to be used

Mice: 2500

Predicted harms

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

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

The complexity of the multiple biological changes that occur during the formation of atherosclerosis and aneurysms cannot be modelled collectively in isolated cells or tissues, and therefore makes the use of living animals essential to predict effective and safe new therapies.

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

Mice will be used in all studies to determine the effectiveness of immune modulating approaches upon atherosclerosis and its complications. The breeding or cross-breeding of genetically altered mice is essential in order to produce appropriate numbers of experimental mice. Upon arrival in the unit, mice will be allowed to acclimatise for a week within standard cages, food, and water. Prior to a change in diet, a small blood sample will be taken from a superficial vessel to provide baseline values of circulating molecules and immune cell numbers. Mice will experience mild and transient discomfort from blood sampling. To induce atherosclerosis, mice will be fed a modified diet. The feeding of a modified diet is not expected to cause distress but may sometimes result in obesity or itchy skin. In some cases, weight loss may occur due to unpalatability. In the event of weight loss, mice will be returned to a normal diet if they lose 15% of their body weight. Typically, mice will experience mild, transient pain and no lasting harm from administration of immune/inflammation-modulating substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal). Where administration is required for prolonged periods, mice will be surgically implanted with slow-release devices such as an osmotic mini-pump. These animals will experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics. The final procedures will be undertaken under non-recovery anaesthesia, during which animals may experience mild distress associated with anaesthetic induction. It will be necessary to assess potential changes in blood pressure and heart rate in animals receiving candidate drugs or genetic manipulations, as such, some animals will have a telemetry chip implanted subcutaneously which will facilitate subsequent analysis of physiological signals including blood pressure and ECG, from conscious, freely moving animals, providing stress- free data collection while enhancing animal welfare.

In studies where gene deletion/induction mice are used for evaluating a potential therapeutic target, we will generate atherosclerosis by inducing the disease through administration (single IP injection) of a transgene (PCSK9) alongside feeding a high fat diet. This will eliminate the need for a complicated breeding strategies of multiple knockout lines to generate experimental mice. Some studies utilising gene deletion/induction mice will require administration of gene inducing or deleting agents, such as five daily IP injections of tamoxifen.

In some studies, we aim to expose animals to an additional associated risk-factor for atherosclerosis, such as infection with a periodontopathic bacteria, and determine if circulating immune subpopulations and/or proteins associated with the presence of periodontitis correlates with atherosclerosis development and progression.

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

The mice used in these studies are not expected to experience more than moderate transient pain or suffering at any point during the study. For the long-term delivery of drugs, mice will have surgery to implant a device under the skin that can release a medicine slowly. They are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in hospital.

However, the induction of atherosclerosis is associated with sudden death in a small number of animals (<5%), due to atherosclerotic arterial wall rupture. The rupture of an atherosclerotic plaque is an unavoidable and inevitable consequence of prolonged high-fat feeding, which cannot be predicted but is essential for scientific results and conclusions ascertaining to neuroinflammation. Moreover, the immediate drop in blood pressure, resulting from the rupture of a plaque, invariably results in loss of consciousness and therefore incurs minimal suffering. As far as possible, suffering will be minimised by closely monitoring the mice and killing them as soon as any signs of clinical disease or suffering become apparent. In addition, a rare occurrence (<2%) in hypercholesterolaemic mice subjected to high-fat feeding is the development of a localised skin rash, which can be irritating and cause the animals to scratch them, breaking the skin. With the application of an anti-septic healing cream under isolation, all mice are expected to make a full recovery within two-weeks.

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

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

The severity of the breeding and maintenance of GA mice is mild: none of the mice are expected to experience more than mild transient pain or distress as a result of the procedures undertaken.

The severity of the atherosclerosis studies is moderate: The majority of animals (>90%) are not expected to show signs of adverse effects that impact significantly on their general well-being. No more than 5% of animals are expected to show moderate clinical signs as a result of hypercholesterolaemia, a novel anti-atherosclerosis agent is administered, or after subcutaneous implantation of sponges/osmotic mini-pumps. Very rarely the severity of these signs may be such that the humane end points may be reached.

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

Killed

Replacement

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

Why do you need to use animals to achieve the aim of your project?

Good in vitro assays for modelling primary immune and inflammatory responses during atherosclerosis formation and progression aren't available. This is because of the complexity of the process which involves the differing accrual, modification, uptake, and processing of cholesterol by immune cells in tissues, alongside their interaction and modulation by other vascular cell types within the vessel wall and lesion, and induction of the primary responses at these sites.

Which non-animal alternatives did you consider for use in this project?

We have explored and considered potential AI and computer modelling systems for atherosclerosis research - with no acceptable models yet developed. An appraisal of in vitro/ex vivo models have also failed to reveal any suitable approaches. However, laboratory studies using isolated cells and tissues from either mice or humans will form a major part of our proposed studies and will be used alongside our recently developed aneurysm model which uses human vessels in a laboratory setting (ex vivo) to determine potential interventions in advance of any work in live animals. Nonetheless, the complex biochemical changes that occur in vascular disease cannot all be modelled in isolated cells or ex vivo tissues, because they are influenced by a wide range of physiological factors that are unique to living animals.

Why were they not suitable?

Due to the multifactorial and complex composition of atherosclerotic plaques, non-animal alternatives are not applicable to this project Neither fruit flies, nematodes, or zebrafish larvae develop atherosclerotic lesions, so are also unsuitable alternatives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Funding is in place in for four studies, with a further six studies planned over the duration of the project. Based on our extensive experience of the mouse models to be deployed and the associated data, experimental group sizes of 15 mice of each gender are required to ensure that the results are reliable and statistically significant. Controls groups will be included when there is a clear scientific need, such as deployment of a novel delivery strategy or use of a vehicle for a new drug that has not been previously used. However, for the purpose of estimating the total number of animals it has been assumed that control groups will be required. To evaluate the effect of potential therapeutics on disease development and progression, two different approaches are deployed.

Planned studies: (treatment + control groups) x 10 targets x 2 approaches (disease development and disease progression) x 30 mice/group = 1200 mice

Furthermore, studies to permit proteomic and single-cell sequencing analysis of atherosclerotic lesions will be undertaken in all studies to facilitate biomarker discovery. The estimated minimum group size for these studies is 6 mice/group = 96 mice.

In addition, we've used our annual return of procedures data and recent extensive experience to estimate the number of mice that we will need to use for breeding.

Five studies require breeding and maintenance of GA mice: 200 mice/line = 1000 mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

As with recent best practice within my research group, we employed the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We will use the EDA diagram and report outputs to support experimental planning with animal users. However, given the limited statistical repertoire of the EDA tool, additional advice was sought from a Senior Research Associate in Experimental Design, alongside utilisation of the Psychometrica platform for the calculation of effect sizes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The number of animals will be minimised by conducting initial studies in cells and tissues in the laboratory, with strictly controlled conditions to minimise experimental variability. Such in vitro work will include studies to evaluate effects of therapeutics upon the behaviour and function (movement, invasion, and survival for example) of inflammatory cells and blood vessel cells. This in vitro work will provide us with the best indication of approaches that are likely to be fruitful in mice, and will be supported by validative expression studies in human tissues.

Our extensive experience of mouse atherosclerosis studies means that we can use historical data to perform power calculations to ensure that the experimental designs are biologically and statistically rigorous. In general, the experimental design will involve comparison of a control group with one or more intervention groups using statistical tests appropriate to the data. We will regularly review our designs in the light of the data generated to ensure that the results are statistically rigorous but do not involve the use of unnecessarily large groups of animals. My research group is already part of a tissue sharing network in place at the Establishment, which we will continue during this project to permit other researchers access to our tissues.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically altered mice that develop severe hypercholesterolaemia, such as the apolipoprotein E (Apoe) knockout mouse, are virtually unique amongst experimental

animals in that they develop spontaneous unstable atherosclerosis, the root cause of most cases of myocardial infarction and stroke in humans, so we have chosen them for our validative studies of candidate therapeutics. In studies where gene deletion/induction mice are used for evaluating a potential therapeutic target, we will generate atherosclerosis by inducing the disease through administration (single IP injection) of a transgene (PCSK9) alongside feeding a high-fat diet. This will eliminate the need for a complicated breeding strategies of multiple knockout lines to generate experimental mice.

To induce hypercholesterolaemia and subsequent atherosclerosis, animals require a modified high-fat diet. In some mice, increasing the fat content of the diet in animals lacking Apoe or with PCSK9 over- expression can lead after several months to the development of cutaneous xanthomas (<2%). Such mice will therefore be inspected regularly by Animal Service Unit staff during routine husbandry for signs of skin lesions. Nonetheless, both the Apoe deletion and PCSK9 over-expression approaches are the gold standard methods for spontaneous atherosclerotic plaque formation, obviating the need for surgical methods to induce atherosclerosis, and therefore limiting the pain, suffering, or distress to the animals.

Some studies utilising gene deletion/induction mice will require administration of gene inducing or deleting agents, such as five daily IP injections of tamoxifen. This method will permit the study of processes that happen in specific cell types and direct future cell-targeted therapy approaches. We will however routinely check the NC3Rs and associated 3Rs newsletters and websites for awareness of improved routes for administration (such as edible gels) and will apply in future studies if possible.

Additionally, the best housing and husbandry practices will be deployed, including provision of cage enrichment and application of no-touch or hand-cup handling to limit distress.

The measurement and characterisation of atherosclerotic lesions requires that the vessels be perfused in situ at normal arterial pressure with a fixative agent. This requires that the animal be exsanguinated prior to fixation, otherwise the fixative clots the blood and blocks the blood vessels. Therefore, animals will be terminally anesthetised for this part of the procedure.

Why can’t you use animals that are less sentient?

Mice have the lowest neurophysiological sensitivity of the animals suitable for the outlined studies. It is not possible to conduct this study in less sentient species as they do not have immune systems comparable to humans or develop complex atherosclerotic lesions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use a new refined approach in our studies by use of a single injection of a substance (PCSK9- AAV) which elevates the blood cholesterol levels of mice when they are fed a high-fat diet. Using our previous breeding data from studies where cross-breeding of genetically altered mice is necessary, deploying the PCSK9-AAV

refinement will greatly reduce (50%) the numbers of breeding mice required to generate experimental mice for atherosclerosis studies. This equates to a reduction of 100 mice per study where cross-breeding is required. Mice are also an appropriate species due to the availability of genetically modified strains and active inhibitors. We have extensive experience in all the models and methods to be used in this project and are confident that they are the most appropriate to address our research questions. The procedures used in atherosclerosis and immune-modulation studies will cause no more than mild transient pain or distress which, where appropriate, will be minimised through the use of general anaesthesia.

Group sizes in ageing experiments will be increased to accommodate for loss of animals and to avoid single housing due to animal losses due to old age. Longer drinking spouts will be used to facilitate easier access to drinking water, and animals will be monitored for adverse effects such as changes in weight, dermatitis, piloerection, paleness, changes in mobility, lumps, eye defects, abnormal respiration, or stools. If these are observed animals will be treated accordingly, and animals that develop severe effects will be humanely killed.

For prolonged drug delivery, we now routinely use smaller mini-pumps, resulting in reduced incidence of wound dehiscence and shorter surgery times. Such refinements deployed during our most recent licence demonstrates our continual endeavours to refine the mouse models we commonly deploy to test validated hypotheses in vivo, which we will continue throughout the life-span of the current license.

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 the NC3Rs Guidelines on best practice for the administration of substance.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly attend NC3Rs events and our annual 3Rs meeting. We will also regularly check information upon the NC3Rs website, and the newsletters from the NC3Rs and our institutes 3Rs team.

32. Circadian clocks in the brain and their dysfunction in 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
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

sleep wake cycles, circadian, neurodegeneration, Alzheimer's disease, glial-neuronal interactions

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

In this project, we will study how ageing and neurodegenerative processes (the decline and death of nerve cells in the brain and nervous system) disrupt the processes which control the daily workings of the body and, in turn, how boosting such processes may enhance healthy well-being during ageing and prevent and/or delay neurodegenerative progression.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

More than 55 million people worldwide have dementia, with almost 10 million new cases every year. Alzheimer's disease (AD) is responsible for 60-80% of these cases. In the UK alone, over 850,000 people live with dementia, and Alzheimer's is the most common type. This disease not only affects those diagnosed but also has a profound impact on their families and caregivers. Alzheimer's progresses over decades, leading to memory loss, anxiety, depression, and sleep problems.

Eventually, patients lose the ability to communicate, become bedridden, and need constant care. With an aging population, the burden of this disease is expected to grow in the coming years unless new treatments are found.

Our internal body clocks, called circadian clocks, help regulate our behaviour and physiology and synchronise it to light-dark cycles. These clocks control our sleep patterns, cognitive abilities, metabolism, and immune responses. Disrupting circadian clocks, whether due to genetics or environmental factors, is linked to various age-related diseases like diabetes, heart disease, and neurodegenerative disorders.

In Alzheimer's disease, disruptions to sleep patterns, such as increased activity and agitation at night (known as sundowning), are common. Recent studies have found that changes in sleep patterns can occur decades before cognitive symptoms appear, and they're associated with a higher risk of developing Alzheimer's. Our modern lifestyles, with widespread exposure to artificial light at night and limited daylight during the day, along with shift work schedules, can further disrupt our circadian rhythms. Despite knowing the association between circadian disruptions and cognitive decline, we do not know which are the molecular, cellular and circuit mechanisms underpinning this link.

This research will thus bring a unique perspective to the dementia field by providing a deeper understanding of the underlying mechanisms which influence circadian function in brain health and how they change in the early stages of dementia. As body clocks are fundamental mechanisms underpinning healthy bodily function, such an approach would have a preventative value: to engage existing body mechanisms to enhance resilience to disease. In doing so, we will complement more radical disease treatments, often accompanied by significant side effects and only showing modest improvements to symptoms and disease outcomes.

What outputs do you think you will see at the end of this project?

Our work will result in the publication of findings related to the aims outlined here in internationally recognised peer-reviewed journals. Our work will be disseminated within the neuroscience, chronobiology (the science of the effect of time, especially rhythms, on living systems) and dementia communities by invited talks and abstracts to national and international meetings. Any potential targets identified through our work will be evaluated for potential clinical application. To achieve this, we will draw from our experience of collaboration with pharmaceutical companies, focused on the integration between preclinical mouse and human models of circadian disruption in Alzheimer’s disease (AD).

Who or what will benefit from these outputs, and how?

Recent evidence shows that sleep and defects with circadian rhythm are closely linked with numerous neurodegenerative conditions and can precede the onset of recognisable symptoms of AD by several years, thus highlighting them as major new targets both for the early diagnosis and prevention/treatment of dementia in the early stages of the disease.

Short-term benefits: In the short term, this project will provide a better understanding of the involvement of circadian clocks in the daily regulation of the processes responsible for the production and the clearance of toxic proteins involved in neurodegenerative diseases and particularly in AD. This will be achieved by genetic and pharmacological (drug) targeting of clock mechanisms in different cell types and brain areas in healthy mice and models of the disease. These experiments will identify the most promising targets to be explored for brain-wide circadian regulation of the processes associated with dementia onset and progression. These candidates might be of interest to pharmaceutical companies interested in leveraging circadian clock mechanisms to prevent/ control neurodegenerative diseases. Moreover, it will provide valuable information to other research groups working in the dementia field.

Medium-term benefits: In the medium term, the most promising candidates will be selected for use in experiments with animals. The effects of the relevant genetic and pharmacological manipulations will mainly be assessed in experiments studying animals over long periods of time, whereby the circadian and cognitive function (ability to understand) of the mouse will be monitored before and after the treatment in a naturally occurring behaviour. This experimental design will ensure a reduction of animal usage while providing evidence that cannot be obtained without using animals (such as effects on brain function, clearance of toxic proteins and behavioural performance). The suggested approach will reveal the best candidates for drug screenings in preclinical models of neurodegenerative diseases.

Long-term benefits: In the long-term, our work will test the manipulation of circadian and sleep function as a way of preventing/delaying neurodegenerative conditions. We will test the idea that clock mechanisms can be engaged to improve brain function in the early stages of Alzheimer's disease, by boosting daily clearance of brain waste (from normal brain processes) and/or reducing the production of toxic proteins. With success, targeting these pathways could be seen as a preventive strategy that will benefit, in particular, people at higher risk of developing Alzheimer's disease because of their lifestyle (i.e. shift-workers, patients). Moreover, such approaches could be used by clinicians as a component in a multipronged approach to disease, with the view of boosting the effectiveness of other therapies based on types of immunisation (i.e. Lecanemab)

How will you look to maximise the outputs of this work?

In our laboratory, we work on both mouse and human preclinical models of AD. This approach enables swift assessment of potential targets which could be relevant to human conditions. We work in close relationship with other research groups that have an interest in exploring circadian alterations of specific processes involved in disease. We strive to publish negative results from our group. Usually, we include these are included as support data in supplementary figures. Finally, we will make

our datasets publicly available to the scientific community by uploading them to freely accessible data repositories.

Species and numbers of animals expected to be used

- Mice: 9000 mice

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We need to use animals in our project because circadian rhythms emerge from a complex interaction involving links between different brain areas and body parts and the environment (i.e. light/dark; feeding/fasting cycles). Moreover, we are interested in following the daily regulation of complex behaviours such as the sleep-wake cycles and cognition (awareness), which cannot be mirrored by in silico (computer-based) or in vitro (non-animal) models. The deep-seated organisation of the circadian system is common to mammals: this motivates using the mouse for our investigations, together with the availability of several tools for refined genetic manipulations. Less sentient animals, such as insects, do not have the same organisation of the circadian system, and therefore any experimental outcomes would not be applicable to Alzheimer's patients. Most animals in our project will be genetically modified but will not undergo any other procedures - they will only have samples of their organs and tissues taken for study after they have been humanely killed. In such cases, we will use young animals but will however use adult forms for these tissue collection experiments when considering the effect of ageing on the preservation of the rhythms. A significant minority of mice will undergo brain surgery to manipulate circadian function and investigate the effects of these genetic manipulations on improving cognition and pathology (disease) in mouse models of brain diseases, such as Alzheimer's disease (AD). As AD and other neurodegenerative diseases are age-related diseases, we will perform experiments across the lifespan in order to investigate the contribution of healthy ageing process vs pathological ageing in the investigated processes.

Typically, what will be done to an animal used in your project?

Most animals in our project will be genetically modified, but will not have any other procedures performed - they will be humanely killed to collect brain samples to be isolated and cultured for live imaging experiments. A significant minority of mice will be single-housed for the assessment of their internal sleep-wake cycles, daily monitoring of cognitive performances and other naturally occurring behaviours (e.g. drinking, eating). This will all be monitored non-invasively (i.e. without injury to the animal) by state-of-the-art in-cage monitoring systems. These experiments will be conducted in our dedicated circadian cabinets and/or circadian room, whereby mice can be isolated from external noise and disruption and light levels can be accurately manipulated to simulate lifestyle disruption of the circadian systems, such as jet-lag,

sleep restriction and shift-working, or diminished light levels. A smaller subset of mice will undergo the administration of drugs and /or biological material to balance circadian function and establish the boosting of the body clock as a mechanism of resilience to disease. The effects of these manipulations on sleep-wake cycles and cognition will be monitored over time within the same animal, which produces more reliable data and reduces the number of animals used. Mice may also be implanted with well-established commercially available lightweight imaging devices to monitor in real-time the processes in the animal's body which are associated with the behaviours under investigation. Typically, these mice will undergo 1-2 surgical procedures (maximum 3) in their lifetime and short bouts of anaesthesia to mount headplate devices during recording sessions. After surgery, these mice will be placed in their home cage and sleep-wake cycles and cognitive function will be recorded non-invasively, and their brain activities (monitored by head- mounted devices), monitored in baseline conditions, or in response to chronodisruptive events (e.g. jet- lag, sleep restriction)

What are the expected impacts and/or adverse effects for the animals during your project?

Our research involves using animals that are genetically homogenous, or carrying genetic mutations which are not expected to significantly harm them, being commonly associated with mild clinical signs, if any. However, it is not always possible to fully predict the severity of any potential defect when circadian and neurodegenerative mouse models are also exposed to environmental changes, combined with naturally occurring ageing. Therefore all mice will be carefully monitored for possible unexpected harmful effects and veterinary advice will be sought promptly if any are seen.

Old age can be associated with an increased incidence of cataracts, arthritis, obesity, spontaneous tumours, gland abscesses and ulcerative dermatitis. Based on our previous experience, we anticipate that no more than 15% of mice in this protocol will exhibit such clinical signs of moderate severity. In such instances, the animal will be monitored closely and veterinary advice sought regarding suitable interventions for prompt resolution of symptoms. If no improvement is seen within 2 days, animals will be humanely killed and tissue collected as appropriate. Aged animals may die unexpectedly without showing prior signs of ill health, we estimate based on prior work that this may occur in between 1 – 2% of aged animals.

Mice are social animals and therefore single housing, which is a prerequisite for detecting internal body clock function, may have a potentially stressful impact on the animal. However, such impact is largely dependent on the surrounding environment, especially for male mice. Stress responses associated with single housing mainly emerge as acute (immediate) responses to group separation and regrouping, rather than prolonged social isolation. Stress and circadian responses in mice that have been individually housed for three or more weeks are in fact indistinguishable from house-grouped littermates. To prevent stress from regrouping, experimental mice that have been singly housed for more than 3 weeks will not be regrouped in their lifetime and will be humanely killed at the end of the experiment. In our experience, placing nesting material and/or wheel effectively mitigates any stress risk. While we constantly monitor singly housed animals for any signs of emerging stress, including

abnormally increased or reduced activity and repetitive behaviour, we have not seen any such instances of distress/ harm whilst using such a setup.

About 20% of animals may undergo brain surgery of moderate severity. Mice will be given multiple types of pain relief during and after surgery. They will recover for several days after surgery and will be checked twice daily for any unexpected signs of pain/distress. Prompt advice from veterinary staff will be sought if needed.

Infections are unlikely (up to 1%) and will be limited by using good surgery techniques. Any mouse exceeding signs of moderate severity (e.g., weight loss more than 15%) will be promptly and humanely killed, in consultation with the veterinary staff.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals (80%) will be only experiencing mild/ subthreshold severity procedures, including breeding, schedule 1 humane killing for tissue collection, or simple low-stress behavioural tests (such as wheel running behaviour, or novel object recognition) to measure circadian and cognitive function, respectively. About 15-20% of animals may undergo brain surgery of moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Circadian control of physiology, behaviour and cognition cannot be fully replicated outside the body, as it requires complex interactions between different brain regions and with the environment, such as light-dark and/or feeding/fasting cycles. Moreover, in vitro models do not yet accurately mirror the ageing process, which is a predominant risk factor for both dementia and impaired body clock function. Animals are therefore the only feasible strategy to study the crossover of disrupted circadian function by genetic and lifestyle factors, and altered body functions in the ageing and neurodegenerative processes, which cannot yet be captured by in vitro assays, computational modelling, stem cell-related work or even post-mortem human brain experiments.

Which non-animal alternatives did you consider for use in this project?

While it is not possible to comprehensively investigate circadian disruption in in vitro models, we extensively use non-animal alternatives in the lab to replace animal

work, whenever possible. In particular, we are establishing the characterisation of circadian function in nerve cells derived from stem cells isolated from Alzheimer's patients and healthy donors (hIPSCs), in collaboration with pharmaceutical partners. We are currently characterising circadian rhythms in these patient-derived cultures by serial live imaging and gene expression profiling and comparing findings in animals. We will use these patient-derived models to enable the fast screening of potential therapeutic compounds, thus replacing most animal usage for this intensive phase of research, critical for the translation of findings from bench to bedside.

Why were they not suitable?

While we do use non-animal cellular models for studies of gene expression and drug screenings, these cannot reproduce the interactions between different brain areas, the feedback from the body and the external environment (light-dark cycles), which are essential to fully model circadian function.

To overcome such limitations, we are collaborating with colleagues working on organoid models, which may reproduce in the near future more complex relationships between cell types and body parts.

Moreover, we are establishing protocols aiming to simulate circadian rhythms of body temperatures as a way to synchronise circadian rhythms in the absence of light-dark cycles. While these approaches bear great potential to replace a bigger proportion of our animal work moving forward, they are not yet established.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our numbers are estimated on our current experimental design, experimental design assistant (NC3R’s) and previous work under the last PPL using similar workflows. We expect an increase in animal usage when compared to past years due to projected increased funding and staffing and the absence of the pandemic hiatus.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We take several steps to reduce the number of animals used.

These include:

- We conduct power calculations to assess the likely number of animals to be used to reach sufficient confidence based on our previous observations, pilot studies and available literature.

- We use online tools, such as NC3R's Experimental Design Assistant and seek ad hoc statistical advice from the Imperial Statistical Advisory Service (SAS) https://www.imperial.ac.uk/research-and- innovation/support-for-staff/stats-advice-service/, when necessary.

- We use an approach that relies on experimental measurements within the same experimental subject before and after treatment, thus allowing for lower experimental variability and increased statistical power (longitudinal approach).

- We detect several readouts from a single mouse where possible, such as sleep-wake cycles in registration with cognitive function and recording of underpinning cellular brain activities, thus allowing to maximise experimental output and improving data quality (multipronged approach)

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- Use of a centralised mouse breeding facility and sharing of surplus animals with other investigators. Sharing of surplus animals is achieved within the laboratory and across groups using the same mouse lines (e.g. mouse models of amyloidosis shared within the UK DRI Imperial Centre, via dedicated internal channels (e.g. Slack), email communications etc

- Establishment of an in-house automated colony tracker app within the laboratory to readily track animal availability and their age, which enables the reduction of surplus animals and careful planning of experiments involving aged animals

- Pilot experiments. Whenever a new experiment is introduced we carry out pilot experiments with low animal numbers (e.g. around 4) to gain preliminary evidence of effectiveness or establish a technique at specific end-points. This allows more accurate estimation of experimental variance and effect size for accurate power calculations.

- We collect brain tissue isolated from pups and adults and perform live imaging and manipulations on this tissue, after the humane killing of the animals. Whereby possible, this reduces the number of animals undergoing brain surgery, as it allows us to monitor circadian function in brain tissue, without the need of implanting head-mounted devices to monitor tissue activities in live animals

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority of the experimental animals will be humanely killed at juvenile (young) stages for tissue collection and will therefore only undergo subthreshold/mild levels of distress.

Animals at adult life stages may be aged to investigate circadian disruption and its link to neurodegenerative diseases and reduced cognition. When performing these experiments, animal behaviour will be mostly monitored using non-invasive methods, including wheel running and cognitive testing. Cognition will be monitored continuously and with minimal interactions with the experimenter, whereby possible, for example by using newly developed "smart-Kages". This refinement will allow us to monitor animals' cognition in their home cages, in an extremely enriched environment, thus minimising disruption of sleep-wake cycles associated with repeated testing and reducing stress levels related to these procedures. No food restriction or fear conditioning testing will be conducted and all the procedures are only expected to generate mild discomfort, if any.

Detection of internally-generated circadian patterns of behaviour in adult mice requires single housing. In our setup, mice are provided with positive enrichment, such as the wheel and neslet material.

Typically, cages are stored in light-tight HEPA-filtered circadian cabinets. In these conditions, they are exposed to familiar smells which minimise potential distress. We have been using this setup for several experiments in the past five years, which has allowed the uneventful recording of sleep-wake cycles for several weeks.

When necessary viral particles and other substances will be injected, or recording devices will be implanted under general anaesthesia into the mouse brain to enable long-term monitoring and manipulation of brain function and evaluate consequences on co-detected sleep-wake cycles and cognitive performances. When substances need to be administered repeatedly, refillable minipumps may be used to mitigate animal distress. After surgery, the animal will be given pain relief and allowed to recover in standard light-dark conditions for at least one week before any further procedures are carried out. The most common adverse effects from the surgery are infections: these are expected to occur in a minimal fraction of animals (less than 1% in our experience) due to the use of sterile techniques and competent surgeons. The animals will be closely monitored throughout and treated humanely in consultation with veterinary staff in the event of pain or infection. Our experimental protocols are typically longitudinal, with behaviour monitored in each animal before and after the intervention. This experimental design allows for the reduction of animals used, thanks to reduced variation of results between experiments and enhanced statistical power. These procedures may expose the animals to moderate levels of distress and pain due to surgical procedures which will be closely monitored throughout the experiment.

Why can’t you use animals that are less sentient?

Mouse is the species of choice for the study of neurobiology and genetic aspects of circadian timekeeping, as they mirror the same underlying circuit structure observed in humans. In particular, mice share the presence of a master clock at the bottom of the brain, called the suprachiasmatic nucleus, which is only present in mammals and not in less sentient species. Moreover, genetic manipulations are easily performed in mice, and several mouse types and tried-and-tested reagents are available which allows for rapid testing of theories and minimises the risk of causing unexpected harm to animals. Expression of sleep-wake cycles requires the animal to be awake (and asleep) and conscious, or asleep and unconscious, a behaviour that cannot be expressed under anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have recently demoed smart-kages for continuous monitoring of animal circadian and cognitive behaviours in home cages, which require minimal interactions with the experimenters and reduce stress levels associated with repeated monitoring of cognition. For pain management after brain surgeries we will provide analgesics for 3 days post-surgery and mice will be checked twice daily. Wherever possible tetherless and battery-less head mountings and wearable recording/stimulation devices will be used for monitoring brain function.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow Morton et al 2001 guidance for volume of substances administered and ARRIVE and PREPARE guidelines and seek further advice and updates from our local AWERB, whereby required. We will also utilise available resources available through the NC3Rs, such as the online Experimental Design Assistant.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will work closely with the NVS, NACWOs and support team. We will consult local AWERB and receive regular updates on 3Rs from it, including conferences and opportunities. Other resources include NIOs, other Named Persons, NC3Rs and NORECOPA. We will also utilise available updated resources available through the NC3Rs, such as the Experimental Design Assistant. Finally, we will stay updated with information disseminated by the Establishment’s 3Rs programme manager and 3Rs advisory group.

33. The interplay between the immune system and the musculoskeletal system

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

immune cells, bone, inflammation, therapy, muscle

Animal types	Life stages
Mice	juvenile, adult, pregnant, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will investigate the interplay between the immune system and the musculoskeletal system both in healthy individuals, during ageing, acute inflammation, chronic (obesity-induced) inflammation and following menopause. Potential therapies will also be tested in these settings with the aim of restoring normal immune or musculoskeletal function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The immune system plays a vital protective response to infection and injury through the movement of immune cells from the blood into the tissue where they mount effective responses to the challenge and aid tissue repair. Similarly, the

musculoskeletal system plays a vital role in mobility and strength of an organism, as well as regulating blood flow back to the heart. Over the life course humans encounter a number of stress events - these can be acute infections, systemic inflammation caused by diet (obesity) or disease, age-related changes in hormones leading to menopause and eventually old age. These stress events affect both the immune system and the musculoskeletal system, but it is unclear who says what to whom and when during such a stress event and what chemical signals are responsible for facilitating this system-system communication. Mounting a good effective response to a stress event indicates healthy physiological resilience, whilst mounting a poor response is often seen in individuals with defects in one or both of the immune or musculoskeletal systems, leading to increased frailty and mortality. Our work aims to provide more detailed knowledge on how the immune system and musculoskeletal systems communicate with one another; how this communication might become dysregulated during or following a stress event; and the factors responsible for these conversations in health, following stress events and with age. Ultimately our aim is to use this knowledge to develop a new type of treatments that restore healthy immune-musculoskeletal system communication pathways to improve resilience to stress events over the life course and thereby maintaining healthy physiological resilience.

What outputs do you think you will see at the end of this project?

Project outputs will include:

- advances in scientific knowledge on the processes controlling both the immune system and musculoskeletal system in health, with age and in disease, and how these communicate with one another;

- scientific and lay publications of our findings and methodology;

- presentation of our data and/or methodology to the wider scientific community through conference oral and poster presentations;

- identification of novel targets or agents that alter immune or musculoskeletal system function, which can be take forward to develop new therapies.

Who or what will benefit from these outputs, and how?

This project has a wide range of beneficiaries. Short-medium term

Information obtained under this PPL will have an immediate impact of other projects undertaken both within the same lab and also those of our collaborators and also further afield upon dissemination.

Moreover, this project aims to improve our understanding the processes across multiple organ systems that drive age-associated pathology and frailty, as well as chronic diseases such as obesity and osteoporosis - an area of intense research interest worldwide. The impact of this is likely to occur in the short-to medium term, during the lifetime of this project and beyond as all publications are released.

Medium-long term

Collaborations – We intend to invite external seminar speakers who have an interest in immunology, musculoskeletal biology, gerontology, ageing and chronic inflammatory diseases to our organisation, with the view of fostering further collaborations based on the concepts and ideas incorporated in this proposal. We envisage that these collaborations will occur during and following the completion of this project, and therefore represent a medium to long-term impact of this work.

Long term

Clinicians and pharmaceutical companies will benefit from advances in our understanding of organ system cross-talk in health, with age and in chronic disease, along with our identification of new targets for drug development. We are in continual conversations with these collaborators with the view to translate our findings into new drugs over the subsequent 10-15 years following the completion of this project.

How will you look to maximise the outputs of this work?

Dissemination of information

We will work with the relevant teams at our Establishment to facilitate communications and resulting impact. We plan to use several routes to disseminate our findings to the wider scientific and pharma community and the public that will facilitate end-user engagement:

- Peer-reviewed publication. We aim to publish high impact papers based on the findings generated during this project licence. In addition, our group has a strong tradition of publishing methodology papers; and negative data to ensure that groups do not unnecessarily repeat experiments that either technically are flawed or biologically yield the null hypothesis.
- Presentations. We and our collaborators will present data at internal seminars along with national and international conferences.
- Dissemination via international societies. We and our collaborators are active members of various scientific societies, allowing our findings to be disseminated to the wider scientific community in societal magazines and training workshops.

Enhancement of public understanding and engagement with research

We will take advantage of several events organised by the Public Engagement Working Group at our organisation and local charities to facilitate the public's awareness our research.

Clinical Collaboration.

My team and I are active members of several multi-institute research centres and will be able to present our findings at least twice annually at ongoing Centre seminars. We are also part of the UK Ageing Network and will be able to present our findings at these quarterly meetings as well as help shape the UK Ageing Strategy/Landscape. We will also attend clinical conferences where we will present our data and foster

collaborative opportunities for translational research across the fields of gerontology, immunology and musculoskeletal biology and chronic inflammatory diseases.

Species and numbers of animals expected to be used

- Mice: 5600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have chosen the types of animals and their age based on our need to model a mammalian immunological and musculoskeletal systems during health, with age and inflammatory disease that occurs in adult humans. As such, adult mice represent the lowest mammalian option available with both an immunological and musculoskeletal system in which we analyse how the two systems communicate with each other in health, with age and in disease, and to test new agents that that could be subsequently progressed through drug development pipelines for human use. Moreover, the majority of the in vivo data within these fields have been obtained using mice, providing baseline characteristics and mechanistic insights. Therefore, using adult mice also allows us to avoid repeating baseline, characterisation experiments in a new species.

Typically, what will be done to an animal used in your project?

Animals will be studied under resting conditions (~20%), where the majority will receive an agent or dye either via injection (typically x16) or via a mini-pump implanted under general anaesthesia (up to 60 minutes) where chronic administration is required and will be killed up to week 6 via Schedule 1 method or non-schedule 1 for withdrawal of fluids (e.g. blood). Approximately 50% of the animals will have undergone natural ageing up to 22 months prior to administration of agents.

Where inflammation is induced, two methods will be used: Acute inflammation (peritonitis) will be induced in 40% via injection of an inflammatory stimuli into young or naturally aged mice. The remainder will be fed an altered diet to induce chronic systemic inflammation (obesity). For all inflammatory models, the majority of animals will also receive an agent or dye either via injection (typically x16) or via a mini-pump implanted under general anaesthesia (up to 60 minutes) where chronic administration is required. Animals will be killed by up to week 6 (acute inflammation) or week 25 (chronic obesity-induced inflammation) via Schedule 1 method or non-schedule 1 for withdrawal of fluids (e.g. blood).

To induce menopause and thus premature musculoskeletal decline in the absence of ageing, 20% of young animals will have their ovaries surgically removed under general anaesthesia (up to 60 minutes), where the majority will receive an agent or dye either via injection (typically x16) or via a mini-pump implanted under general

anaesthesia (up to 60 minutes) where chronic administration is required and will be killed up to week 8 via Schedule 1 method or non-schedule 1 for withdrawal of fluids (e.g. blood).

What are the expected impacts and/or adverse effects for the animals during your project?

Animals experiencing acute inflammation may lose weight or show signs of abnormal subdued behaviour for a short duration before they recover or are humanely killed. Animals fed an altered diet to induced low-grade systemic chronic inflammation (obesity) will steadily gain weight and may experience early signs of insulin insensitivity (diabetes). In all cases, animals be monitored closely before humane endpoints are actioned.

Aged animals may experience age-associated physiological changes and may spontaneously develop tumours which will be monitored closely before humane endpoints are actioned.

For surgical procedures it is expected that animals will experience a moderate level of pain shortly after the surgical procedures which will be managed with pain relief. Animals will be closely monitored and killed if humane endpoints are reached.

Whilst there are no adverse effects anticipated from the agents themselves, the route of administration itself causes transient pain each time the reagent is administered.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate severity to approximately 95% of animals Mild severity to approximately 5% of animals.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Multiple physiological processes are involved in a variety of stress events that affect humans throughout their life-time. Such events can be localised to a single tissue (e.g., an infection) but may have wider felt effects. Others are experienced more broadly across the entire body (e.g., obesity, menopause, ageing). Both the

musculoskeletal and immune systems are crucial for maintaining physiological resilience to these stress events, however, how these systems communicate with one another to facilitate this resilience is poorly understood. Given many of these stress events are not static and confined to a single tissue or time point, modelling the effects of specific regulatory processes and new therapies inevitably involves the use of whole organisms and, in particular, the use of animal models of the stress event. However, where possible, cells from patients will be used to address questions relating to the mechanisms of action of therapeutic targets (see below in vitro models).

Which non-animal alternatives did you consider for use in this project?

We have pioneered a range of in vitro multi-cellular 3D constructs, incorporating primary human cells/tissues from healthy young and old subjects, patients with obesity, obesity-induced musculoskeletal diseases or individuals pre and post menopause. This has allowed us to further our understanding of the mechanisms regulating how the immune and musculoskeletal systems communicate with one another, and how this communication might be altered following a stress event common in the life course (e.g., infection, obesity, menopause, ageing). Indeed, for the majority of our work to date, we have used these in vitro tissue culture techniques to provide information about molecular and cellular mechanism(s) involved in the pathogenesis of disease or therapeutic effects.

These cell culture assays will be continued to be used throughout this licence to assess potential therapeutic molecules in functional assays such as expression, activation, proliferation and cytotoxicity. Molecules shown to be inactive in these assays will not be examined further in vivo. This utilisation of in vitro assays is an important replacement of in vivo experiments. Moreover, key molecules and/or processes identified using human and patient material will be used to inform the in vivo studies, focusing on those aspects relevant to the disease setting we are modelling.

Why were they not suitable?

Our in vitro models are unable to fully recapitulate the blood and lymphatic vasculature, the movement of cells through complex organs, or the physiological impact of changes in one organ system (e.g., musculoskeletal) on a different and anatomically distinct organ system (e.g., immune system). On this basis, whole body systems are required to provide a setting for studies in which the efficacy of possible novel therapeutic agents can be determined. There are no other in vitro or in vivo alternatives to this work. We will continue to collect as much in vitro evidence as possible before embarking on animal experiments, using it to inform and refine experimental design.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any.

These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used specific mathematical calculations based upon previous studies and the likelihood of our interventions producing positive results, to estimate the number of animals we will use in our study. For all experimentation, the lowest possible number of animals will be tested whilst ensuring that the experimental result is robust.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used statistical analysis to calculate the minimum number of animals necessary for this project.

We will continue to use the NC3R's experimental design tool to aid experimental design and consult available trained statisticians before using any new protocols.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have used statistical analysis to calculate the minimum number of animals necessary for each experiment within this project.

New interventions will first be tested for efficacy using in vitro models prior to use in vivo. Where new routes of administration or new interventions are being examined, pilot studies will first be established in 2-3 mice prior to full experiments. Subsequently these pilot data will used in the specific mathematical calculations described above to ensure that we use the minimum number of animals needed to obtain statistically significant results.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used in models of specific stress events experienced over the life-course – inflammation, obesity (chronic inflammation), ageing and musculoskeletal decline (menopause). Each model has been selected based on its ability reduce the suffering and harm whilst simultaneously exhibiting either a functional immune system or musculoskeletal system to allow us to investigate how these two organ systems talk to one another. This species and procedures have been chosen as they

represent the lowest animal models with an immune and musculoskeletal system in which it is possible to study their communication, how these changes with a stress event and the effects potential therapies may have on this.

Why can't you use animals that are less sentient?

Less sentient animals do not possess the same sort of skeletal structure that composes the joints, and often their vascular tree and immune system do not fully represent that of humans. Small rodents are the lowest mammals that can be used to recapitulate the human immune and musculoskeletal systems response to a stress event (inflammation, menopause, ageing). As such stress events occur in adulthood through to older age and the impact of these challenges can take several weeks to months to manifest within the immune and musculoskeletal systems, we are unable to use immature or anaesthesia animals to answer our research questions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Each experimental model will be monitored daily following intervention and mice will be assessed for any signs of distress such as pain and inability to feed. Surgical interventions will be undertaken using the most appropriate anaesthetic and analgesia will be given.

The mode of substance administration will be chosen to cause the least harm and distress to the animal. Any new substances or route of administration will be tested in a small pilot study and the mice monitored daily for signs of distress. Where substances need to be administered over several weeks (e.g., chronic inflammation), these agents will be delivered using surgically implanted slow release mini pumps rather than repeated injections.

We will also systemically review each experiment on completion to see what lessons can be learned from the study in terms of endpoints (scientific and humane) and any animal welfare issues that may have arisen during the experiment that could then guide the subsequent experiments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Animal welfare is a key consideration in all of our protocols, and we will be guided by our NACWOs and NVS in always ensuring that we are using best practice and the most refined techniques.

Following every experiment and regularly during group meetings we will review our procedures from a welfare standpoint to identify any potential for refinement.

If undertaking a systematic review, we will use SyRF, the free online platform for researchers, to perform a systematic review and meta-analysis of animal studies. This will allow us to keep up to date with any improvements in protocols and techniques which may reduce or replace the use of animals.

We will follow the LASA guidelines on and Guiding Principles for Preparing for and Undertaking Aseptic Surgery (www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf) when undertaking aseptic surgery and providing analgesia and on dosing for substance administration.

Finally, we will use ARRIVE 2.0 to ensure transparency and reproducibility in our published data.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continue to engage with establishment efforts to promote the 3Rs and workshops; and receive the NC3Rs newsletter.

34. Improving immunotherapy strategies for paediatric cancers

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

immunotherapy, neuroblastoma, childhood cancer, drug repurposing, inflammation

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

In this project we aim at improving cancer immunotherapy in the context of childhood malignancies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Immunotherapy has improved the outcome of childhood malignancies such as neuroblastoma. However, some patients are poor responders and a leading hypothesis is that the mechanism by which the tumours becomes resistant to immunotherapy is the modification of host immune cells induced by the cancer. The goal of this project is to repurpose commonly used drugs to re-activate the anti-

tumour immune response, enhancing immunotherapy and improving patients survival.

What outputs do you think you will see at the end of this project?

The outputs produced by this study will be (a) new knowledge on the mechanisms governing the interaction between cancer and immune cells (b) publications in which we will describe the outcome of the research and the potential clinical benefits and (c) clinical trials in which the methods pioneered in the animal study will be validated in children with cancer.

Who or what will benefit from these outputs, and how?

The ultimate beneficiaries of this research will be cancer patients. We predict that at at the end of the 5 years research, clinical trials will be started to validate the hypothesis that combining immunotherapy with the drugs identified in this study will be safe, extending the life expectation of children with cancer.

How will you look to maximise the outputs of this work?

We will maximise the outputs by publishing our results in reputable scientific journals and attending national and international conferences. We will also promote the research by interacting with a network of collaborators in paediatric cancer research, we have established strong ties with local and international childhood cancer research charities such as the Children Cancer Leukaemia group, the Associazione Italiana per la Lotta al Neuroblastoma and the Little Princess Trust.

Species and numbers of animals expected to be used

- Mice: 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 full complexity of cancer growth can only be replicated using a mouse model, since contact with the tumour microenvironment and the host immune system is often critical for tumour growth. The host- tumour interaction, which is a fundamental aspect of cancer growth, can only be adequately investigated in developed (i.e. adult) mice - using newborn mice would be unpractical and present welfare issues related to the growth of xenotransplanted tumour masses relative to the recipient size.

The main objective of this investigation is to develop new therapeutic interventions for childhood cancers, and a child's immune system is sufficiently developed to be compared to an adult mouse. The focus of our research is translational and it is essential to demonstrate that the potential therapeutic substances previously

validated in vitro or as single agent are effective in the context of combinations with standard therapies in appropriate disease models. This step is required before any drug is considered for clinical trials in human, making the use of animals, and mice in particular, a necessity.

Typically, what will be done to an animal used in your project?

Tumour cells will be transplanted under the skin of mice by subcutaneous injections and allowed to grow until they reach a palpable stage. Next, mice will be injected in the peritoneum with drugs either used as single agents or in combination with immunotherapy. The duration of the experiments will be generally around 4-5 weeks after which the mice will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Subcutaneous tumour transplantation procedures are well established and will cause transient discomfort but not pain to the animals. Mice will be able to feed and behave normally because tumour volumes will not be allowed to grow over values that could cause pain or abnormal behaviour. Dosing mice with drugs by intraperitoneal injections should only cause a very transient discomfort at the injection site which can be mitigated by using very fine needles.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for the tumour transplantation procedure, tumour growth phase and drug dosing is moderate for the totality of mice.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cancer development is regulated by intrinsic and extrinsic factors. Single features of cancer cells such as their ability to proliferate, invade and respond to drugs can be studied using methods alternative to animals such as invasion chambers, flow cytometry, classical proliferation and death/live cell assays and other in vitro techniques. We have used and will continue to use these in vitro analyses as proof of principle studies before starting animal experimentation. However, the full complexity of cancer growth can only be replicated using an animal model, since contact with the tumour microenvironment and the host immune system is often

critical for tumour growth. The host-tumour interaction, which is a fundamental aspect of cancer growth, cannot be adequately investigated in vitro. The focus of our research is translational and it is essential to demonstrate that the potential therapeutic substances previously validated in vitro are effective in the context of a living organism. This step is required before any drug is considered for clinical trials in human, making the use of animals a necessity.

Which non-animal alternatives did you consider for use in this project?

Unfortunately there are no useful alternatives to the use of animals in this case.

Why were they not suitable?

In vitro techniques can't reproduce the interactions between tumour cells and the tumour microenvironment, which is critical for the anticancer immune response. Immunotherapy acts on host immune cells that are activated against the tumour. We can only study the effect of drugs and immunotherapy in the context of a living organism with a functioning immune system. There are no in vitro systems that are able to faithfully replicate the interactions between immune cells and the tumour.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For experiments with tumour injections, animal group size of usually around 10 animals per experiment is necessary, determined on the basis of own previous experimental work and statistical calculations.

The relatively high number of animals in each experimental group is due to a certain degree of variability of the parameters (variability of tumour injections and the incidence of tumour formation, proliferation or apoptotic rate of tumours of different genotype, etc) that will be analysed. Experimental variability is inherently difficult to predict and can't be totally eliminated

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The main step to reduce the number of animals required for this project has been adopting a screening based on cells cultured in vitro and non-regulated zebrafish embryo system to prioritise the drug(s) that will be used in experiments with mice. In other words, mouse cells or zebrafish embryos will be cultured in petri dishes and exposed to a panel of drugs to select the most effective top candidates that will be used in mice. Furthermore, reduction is also achieved by adopting robust statistical analyses to calculate the minimum number of mice required to obtain reliable results.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Before embarking upon any in vivo experiments, hypothesis will be tested in in vitro models. When in vivo experiments are appropriate, small pilot studies will be carried out to estimate the variability of the experimental data so appropriate statistical power analysis can be used to minimize the numbers of animals required for a validated result. The knowledge acquired in previous projects will be helpful in minimising animal suffering. For example, we will only use neuroblastoma cell lines that we have already validated in vivo and of which we know the growth kinetics. This will reduce the number of animals to be used in preliminary growth tests.To minimise animal wastage, prevent the unnecessary production of animals showing adverse effect and to ensure that animal breeding is inextricably linked to research requirement, the project licence holder will:

● Ensure high standards of animal care, welfare and utilise the most appropriate breeding methods.

● Ensure that Personal Licensees working on this project are appropriately trained and suitably competent to enable a high success rate to be achieved and thus minimise the number of animals used.

● Ensure that Personal Licensees will work alongside highly qualified and skilled technical staff who will assist with best breeding practices and experimental procedures

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The greatest potential source of suffering for animals is engraftment and growth of human tumour cells subcutaneously. Animal suffering is minimised by limiting upper tumour size to 12mm maximal diameter in subcutaneous tumours. Differences in subcutaneous tumour growth will be quantified by measurement of tumour diameter using callipers. This method is widely used and it is the gold standard for tumour growth assays. Dosing of mice will be carried out using intraperitoneal injections because this method is less invasive and more comfortable for animals than oral gavage. Spontaneous oral supplementation does not allow precise dosing of the drug, which would imply using a larger number of mice to compensate the increased experimental variability.

Why can’t you use animals that are less sentient?

As explained before, we need to study the interactions between tumour cells and the host immune system during immunotherapy, replicating a real life clinical scenario. This is only possible using an adult, fully developed organism with a functioning, mature immune system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Refinement methods that will be adopted are the use of fine needles for injections to minimise discomfort at injections sites and frequent inspections of mice subjected to the transplantation and drug treatment procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

High quality studies on immunotherapy for paediatric cancers have been published in respected scientific journals which require high ethical standards in animal research. We will only use these key scientific publications as guidance for our experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We can stay informed about advances in the 3Rs by consulting the NC3R website, reading the latest scientific literature, and attending conferences. Furthermore, the local AWERB meetings have a session dedicated to the latest developments in 3Rs in which scientists involved in animal research share their updates.

35. Targeting innate and adaptive immune responses in immune-mediated kidney disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Chronic Kidney Disease, Glomerulonephritis, Vasculitis, Cardiovascular Disease

Animal types	Life stages
Rats	adult, embryo, neonate, juvenile, pregnant, aged
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Identification of more targeted and effective treatments for immune-mediated kidney disease (glomerulonephritis).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic kidney disease and (CKD) and end-stage kidney disease (ESKD) are important global health problems with increasing numbers of people affected by these conditions over the last 30 years.

Around 10% of the world's population have a diagnosis of CKD - this is almost double the number of people living with diabetes, and 18 times the number of people living with cancer. In the UK, there are around 70,000 patients with ESKD requiring dialysis treatment or a kidney transplant. Immune- mediated kidney disease (glomerulonephritis, GN) is a leading global cause of CKD and ESKD - it accounts for approximately 20% of patients with kidney failure in the UK. Current treatments for GN, typically with steroids or other drugs to suppress the immune system, are sometimes ineffective and often lead to significant side effects such as infection (which may be fatal), diabetes, heart disease, and osteoporosis. In addition, there are no treatments to cure GN, and many patients experience multiple disease flares, leading to the accumulation of disease- and treatment-related damage over their lifetime. More effective, less toxic treatments are urgently needed for these patient groups. In order to identify these therapies, or potentially curative treatments for GN, we need a better understanding of how and why disease happens, and to test potential new drugs under experimental conditions.

What outputs do you think you will see at the end of this project?

We anticipate that we will generate:

- new data on the causes, progression and treatment of immune-mediated kidney disease
- improved animal models of kidney disease for use by other researchers or in other projects
- potential drug candidates or therapeutic approaches to be studied in clinical trials in patients
- conference presentations, manuscripts and datasets that will be shared with the scientific community

Who or what will benefit from these outputs, and how?

Potential beneficiaries of this research include:

In the short-medium term:

- the renal research community, through generation of new data on the causes, progression and treatment of kidney disease
- pharmaceutical and industry collaborators, through identification of potential drug candidates or therapeutic approaches to be studied in clinical trials in patients
- animals and animal researchers, through refinement of current kidney disease models, and the development of new models

In the long term:

- patients with kidney disease, through development of new therapies

How will you look to maximise the outputs of this work?

We will present new data at scientific meetings for the renal research community - including local, national, and international meetings.

We will publish our findings in high-impact scientific journals, in accordance with funder and our institutional Open Access requirements in order to maximise availability of data to the research community.

New datasets will be made available on publicly available repositories, to be available to other researchers studying kidney disease.

We will report our findings to our patients and the public. Our research group has an established Patient and Public Involvement Group and we regularly write research summaries and reports for charitable patient partners.

Species and numbers of animals expected to be used

- Rats: 2225
- Mice: 1275

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 application includes both rat and mouse models of kidney disease, since each species has advantages for certain studies.

In general, rats are our preferred species for studying kidney disease, as some important aspects of physiology and kidney disease in rats are more similar to humans than in mice. For example, in our vasculitis (inflammation of blood vessels) model, rats develop the same pattern of kidney disease which is caused by the same mechanisms (an antibody which attacks and activates some white blood cells) that occurs in humans with vasculitis, whereas vasculitis models in mice are often induced by different mechanisms (without the same antibody that is present in humans) and the kidney injury is milder than in humans. For this reason, the majority of our project work will be conducted using rats.

One advantage of mouse models is the availability of a wide range of genetically modified mice (e.g. “knock out” mice) which can be used to study the effect of specific genes on the disease being studied. For this reason, we will use mice when suitable genetically altered rats are unavailable, or to validate data derived using non-genetic approaches (e.g. using a drug) using a genetic approach to confirm our findings.

We will use young adult rodents (age 3-6 months) for the majority of experiments. We will use this age of adult rodents, rather than younger rodents, as changes in physiology, hormones and immune function in younger, growing rodents can influence the development of kidney disease, and the relevance of data to human adults. We also know from published data that aging has an important effect on the development of autoimmunity and kidney disease (both in rodents and in humans).

Therefore, for a proportion of the studies, we will used older rodents up to the age of 18 months.

Typically, what will be done to an animal used in your project?

Day-to-day, animals will be housed in groups in individually ventilated cages, with free access to food and water and environmental enrichment. Their welfare will be monitored daily by staff of our animal research facility. If animals have not been bred in our facility (i.e. imported from another place) they will have a period of acclimatisation (typically 7 days) before they undergo any experimental procedures.

The majority of rodent strains we use do not spontaneously develop autoimmunity or kidney disease. We will therefore use a number of approaches to induce kidney disease. The most commonly used approach will be administration of proteins or chemicals (e.g. antibodies, autoantigens, kidney toxins, adjuvants) using injections via standard routes (i.e. under the skin, into the abdominal space, into a vein). In general, injections under the skin or into the abdominal space will be administered using manual restraint (e.g. scruffing), unless the animals are receiving a general anaesthetic for another reason. Injections into a vein in mice require a restraining tube or similar equipment. Injections into a vein in adult rats require a short period (<10 minutes) of general anaesthesia to ensure consistency of the injections. Typically, animals experience mild, temporary discomfort and no long-lasting harms from injections using these standard routes. For experiments in which we use surgery to cause dilation of the kidney which then allows us to image the kidney, a short surgery (<1 hour) is needed - this requires a small incision in the side of the animal and tying off the ureter (the tube which drains urine from the kidney into the bladder) on one side. Surgery is performed under general anaesthesia. These animals will experience some discomfort after surgery which will be treated with pain relief medications.

Before or after the induction of kidney disease, animals may be given an altered diet (e.g. high salt or high fat diet). This allows us to study the important influence of diet on the progression of kidney disease, which will increase the relevance of our findings to humans. This is not expected to cause distress but may sometimes results in obesity or itchy skin. Some diets may result in weight loss as the animals may find them less enjoyable to eat than standard diets; animals we be placed back onto a normal diet if they lose up to 15% of their body weight. Before or after the induction of kidney disease, animals may undergo interventions to modify the severity of disease (e.g. administration of drug treatments, antibodies, cells). These will be administered using the standard injection approaches described above. Where administration is required for prolonged periods, animals will be surgically implanted (under general anaesthesia) with slow-release devices such as a mini-pump to avoid repeated injections. Compounds may also be administered orally.

For the study of kidney diseases, it is essential to examine the urine produced by the kidneys in each individual animal undergoing an experimental procedure. To collect urine specimens, animals will be placed in individual cages containing hydrophobic sand (i.e. a sand that does not absorb liquid, thus allowing collection of urine) or individual metabolic cages for 24 hours, with free access to food and water. To collect blood specimens, animals will be placed in heating box (to enlarge the vein

and allow easier sampling) for up to 10 minutes, and blood samples will then be drawn via the tail vein using manual or plastic tube restraint. These procedures may cause mild, temporary discomfort. As highlighted above, acclimatisation to these procedures will be performed where appropriate, in order to reduce the stress associated with them during experimental protocols.

The final procedures will be undertaken under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain.

The most common experimental protocol used in this project will be an injection of a substance to induce kidney disease followed by a 4-6 week experimental period. We commonly use daily administration of a substance (e.g. a drug) designed to modify disease for up to a 2-3 week period (usually either by an injection into the abdomen or administered orally) with weekly blood and urine collections. A small number of animals will undergo an additional procedure to these steps, such as a single surgery to enable imaging of the kidney, ageing, or a modified diet.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will undergo injections by standard routes and receive drugs by oral administration; they may experience mild, transient pain and no long-lasting harms from these procedures.

Injection of agents which boost the immune system (called adjuvants) can rarely be associated with ulceration at the injection site (incidence <0.5%; under our previous licence, we did not observe any events in >200 rodents which received adjuvant injection). Any lesions that do occur are expected to be minor and where appropriate will be promptly treated under the guidance of the NACWO and NVS. Animals with lesions that are ulcerated or infected, or that do not respond favourably to treatment of minor lesions, will be killed via a schedule 1 method.

Animals may undergo minor surgery to implant minipumps (a device under the skin that can release a drug slowly) or surgery to cause dilation of the kidney by tying off the ureter (the tube which connects the kidney to the bladder). They are expected to recover quickly from these procedures, and will be given analgesia and post-operative care. We estimate peri-operative survival after kidney surgery to be >99%.

In some studies, it will be necessary to use older rodents (e.g. >15 months) as we recognise that aging has important influence in the development of kidney disease, in both rodents and humans. This will increase the relevance of our experimental findings to older patients with kidney disease. We do not anticipate that animals will experience significant distress from the aging process.

For the study of kidney diseases, it is essential to examine the urine produced by the kidneys in each individual animal undergoing an experimental procedure. We do not anticipate that animals will experience significant distress by being housed individually for 24 hour periods to allow urine collection, and the total number of urine collection periods has been limited in each experimental protocol to limit any distress that animals might experience.

A small proportion of the work we will perform will be conducted in genetically altered rodents. To date, the work we have conducted has not used rodents which experience adverse effects due to these genetic alterations (indeed, this would be undesirable as it may affect the interpretation of data relating to kidney disease).

We do not expect animals to develop symptoms of kidney disease under these protocols, as all procedures are completed before the onset of kidney failure. Kidney failure may be evident by non- specific signs of distress, anorexia, weight loss, and development of ascites (fluid accumulation in the abdominal cavity). Animals that develop signs of kidney failure within the time frame of these protocols will be assessed with the NACWO and NVS and be killed via a schedule 1 method if thresholds are met (e.g. >15% loss of body weight). We estimate that this will be necessary in <1% of animals. In work conducted under our previous licence, no animals met these thresholds.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Around 25% of animal will undergo subthreshold procedures (i.e. breeding without an adverse phenotype. Around 25% of animals will undergo mild procedures (e.g. breeding with a mild phenotype, intravenous injection, administration of substances for a limited time period). Around 50% of animals will undergo moderate procedure (e.g. surgery, repeated administration of substances by injection).

None of the protocols are classified as severe.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The disease processes involved in the development of immune-mediated kidney diseases such as vasculitis are complex, and involve many different cell types, tissues and organs. For example, some immune cells involved in disease onset originate in the bone marrow and subsequently develop in lymphoid tissue, where they produce autoantibodies or become mature cells that then travel to distant organs including the kidneys and lungs. In these organs, autoantibodies and/or mature immune cells interact with many different cell types, including blood cells, other immune cells, and the tissue of the organ itself, ultimately leading to inflammation and eventually damage and scarring. These interactions are likely to

involve different mechanisms in each target organ or tissue, and to vary over time as organ injury progresses. These processes are so complex that currently there is no in vitro, organoid or computer modelling system that is able to replicate them.

Which non-animal alternatives did you consider for use in this project?

We have explored several alternatives to using protected animals in this project:

- Use of non-protected species
- Organoids
- Existing datasets
- In vitro studies

Why were they not suitable?

Use of non-protected species: the immune systems and organ systems, specifically the kidney, of non-mammalian species do not sufficiently reflect the diversity and complexity of their respective human systems in order to address or research questions.

Organoids: there have been significant advances in the development of kidney organoids in recent years, generated from stems cells in 3D structures, and they exhibit a degree of similarity with native tissues in terms of cell type and function. However, work to date has focused on the biology of cells intrinsic to the kidney (and thus of genetic kidney diseases such as polycystic kidney disease). To date, kidney organoids have not been used to study autoimmune kidney disease or the role of circulating and tissue immune cells in kidney injury. This may become possible in the future, but establishing these models is currently outside the scope of our expertise and the beyond the time frame of this project.

Existing datasets: we have explored publicly available datasets derived from mouse models and clinical cohorts with immune kidney disease (glomerulonephritis) and vasculitis (inflammation of the blood vessels), and did not find significant data relevant to our research questions. Searching the GEO Accesssion database, for example, for datasets related to 'glomerulonephritis' identified 19 projects: 5 human, 11 mouse, 4 rat. Analysis of these datasets identifies alterations in immune response pathways during glomerulonephritis, consistent with our project hypothesis and supporting our plan of investigation. However, there is no existing data regarding using treatments or other ways to alter these pathways using the approaches we have proposed.

In vitro studies: there are aspects of cellular function and intercellular interactions that it is possible to study using in vitro approaches. Indeed, the reduced experimental conditions of an in vitro experiment often provides more clear-cut conclusions than are possible in vivo. Our approach is to therefore use in vitro and in vivo experiments in a complementary manner, and to replace in vivo work with in vitro methodology wherever possible. For example, using cultured cells exposed to candidate drug compound screening prior to performing an in vivo experiment, and use of co-culture systems to study some cell interactions in isolation.

Wherever possible, we will use alternative approaches alongside animal experiments in this project. We currently have a number of in vitro models that complement our animal models, which employ rodent or humans cells and antibodies to replicate specific aspects of disease. We will continue to use these, and develop additional models where possible. We will continue to interogate the scientific literature and publicly available datasets throughout the project, to avoid reduplication of existing work and to refine our experimental plans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our experimental and analyses plans were designed with the aim of producing robust and reproducible results, whilst minimising the number of animals used. We have used resources including the NC3R's experimental design assistant and the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Statistics software packages have been or will be used to estimate groups sizes based on previous data where available. We have also used our annual return of procedures data to estimate the number of animals that we will need to use for breeding and experimental protocols. Specific considerations in our experimental design are discussed below.

Study Design: The majority of experiments will have a simple hypothesis-testing interventional design (e.g. placebo versus active treatment with a drug). Negative and vehicle (i.e. the solution used for delivery of drug, not containing the active compound) controls will be used to determine if a difference between groups is caused by the drug intervention. Positive controls may be used to support the interpretation of negative results or determine if an expected effect is detectable. Where possible, repeated measures of non-fatal endpoints (e.g. urinary abnormalities, blood sampling) in the same animal will be taken to minimise total animal use.

Group Size: Where available, we have used existing data from previous experiments in our laboratory to assess variability in results, and to predict of the number of animals needed to generate statistically robust findings. These calculations typically show that group sizes of 4-8 are required, depending on the model used, in order to achieve the quality of results that we want to deliver.

Randomisation: stratified randomisation will be used to minimise variation related to animal traits that may affect response (e.g. body weight, age, sex). Online random number generators will be used to allocate animals to treatment groups according to these strata.

'Nuisance' variables: for variables that may affect outcome, but that cannot be accounted for in stratification, we will take steps to minimise the introduction of bias. For example, performing all immunisations for disease induction at the same time, on the same day, by the same investigator. For interventional drug studies, the investigator will be 'blinded' during drug administration.

Sample Analysis: for the analysis of samples obtained during in vivo experiments (e.g. flow cytometry, tissue immunohistochemistry), procedures will be performed on the same day in complete sets, and in a blinded fashion, to minimise variability and introduction of bias. Where this is not possible, stratification for analysis batch or 'block' may be used in subsequent analysis.

Exploratory studies: for some exploratory studies (e.g. studying how nanoparticle therapeutics are spread across different organs) we cannot choose sample size in advance, as there are no existing data to assess variability or to perform statistical prediction. We have therefore estimated numbers of animals taking into account previous experience (over 10 years) and using comparable published work from our group or collaborators.

Use of pilot or feasibility studies: prior to undertaking definitive studies, it may be appropriate to conduct pilot studies (e.g. dose-ranging for novel drug compounds) or feasibility studies (e.g. to confirm that all outcome measures can be measured as expected, such as obtaining and processing all samples for time-critical analyses such as flow cytometry). The information we obtain will be used will be used to inform the design of large definitive studies, which may increase the likelihood of their success and reduce the need for them to be repeated.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used resources including the NC3R's experimental design assistant and the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. We have also referred to the recently published International Society of Nephrology TRANSFORM guidelines for preclinical animal studies in translational nephrology (Kidney International, 104(1), 36-45), to ensure that design considerations specific to kidney disease have been included.

Statistics software packages have been or will be used to estimate groups sizes based on previous data where available. These calculations typically show that group sizes of 4-8 are required, depending on the model used, in order to achieve the quality of results that we want to deliver.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animal colonies will be closely monitored to avoid excessive breeding.

If appropriate, we will plan mouse crossings which can provide us with the experimental mice and the controls.

Where possible, we will use one control group to investigate multiple treatment interventions (e.g. one control group treated with vehicle with two intervention groups treated concurrently with different investigational drugs).

Prior to undertaking definitive studies, it may be appropriate to conduct pilot studies (e.g. dose-ranging for novel drug compounds) or feasibility studies (e.g. to confirm that all outcome measures can be measured as expected, such as obtaining and processing all samples for time-critical analyses such as flow cytometry). The information we obtain will be used will be used to inform the design of large definitive studies, which may increase the likelihood of their success and reduce the need for them to be repeated.

Postmortem tissue from as many organs as possible will be harvested at the end of experiments for histological or biochemical analysis. This will negate the need for additional experiments solely to obtain such tissue. If we do not need to analyse these samples immediately, they will be frozen and made available for future studies or to other researchers working in similar research areas.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Choice of animal model and refinements:

We propose using several models of kidney disease, that provide by complementary information by modelling different human diseases (e.g. vasculitis versus anti-GBM disease) or by modelling different aspects of kidney injury (e.g. inflammation versus scarring). We will choose the most applicable model for addressing each of our research questions, taking into consideration the type and quality of data required, animal usage, and expected severity. Where possible, we will use models that reproduce multiple aspects of disease pathogenesis (e.g. autoimmunity, inflammation, and scarring) to avoid repeating unnecessary experiments in several models.

In the course of work completed under our previous licence, we optimised our procedures to enable repeated measures of important outcomes without the use of general anaesthesia (e.g. for serial measurement blood tests), and also to minimise animal usage and maximise data generation.

We recently refined some of our existing models to make them more reproducible and of shorter duration, so that fewer animals are needed to generate statistically robust results. This increased the acuity, severity and consistency of kidney disease in the model (without increasing the overall severity of the protocol or compromising

animal well-being), meaning that peak kidney disease developed earlier, and that variance in phenotype was reduced such that group sizes for interventional studies are reduced, on average, from 8-10 to 4-6 animals.

Welfare considerations and improvement:

All the protocols proposed here are all either mild or moderate and none are severe. In all procedures, as outlined in the individual protocols, we will monitor the condition of the animals regularly according to the protocol used. If unexpected distress occurs, animals will be humanely killed.

Acclimatisation periods will be maintained after import or movement of animals, and where appropriate we will perform acclimatisation for experimental procedures including restraint for venesection, housing in metabolic cages, etc.

All animals used for protocols involving recovery surgery will receive pre- and post-operative analgesia to treat any apparent discomfort, and short-acting general anaesthetic will be used for recovery surgery. In some circumstances, anti-inflammatory analgesia cannot be used, since assessment of inflammation is an important outcome measure in our studies. In this case, alternative analgesia (e.g. opioids) will be used.

We do not expect animals to develop symptoms of kidney disease under these protocols, as all procedures are completed before the onset of kidney failure. In work conducted under our previous licence, no animals met thresholds related to signs of kidney failure.

Animals will be housed in groups and environmental enrichment will be provided. Our study protocols do not require that this is withheld, except for isolated 24 hour periods when the animals are housed in individual cages with hydrophobic sand or in metabolic cages, to enable collection of urine samples.

The total number of urine collection periods has been limited in each experimental protocol to limit any distress that animals may experience during this procedure.

Why can’t you use animals that are less sentient?

The immune and kidney organ systems of non-mammalian species do not sufficiently reflect the diversity and complexity of their respective human systems in order to address or research questions.

The majority of our studies will employ rats, rather than mice, as important aspects of rat physiology (e.g. susceptibility to high blood pressure) and kidney pathology (e.g. development of inflammation in the kidney) more closely resemble features of human disease, thus increasing the relevance of our findings clinical application. In general, we will use adult rats, since changes in physiology, hormones and immune function in younger, growing rodents can influence the development of kidney disease, and the relevance of data to human adults.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals used for protocols involving recovery surgery will receive pre- and post-operative analgesia to treat any apparent discomfort, and short-acting general anaesthetic will be used for recovery surgery. In some circumstances, anti-inflammatory analgesia cannot be used, since assessment of inflammation is an important outcome measure in our studies. In this case, alternative analgesia (e.g. opioids) will be used.

We do not expect animals to develop symptoms of kidney disease under these protocols, as all procedures are completed before the onset of kidney failure. Animals will be monitored daily for signs of distress and significant kidney impairment (e.g. anorexia, piloerection, development of ascites [fluid in the abdominal cavity]). Body weight will be measured at least weekly (and more frequently in animals showing any signs of kidney impairment. Animals that develop signs of kidney failure within the time frame of these protocols will be assessed with the NACWO and NVS and be humanely killed if thresholds are met (e.g. >15% loss of body weight). We estimate that this will be necessary in <1% of animals. In work conducted under our previous licence, no animals met these thresholds.

Animals will be housed in groups and environmental enrichment will be provided. Our study protocols do not require that this is withheld, except for isolated 24 hour periods when the animals are housed individually for 24 periods to enable collection of urine samples. We have previously employed housing in individual metabolic cages to enable collection of urine samples, and in the current licence plan to investigate the use of hydrophobic sand for urine collection (which may limit any discomfort associated with housing in metabolic cages). The total number of urine collection periods has been limited in each experimental protocol to limit any distress that animals may experience during this procedure.

We recently refined some of our existing models, which previously induced very mild histological features of kidney disease, to increase the degree of kidney inflammation. This means the models are more in keeping with human disease, more reproducible and of shorter duration, so that fewer animals are needed to generate statistically robust results. We have published these protocols to make them widely available to the research community, and we will preferentially use them for future studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will refer to published guidelines for the planning and design of animal experiments. Specific examples include:

The PREPARE guidelines (https://norecopa.no/prepare)

The ARRIVE guidelines (https://arriveguidelines.org/). Whilst these are focused on best reporting of in vivo experiments, early reference to these guidelines may improve experimental design

For information relevant to pre-clinical models we will refer to outputs from the recently convened TRANSFORM group (International Society of Nephrology consensus group for preclinical animal studies in translational nephrology)

Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement (Refining procedures for the administration of substances, Morton et al 2001)

NC3Rs guidance for blood sampling (https://www.nc3rs.org.uk/3rs-resources/blood-sampling)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check information on NC3Rs website.

All members of our group have signed up to the NC3Rs newsletter and will be invited to attend local and regional 3Rs symposia.

Advances in the 3Rs will be discussed at our laboratory group meetings, and all members of the group will have access to our establishment's resources and meetings on 3Rs and experimental design.

We will refer to updates from the International Society of Nephrology TRANSFORM Group (consensus guidance for preclinical animal studies in translational nephrology) so that we are aware of developments and recommendations relevant to study of kidney disease.

36. Mechanisms of neural development and regeneration

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Development, Regeneration, Neurobiology, Cell Signalling, Zebrafish

Animal types	Life stages
Zebra fish (Danio rerio)	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to investigate how communication amongst cells drives the formation of the nervous system and its repair after injury in the zebrafish.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Neural tube defects (NTDs) are one of the most common birth defects, leading to premature termination, stillbirth, and disability; however, the causes underlying these defects are often unknown. On the other hand, spinal cord injuries cause paralysis in one individual every four hours in the UK. These patients will require long hospital stays and expensive treatments. This is because neither the brain nor the spinal cord can regenerate and recover their function in humans.

We and others have observed that cells communicate with their neighbours in a similar way during the formation and repair of the nervous system. A better understanding of how cells communicate during the formation of the nervous system

will provide insights into why NTDs occur and open the door to better diagnosis and treatments; while new insights on how neural repair occurs in regenerative species such as the zebrafish will enable us to design strategies to promote neural repair in patients suffering neurodegenerative conditions or traumatic lesions in the brain and spinal cord.

What outputs do you think you will see at the end of this project?

The outputs of this project will include:

- New information that will contribute to expanding our basic knowledge on the mechanisms of neural development and regeneration.
- Our findings will be published in our establishment research portal and in Open Access journals whenever possible. Dissemination will also involve presenting at scientific conferences and outreach events.
- New information about zebrafish genes will be made available via public repositories such as ZFIN (https://zfin.org/). Zebrafish lines used in this project will be shared on demand.
- New reagents will be published in line with Open Science guidelines.
- Large sets of data will be made available via public repositories such as FigShare.

Who or what will benefit from these outputs, and how?

The academic community will benefit from our new knowledge, zebrafish lines and reagents, which will be disseminated via publications and presentations at national and international conferences.

The lay public will benefit from our findings via outreach events and by accessing the publicly available information repository at the our establishment.

Neural tube defects (NTDs) rank among the most prevalent birth defects, leading to premature termination, stillbirth, and disability. Simultaneously, spinal cord injury (SCI) stands as a primary cause of paralysis. In the UK, a new case of SCI emerges every four hours, imposing a substantial financial burden on the NHS. Our research delves into the mechanisms governing neural development and regeneration, aiming to enhance our understanding of NTD pathogenesis and facilitate neural regeneration in human tissues. Thus, beyond its academic implications, and at a longer term, our findings hold the promise of influencing applied research projects aimed at identifying novel therapeutic strategies that can directly benefit clinicians, NTD and SCI patients. Moreover, such advancements have the potential to reduce recovery times and prolonged hospital stays, thereby mitigating NHS treatment costs.

How will you look to maximise the outputs of this work?

We have developed a range of strategies to maximise the outcomes of our project:

- Establishing Interdisciplinary Collaborations. Internal and external collaborations have been already initiated; colleagues from within our institution will bring experience in inflammation and neural repair, which will be invaluable in

validating some of our findings in human cultured cells. We are also working with colleagues with expertise in transcriptomic analyses; they will contribute to the sample preparation and analysis of large data sets. Additionally, we have the privilege of collaboration with renowned academics from other establishments with extensive knowledge in zebrafish immunology, who have generously agreed to provide key transgenic zebrafish lines crucial for the completion of our project.

● Securing Funding. To support our research programme, we have already secured internal funding from our host establishment. Additionally, applications have been recently submitted to several funding bodies and in the coming three years, we plan to apply for various grant programmes.

● Dissemination of Findings. Our findings will be shared internally within our institution to gather valuable feedback from colleagues. Furthermore, we intend to present our work at national and international conferences such as the British Society for Developmental Biology (BSDB) Meetings and the European Developmental Biology Congress. Additionally, we will actively engage in outreach events and educational settings to raise awareness and foster interest in our research.

● Data sharing. Our commitment with transparency aligns with Open Science principles. We plan to make our research approaches, methodology and outcomes accessible to the broader scientific community.

● Careful Experimental Design. We emphasise rigorous experimental design, including formulating clear hypotheses and predictions, determining appropriate sample sizes, maintaining consistent control of variables, and meticulous record-keeping. These practices are essential to ensure robustness and reproducibility of our data while optimising resource use.

Species and numbers of animals expected to be used

Zebra fish (Danio rerio): An estimate of 3,000 adult fish will be bred in the facility during this project to generate embryos/larvae for experiments. Of those, 250 will correspond to wild type fish and the remaining to GA fish.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Unlike many other species, zebrafish can regrow damaged parts of their bodies such as the brain and the spinal cord at different stages of their lives, providing a window into the cellular and molecular mechanisms governing tissue healing and repair. Further to this, the zebrafish genome has been fully sequenced making it easier to study the genes involved in this process.

A number of transgenic zebrafish lines are available to us, which express fluorescent proteins in specific cell populations. This will allow us to study the behaviour and the contribution of these cells during neural development and repair.

Our experiments focus on young zebrafish, specifically embryos and larvae, and we stop our observations by the fifth day of their development at the latest. This approach helps us reduce the number of animals used in research in line with the 3R principles.

Young zebrafish are great for our research because they grow quickly, and their bodies are clear, which means we can easily see what is happening inside them under a microscope. We can also make changes to their genes and proteins using special techniques, which helps us understand how genes work when tissues are forming. Another remarkable capacity of this model is that zebrafish larvae can regrow a damaged spinal cord in just three days, while it can take adult fish up to four weeks.

Traditionally, scientists have looked at how animals like rodents, frogs, and adult fish can regrow body parts. But we're taking a different approach by studying zebrafish larvae. Other researchers have already shown that this is a good way to study how nerves and tissues regrow because the basic rules are the same between larvae and adult fish (Alper and Dorsky, 2022).

Reference:

1. Alper, S. R., & Dorsky, R. I. (2022). Unique advantages of zebrafish larvae as a model for spinal cord regeneration. Frontiers in Molecular Neuroscience, 15, 983336. https://doi.org/10.3389/fnmol.2022.983336

Typically, what will be done to an animal used in your project?

We will keep two groups of zebrafish in our facility: regular or wild type fish and genetically altered (GA) fish which contain changes in specific genes. We will breed these fish to produce embryos for our study.

The maintenance of GA zebrafish colonies requires new adults to be screened for the presence or absence of genetic abnormalities. To do this we try to collect samples from the surface of the fish using non-invasive methods. Sometimes we have to take a small piece of tissue from the fin of anaesthetised fish. When this is the case we treat the fish with pain killers to alleviate any discomfort.

To understand the function of genes and proteins, fish embryos will be collected from the water where they are laid, and injected with reagents that interfere with the way specific genes carry out their function.

For those experiments aimed at showing how the zebrafish spinal cord heals, the spinal cord of 3-day old anaesthetised fish larvae will be lesioned. We will then observe the behaviour of these fish for two days to check how quickly they can recover their ability to swim. Fish larvae will be fixed at 120hpf, before they become protected by the 'The Animals (Scientific Procedures) Act 1986'.

Young stages of fish (embryos and larvae) will be used for different experiments, including watching them in special microscopes while they are anaesthetised, to obtain genetic information or to visualise the products of their genes, which may shed light into the way the brain and spinal cord form and heal in the zebrafish.

Often zebrafish lay hundreds of eggs in the water. In the case an excess of embryos is produced we will either freeze them for further experiments to reduce the use of animals or we will dispose of them using humane approved methods.

On occasions, we will grow some of the embryos produced for breeding purposes.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are anticipated from breeding GA fish.

Non-invasive tissue collection involves wiping a sterile swab over the surface of the fish and is not expected to cause adverse effects.

Tail biopsies will be performed by very experienced staff and may cause discomfort to the animal; however, fish are expected to recover their normal swimming patterns a few minutes after the procedure. Their fins regrow within two weeks, consequently, this process will not cause any long-term adverse effects. However, there is an increased risk of infection and on rare occasions, this procedure could lead to excessive bleeding. Further to this, behavioural changes and a stress response may be observed in the fish after this procedure.

Healthy fish will be maintained only to the age of 18 months, after which they will be culled using an overdose of anaesthetic. This will minimise the risk of infections and disease and contribute to the well- being standards of the whole colony. At the end of the project, fish lines will be assigned to other projects or cryopreserved and disposed of.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Breeding GA fish. Most GA adult fish will be used for breeding and the expected level of severity is sub-threshold.

Tail biopsies. 40% of our tanks need to be genotyped to ensure the correct fish are used for experiments. The prospective severity of tissue collection or tail biopsies is mild.

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project aims to study the mechanical and chemical changes occurring in the brain and the spinal cord during the formation of these tissues and their repair after injury. While progress has been made in the recent years to study aspects of neural development and regeneration in cell cultures, these are not yet able to reproduce the complexity of cell types present in the animal nor their spatial organisation. Consequently, the chemical and mechanical signals the neural tissue is exposed to, which is the subject of the study of this project, are not equivalent in cell cultures and in embryos.

To maximise the impact and relevance of our studies, we propose to use the zebrafish embryo/larvae as a model system. As opposed to cultured cells, animal models are the natural environment of diverse cell populations making them excellent systems to study complex cell-to-cell interactions in physiology and disease.

For example, zebrafish larval forms have immune and circulatory systems which are key to understand how these organisms respond to injury, in a way similar to humans. The presence of immune and circulatory system will allow us to study how inflammation contributes to the process of tissue repair.

Studies using embryonic and larval forms of zebrafish also make it possible to explore vertebrate biology while contributing to the reduction of protected animals in scientific research.

Which non-animal alternatives did you consider for use in this project?

As an alternative to zebrafish larvae, we considered the use of primary cell cultures. However, these still require using mice to establish initial cell cultures. Furthermore, due to limited capacity of division of these cells, they can not be indefinitely propagated in the lab, meaning that a constant supply of animals would be required throughout the project.

As an alternative to those, we also considered the use of the following models:

- established cellular lines, such as BV2 cells
- combinations of BV2 cells and neurons (co-cultures)
- 3D cultures such as organoids

Why were they not suitable?

• Cell lines such as BV2 have the advantage that can be indefinitely propagated in the lab, thus providing biological material for studies indefinitely without the need of using animals. However, cultures including only BV2 cells are too simple and would not represent the complexity of injured tissues, which include a large number

of different cell types. Further to this, recent studies have shown that BV2 cells behave differently from normal cells, thus, we have to be careful when interpreting data from them. Still, we are planning to use these cell lines for specific sections of the project, such as validating some of the findings in fish.

- Co-cultures - While co-cultures allow us to study the interaction between a small number of cell types, they do not include all types of cells that are important for tissue regeneration. Also, these cells grow in flat environments and they do not reproduce the shape nor the mechanical properties of tissues, which are critical factors in tissue formation and repair. Thus, these types of models are not appropriate to understand how cells interact chemically and mechanically during neural development and regeneration, which is the main research interest of this project.

- 3D cultures - Three dimensional cultures called organoids have recently been developed and are used an alternative to animal models. They are more complex than cell cultures and reproduce to some extent the morphology of tissues but they are still not as complex as real tissues. For example, brain cultures do not form blood vessels which are essential to nutrients needed for tissue repair. Also, 3D cultures lack some immune cells required for healing. Similarly, since the complexity of tissues has not been yet replicated in 3D cultures, these cannot mimic how tissues interact during nerve formation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any.

These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

My estimate has been calculated based on the number of lines that we will require initially for the first part of the project (24 GA lines + 1 wild type line), with a total of two 2.6L tanks per line at a density of 5 adult fish per litre (13 fish per tank).

Fish will be replaced every 18 months to minimise disease and ensure health of the colony. Thus, a total of 2160 fish will be used for the duration of the project.

My estimate also includes capacity to grow additional fish lines generated by intercrossing of existing lines (840 fish).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In line with the NC3R's guidelines all our experiments will use embryonic or larval forms of zebrafish from 0 to 5 days old, before they start feeding independently and become protected by the Act.

The number of embryos to be used in each experiment will be defined using power calculations or from estimates from previous experiments. Positive and negative controls will be included when appropriate.

To minimise bias, automatic methods of analysis will be favoured over manual methods and instruments will be frequently calibrated. Randomisation and double-blinded analysis will be performed when possible and critical experiments will be analysed by a second member of our team.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Whenever possible, embryos will be obtained from mass-spawns using 5-7 pairs of fish. Embryos will be randomly assigned to treatments and incubated in defined media in the same refrigerated incubator to ensure consistency and reproducibility.

Most of our GA lines have been generated in the same genetic background. We will continue using the same control line to ensure reproducibility across experiments.

Further reduction in our projects will result from freezing any excess larvae or embryos that can be used at a later time for other experiments and re-using adult fish for intercrossing.

Similarly, in recent years we have managed to refine our genome-editing technique which now allows us to introduce changes in genes and proteins in zebrafish larvae without the need of growing them beyond the 120 hpf. We will continue making use of these extremely useful pilot studies to minimise the number of GA animals used in our research.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will make use of zebrafish. Adult fish are kept in enriched environments and are bred to obtain embryos. The embryos will be collected and incubated to the desired stages for all experimental procedures. All experiments will be concluded by day 5 post-fertilisation. These experiments will include staining of tissues and imaging to analyse the way cells and tissues behave. On occasions, some larvae will be lesioned in their spinal cord for the study of tissue regeneration. Lesions will be introduced using sterile equipment in anaesthetised larvae, which will be regularly monitored after the procedure until the time of analysis.

On a different set of experiments we will study the behaviour or larvae. For this, we will analyse their movement at different stages after injury.

It is unlikely that any of these procedures will distress the fish. Only embryos that have not been subjected to any of the previously mentioned procedures will be grown to adults and used for breeding.

On occasions, injection of embryos aiming to interfere with the normal functioning of genes will be performed in order to investigate gene function. These embryos will be used for analysis no later than 120 hours post-fertilisation (hpf).

Often, we need to take biopsies of genetically altered adult zebrafish to check if they contain the expected genetic mutation. In this case, animals are previously anaesthetised and treated with pain- killers before and after the procedure. While the risks associated to this procedure are low and animals do not generally show signs of distress, I conducted pilot studies in the past to develop a less invasive way to obtain genetic material from fish skin. However, our studies were only partially successful, indicating that we need to further improve the way we use this method in the laboratory. We are planning to continue developing this technique, with the aim of replacing fin biopsies in the future.

Whenever possible, we use visual examination to check for the presence of a genetic change. This avoids the need of biopsies or skin swabs.

Why can’t you use animals that are less sentient?

In all our experiments we will use the most immature life stages of zebrafish when the nervous system is not fully developed. Adult zebrafish will be uniquely used for breeding purposes.

As discussed above, the advantages of zebrafish larvae make it the perfect model for our research project and our procedures are unlikely to distress the animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All tanks in the facility will be checked daily for any signs of health deterioration in order to monitor the general health of the colony. Diseased or injured fish, altered behaviours and mortality rates will be recorded for each tank.

Animals will be maintained up to the age of 18 months to minimise health problems.

Further pilot studies will be performed in order to optimise skin-swabbing techniques, with the aim to replace tissue biopsies in the mid-term.

After tissue biopsy is obtained, fish are transferred to a recovery tank in the presence of analgesia. Recovery of the fish is closely monitored for the next 10-30 minutes. After that, fish will be monitored at regular intervals until they can be transferred to their home tanks.

The accidental introduction of new pathogens will be minimised by importing bleached embryos from reliable sources and growing them in a quarantine unit in the first instance.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adopt the ARRIVE 2.0 (https://arriveguidelines.org/arrive-guidelines) and the PREPARE guidelines (https://norecopa.no/prepare) to plan, conduct and report our research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We obtain regular updates from the AWERB that include advances in the 3Rs. Further to this, I am currently subscribed to the NC3Rs newsletter and receive regular email updates.

We will attend NC3Rs meetings in order to keep updated on the best practices for husbandry and experimentation with zebrafish.

37. Characterisation of haematopoietic stem and progenitor cells and their niches

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Blood, Transgenic, Stem Cell, Therapy

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the processes underpinning the development and biology of the blood system and to use this knowledge to advance methods for generating cells of the blood system in the lab.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This research will further the understanding of blood stem cell biology and the blood system, which is of fundamental scientific and medical importance. Blood stem cells, found in the bone marrow of the adult, can supply all the cells of the blood system throughout life. These cells are widely used in treatments for blood disorders and cancers, but there is limited availability of suitable donors for some patient groups. Therefore, many laboratories around the world are looking for methods to

generate transplantable cells in the laboratory. In this project, a variety of specialised approaches will be used to investigate which genes and tissues are important in the formation of blood stem cells. This knowledge will help in the development of methods for producing blood stem cells and mature blood cells, for example from human embryonic stem cells or reprogrammed “stem cells”. Establishing robust methods for producing and expanding transplantable cells in the laboratory is a major goal in the field, as it could ultimately help address the shortages of donors in the clinic.

What outputs do you think you will see at the end of this project?

This research will provide important insights into the origins and regulation of mammalian cells of the blood system, including blood stem cells. This project will advance the field of stem cell biology, haematology and blood cancers. It will reveal the pathways of normal development of these cells, their origin, interactions with other cell types, their genetic regulation and pathological transformation. The research results and datasets would be published as appropriate for the field, to facilitate dissemination of findings and to inspire and support further studies.

Who or what will benefit from these outputs, and how?

The outputs of this research will enrich the knowledge of academic scientists within the fields of stem cell biology, haematology and cancers. In the long-term, our study will help medical researchers and clinicians to facilitate development of more treatment options for patients.

How will you look to maximise the outputs of this work?

The research environment is highly collaborative and wider knowledge transfer is further facilitated through open-access publications, networking and presentations at scientific conferences. New collaborations may be established as appropriate as the project progresses.

Species and numbers of animals expected to be used

- Mice: 65,000 mice over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are standard model for analysis of the blood system as they are mammals and evolutionarily close to the human. Genetically modified mice useful for our studies are broadly available and can be generated de novo, expanding our capability to address important scientific questions. Mice are one of the best models for analysis

of transplantable blood stem cells. We will study all life stages from the egg to late adulthood.

Typically, what will be done to an animal used in your project?

Animals will be bred and housed in accordance with Home Office and local regulations and best practice guidelines. Mice will be bred and humanely culled to obtain cells and tissues for analysis. In transplantation studies, mice will be irradiated to dampen their blood system before test cells are transplanted, by one of the following routes, for example, intravenous, intrauterine, intrafemoral, subcutaneously or under the kidney capsule. Recipients are closely monitored and typically kept for 3.5-6 months to assess contribution of donor cells in blood. In the end of the study, the mice will be humanely culled.

What are the expected impacts and/or adverse effects for the animals during your project?

Few adverse effects are expected in the mouse breeding colony, which is the main source of study material. The transplantation procedure has a mild to moderate severity, the majority of transplant recipients would be in mild category. We expect 2-5% to undergo surgical implantation, typically into the kidney capsule or administered directly into an embryo/foetus. Where surgery is performed, animals will experience pain which will be controlled prophylactically by administering analgesics.

Some mice will be irradiated which can make mice feel sick and they may temporarily lose some weight. Mice will be supported with mashed diet and prophylactic antibiotics to prevent infection and to support mice during this period.

Recipients are closely monitored. Animals may experience mild transient discomfort during blood sampling. In some instances, the mice will be transplanted under general anaesthesia and given analgesics. They will be humanely culled at the end-point, which is typically after 16 weeks, or sooner if they display signs of sickness where recovery is not expected.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Breeding and maintenance of genetically altered mice: 96% sub-threshold, 4% mild. Recipient mice: 96% mild, 4% moderate, including non-irradiated surgically transplanted mice.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The study of blood development in mouse models is well established and, coupled with the power of transgenic technologies, there is no better system to investigate genes important in determining the blood system in mammals. The long-term repopulation (transplantation) assay is also the only means of detecting the presence of functional blood stem cells and no alternatives are available.

Which non-animal alternatives did you consider for use in this project?

The processes under investigation are highly complex, no current in vitro system can entirely replace the animal model. However, we have and continue developing in vitro models trying to gain an insight into the in vivo processes and enhance the culture so that it mimics closer the situation in vivo.

Why were they not suitable?

We are looking into biology of the blood system in the body. Culture systems are still not good enough to fully reproduce haematopoietic development and maintenance in vivo.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animals are bred according to current established guidelines in order to achieve a colony size that is sufficient to meet research needs and to maximise efficiency. We have considerable experience in blood stem cell transplantation and robust statistical methods are used to calculate the number of animals needed to ensure that meaningful data are obtained. Power calculations, based on preliminary data when possible, will be used. Testing effects of biological impacts on production of long-term repopulating HSCs requires quantitative approach. Where appropriate we will use limiting dilution analysis, normally with 3 replicates (usually several recipients per condition will be tested to achieve statistically significant results). Blinding and randomisation will be used to ensure robustness.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Studies are carefully designed in line with relevant guidelines, best practice, and where appropriate, with the aid of specialised software. Studies are combined where possible, so that controls do not need to be repeated. This ensures that appropriate minimal numbers of animals are used. Where possible we use in vitro cultures to inform our in vivo studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Improving methods of growing cells of interest in the laboratory, which is one of the goals of this research, will further reduce the amount of embryonic material required for studies. We routinely use currently available methods to grow cells in the laboratory and study them in this way wherever possible, so that fewer animals are used. This research will inform the design of methods to produce blood stem cells from other sources, such as embryonic stem cells or "reprogrammed" stem cells, which will ultimately reduce the requirement for mice. When appropriate software is available for our studies, we will use it to make in silico predictions to inform experimental design.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice, including genetically altered, will be used as models to study blood development, to provide experimental material and as transplant recipients. These methods are well established in the field and performed according to best practice guidelines to minimise and mitigate adverse effects. Appropriate transplantation routes with the least invasive options will be chosen for each study. Mice will be monitored closely, so that human endpoints can be applied as appropriate.

Why can't you use animals that are less sentient?

Mice are the most accessible mammals for biological studies. Use of genetically altered mice and material enables detailed investigation of the roles of specific genes, mechanisms and cell types involved in blood development. Blood stem cells can only be reliably detected and characterised by transplantation assays, whereby their functionality can be confirmed by their contribution to the recipient blood system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

This research will be conducted in state-of-the-art facilities and all researchers are fully trained in the procedures they perform and follow recommendations given by the facility staff to further refine techniques. Experienced facilities staff, animal welfare officers and named vets support research. Mice are bred according to current guidelines to minimise numbers. If substances are administered, only approved and appropriate routes and doses are used. Newly published refinements are routinely monitored and implemented wherever possible. In transplantation studies, the irradiation dose is given in two parts in order to minimize stress and antibiotics is used afterwards to prevent infection. Health status is closely monitored throughout using a vet approved system. Animals displaying signs of sickness where recovery is not expected will be humanely culled.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow ARRIVE, PREPARE, NC3Rs, local AWERB guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

NC3Rs guidelines and latest published refinements are routinely monitored and implemented during the course of the project. This will be achieved by engaging with animal welfare committee and attending relevant conferences.

38. Information processing in the mammalian brain and investigation and treatment of Traumatic Brain Injury

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Traumatic Brain Injury, Sensory neuroscience, Auditory neuroscience, Blast injury, Neurorehabilitation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how the mammalian brain processes information with a heavy focus on how the brain processes sound. We will then use this knowledge to understand how the act of hearing contributes to a healthy brain, as well as to study and help treat the effects of different forms of brain injury.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The processing of sound is an incredibly complicated process that involves the entire brain. This makes the hearing pathway an ideal candidate model for probing healthy brain activity as well as to uncover how injury can damage a brain.

Hearing is vital to our health, and it has been shown that hearing is very important to healthy ageing. For example, hearing loss is a big risk factor for developing dementia. This is concerning because as we age we lose our ability to hear, with approximately one third of the population aged between 60-70 years and three quarters of the population aged over 70 experiencing hearing loss. The aged are already at higher risk of social isolation, which in turn is a risk factor for a number of negative outcomes such as dementia and depression, and the risk of social isolation can be increased by a deterioration in the ability to hear. Furthering understanding how the healthy brain processes complex sounds such as speech will aid our development of therapies to improve the impaired ability to process these complex signals due to injury and the natural ageing process.

In order to understand how the brain processes complex sound, it is important to investigate the 'neural code' of the brain from single neurons (grey-matter cells) to large populations. In humans, we are only able to look at brain activity from the outside with electrodes placed on top of the scalp, and this restricts our view of the brain to the activity of large neural populations. To see what is happening in the brain at a single neuron or small neural population level, the placement of electrodes inside the brain are required, something that can only occur with animal studies.

These animal studies then increase our ability to interpret experimental findings and therefore increase the value of the recordings that are obtained from human studies.

Astrocytes (white matter cells in the brain) are often overlooked when considering the different cell types that are found in the brain, and yet they almost equal the number of neurons present. To understand how the healthy and damaged brain functions it is therefore crucial to understand how astrocytes interact with neurons and how that interaction affects brain function. Therefore, part of this research will involve investigating this interaction using the processing of complex sound as a model of healthy and injured brain activity.

Traumatic brain injury (TBI) can induce profound disability and severely impact quality of life. A traumatic brain injury (TBI) will be experienced by approximately 50-60 million of the world’s population in their lifetime and is the biggest cause of death and disability in those under 40. The long-term effects of TBI include the raised risk of developing conditions such as Chronic Traumatic Encephalopathy (CTE), Alzheimer’s disease (AD) and other forms of dementia later in life. A TBI event can also often escape typical clinical assessment measures. This is concerning given that a cumulative effect exists where multiple TBI events can result in more severe and long-term injury if a recovery period is not maintained, therefore increasing the risk of permanent brain damage. Together, these challenges highlight the urgency to find an effective method to identify the initial occurrence of even a mild TBI to manage injury and recovery. What makes this more challenging is that when considering blast injury a large degree of the injury only appears much later (months)

after the initial trauma. Given the length of time before actual injury appears, TBI serves as an ideal candidate for understanding the development of brain damage before it can be observed in behaviour, as well offering rich opportunities for treatment and the development of better tools for identifying early signs of brain damage.

A further high-risk factor for hearing loss is noise-exposure, and living in a city is a major risk factor for this. When living in a city, you can expect to be regularly exposed to noise above 85 decibels from sources like traffic, subways, industrial activity, and airports. This can cause significant hearing loss over time. Furthermore, it's been shown that 10 decibels more daytime neighbourhood noise is associated with 36 percent higher chance of mild effects on memory, concentration, learning ability, and making decisions in everyday life as well as 30 percent higher chance of developing Alzheimer's disease. It's important to understand how this increase in noise exposure can lead to future conditions such as Alzheimer's disease and to also understand the similarities and differences between brain damage caused by noise exposure, versus that caused by other forms of injury.

What outputs do you think you will see at the end of this project?

- Our investigation will result in extensive datasets of recordings from single neurons and neural populations as well from astrocytes in healthy and injured brains. This data will be made available to all members of the lab as well as undergraduate and masters students for analysis.
- These data will form the basis for new publications in peer-reviewed journal articles and presentations at relevant scientific conferences and meetings.
- The insights gained from the research will help in the development of new rehabilitation strategies for patients, as well as advance the scientific understanding of healthy brain function when using our senses and how that can be disrupted by injury.
- Confirm potential new treatment options for TBI in animal models that can then enter the clinical testing pipeline.
- Findings from our research into astrocyte function will yield valuable information regarding how neurons and astrocytes interact to produce healthy brain function and as such will provide targets for possible therapeutic treatment after injury.

Who or what will benefit from these outputs, and how?

- Insight into neuronal interactions obtained from our research will be an important step towards understanding how sensory information is processed in the brain. This will be of benefit to the entire neuroscience community.
- We will gain valuable insight into the nature of traumatic brain injury, how different forms of brain injury are similar and distinct from one another, and give us direction regarding the best treatment options. This will benefit doctors looking to treat patients with brain injuries.
- In the short term, all findings from this research will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences. The data we will obtain will help progress research into the complex mechanisms underlying traumatic brain injury, and how the severity of

injury affects the development of TBI symptoms over time. In the longer term, this knowledge will help in the development of a way for clinicians to assess TBI severity and eventual clinical outcome following injury. As symptoms differ vastly between individuals suffering seemingly similar injuries, the identification of important symptoms and biological indicators of injury (such as markers in the blood or neural recordings) which might determine the future course of symptoms is of vital importance to doctors.
- We will also test a range of proposed treatment strategies and make the results publicly available. In the longer term, these insights will be used to develop targeted treatment options which act on the complex mechanisms that occur in the weeks to years following TBI. These treatments will be relevant in a wide range of settings, potentially including blast-injured veterans in the military setting, professional athletes which experience sports-related concussions, and civilian accident- or abuse-induced TBI.
- The insights obtained from our research are an essential step for the development of brain- machine interfaces and will be of great value for scientists working in the field of artificial intelligence, robotics and computational brain models and for software developers making use of these approaches.

How will you look to maximise the outputs of this work?

- We will share our results with the general scientific community through peer reviewed publications. We will also present our findings at scientific conferences where we will assess the state of the field and ensure we minimise any duplicate lines of research being carried out by other groups.
- We will share our results with the clinical community through peer reviewed publications as well as clinical conferences.
- We will collaborate with several other labs at our institution as well as other institutions to test new potential therapies and make any available data available to them.

Species and numbers of animals expected to be used

- Mice: 3000
- Rats: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

- The hearing pathway and brain structures involved in hearing are similar across mammals, which means that both mice and rats share significant similarities across their brains to humans, and this makes them ideal candidates for our research.
- We have also chosen rats and mice as they are the most common mammalian

species used for conducting invasive (inside the brain) brain research, which gives us an abundance of existing research literature to build upon for our investigations.
- Genetically altered mice and rats allow us to investigate specific populations of neurons and astrocytes as well as their cellular processes that we wouldn't be able to observe in non- genetically altered species. Typically there are many more types of genetically altered mice than rats, so we would predominantly use mice for these types of investigation.
- Rats, compared to mice, have a larger brain volume and as such are more favourable for imaging and allow for more flexible data collection with the option for recording brain activity from many more locations simultaneously. This allows us to gain a better view of what is happening across the entire brain at any one time.
- Due to this shared brain structure with humans, and despite their size difference to us, rodent models of traumatic brain injury allow the complex cascade of events after TBI to be observed. In addition, this shared structure allows us to also investigate potential therapeutic targets of TBI.

Typically, what will be done to an animal used in your project?

There are 3 typical scenarios that animals will experience in this project.
Experiments under non-recovery anaesthesia:

A typical scenario will see an animal undergo general anaesthesia for the entire duration of the procedure, which can last up to 15 hours. Once under anaesthesia, surgery will be performed to expose the skull, and a window or two will be drilled into the skull to expose the brain. Electrodes will be placed in the brain as well as on top of the skull and on top of the exposed brain. We will then place the animal into a specially designed recording chamber that is shielded from outside noise. Whilst in the chamber, we will play different types of sound to the animal and record the responses to these sounds in the brain. We will also record brain activity during silence for comparison. At the end of the procedure, and whilst still under general anaesthesia, the animal will be humanely killed.

Experiments under recovery anaesthesia to inject viral tracers:
A typical scenario will see an animal undergo general anaesthesia for the entire duration of the procedure and then it will be allowed to recover. Once under anaesthesia, surgery will be performed to expose a section of the skull, and a window will be drilled into the skull to expose the brain. A specially designed virus that is harmless in terms of disease will then be injected into the brain. This virus will multiply in the area that has been injected, and will typically program cells to produce fluorescence (ie. give off a certain type of light) when a specific type of activity in the brain occurs. The window will be covered with glass, the scalp will be sutured closed, and the animal will be allowed to recover and returned to its cage.

Two weeks after injection of the virus, the animal will undergo non-recovery anaesthesia, the window will be exposed, and a specific type of activity in the brain will be imaged using the fluorescence that has been created by the virus. The rest of the non-recovery procedure will record this activity during exposure to different types of sound. We will also record this activity during silence for comparison. At the end of

the procedure, and whilst still under general anaesthesia, the animal will be humanely killed.

Experiments investigating traumatic brain injury:
A typical scenario will see an animal undergo general anaesthesia for the entire duration of the procedure, and then it will be allowed to recover. Once under anaesthesia, an animal will be given some precautionary pain relief, and then subjected to one of the following models of brain injury: Either blast injury by exposure to a shockwave, or prolonged exposure to a loud noise. The total anaesthetic duration will typically be less than 1 hour. The animal will then recover and be returned to its cage.

After exposure to the injury, an animal might receive a drug (typically delivered via injection into the abdominal space) with the aim of treating and reducing the effects of the traumatic brain injury. This may be injected into the animal, and may be administered multiple times at several time points in the weeks and months following injury. An animal will undergo behavioural tests to find any observable effects immediately after injury. The animals may be introduced to a behavioural testing environment several times in the week before exposure to the initial injury to act as a benchmark for comparison, and the animal will then typically be assessed for behavioural effects once a week until the end of the experiment. Two types of behaviour tests will be performed. The first is called a rotarod test (~1hr) which investigates an animal's balance. This test doesn't require any training, and will be performed a maximum of 10 sessions (max freq once a day) prior to injury. Maximum once every day for first 7 days post injury, thereafter once every 3 days. The second test will test for ringing in the ears, called tinnitus. This will involve the animal listening to different 'startling' tones overlaid on top of a background noise. If they hear the tone and are startled (ie. stand still) then they don't have tinnitus. Total frequency: maximum 10 sessions (max freq once a day) prior to injury. Maximum once every day for first 7 days post injury, thereafter once every 3 days. Total duration per experiment: ~1 hour 10mins

Typically a month after injury, an animal will be placed under general non-recovery anaesthesia as per the first scenario (***Experiments under non-recovery anaesthesia).*** Here***,*** the animal undergo general anaesthesia for the entire duration of the procedure, which can last up to 15 hours. Once under anaesthesia, surgery will be performed to expose the skull, and a window or two will be drilled into the skull to expose the brain. Electrodes will be placed in the brain as well as on top of the skull and on top of the exposed brain. We will then place the animal into a specially designed recording chamber that is shielded from outside noise. Whilst in the chamber, we will play different types of sound to the animal and record the responses to these sounds in the brain. We will also record brain activity during silence for comparison. At the end of the procedure, and whilst still under general anaesthesia, the animal will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Recovery surgery procedures:
Animals are expected to make a rapid and unremarkable recovery from any recovery

procedure within 2 hours. Any animals that fail to do so or exhibit signs of pain and distress or of significant health concern will be humanely killed unless a programme of enhanced monitoring and care can be instituted until the animal fully recovers. Any animal not fully recovered from the surgical procedure within 24 hours will be humanely killed. In the case of wound opening post-surgery a vet may be consulted and re-suturing may be done of minimally inflamed and uninfected wounds under general anaesthesia on no more than one occasion. Animals will be monitored for any infection that may develop after electrode insertion, injection of substances in the brain, or placement of transparent window to replace a section of skull under recovery anaesthesia. A vet will be contacted should infection occur and treatment advised accordingly. If the course of treatment is ineffective then the animal will be humanely killed.

Behavioural tests:
We will conduct behavioural tests that require no pain or training, as such we are not expecting any significant expressions of stress from the animals during or after the tests. However, any animals demonstrating persistent (>3 hours) signs of stress (hunched posture, facial grimacing and/or raised fur) after completing a behavioural test will be moved to a control group or humanely killed.

Blood sampling:
We do not expect anything more than a short duration of pain during blood sampling. Sampling of blood will be carried out in surface vessels in order to minimise discomfort, and a vet will be contacted should infection occur and treatment advised accordingly.

Blast-induced traumatic brain injury:
Animals are not expected to display any signs of pain or distress lasting longer than 6 hours after blast injury. Animals will be anaesthetised and given painkillers during the production of injury. Death from blast injury is incredibly rare (<1%). All animals will be closely monitored (at least 2x per day) during the 72 hours after injury and regularly (once a day) thereafter (animals will be weighed once a week for two weeks). But as we are intending to cause a brain injury, animals may display trouble with balance and/or walking with the development of TBI (which is an intentional outcome of the blast treatment).

This might occur immediately after injury and can also be expected to resolve within 6 hours. This should not be painful, and animals will be carefully monitored for any signs of distress, and if distress is observed, pain relief will be provided immediately or increased. Any animal exhibiting persistent side- to-side weaving behaviour or the inability to rise to their feet by the end of the working day will be humanely killed.

Some animals might experience damage to the edge of the ears due to the movement of the head against cushioning during blast. This is most often prevented by enclosing the ears in tape during the blast, however if it does occur, animals will be managed under the advice of a vet- and topical treatments and or additional pain relief will be used as required. If the ear injuries cause distress or the prognosis for recovery is poor in the opinion of a vet, the animals will be humanely killed or terminal anaesthesia will be immediately applied. It is possible that an animal might experience ‘blast lung’, which is detectable by changes such as laboured and/or

rapid breathing and any animals displaying this during recovery will be humanely killed.

Noise-induced traumatic brain injury:
Animals are expected to recover normally from this procedure within 2 hours. However, as this procedure is designed to cause tinnitus (a ringing in the ears), it might create some slight discomfort and animals will be given pain relief during and after a procedure for at least 24 hours. All animals will be closely monitored (at least 2x per day) during the 72 hours after injury and regularly thereafter.
Animals will be monitored for any persistent signs of reduced movement, hunched posture, raised fur, or facial grimace. In the event that these signs remain for longer than 24 hours, animals will be humanely killed.

Administration of therapeutic drugs:
These will cause no more than a short duration of pain, suffering, distress and no lasting harm. Such drugs will be used that are effective and cause minimal damage (if any). Animals will be closely monitored for a minimum of 4 hours after the administration of a drug, and then daily for 2 days afterwards to ensure that there are no lasting adverse effects on behaviour. Any persistent behavioural changes for 24 hours will require the animal to be humanely killed. Some drugs might cause an initial increase in grooming and sniffing movements. This can be followed by a period of lying down where animals may lie on their abdomens making swimming movements of the limbs. Mild twitching and abdominal contractions are also possible. Animals will be monitored closely and if these effects as well as any hunched posture, raised fur and impaired movement persists beyond 5 hours after the drug is administered and show no sign of disappearing we will humanely kill the animal.

Multi-sensory stimulus presentation:
No adverse effects are expected. Any animals demonstrating persistent (>3 hours) signs of stress (hunched posture, facial grimacing and/or raised fur) after exposure to multi-sensory stimulus presentation (repeated sounds or light flashing for an hour) will be moved to a control group or humanely killed.

In all cases, animals will be kept warm and given wet mash if necessary to aid recovery.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

mice: non-recovery: 20% sub-threshold: 10% mild: 20% moderate: 50%
rats: mild: non-recovery: 20% sub-threshold: 10% mild: 20% moderate: 50%

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We require the use of an animal model to uncover the complex and unknown ways by which natural sounds (such as speech) are processed by the brain. Currently there exists no computational model (computer based model of the brain) capable of explaining how sound is processed by the brain.

The pathway for hearing is a complex series of processing centres in the brain that can interact both up (feedforward) and down (feedback) the auditory pathway in the brain. In addition, there is evidence that the entire brain can be affected by the processing of sound. For example, in an animal model of Alzheimer’s disease it has been shown that the repeated presentation of simple sounding clicks at a particular frequency can actually reduce the presence of 'beta-amyloid plaques' (physical indicators of Alzheimer’s disease) in regions of the brain not typically associated with the processing of sound.

Therefore, a whole and intact living brain is required for this research. In addition, it is important to find a model of hearing that is similar to that found in humans, and for that we require a mammal model, as the hearing pathway and how the brain processes sound is very similar across mammals.

TBI is a very complex injury involving many processes which evolve over time scales ranging from seconds to years after injury. The use of animal models is therefore essential for studying these complex mechanisms. Animal models also allow the injury to be related to behavioural changes and sensory processing deficits, which are not possible in non-animal models. In the case of blast injury, the “primary” injury during trauma initiates a “secondary” injury consisting of a complex cascade which only present some of its effects months after injury. This would be impossible to study outside of a live animal.

Substantial human clinical data exists on TBI, however the inability to perform recordings within the brain except for under rare circumstances makes it difficult to relate changes in neuronal activity to behavioural and sensory deficits.

Furthermore, an animal model allows for a reproducible model of injury, something that is not available in the clinical population.

Which non-animal alternatives did you consider for use in this project?

computer modelling : a review of the literature revealed that although certain aspects of injury are able to be modelled computationally, there is currently no available model complex enough to capture how the brain processes sensory information across all regions. In addition there is no model complete enough to model how the brain changes over time in response to a TBI.

non-protected species: a review of the literature revealed that the brain and hearing pathway of non-protected species are so different to that of mammals to make it almost impossible to infer any meaningful clinical conclusions regarding their study.

brain slices and cell cultures: brain slices and cell cultures will be used extensively by the lab. However, they do not allow us to probe the large scale neural networks that are typically affected by TBI.

Why were they not suitable?

Where possible, we will use computational models to test theories before doing experiments on animals. However, scientists do not yet know enough to simulate the entire brain and how it reacts to a sound or an injury such as TBI or responds to a drug, so we still currently need animal models to explore these theories.

Non-protected species have brains and sensory pathways which are very different from ours, so they are not suitable for helping us understand the processing of sound, the complexity of brain injury, or the response to a therapeutic treatment in humans.

Cell cultures and brain slices allows us to observe specific aspects of how the brain operates, and we will use them where appropriate. However, for us to understand something as complex as the processing of sound, the progression of an injury, or the recovery of an injury through therapy, all of these involve the entire brain often over long periods of time. Therefore, it is vital to investigate an intact, living brain that is adapting to an injury or therapy, and also importantly one that can hear.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A statistician helped us with calculations using typical variations from our own earlier experimentation to calculate minimum numbers of animals to be used whilst ensuring that the results are statistically significant. Sample sizes for our experiments are estimated from past experiments conducted on the current PPL, as well as estimates based on the standard number of animals used to show statistically significant effects in our field. Given the effect sizes we observe across the different recording modalities we used, calculations typically show that we need group sizes of 20 to achieve the quality of results we need. In addition, for behavioural studies, we will often need to increase group sizes to 30.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We employed the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We will use the EDA diagram and report outputs to support experimental planning with animal users.

We carefully plan all our experiments to minimise the number of animals we need to use to get the most information possible. In many cases, we will obtain answers to multiple research theories within each animal, for example assessing the effectiveness of a drug through behavioural testing whilst simultaneously recording other data at various stages post-injury. This will allow us to maximise the data generated by one animal, thus reducing the overall number of animals needed without increasing the amount of suffering of each individual animal.

Sham control groups will be used for both injury and potential treatments. Variability is an inherent feature of the injury models. However, by ensuring that the protocol as well as experimental environment has been rigorously tested and is highly controlled and reproducible, this will result in minimal possible variability. Where possible, studies will be randomised and blinded by using conventional techniques such as random animal selection from cages and animal marking conventions that are blind to the experimental group.

All experiments will be designed in such a way as to be publishable under the NC3Rs ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will take all reasonable steps to reduce the number of animals used in our project. We will collect as much information as possible from every animal, for example, by making many measurements over different experimental sessions. In addition, we will maximise the data collected for each individual animal by using multiple recording methods simultaneously. This has been shown to maximise the number of neurons that can be recorded from simultaneously, thus reducing the number of animals required to record from a given number of neurons. We will use consistent experimental techniques across studies, thus reducing variability. We will also make use of pilot studies to give an early indication of the merits of a specific scientific hypothesis (such as the effectiveness of administering a drug at a particular time point post-injury) before including more animals to reach statistical significance.

Importantly, we build this research on established protocols which we have developed over the past 5 years for models of the processing of sound in both neurons and astrocytes, of injury, as well as injury treatment.

We also plan on using brain slices and brain cultures from the terminal anaesthesia experiments to test and develop hypotheses.

Any tissues not used in our investigation will be made available to other groups at our institution.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use established rodent models of healthy hearing as well as TBI, including blast injury and noise-induced TBI. We will use mouse models wherever possible, however there are certain instances that require the use of rats, such as where we need to record neural activity using multiple probes concurrently (due to their larger brain size).

Wherever possible we will use terminal anaesthesia when conducting experiments to investigate how the brain (astrocytes and neurons) processes sound in health and after injury. However, in certain instances, such as when performing 'calcium imaging' of astrocytes, we will need to perform recovery experiments. This is because we need to inject harmless viruses to make certain cell types fluoresce when they are active and this harmless 'infection' takes time to adequately develop.

In these instances, we will use mice as the preferred option. When subsequently recording data from these animals, we have placed a limit of two recording sessions (ie two times under recovery general anaesthesia) per animal in order to minimise suffering whilst allowing us to obtain more data per animal and also capture the natural variability in neural activity that occurs over time. All surgical procedures and procedures performed under recovery anaesthesia will require the appropriate delivery of pain relief to ensure that animal suffering is minimised. Sterile procedures will be used throughout. All animals will be closely monitored (at least 2x per day) during the 72 hours after surgery and regularly (once a day) thereafter.

All injuries will be performed under a sufficient anaesthetic depth such that the animal experiences no pain or discomfort, and this will be continually monitored by appropriate methods throughout the procedure. Appropriate pain relief will also be administered to ensure the animals experience minimal pain, suffering and distress during recovery. For both the blast injury and noise injury models, animals will receive pain relief during the injury and for at least 24 hours after the injury. We require a number of time points to be investigated as brain injuries tend to evolve with time after the initial injury, and its important to understand how conditions such as dementia can develop from these. All animals will be closely monitored (at least 2x per day) during the 72 hours after surgery and regularly (once a day) thereafter.

These animals may also experience another brief recovery anaesthesia in order to test the brain's ability to hear (called auditory brainstem responses) in the week prior to the injury and once a week after the injury. We will avoid the traditional method of

collecting these responses that requires placement of electrode wires under the scalp, by placing an electrode array on top of the shaven scalp of a lightly anaesthetised animal (therefore reducing suffering). Another electrode will be attached to the ear or another part of the animal (as a reference electrode).

Why can't you use animals that are less sentient?

This project investigates how the brain processes sound in health and injury. To do this we need to have an intact auditory system spanning from the moment the sound reaches the ear to its subsequent processing by the brain. Rodents have auditory systems comparable to humans and share similar ways in which such systems communicate which require processing of complex sounds in time. The use of species with a less complex auditory processing ability would therefore result in a poorer translation of results to understanding human sensory processing and its deficits. Similarly, an adequate level of sentience (cognitive development) is required to observe complex behavioural expressions of injury associated with TBI such as memory loss and anxiety. Lastly, although differences exist between species, the basic mechanisms in the brain are likely to be common for all mammals, and as such our investigation will build upon a large body of existing data in mice and rats, which minimises the need for duplicate investigations.

Wherever possible we will use mice for our investigations as they are commonly accepted to be of a lower sentience than rats. However, in our injury models, we are first using a rat model, this is due to their larger brain size which allows us to place more electrodes in and over the brain to record neural activity from a larger area and higher number of neurons. This will allow us to better understand how injury develops over time after the initial injury and how different regions can affect one another in the brain.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be acclimatised to any new location or housing environment for a minimum of 7days. After the procedure animals will be very closely monitored and given anti-inflammatory and pain kililng drugs to reduce pain. All animals will be closely monitored (at least 2x per day) during the 72 hours after surgery and regularly (once a day) thereafter. Animals will be weighed once daily for the first 7 days post procedure, and once weekly after that. Any animals showing signs of pain or high levels of distress such as raised fur, hunched posture, reduced mobility or eye or nasal discharge will be referred for examination by the NACWO and/or NVS at the earliest opportunity. If the animal fails to respond promptly to simple treatment or their condition deteriorates, it will be humanely killed. Any animals which experience a weight loss of over 15% body weight will also be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are published guidelines to assist with planning animal research and testing, such as the PREPARE guidelines:
http://journals.sagepub.com/doi/full/10.1177/0023677217724823

Further information about the PREPARE guidelines can be found here:
https://norecopa.no/prepare
For the administration of substances, we will follow guidelines from: Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. British Veterinary Association Animal Welfare Foundation/Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare (Morton et al 2001)
Other resources are available including guidance and publications from the NC3Rs and Laboratory Animal Science Association.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check information on NC3Rs website, we’ve signed up to the NC3Rs newsletter, we will meet the NC3Rs Regional Programme Manager, and attend Regional 3Rs symposia.

39. Neural basis of circadian rhythms in feeding and drinking

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Circadian clock, Drinking, Feeding, Obesity, Diet

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To advance understanding of the neural mechanisms regulating food and water intake throughout the day and how these alter in response to diet with the aim of identifying strategies that could be used to modulate dietary intake to a healthy level.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Obesity is a major contributing factor to a wide range of health issues including heart disease, strokes and cancer. Throughout the developed world, obesity has reached epidemic proportions, especially among the poorer members of society, and dealing with the associated health issues is presenting a major challenge to health care providers like the NHS. The purpose of the outlined work is to advance understanding of the neuronal mechanisms involved in regulating food and fluid

intake throughout the day with the ultimate aim of identifying strategies that could be used to modulate dietary intake to healthy levels.

What outputs do you think you will see at the end of this project?

The principle output from the outlined studies will be data on the neuronal mechanisms regulating dietary intake throughout the day including how these mechanisms are influenced by the composition of the diet, the time of day, and the impact of targeted neuronal stimulation on dietary intake. The data generated will be presented at conferences and published in peer reviewed scientific journals.

Who or what will benefit from these outputs, and how?

In the short term, the primary beneficiaries of this work will be scientist working to understand the neuronal control of appetite. In the medium term the work is expected to benefit scientist and physicians working on strategies that could be used to modulate dietary intake to healthy levels. In the long term the work is expected to contribute to the development and implementation of policies, statutes and health care advice to tackle the obesity crisis facing developed societies throughout the world.

How will you look to maximise the outputs of this work?

The findings of the outlined work will be presented at scientific conferences and published in peer reviewed journals. The work will be undertaken by a research group with a strong publication record, enhancing the prospects for the effective dissemination of the findings. This dissemination will be further facilitated by our links to an international network of collaborators engaged in this field. Given the recent success of novel and promising obesity medications, the insights gained during this project are expected to be readily translatable to the clinical setting. Unsuccessful approaches, ‘null data’ and negative results will be disseminated through data presentations at conferences and pre-printing services, including bioRxiv, Harvard Dataverse, and Open Science Framework.

Species and numbers of animals expected to be used

- Mice: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In order for the data generated to be meaningful, the study requires an animal with a physiology, neuroanatomy and daily feeding behaviour similar to that of humans.

Mice have been selected for these studies as they have the lowest neurophysiological sensitivity of the species that meet the required scientific criteria.

In addition, mice have the advantage that their genotype can be easily manipulated to facilitate the investigations being undertaken. Animals of a lower neurophysiological sensitivity which are not protected by ASPA (e.g., invertebrates, or other less sentient animals to rodents) are unsuitable for these studies due to the marked dissimilarities in their regulatory mechanisms of homeostasis and behaviour.

Typically, what will be done to an animal used in your project?

The vast majority of the mice used in the outlined studies will carry genetic alterations that do not result in harmful phenotypes. Most mice (80%) will have their access to food and water restricted to specific periods of the day to evaluate the influence disrupting the normal circadian pattern of feeding has on dietary intake. A few juvenile mice (20%) will be fed a modified diet reflective of so called 'fast foods' (standardized rodent high-fat diet, high-sugar diet, or a mix of both) to assess the influence this has on dietary intake in adulthood. Some mice (10%) will be kept on study for up to 2 years. None of these manipulations are expected to induce any clinical signs.

Around 75% of the mice (excluding aged animals) will undergo an aseptic surgical procedure, conducted under general anaesthesia, to either inject viral vectors carrying genetic constructs into the brain or to implant devices required for the recording or stimulation of neuronal activity. Following surgery, the mice are expected to make an uneventful recovery and to resume normal behaviour within a few hours. Upon recovery, the mice will be given pain killers until they show no detectable signs of pain. Approximately two weeks post surgery, the mice will be transferred to automated cages equipped for the continuous monitoring of feeding, drinking and voluntary physical activity (via an installed running wheels).

Throughout the study period, the mice will undergo weight checks, daily observation, and assessments conducted by both experimenters and qualified technicians. At the end of the study the mice will be killed using a humane method so that their tissues can be analysed.

What are the expected impacts and/or adverse effects for the animals during your project?

None of the animals are expected to experience any adverse consequence as a result of being genetically altered.

Animals undergoing surgery are expected to experience some level of pain upon recovery however, this will be minimised by giving pain killers and all animals are expected to recover uneventfully and to resume normal behaviour within a few hours.

Some animals are expected to develop obesity, which may increase their susceptibility to developing obesity related conditions however, the state of obesity is

not in itself expected to induce any clinical signs and the limited duration of the studies means that it is unlikely that obesity related conditions will occur. Throughout the study the condition of the mice will be closely monitored, including by weighing and body score assessment and any mouse showing signs of overt suffering will be killed humanely.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 10% subthreshold, 20% mild, 70% moderate.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The purpose of the outlined studies is to advance understanding of the neural mechanisms regulating food and water intake throughout the day and how these alter in response to diet with the aim of identifying strategies that could be used to modulate dietary intake to healthy levels. It is not possible to conduct these studies without the use of animals as current understanding is insufficient to enable in-silico modelling and simplified systems, such as cell cultures, unprotected animal species and non- animal life forms, are unable to replicate the complexity of the neuronal mechanisms involved in regulating dietary intake.

Which non-animal alternatives did you consider for use in this project?

In-silico modelling and simplified systems, such as cell cultures and unprotected animal species and non-animal life forms.

Why were they not suitable?

Current understanding of the neuronal mechanisms involved in regulating dietary intake is insufficient to enable in-silico models to be developed and simplified systems, such as cell cultures, unprotected animal species and non-animal life forms, lack the neuronal mechanisms involved in regulating dietary intake.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Protocol 1 is exclusively designed to generate mice for use in Protocol 2. To ensure an ample supply of 400 mice for subsequent procedures (of which a minimum of 360 will come from local breeding), we plan to employ 500 mice in the breeding protocol. Although every effort will be made to utilize each mouse generated in Protocol 1 for Protocol 2, an estimate accounting for normal survival rates of juvenile rodents, and some animals used purely for breeding and not proceeding to Protocol 2, necessitates the consideration of a larger initial number. Excess animals generated in Protocol 1 will be aimed to be utilised through the internal Tissue Sharing Network.

The specific group sizes within the procedures outlined in this project license vary significantly. For surgical models, 6-8 animals are required per group, with this project aiming to employ at least 6 different models across two diets (obesogenic vs. balanced diet): 8 x 6 x 2 = 96. While ongoing efforts will be made to reduce these numbers based on results, an initial estimate of 100 animals is planned for this set of procedures.

Another set of models involves drug injections at four different time-points across the day (n=6 per group), with each animal receiving one of up to 6 drugs, including obesity medications and drugs used to evoke a phenotype of a genetic model. Some of these animals will also undergo surgery, and approximately half will experience a change in diet to an obesogenic one: 6 x 4 x 6 x 2 = 188. Initial estimates suggest 200 animals for this set of procedures, with ongoing adjustments based on results.

Additionally, some animals may undergo surgery, drug injection, and/or diet modification, and will be subjected to time-restricted feeding. Quantification of results will occur at the behavioural level, and post-mortem tissue will be utilized for ex-vivo experiments and molecular studies of gene expression. For these protocols post-mortem tissue use dictates the number, and n=4 animals will be required per group.

Animals will be humanely killed at four daily time points. We anticipate at least 6 cohorts of animals undergoing time-restricted feeding: 4 x 4 x 6 = 96. The estimate for this set of procedures is 100 animals, with ongoing efforts to reduce this number based on evolving results.

In total, we estimate using up to 400 animals for all procedures outlined in Protocol 2, with ongoing efforts to refine and reduce these numbers as the study progresses.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Number of animals for each procedure is based on our previous experiments 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?

The statistical power of each experiment will be recalculated based on ongoing experiments, ensuring the use of a pertinent number of animals in the study. The figures specified in this project license represent the maximum numbers that might be required to achieve the study objectives. It is highly likely that these exact figures will be adjusted based on ongoing assessments, as they were initially calculated with a margin of assurance to ensure the attainment of study objectives. Adjustments will be made as necessary to align with the evolving needs of the research while maintaining scientific rigor.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetic alterations: The vast majority of mice used will carry genetic alterations however, these do not result in harmful phenotypes.

Restricted access to food and water: Most mice (80%) will have their access to food and water restricted to specific periods of the day to evaluate the influence disrupting the normal circadian pattern of feeding has on dietary intake. In all cases, the time available for the animals to eat is sufficient to meet their daily need and is not expected to result in hunger or thirst. Throughout the study period the animals body condition and weight will be measured regularly and if any animal is found to have lost condition or its body weight drops by over 15% (matched to the normal growth curve) it will be given free access to food and water until it returns to its normal body weight .

Dietary manipulation: A few juvenile mice (10%) will be fed a modified diet reflecting of so called 'fast foods' (e.g. commercial rodent high-fat or high-sugar diet) to as sess the influence this has on dietary intake in adulthood. Some of these animals may become obese however, this is not expected to result in any welfare issues for the animals given the limited duration of the study period. Nevertheless, these mice will be closely monitored, as outlined below, and promptly killed using a humane method in the event that their wellbeing becomes adversely affected.

Aging: Some mice (10%) will be kept on study for up to 2 years to evaluate the impact of the dietary manipulations on later life. Towards the end of this period these

animals will be at heightened risk of age-related conditions and will therefore be closely monitored, as outlined below, and promptly killed using a humane method in the event that their wellbeing becomes adversely affected.

Surgery: Around 75% of the mice used will undergo a surgical procedure, conducted under general anaesthesia to either inject viral vectors carrying genetic constructs into the brain or to implant devices required for the recording or stimulation of neuronal activity. Surgery will be conducted using full aseptic precautions and in line with LASA guidelines. Upon recovery, the mice will be closely monitored and given pain killers until they show no detectable signs of pain.

Monitoring: Throughout the study period, the mice will undergo weight checks, daily observation, and assessments conducted by both experimenters and qualified technicians.

Animal handling and delivery of substances: All animals will be handled and dosed according to refined methods proved to cause minimal distress.
Why can’t you use animals that are less sentient?

Mice have the lowest neurophysiological sensitivity of the animals suitable for these studies. It is not possible to conduct this study in less sentient species as they do not have complex feeding/drinking behaviours and the central nervous system comparable to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Handling and housing: The procedures required to undertake the outlined work have all been refined previously to minimise any associated suffering. Care will be taken to habituate the mice to the research staff and at all times they will be handled using non-aversive methods. The mouse's home cage will be provided with environmental enrichment at all times.

Genetic alterations: The vast majority of mice used will carry genetic alterations however, these do not result in harmful phenotypes.

Restricted access to food and water: Most mice (80%) will have their access to food and water restricted to specific periods of the day. The time available for the animals to eat is sufficient to meet their daily need and is not expected to result in hunger or thirst. Throughout the study period the animals body condition and weight will be measured regularly and if any animal is found to have lost condition or its body weight drops by over 15% it will be given free access to food and water until it returns to its normal body weight.

Dietary manipulation: A few juvenile mice (10%) will be fed a modified diet reflecting of so called 'fast foods'. Some of these animals may become obese however, this is not expected to result in any welfare issues for the animals given the limited duration of the study period. Nevertheless, these mice will be closely monitored, as outlined below, and promptly killed using a humane method in the event that their wellbeing becomes adversely affected.

Aging: Some mice (10%) will be kept on study for up to 2 years, towards the end of this period these animals will be at heightened risk of age-related conditions and will therefore be closely monitored, as outlined below, and promptly killed using a humane method in the event that their wellbeing becomes adversely affected.

Surgery: Around 75% of the mice used will undergo a surgical procedure, conducted under general anaesthesia. Surgery will be conducted using full aseptic precautions and in line with LASA guidelines. Upon recovery, the mice will be closely monitored and given pain killers until they show no detectable signs of pain.

Monitoring: Throughout the study period, the mice will undergo daily observation, weight checks, and assessments conducted by both experimenters and qualified technicians.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All procedures will be performed in accordance with guidance published by NC3Rs UK and Norecopa. Surgical procedures will be conducted in compliance with LASA guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I receive regular updates on 3Rs advancements through the newsletters provided by my institutes Named Information Officer which includes valuable information about events and training sessions organized by entities such as NC3Rs UK and Norecopa. In addition, I actively engage in the 3Rs events hosted by my institute, including its Early Career Researcher Group, which actively promotes the uptake and development of 3Rs initiatives. I am committed to incorporating all relevant 3Rs advancements into my research as soon as possible.

40. Neurophysiological mechanisms of pain

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Pain, Sensory neurons, Nociception, Analgesia, Neuroimmune axis

Animal types	Life stages
Mice	aged, neonate, juvenile, adult, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Investigate neuronal and non-neuronal mechanisms of pain and somatosensation to define nociceptive circuitry and new drug targets to treat chronic pain

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic pain remains, arguably, one of the biggest clinical challenges of the age. The problem of chronic pain continues to grow with ageing populations. The prevalence and impact of chronic pain are far reaching with a devastating socioeconomic impact. In a telephone survey of over 46,000 people across 15 European countries, data showed that one in five adults suffer from chronic pain.

Chronic pain was defined as pain lasting 6 months or longer, occurring at least twice a week with a moderate- to-severe intensity of more than 5 on a 10 point numeric rating scale. Nearly one-third of chronic pain patients reported that their pain was intolerable and more than 20% were diagnosed with depression. In this study, ca. 15 working days were missed annually due to pain, and other studies have shown that in Europe the national healthcare and socioeconomic costs of conditions associated with chronic pain amounts to 441 billion Euros, representing anywhere between 3-10% of gross domestic product. The socioeconomic consequences of chronic pain are substantial and the impact of chronic pain on quality of life is detrimental. Clearly, there is an unmet need to investigate the physiological mechanisms of chronic pain to develop more effective and targeted analgesic therapies for patients that suffer from it.

Animal models of pain are essential in order to dissect the sensory pathways and the mechanisms that lead to neuronal hyperexcitability that underlies the initiation and maintenance of chronic pain. More recent functional imaging studies in humans have re-affirmed the similarity in the nervous system structures that are involved in pain processing between rodents and humans. We have previously published data showing the correlation between neuronal activity in rodents and human pain perception. More recent studies have demonstrated the translatability of neuronal and immune mediated mechanisms of pain across mouse models of chronic pain and in patients with chronic pain, and we have been able to back-translate patient findings into mouse models to dissect mechanisms, e.g. the role of neutrophils sensitising neurons in the dorsal root ganglia to driving chronic widespread pain.

What outputs do you think you will see at the end of this project?

This programme of work aims to (1) define the cellular networks and molecular mechanisms that acute and chronic pain; and (2) determine new therapeutic approaches and targets for pain relief. Our research involves a range of transgenic mouse strains to use for intersectional genetics, as well as murine models of pain states that reproduce clinically relevant features of pain syndromes. These tools will allow us to assign precise functional roles distinct subsets of neurons and non-neuronal cells in models of different pain syndromes using neurophysiological analyses. Our approach in exploiting in vivo assays in animal models of chronic pain is fundamental to acquiring insight of the nervous system organisation for nociceptive processing, i.e. we can manipulate the nervous system at the molecular level and monitor at the whole system level. Through these approaches our studies will reveal what cellular and molecular processes can be targeted for novel analgesic therapies. We expect key outputs to include publications that will guide our and other clinical studies in pain research to develop and test pain relieving drugs and approaches.

Who or what will benefit from these outputs, and how?

With at least 7% of the population in excruciating pain, the utility of identifying new therapeutic targets for pain relief is self-evident. There is a huge unmet clinical need for improved and targeted analgesia, but many chronic pain conditions are currently addressed with similar classes of centrally-acting drugs that do not differentiate between distinct diseases and classification of pain states. Moreover a substantial

programme of our work is dedicated to investigating the mechanistic basis of sex differences in pain, which is highly pertinent to women, who are more predisposed to developing persistent musculoskeletal pain with disease. Our experimental approaches to define distinct cellular and molecular targets in different pain conditions are essential to providing candidate drug targets for more effective pain relief. Our experiments will define new analgesic targets within the lifetime of the licence. However, drug development can require considerable time due to regulatory constraints, but the potential reward for pain relief remains very high.

The short term benefit of our work is the provision of new insights into the cellular mechanisms that underpin different pain syndromes in rodents. The long term benefit of our work is identification of key genes and cellular interactions that cause distinct pain syndromes and that present new targets for the development of therapeutic drugs. We are also working closely with clinical colleagues (rheumatologists, anaesthetists and pain physicians) to ensure the translational value of our findings.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work by sharing expertise in the pain models that we have refined and tested, as well as by having a range of relevant available mice for intersectional genetics to be applied in models that are well established in our laboratory. These tools will allow us to assign particular subsets of neurons to precise functional roles in models of different pain syndromes, through electrophysiological, imaging and behavioural analyses. The data generated from the mice that we characterise will be invaluable for use by other researchers and the pharma industry to examine pain mechanisms. Given that pain is a multidimensional experience and requires an intact nervous system to manifest, our approach to exploit in vivo assays in animal models of chronic pain is fundamental to acquiring insight of the nervous system organisation for nociceptive processing. We will analyse functional data at the molecular level (that we can manipulate) and at the whole system level (that we can monitor) to provide novel insights into the mechanisms underlying the generation of pain and approaches to treating chronic pain.

Our laboratory also runs clinical studies in parallel with the animal models of diseases, which means we are in a in a position to expedite changes to clinical pathways for treating pain depending on what our results show int terms of analgesic efficacy of novel compounds and mechanisms that may inform on novel pain biomarkers.

Species and numbers of animals expected to be used

- Mice: 20000 mice

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used for this project because they represent the most appropriate, genetically amenable species in the proposed studies in pain. Mice share substantial homology for physiological systems of pain processing with humans. All forms of analgesia used in humans are also effective in mice as tested with the behavioural, electrophysiological and imaging based readouts we have established in the laboratory. Many key physiological systems and structures involved in pain processing were originally identified in rodent and other models and validated in more recent human studies since the turn of the century. Importantly, the mouse is highly amenable to genetic modification, allowing transgenic identification of specific cell types crucial to the fulfilment of the project where we aim to identify the neuronal and non-neuronal mediators of peripheral and central sensitisation. Over several years of working with rodent models of pain, we have established protocols to minimise stress in rodents in order to ensure the best welfare of all animals and maximise scientific output from our studies that otherwise require consistent animal handling and the listed interventions in our protocols. We have also developed new techniques to replace conscious animals with unconscious animals (e.g., in imaging experiments to assess cell activity) to reduce suffering. In this licence we are using adult mice only because it has been established that the development of the nociceptive system continues in neonatal mice, e.g. activity dependent development of inhibitory neurotransmission in neonates.

Typically, what will be done to an animal used in your project? Testing acute pain thresholds.

We will use assays to measure pain in rodents using behavioural assays that involve quantifying withdrawal reflexes — these correspond to pain thresholds and are indicative of changes in sensory and nociceptive function. Threshold stimuli are applied such that the intensity is increased until the animal withdraws. The withdrawal reflex exists to protect tissue from damage and is generated when a minimal amount of pain has been detected by the animal. The attenuation of these nocifensive behaviours by analgesics indicates they are a reliable measure of pain in animals. Animals can also indicate discomfort, such as altered gait or weight bearing asymmetries as deficits in motor function.

Assessment of motor function allows us to ensure we are quantifying a pure change in nociceptive sensory function rather than general motor movement of the somatosensory system.

Importantly, the acute pain responses measured here are only temporary and generally mild severity.

Specified tests do not damage tissue and cut-offs are applied to each test to ensure this so that they may be repeated (typical time courses are listed in each protocol).

We perform a phenotypic analysis of mice in terms of pain, sensory and motor function following genetic manipulation and/or interventional treatments:

Thermal sensitivity (application of radiant heat to the hind paw or cooling stimuli using the following standardised assays: Hargreaves', hot plate, acetone, cold plate, dry ice test)

Mechanical sensitivity (application of a mechanical stimulus to the hindlimb such as von Frey hairs or a mechanical probe in the Randall Selitto tests)
Motor function (assessed using rotarod apparatus, monitoring of limb use, assessment of forelimb/hindlimb grip force, as well as gait analyses)

Pain models
The study of mechanisms underlying chronic pain requires the use of animal models that involve induction of some sustained tissue injury. Without these models, it would not be possible to fully understand the pathophysiology of chronic pain that occurs in the intact nervous system. There is a considerable overlap between humans and rodents for the known nervous system pathways involved in nociceptive processing and transcriptional profiling of sensory neurones, which makes the rodent an excellent model for the investigating the mechanisms that underlie the development and maintenance of chronic pain in humans.

An investigation of pain mechanisms at the level of signalling cascades involves the need to test agonists and antagonists for distinct proteins within pain pathways in vivo. In this licence we will use various routes of administration (appropriate and relevant for the drug and site of study) to determine the effects of a compound on behavioural pain thresholds and neuronal excitability in acute pain and chronic pain models - progressing from mild to temporarily moderate only. We will additionally compare changes in gene and protein expression due to the induction of a pain state in wildtype and genetically altered animals, and no pain model will exceed 12 weeks post induction. Individual protocols will use anaesthesia to mitigate suffering or distress associated with the performance of specific surgical and/or other procedures like imaging. Analgesics will be used to relieve pain, except for those studies where the primary functional outcome for the pain models will be the development and assessment of pain itself, and so the administration of analgesics can intervene in the objective of our studies.

This approach of using *in vivo* studies is essential to meet our project objectives to determine the role of candidate genes in chronic pain using neurophysiological analyses, and to provide data on potential drug strategies that would be useful to treat distinct pain syndromes in humans. Importantly, our research funding also includes investigation of sex-specific processing of pain, and so we study pain mechanisms in both male and female mice.

Inflammation
We will use models of inflammation to assess the following functional outcomes:
Allodynia (hypersensitivity to non-noxious inputs)
Hyperalgesia (exaggerated hypersensitivity to noxious inputs)

Spontaneous pain (nocifensive signs, postural changes, altered gait)
A number of different models are used to represent the different types of inflammatory pain (e.g. in the skin, joint or muscle) in humans in terms of duration and severity. Rodent models of inflammatory pain were established to produce the

minimum discomfort possible for the animal, i.e. by restricting inflammation to one body part and being short lasting, while still producing a model of a clinically relevant mild to moderate pain state. Moreover, different models allow us to minimise testing required for sufficient data, e.g. if just a single time point is required, then a shorter acting model can be used to minimise discomfort. Likewise, if many time points are needed, a longer acting model and fewer animals can be used as required in power calculations. In the model of 'hyperalgesic priming" animals will be administered an inflammatory agent, and following the resolution of this initial inflammation and hyperalgesia, animals will receive another administration of an inflammatory agent.

Whereas the second agent would normally produce a short-lasting hyperalgesia, priming the animal with a prior exposure to inflammation produces a hyperalgesia that is longer-lasting and so produces chronic, rather than acute, pain. This is important for mimicking the development of long-lasting hypersensitivity of nociceptors that underlies the transition of acute to chronic pain.

Arthritis
We will use models of arthritis that involve joint surface injury. These models induce a mild degree of instability of the joint and lead to slow and progressive cartilage degradation to mimic the human condition. Pain is a primary disabling feature of arthritis, and so it will be essential to determine outcomes on pain as well as correlating this to the structural integrity of the joint.

We will assess the following structural outcomes:
Cartilage degradation (histological scoring, imaging)
Bone integrity (using in vivo or ex vivo microCT imaging)

We will also assess functional outcomes on sensory function and pain:
Allodynia (hypersensitivity to non-noxious inputs)
Hyperalgesia (exaggerated hypersensitivity to noxious inputs)
Motor function (limb use, altered gait)
Different models involve either indirect or direct damage to the joint surface. Indirect damage induces instability, and these are the gold standard models of osteoarthritis. Other models involve acute cartilage or osteochondral defects that are surgically generated, and are instead useful to study the mechanisms of the repair of acute injuries and associated pain.

Cancer-induced bone pain
We will use a model of cancer-induced bone pain by introducing cancerous cells or substances to induce resorption into a bone, usually the femur. Pain behaviour will be assessed following the surgical procedure and throughout the duration of the focal tumour development where animals will be monitored according to the moderate protocol severity limits.

Functional outcomes on sensory function and pain will be assessed:
Allodynia (hypersensitivity to non-noxious inputs)
Hyperalgesia (exaggerated hypersensitivity to noxious inputs)
Motor function (limb use, altered gait)

We will also examine bone morphology using imaging and histology to evaluate

cancer growth (using fluorescently tagged cancer cells, where possible, for *in vivo* imaging) and bone changes as a readout of structural damage caused by tumour growth to be correlated with pain outcomes.

Post-surgical pain
Induction of local hypersensitivity occurs following a surgical procedure as surgical intervention, and is an important risk factor for the development of persistent pain.

The Brennan model of incisional pain in rodents is well established, which involves incision of cutaneous, fascia and muscle tissue followed by suturing to mimic a surgical intervention in humans and produce acute post-surgical pain. For chronic post-surgical pain we will use 'hyperalgesic priming' models as described previously in the 'inflammation' section. Surgical pain can serve as a priming factor to produce prolonged pain after a subsequent exposure to an inflammatory stimulus, and likewise the prior exposure to an inflammatory stimulus can serve as an important risk factor for developing chronic pain after surgery.

We will also assess the following functional outcomes on sensory function and pain:
Allodynia (hypersensitivity to non-noxious inputs)
Hyperalgesia (exaggerated hypersensitivity to noxious inputs)
Motor function (limb use, altered gait)

***In vivo* electrophysiology**
We describe will use *in vivo* electrophysiological techniques to assess the activity of individual neurones in the peripheral and central nervous systems to similar stimulus parameters used in behavioural tests described above. This enables us to assess the functionality of discrete peripheral and central mechanisms involved in pain processing, where we can study populations of sensory neurones in response to noxious and non-noxious peripheral stimuli. The continued use of animals from will help reduce total numbers of animals by permitting multiple approaches (i.e. behaviour assessments, induction of models and pharmacological interventions) for assessment of pain and sensory function in our studies.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are expected from application of acute stimuli to test sensory and pain behaviour, but importantly, maximum intensities of stimuli are listed in the protocol to avoid any tissue damage. The behavioural tests in this protocol provoke mild degrees of pain that do not persist after testing. It is likely that some animals used in these experiments will be analgesic from genetic modification or drug treatment and that they will therefore fail to respond to the stimuli which they are exposed to. For this reason, each test has a conservative cut off point that will prevent tissue damage from occurring. In order to avoid adverse effects from occurring, the number of testing periods will be kept to a minimum. Mice will be subject to no more than two tests for each modality per day with a maximum of three tests per animal per day. There will typically be three testing days per week.
When compounds are administered the minimum effective dose will be used to minimise the chance of adverse effects. The animals will be observed in the period immediately after injection and will be monitored at least once daily afterwards

including as part of daily checks by animal house staff. Any signs of reactions such as infection or swelling at an injection site will result in additional monitoring or, if appropriate according to our humane endpoints listed below, the animal will be culled immediately with a Schedule 1 method.

We will use tamoxifen injections to induce Cre recombination in transgenic animals expressing Cre- ERT2 under promoters for genes of interest. This Cre recombination approach is a useful tool to temporally restrict the study of specific genes in adult animals, without congenital deletion of genes from birth that may impact the development of the nociceptive system. The reported capacity of tamoxifen to induce side effects, such as body weight loss and general malaise, are only temporary because of the short duration of the treatment (5 days maximum). Throughout the course of dosing and any post-dosing period, mice will be closely monitored for any adverse reactions to the treatment. The protocol should produce little direct effect in itself as animals are expected to recover after the temporary dosing effects.

For surgical procedures, animals are expected to recover uneventfully from anaesthesia and surgery. Any animal that has not resumed normal mobility and started to eat and drink by the end of the working day of surgery will be killed or a schedule for regular out of hours inspection will be instituted so that it may be euthanized if its condition deteriorates. Any animal that has not resumed normal mobility, eating and drinking by the day following surgery will be killed. Occasionally (<1:70), some mice may not recover from anaesthesia. All surgery will be conducted using aseptic technique that meets at least the standard set out in the HO Minimum Standards for Aseptic Surgery. Should an infection occur it will be treated in accordance with advice from the NVS or the animal will be killed by a Schedule 1 method.

We expect that the mild sedation that follows anaesthesia could reduce activity levels for the acute post-operative setting for a few hours (also enabling wound margins to adhere better, limit dehiscence of sutures where applied and allow repair responses). All animals undergoing a procedure will be monitored carefully with welfare scoring sheets. Monitoring will include gait analysis, as we expect in some models (e.g. cancer induced bone pain and surgically-induced arthritis), for animals to reduce some weight bearing and functional activity of the affected leg. We will also expect to observe the development of persistent mild to moderate pain develops later on. Abundant bedding and enrichment will be provided to minimise discomfort and distress, as well as wet mash available on the cage floor to facilitate access for food and water. In our experience, skin wounds on the leg for arthritis or bone cancer models usually heal without complications within few days. Occasionally (1:30) the animals manage to remove the suture points. If this happens after 2-3 days this is inconsequential. However, if it happens within the first 24 hours, this may result in a gaping wound. If this is immediately recognized and is still wet, it will be re-sutured. If it is dry, however, it will be left to heal of second intention because there is no benefit in re-suturing at this stage. If the wound is still open, without a scab and is clearly infected (frequency <1:300) local antibiotics will be applied if advised by the NVS or the animal will be killed by a Schedule 1 method.

In all cases, the following humane endpoints will determine whether animals are no longer suitable for this protocol, necessitating veterinary advice or Schedule 1

termination:

Transient hunched posture, i.e. after dosing or stimulation
Piloerection
10% weight loss
Transient tremors
Prostration
Self mutilation leading to autotomy
Reduced peer interaction

Animals exhibiting any unexpected phenotypes approaching the humane endpoints will be killed by a Schedule 1 method, or in the case of individual animals of particular scientific interest, advice will be sought promptly from the NVS or local Home Office Inspector.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We do not expect any of the protocols in this licence to exceed moderate severity. Protocols for breeding and for acute behaviour testing will fall under mild severity. Other protocols that involve administration of substances or a surgical intervention fall under a moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of animal models of acute and chronic pain conditions is fundamental to providing insight of cellular, molecular and systems organisation of nociception that we can otherwise not determine clinically. Over the last 2 decades functional imaging has been proposed to study human pain mechanisms, but the relevance of a nominal pain matrix in the brain remains ambiguous. Currently only self-reporting of pain intensity from humans remains a useful guide to drug efficacy. On the other hand, animal models offer the ability to produce fine-tuned characterisation of anatomical components of neuronal pathways for nociception, with excellent temporal and spatial resolution provided by electrophysiological recordings of neuronal activity. We have also previously reported essential findings that functional activity of sensory neurones in vivo is lost when these neurones are excised and studied in culture i*n vitro*. This has important implications for the study of sensory

neuronal cell lines, and results from cultured neurons must be considered with need caution when inferring in vivo function. We fully acknowledge their strengths and appreciate their limitations as these studies may be valuable, but only neurophysiological studies in the intact nervous system will inform on both peripheral and central nociceptive signalling pathways.

Which non-animal alternatives did you consider for use in this project?

We are working with patient derived tissue and cell samples directly, where available to us through our clinical programmes of work. For example, our mouse studies in a model of fibromyalgia revealed an important role for neutrophils in driving chronic widespread pain, and current work now is building on the earlier mouse studies to define the pathological phenotype of neutrophils derived from patients using *in vitro* assays.

Why were they not suitable?

Animal models offer the ability to produce fine-tuned characterisation of anatomical components of neuronal pathways for nociception, with excellent temporal and spatial resolution provided by electrophysiological recordings of neuronal activity. We have also previously reported essential findings that functional activity of sensory neurones *in vivo* is lost when these neurones are excised and studied in culture *in vitro* - this is an important example illustrating why pain studies in the intact nervous system are essential to determine neuronal excitability to noxious stimuli.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Using our previous Home Office returns as an indication of how many animals are used for each protocol, as well as having more than 5 colonies that we maintain. Moreover, for our experimental studies we use online tools such as Gpower and the NC3Rs research design assistant to plan experiments and estimate required sample sizes for our studies with appropriate power. Our expertise with animal models allows us to perform accurate power calculations to determine the minimum number of animals necessary to achieve a statistical power of 80%, generally using a significance level of 5%, in accordance with ARRIVE guidelines using our own previous data to derive variability and expected effect size.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We routinely use online tools such as Gpower and the NC3Rs research design assistant to plan experiments and estimate required sample sizes for our studies with

appropriate power. This is usually a necessity for all our funding application too. Our expertise with animal models allows us to perform accurate power calculations to determine the minimum number of animals necessary to achieve a statistical power of 80%, generally using a significance level of 5%, in accordance with ARRIVE guidelines using our own previous data to derive variability and expected effect size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For experimental design of studies reduce the number of animals by using (1) factorial designs will be used where relevant to maximise the information acquired from the minimum number of animals. (2) Multiple validated readouts acquired at repeated time points; this approach increases the statistical power, such as behavioural readouts or in vivo imaging at multiple timepoints. The use of longitudinal measurements at multiple time points avoids in many cases the need to set up multiple groups of mice to be killed at different time points. We also acquire nervous and musculoskeletal tissues from the same mice to reduce the number of animals used for studies where we can share across projects in the lab where possible, but also as internal controls.

For breeding we use the following considerations to reduce the number of animals used where possible: (1) We will optimise our breeding strategies to produce minimal numbers of animals. For example, we will breed homozygous floxed mice with homozygous floxed mice that express one copy of a Cre-recombinase, so that all the progeny of the mating will either be experimental animals (i.e. genes will be deleted) or appropriate controls (i.e. floxed genes will be present but not deleted). It is important to emphasise that gene deletion studies are infinitely more reliable and reproducible than gene knock-down studies using antisense oligonucleotide or siRNA approaches. (2) Using inbred strains allows for much smaller variability and we can therefore to achieve a higher statistical power and smaller experimental groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our project uses mouse models of pain and transgenic mice as they provide the best potential to explore the role of specific genes and mechanisms involved in pain. It is possible to generate transgenic mice more easily than other species because totipotent embryonal stem cells from mice can be maintained in culture, and widely available technology for production of transgenic mice allows our results to be

comparable with the wider community of pain researchers that also use similar models. In our studies, animal suffering will be limited by strict monitoring with reference to severity limits of associated protocols and welfare scoring sheets that we maintain in our records. Our protocols have been carefully designed to minimise trauma and suffering. We have chosen different pain states to study in this licence, as it is clear from prior research that a single pathway does not exist for all types of pain. Thus we can use different pain models to reach our objectives of identifying distinct pain pathways. Integral to the different pain models is the surgical or chemical induction of pain, where e.g. the use of post-operative analgesia would interfere with the full development of chronic pain and thereby confound results. The stress response following procedures is also influenced by nociceptive drive in the peripheral and central nervous systems, and so the use of analgesics or stress-modulating compounds to address this would also affect the full expression of chronic pain.

Why can't you use animals that are less sentient?

Several decades of research have demonstrated that rodents and humans share evolutionarily conserved neuronal pathways for pain. All analgesics that work in humans also produce pain relief in mice. Pain is a complex sensory and affective experience that require cortical and top down modulation, which is not present in less sentient beings. Although mice at embryonic stages do not have a fully developed cortex and so are not thought to experience pain (but may have capacity to elicit nocifensive responses that require subcortical processing), there is also growing evidence that the developing nervous system is starkly different to the adult nervous system, requiring touch dependent maturation of nociceptors in the early life stage. There is no evidence that less sentient animals have developed cortical networks to consciously experience pain.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The welfare of animals essential and we will ensure to take the following measures to reduce suffering and distress alongside regular monitoring of animal welfare: Sufficient nesting material to keep comfortable, cushion sore joints or limbs and enable thermoregulation.

An appropriate group of cage-mates for social animals, depending on age, sex and strain.

One or more refuges to permit natural behaviour and enrichment materials to alleviate potential anxiety in animals with compromised mobility.

Effortless access to food and water.

Animals will be culled immediately after the last time point of testing or as soon as social behaviour, grooming, weight loss/gain and/or wound healing indicate the animal is suffering according to severity monitoring sheets.

What published best practice guidance will you follow to ensure experiments

are conducted in the most refined way?

We will continue to adhere to NC3R, PREPARE, EDA-NC3Rs, BPS (British Pharmacological Society) and ARRIVE 2.0 guidelines for all our experimental design and reporting in order to ensure that all experiments are conducted in the most refined manner and with most scientific impact. As part of experimental design we ensure the experimenter is blinded to intervention/genotype from testing to data entry until data analysis is performed.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continue to work closely with our colleagues at the Biological Services Unit and our veterinary surgeon to keep updated on guidelines for animal research and advances in the National Centre for the Replacement, Refinement and Reduction of Animals in Research. Our institution regularly hosts events, through our veterinary surgeon convening researchers involved with animal studies to discuss approaches to achieve 3Rs. For example, the licence holder is a member of various scientific training schemes that support seminars and annual events for trainees to discuss approaches and obstacles in animal research.

41. Production of Surgical Animal Models

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Vascular catheterisation, Gonadectomies, Surgical Models, Surgical Services, Biomedical research

Animal types	Life stages
Mice	adult
Rats	adult
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The direct goal of this project is to produce high-quality surgically prepared animal models (various cannulations, catheters and gonadectomies). These animal models will be used for biomedical research that is important for the health and welfare of humans or animals or for the environment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit -

these could be short-term benefits within 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 scientific interest of this project proposal is to introduce as little variation between animals as possible, so that fewer animals are needed to generate reliable results and the results are more reproducible. We anticipate this will help reduce animal numbers. Experienced surgeons should have fewer complications and less bruising and inflammation which should provide a refinement for the animals.

In addition, we are able to work across multiple research fields which may not occur within a traditional research setting, allowing us to produce surgically altered animals to a very high standard.

What outputs do you think you will see at the end of this project?

The surgically altered animals produced will be used in a variety of models. The high-quality surgical model outputs will be used in studies required by regulators, and other studies supporting this aim. This will help progress drugs into clinical trials, or prove the chemicals produced will be safe to be used by the public. The results from these models will be used as source of information in peer reviewed publications within the scientific community.

It is possible that any surgical model refinements that come out of this project may be published and shared with the wider scientific community, to enable better surgical techniques/aftercare to be performed and thus better animal welfare. Animals produced within this licence will contribute to further scientific benefits across other licences.

Who or what will benefit from these outputs, and how?

The scientific interest of this project proposal is to introduce as little variation between animals as possible, so that fewer animals are needed to generate reliable results and the results are more reproducible. Because our surgeons are well trained in the production of the surgical animal models, these models will be produced to a high, uniformed standard.

The social importance of this project proposal is as follows; because these surgical animal models are produced by surgeons who perform these procedures frequently, fewer animals are needed and the animal harms will be reduced, compared to if these animals were produced within an institute where the surgeons are less experienced in a surgical procedure or where the specialised facilities are not (sufficiently) present.

There is a need for high-quality, standardised produced surgical models for research. This contributes to reliable and reproducible results.

How will you look to maximise the outputs of this work?

The surgical models produced will be used in studies that will inform clients who will,

in turn, use it to determine their future strategy, or for submission in documents required by regulatory authorities.

Surgical models will be used by our clients in studies to support drugs progressing to clinical trials, or to show that a certain chemical is safe for human exposure, for example.

We will maximise outputs through collaborations with our customers for the best surgical outcomes. We will be able to monitor our outcomes with our clients to ensure that we are able to meet their needs and how we can minimise the number of animals used. Through these collaborations we will be able to share data in the form of scientific publications that will be in the public domain.

We will remain abreast of new developments through journals, attendance at meetings and will introduce refinements to existing models as information becomes available.

We will follow NC3R's guidelines throughout (National Centre for Replacement, Refinement and Reduction of Animals in Research).

Species and numbers of animals expected to be used

- Mice: 2950
- Rats: 5220

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodent models are widely used in research in the development of novel compounds and are increasingly important in the study of human diseases. Adult animals will be used in this Project Licence as studies performed after the surgical modification will require the use of adults.

Typically, what will be done to an animal used in your project?

Specific Pathogen Free (SPF) or Specific Opportunistic Free (S(O)PF) animals are transported from the barriers to the surgical unit for acclimatization at least 7 days before the start of the operation to allow recovery from any stress caused by transport. Before the start of the operation, the animals are checked again for physical abnormalities/general health status.

Animals will be surgically prepared under a general anaesthetic. These surgical preparations will differ depending on the specifics of the study the animal will be subsequently used in. Surgeries may include the removal of the reproductive organs

or the implantation of a cannula or catheters.
Prior to surgery, animals will be administered pain relief prior to being anaesthetised.

Animals maybe anaesthetised using injectable anaesthetics or through a gaseous set up. After the surgery, all animals will be given appropriate analgesics as advised by the NVS. Some surgeries may require the use of antibiotics. However, these will only be given under the guidance of the NVS and or a deputy that can advise on the NVS's behalf.

Animals will be allowed to recover in a designated recovery room. The lighting and temperature will be controlled to aid recovery. All animals will be monitored regularly until they show signs of recovery.

Veterinary guidance will be sought when planning the analgesics required for pain relief.

Once the animals have recovered from the surgery and have passed all health and body condition checks they may be transferred to other projects.

What are the expected impacts and/or adverse effects for the animals during your project?

Surgical procedures in rodents can result in some adverse effects such as bleeding/blood loss, post- operative pain and weight loss. Most animals are expected to have recovered their pre surgical bodyweight fully or partly within 72h of surgery ending. Bleeding is minimised with good surgical technique by an experienced surgeon. Post-operative pain is reduced by the administration of pre- and post-surgical pain relief.

Once animals have recovered sufficiently from their surgical procedures, animals may be transferred to another licence normally 7 days after surgery. During this time the animals will have received their post operative medications, and the surgical site will have healed sufficiently.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals will undergo a surgical procedure. We expect 100% of Rats and Mice used in this project licence to be classified within the moderate severity limit.

What will happen to animals at the end of this project?

- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

Surgically altered animals have physical transformations (e.g., implants of catheters/devices or removal of tissues) which can currently not be achieved by chemical or other means.

In the UK before carrying out surgical procedures we will assure ourselves that the appropriate authority exists for the continued use of these animals. Records will be maintained of transfer between licences.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives to these surgical models. Surgical models cannot be modelled in- vitro or through other non-animal models.

Why were they not suitable?

Surgical models maybe required by regulators after non animal alternatives have identified issues with drug compounds or chemicals which means these models must be tested in an animal model. Animals may be required to test novel compounds to prove that they cause no harm to human health prior to clinical trials. Testing these models are required by law.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers we have used are based on figures of previous usage over the past 5 years under an alternative authority used over 2019-2023, or a projection thereof (based on estimated incidence) based on requests received in the past.

For the most realistic estimate we would use the number of animals from the year in which the procedure to the respective animal species was maximum.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Surgically altered animals will only be supplied to specific numbers and orders, high level of aseptic techniques will also ensure the highest standards of surgery are carried out, minimising losses.

As animals surgically altered under this licence are supplied to a specific request, the principles of experimental design will have already been addressed by the receiving AWERB (local ethical review process).
The numbers of animals produced to fulfil the order and to achieve the scientific aims will be kept to a minimum. The number of animals surgically prepared will aim to ensure that the study can be completed, allowing for any post operative issues such as patency issues (i.e., blocked catheters) and other issues that might prevent dosing or sampling.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Without compromise, all surgeries will be conducted under strict aseptic techniques that will exceed the minimum standard set out by the Home Office.

Good Surgical Practice will be observed for all surgical procedures. Surgical procedures will be carried out in accordance with the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017).

An appropriate anaesthetic and analgesic regime will be determined in consultation with the Named Veterinary Surgeon (NVS) prior to the start of surgery. Animals will be given heat supplementation during surgery and recovery and will be monitored closely until the animals display appropriate behaviours. Animals will be checked at least twice daily before they are shipped to our client. In addition, care is taken to provide as much environmental enrichment as possible. Environmental enrichment may include but not be limited to plastic shelters, blocks to gnaw on, extra bedding for warmth and any supplementary foods (mash or recovery gel packs) after surgery.

Long spouted water bottles or easy to access hydration packs maybe used during the recovery period. Easy access to hydration is especially important for animals that have undergone abdominal surgery.

All surgeons will be trained by an experienced surgeon who is already competent to assess a specific technique. Training records will be regularly updated and will show competencies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The species and model of animal used under this project licence is research demand

driven and authorised in the receiving project licence. Surgically altered rodents will only be produced to order for supply to establishments in the UK.

The choice of surgery technique (method) will always be aligned with good surgical practice for example, where possible our first option will be to use the scrotal route when performing vasectomies and we will only use the abdominal route when the age or size of animal requires this method.

Materials will be chosen which allows the animals to be as mobile as possible, high standards of enrichment will be provided and the animals will be group housed wherever possible.

In order to minimise animal suffering, we will maintain the most up to date choice of both pre and post operative analgesia combined with the most suitable choice of anaesthesia and other appropriate perioperative care.

Any major changes/refinements to surgical technique or pain relief/anaesthesia protocols will be peer reviewed and discussed and carried out by the NVS and AWERB when applicable. The animal technicians and surgical technicians will be in regular discussion. Group discussions with the NVS and NACWO will ensure that best practice is followed and will ensure that there is no issue from the perceived conflict of interest with the Project Licence holder who will be the lead surgeon within the facility.

Why can’t you use animals that are less sentient?

Rodents (rats and mice) will be used in all of the studies conducted under this licence.

Rodents are commonly used in research as they are considered to be the species of the lowest neurophysiological sensitivity (have a brain and physiology similar to humans) that will allow us to achieve the study aims and are considered suitable for predicting what is likely to happen in humans. Using these species will allow us to achieve the objectives and aims of this project licence. The models used are considered suitable for predicting the likely effects to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All Surgical procedures will be conducted using the highest standards of aseptic techniques set out in the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017). These standards ensure that animal welfare is minimally compromised. In addition to aseptic techniques, all surgeries will be conducted under the guidance of Good Surgical Practices.

Before the commencement of any surgical procedures we will assure ourselves that the appropriate authority exists for the specific use of the surgically altered animals by requesting the details of the respective Project Licence(s) and justification for each request. The justification will be reviewed by the Animal Welfare Ethical Review Board (AWERB).

In agreement with the NVS we will provide an appropriate anaesthetic and analgesic regime suitable for the procedure performed. Post operative care will include adequate analgesics, extra warming and all animals will be monitored closely until recovery and normal behaviours are observed. Animals will be closely monitored for signs of pain and discomfort and all observations will be recorded using appropriate monitoring proforma. The use of appropriate pain and monitoring checklists will ensure that monitoring is consistent. All technicians will be trained and be familiar with the adverse effects noted in this licence. We will strive to group house all animals where possible. In the event that animals are singly housed we will ensure that the animal is provided with as much environmental enrichment as possible.

Additional enrichment may be added if needed.

Individual housing may be necessary for example, an externalisation of the cannula / catheter by means of a button without a protection cap, pin port or harness (danger of damage / eating by cage- mate) or when joining is not possible, such as in the case of certain aggression-sensitive strains where there is a risk of fighting.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. A report by the LASA Education, Training and Ethics section. (E Lilley and M. Berdoy eds.).
Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Procedures. (2014) London: Her Majesty's Stationary Office.
Home Office Minimum Standards for Aseptic Surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This will be achieved by regular discussions with our Named Information Officer, Named Training and Competency Officer and other colleagues in Animal Technology, and by attending appropriate training courses and conferences.

We will keep abreast of developments concerning surgical techniques with emphasis on asepsis, post operative care, and the use of analgesia and anaesthesia.

42. Zebrafish models of inherited neurological diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Inherited neurological disease, Brain, Muscle, Disease mechanism, Treatment

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our overall aim is to examine in zebrafish animal models how some changes identified in the DNA of patients lead to the development of some rare neurological diseases affecting the brain, peripheral nerves and skeletal muscle.
In addition to investigating the disease mechanism of these rare inherited neurological conditions, we will also study the effect of potentially beneficial treatments in our zebrafish models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We study some rare childhood onset severe inherited neurological diseases, called

mitochondrial disease. Mitochondrial diseases affect 1 in 5000 people, they affect the brain, skeletal muscle, peripheral nerves, heart and liver, leading to severe disability or death in children. In these diseases the brain, the nerve cells and the muscles don't function properly, and patients have difficulties with movement and coordination. The cause of the diseases lies in the DNA of the patients, which is the material in the cells of all living things that contains the information for their characteristics and appearance. These diseases are currently incurable and have a devastating impact on patients and their families as well as requiring substantial resources from the national health service. The lifetime treatment cost for a patient with serious mitochondrial disease is around £1.3M (NHS, 2016).

In our laboratory we use human cellular models (cells from patients) to study these rare neurological conditions, however, for understanding how the gene affects the whole organism (a person) we need animal models (an animal that represents the patients` condition) as we currently cannot fully recreate a representative biological system using only human cells. Further, it is currently required by regulatory agencies to trial the effect of treatments in animal models before giving them to patients.

What outputs do you think you will see at the end of this project?

Our aim is to better understand the disease mechanisms in fish, which will help us to define which treatment can be beneficial. This informatiion will contribute to the development of new and improved treatments for patients in the long term. We also aim to test drugs for their ability to correct defects in fish, which will inform drug development programs for patients with mitochodnrial disease. Our aim is to provide better and effective treatments for patients with mitochondrial disease. We also plan to publish our results in scientific journals, so that other researchers can learn about our findings to help move treatments closer to reaching patients.

Who or what will benefit from these outputs, and how?

The major short term benefit of this research is to the scientific community by progressing our understanding of how changes in specific parts of the DNA lead to a neurological disease. For example, if we can show that the same mutation (change in the DNA sequence) in a gene of a patient also results in zebrafish showing similar disease characteristics, we can use this information to indicate that mutations in this gene are responsible for the patients disease. When we recreate mutations in fish, we observe if they have a muscle weakness or other typical symptoms seen in the patients. This helps link the patients disease to a genetic cause.

Longer term benefits will be that our research will generate new and better treatments for patients with mitochondrial disease. The information will guide clinicians treating patients and will be also useful for scientists in the field. This will result in improved diagnosis and treatments of patients. For example, it has been indicated that in patients with certain mutations that lead to lower amounts of mitochondrial DNA (the DNA inside mitochondria) may respond to treatment with nucleosides (DNA building blocks). If we can show that the fish with the same mutations respond to this treatment, this may inform the clinicians treating patients that this is an effective treatment.

How will you look to maximise the outputs of this work?

We will publish our data in open access (free for anyone to read), peer reviewed (reviewed by other reputable scientists) journals and present at national and international scientific conferences. We widely collaborate with clinicians and scientists world-wide on this research to increase the success of our work and also to enable very specific experiments in collaboration with our partners.

We try to maximize the success of our research by following the most advanced technologies. In the event some approaches are unsuccessful, we have the knowledge and experience to troubleshoot, or use alternative model systems. In our lab we also work with human cellular models.

In the cases that we have negative results or unsuccessful approaches to the research, we aim to make this information known to the scientific and wider community. This can be done via communications in group meetings/conferences or in publications, making these important negative results available to as wide an audience as possible.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 25500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Muscle, brain, heart and eye are the organs affected by these neurological diseases early in life. These are complex organs made up of multiple cell types, which means that cell culture models that generally consist of a single cell type, have limited applicability to patients. Therefore, many pre-clinical studies (research performed before treatments reach testing in patients) can realistically only be achieved in whole animals. Zebrafish are a good model system as they share over 80% of human disease genes, meaning there is a high chance that we can study the same human disease gene in zebrafish. While zebrafish are not mammals, they are vertebrates, sharing many of the organ systems and tissues we are interested in studying. In addition, the small size of larval zebrafish allows us to perform high throughput analysis (the ability to study lots of samples in a short timeframe), which allows for statistically robust data, something which is more challenging to achieve in higher organisms.

We plan to generate genetically modified zebrafish carrying the same changes in the DNA as identified in patients and investigate how these changes impact the development and function of the different organs. Primarily, we plan to do most of the research on zebrafish less than 5 days post fertilisation (dpf), as most of the

organs of interest are developed by this stage. However, based on our recent data, sometimes we need to work on slightly older, but less than 14 dpf fish to study the effect of some treatments. This is due to how the diseases manifest, as they only start to show signs of disease after all the cells have finished rapid development, which is usually around 5 – 7 dpf. So to fully understand how the mutations affect the fish, sometimes we need to study them up to 14 dpf.

Typically, what will be done to an animal used in your project?

When drug tests are performed on zebrafish, the substances are administered by dissolving them in the tank water for up to a maximum of 3 months but more typically for 14 days, which is naturally ingested. No invasive methods are required, minimizing the stress for the animals during the experiments. This is one of the big advantages of using zebrafish to test potential drug treatments. Most animals will be humanely killed at the end of the experiments except those required for breeding who will be expected to be only mildly affected, if at all.

The only non-experimental surgical procedures performed on zebrafish would be the caudal fin biopsy (taking a small amount of the tail to determine if a fish has a mutation). This is a routine procedure that is well established, although there are also options to perform alternative, non-surgical, methods (swabbing) which we may explore.

What are the expected impacts and/or adverse effects for the animals during your project?

The experiments that we undertake involve the use of animals as models for human neurological diseases and so the effects on the animals in part reflect those diseases. However, we will limit the effects on the animals to the first 5 days of life (embryonic and early larval period) wherever possible. During these first 5 days, zebrafish are believed to have less capacity to experience suffering and are not considered protected animals by the Home Office legislation.

Some of our zebrafish models failed to inflate their swim bladder (an air filled organ, which helps the fish to maintain their position in the water), which may be associated with different genetic defects. This defect prevents the animals from normal swimming behaviour however, this does not cause the animals any additional suffering. It is necessary to keep the fish longer than 5 days in some of the experiments we do to determine the full consequence of mutations and effect of treatments. When treating fish longer than 5 days, we will need to withdraw feeding up to 14 days, which has been shown to not cause any additional harm to the animals, as has been previously shown by our colleagues. We will not perform food withdrawal beyond 14 days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Zebrafish: Mild 100%
Moderate 0%
Severe 0%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Muscle, brain, heart and eye are the organs affected by the diseases we are studying; all of these are very complex organs made up of multiple cell types. This means that cell culture models, which generally consist of a single cell type, have limited applicability to patients. Therefore, many pre-clinical studies can realistically only be achieved in whole animals.

Which non-animal alternatives did you consider for use in this project?

Where possible, we also use primary cells derived from patients in parallel to investigate aspects of the diseases. In the last few years we have started to utilise stem cell derived organoids, particularly brain organoids, to investigate the diseases of interest. These allow us to go beyond simple primary 2D cell monocultures and investigate how mutations affect neurodevelopment and neuronal function in 3D models that more accurately represent the human brain. We also plan to start trialing therapies in these organoid models. Utilization of organoids has resulted in less reliance on animal models and they now make up the large majority of the research we do.

In addition, if muscle samples become available from patients, we will use this tissue instead of animals to study the consequences of the disease in this tissue. Tissue samples from patients will also be archived for future use to replace animal experiments. We also considered using invertebrates including fruit flies and nematode worms, as their use is widespread throughout science for certain applications.

Why were they not suitable?

The detailed structures of interest for us are too different in invertebrate species such as worms and flies, especially the contact sites between nerve and muscles (called neuromuscular junctions) which are of particular interest for the diseases studied by our group.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated our animal numbers from our previous experience, generating mutant zebrafish models and trialling compounds. Our experience has now shown us that we may need to focus on juveniles up to 14 dpf, which means we have to increase numbers slightly to accommodate working with more over 5 dpf.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We reduce animal numbers wherever possible by reviewing our experimental data rapidly following an experiment and planning follow up experiments to resolve outstanding experimental questions. In this way the information generated by our research is maximized while experimental animal use is minimized.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use the PREPARE and ARRIVE guidelines recommended by the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) for designing our experiments and publishing our results.

Based on our previous work and if needed pilot studies we will minimize the number of fish used in the project. In addition, for lines we are not currently using, we have started to freeze sperm for future in- vitro fertilisation (IVF) if required. This allows us to reduce our animal numbers by approximately 100 animals per line. Previously these lines would have been maintained in the system.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 protocols on this license are designed to minimize any effects on zebrafish after 5 days of development, where the animals have more substantial capacity for suffering. We aim to restrict any harmful effects where at all possible to the early

stages before 5 days where we can, closely monitor the health of embryos and humanely kill severely affected individuals before they develop into hatchlings.
In instances where we use fish up to 14 days of age or adults, we will monitor the animals regularly for any abnormal behaviour (hyper/hypoactivity, trouble swimming) or morphological defects (differences in the way they look). If we detect any such abnormalities, which is not due to the modified genetic environment, the experiment will be terminated and we will inform the Named Animal Care and Welfare Officer (NACWO).

Why can't you use animals that are less sentient?

Muscle, brain, heart and eye are the organs affected by the diseases we are studying; all of these are very complex organs made up of multiple cell types. This means that cell culture models, which generally consist of a single cell type, have limited applicability to patients. Therefore, many pre-clinical studies can realistically only be achieved in whole animals. The detailed structures of interest for us are too different in invertebrate species such as worms and flies, especially the contact sites between nerve and muscles (called neuromuscular junctions) which are of particular interest for the diseases studied by our group.

We previously focused mainly on larvae up to 5 dpf, but our recent work has informed us that working with fish up to 14 dpf will be necessary due to how some of the diseases we study manifest (after rapid development). Despite this, we will still aim to focus our studies, where possible, on larvae 5 dpf or less.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In our zebrafish facility, the environmental conditions such as water temperature, food and lighting are strictly monitored according to Home Office guidelines to ensure the health of the animals. All fish are inspected daily and obtain daily live prey (brine shrimp) feeding to allow them to express natural feeding behaviours.

Together with the animal technicians in our facility we also test new environmental enrichment options for the fish tanks and use them if they are compatible with routine husbandry and tank cleaning procedures. As zebrafish are social animals, single housing of individual fish during experiments will be kept to an absolute minimum.

When the fish are moved to new tanks or put together with other unfamiliar individuals, we will allow sufficient time for them to adapt to the new environment before starting the experiments. Handling of the animals by using a net is kept to a minimum to avoid unnecessary stress and damage to the animals.

Analgesic treatment (pain killers) is not routinely given after caudal fin biopsy, unless the fish is displaying abnormal behaviour, in which case we would seek advice from the named veterinary surgeon (NVS) about applying analgesics.

Animals will be regularly monitored after caudal fin biopsy by a technician.

As an alternative to fin clipping for genotyping, skin swabbing has been shown to be

effective at collecting DNA, while reducing the welfare impact seen with fin clipping.

We currently have no practical experience in this technique, however this would be something that we would like to explore and will discuss with the facility NACWO.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow PREPARE and ARRIVE guidelines recommended by the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) for designing our experiments and publishing our results.

We will also follow the newest guidelines provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R).

We also follow new research on animal studies and will use the most efficient and humane methods available.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will always follow the newest guidelines and information provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R). In addition, online resources including the RSPCA's 'focus on fish' online event (which can be accessed anytime: https://focusonfish.co.uk/) and the zebrafish section of the Norecopa website (https://norecopa.no/species/fish/) also provide valuable information to stay informed about advances in the 3Rs.

In addition, we regularly communicate with the NACWO and other members of the aquatic facility team, who often inform us on best practices working with zebrafish.

We also follow new published research on animal studies and will use the most efficient and humane methods available.

43. Investigating the role of redox signalling in endothelial dysfunction and cardiovascular disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Redox, cardiac, Vascular, female, endothelial

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The research project aims to understand how changes in the balance of reactive oxygen species (ROS) and antioxidants, collectively known as redox balance, influence biological messaging in cardiovascular cells. Reactive oxygen species are highly reactive molecules generated in all cells and have the potential to cause damage to body organs. Antioxidants, on the other hand, help to neutralize these harmful molecules.

However, at low levels reactive oxygen species are able to cause changes in other proteins in the body and play an important part of normal physiological responses.

In the context of cardiovascular disease, an imbalance in redox communication within the cell and the body has been implicated in various cardiovascular diseases, such as atherosclerosis, heart failure, and hypertension.
Obesity, diabetes and age all contribute to imbalance in redox signalling

Understanding the role of redox communication pathways in cardiovascular disease could potentially lead to the development of targeted interventions and treatments to improve heart health and reduce the burden of cardiovascular conditions.

The main objective is to identify proteins and biological pathways which are modified by redox signalling pathways. Then to try and understand the consequence of redox modification on cardiovascular disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cardiovascular disease is one of the main reasons people die. People with cardiovascular disease find it difficult to work and there is a significant social and economic impact.

It is becoming more common for people to have diabetes or obesity, and the general population is becoming older, all of which contribute to higher levels of cardiovascular disease. Cardiovascular disease is becoming more common in women therefore, understanding the gender differences and similarities in the biological messaging which cause cardiovascular disease is critically important. Many treatments for cardiovascular disease are used to prevent progression of the disease. These are just buying time rather than preventing or reversing the damage.

We need to identify ways to reverse the detrimental effects and/or stop cardiovascular disease earlier.

What outputs do you think you will see at the end of this project?

We expect to:

- publish several research articles about our investigations into redox signalling pathways and the impact on the cardiovascular system in health and disease.

- provide foundation studies in how redox signalling is involved in stresses such as obesity, diabetes, western diet in cardiovascular disease.

- identify biological pathways that underpin cardiac failure or peripheral artery disease which can be further explored or utilised as biomarkers (e.g. measurement of a protein in urine or blood that can predict the development of onset or likelihood of developing disease) in human cardiovascular disease

- link physiological data with large molecular (gene and protein) data to characterise the development of cardiovascular disease such as heart failure or peripheral artery disease in both male and female genders.

Who or what will benefit from these outputs, and how?

The main output of this project is to publish several research articles about our discoveries in redox signalling in cardiovascular disease in different genders.

We expect our research of cardiovascular physiology coupled to molecular signalling pathways will advance our understanding in how, when and why disruption in redox signalling in the cardiovascular system impacts on cardiovascular disease leading to heart failure, hypertension or peripheral artery disease. Our findings will be relevant to clinicians and our collaborators running clinical trials since our in vivo (in body) physiological measurements will match clinical measurements. Our findings with molecular pathways will help identify clinical biomarkers that can be confirmed in human studies.

We will present our findings at international science conferences.

We will engage with patient group, specialised key workers (e.g. echocardiographers) and public to share our discovery.

Our findings will help shape in vitro replacement models that do not use animals such as 3D organ on a chip models.

A major training output will provide early-stage researchers to engaged and be trained to carry out in vivo physiological techniques.

How will you look to maximise the outputs of this work?

A number of scientists in different stages of their career will benefit from working on this project this includes, undergraduate biomedical and medical students, people studying for their PhD or those in the stages before lecturers. They will develop specialised techniques under my instruction. These people involved with the project will be involved in publishing and talking about the research.

The scientific outputs will benefit researchers including clinical and basic scientists interested in maternal and foetal medicine. We will develop and publicly share data sets from our work. This will help other researchers look at their area of interest in our datasets.

In the medium-term outputs will benefit human studies. Our potential research on impact of obesity will inform policy makers and public health assessment. A wider scope of researchers could also benefit for example those researchers that are conducting field research into obesity and advise the molecular signals to measure the impact.

In the long-term identification of drug targets that are crucial in heart failure or peripheral artery disease can be used to design future therapy. Moreover, the development of techniques that can test the new therapy that are closely matched with the clinical situation will allow for efficient drug discovery.

Species and numbers of animals expected to be used.

- Mice: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures. Explain why you are using these types of animals and your choice of life stages.

We aim to study how redox signalling contributes to cardiovascular disease using adult mouse models.

The mouse is a good model as we can manipulate the genetics to control the expression of one protein, either removing or increasing the expression. We can do this in certain cell types and alter the expression in a time dependent fashion. In some cases, we can modify a small part of a protein to discover which part of the protein is important for its function. This control of the genes allows scientists to determine the importance of a protein in a particular disease setting.

We also have cutting-edge equipment that can make accurate measurements of the mice very similar to the measurements in hospitals of patients. This includes a high-tech ultrasound imaging that can see inside the body of the mouse. A mouse heart is about the size of a human little-finger nail. The ultrasound allows us to image the moving heart and even measure the thickness of the heart wall (10th of a mm). This allows us to match the clinical signs in the mice with the same clinical signs in a human.

Typically, what will be done to an animal used in your project?

Where possible and appropriate we will investigate both genders and have controlled mice of similar age.

Modelling peripheral artery disease
Peripheral artery disease will be modelled by surgically removing a small section of blood vessel in one leg.
Sub Non-invasive phenotyping will take place which includes
Non-invasive laser imaging will be able to measure the amount of blood perfusion /flow in the paws in a live animal. The device can be held on the skin, the mouse may be briefly anaesthetised to allow the paw to be held still for the duration of the measurement. By monitoring blood perfusion on a regular basis we can measure how quickly the peripheral artery disease recovers.

Modelling heart disease
Early stages of heart failure will be modelled by, either.
slightly constricting the aorta to cause the heart to work harder
implanting an osmotic pump under the skin that slowly releases a drug that induces increase in blood pressure.
To model obesity we will feed the mice a westernised diet (high fat high sucrose).

Followed by monitoring changes in physiology:

The changes in blood pressure will be monitored by tail-cuff blood pressure measurements or by implantation of radio devices that measure blood pressure continuously by sending the data to a receiver outside the cage.
Changes in blood vessels or heart shape or function will be monitored using ultrasound that is non- invasive.
Fat content will be monitored using a non-invasive technique called echoMRI.
Under terminal anaesthesia a catheter will be inserted into the heart to provide highly accurate heart and vascular pressure and volume measurements.
The number of non-invasive anaesthesia procedures conducted on a single mouse will be limited (<20) to ensure no cumulative adverse effects.
All conditions will be carefully controlled. Our monitoring techniques match clinical measurements to be able to link with humans. Subsequently, organ and blood samples will be intensively studied to provide molecular and biochemical signals.
We will use genetically modified mice with the techniques above to be able understand the role of a particular pathway in disease development.

What are the expected impacts and/or adverse effects for the animals during your project?

Surgically removing a blood vessel in the leg to mimic peripheral artery disease causes transient lameness which usually resolved in days. In most cases the blood flow returns to the limb in a week with no adverse effects.

Surgically constricting the aorta and or treatment with hypertensive drug, initially changes blood pressure and causes heart to work harder. Over a longer period 6-8 weeks the heart will start to transition into failure. However, we are interested in the early stages and the experiments are usually stopped before this point.

In rare cases mice may randomly and rapidly die due to aortic aneurisms caused by the increase in blood pressure.

Mice fed a westernised diet gain weight but do not show any abnormal behaviour.

In some cases, we will implant small devices (telemeters) into the blood vessel of the mouse, which radio's the readings back to a receiver plate. This is used to take very-accurate blood pressure measurements when the mouse is free to move around its cage. There is a risk that blood clots can form after this procedure causing stroke or paralysis.

The non-invasive measurements often require hair removal for imaging which sometimes induces irritation of the skin. Cream and ultrasound gel will be applied to provide moisturising as a preventive measure.

To accurately administer drugs we may implant a small pellet size pump. This can give very accurate doses but requires quick surgery while the pellet size pump is placed in a pocket made under the skin. In some cases, the pump rubs if the pocket is too tight and can cause the skin to break down. The advantage of pumps are that the mice do not have to go through a daily procedure like injection or gavage, which can be stressful.
For surgical procedures, animals will be provided with pain killers as required and

recommended by the NVS to minimise the harms.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

70% of animals will be under moderate severity

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To understand the complexity of cardiovascular disease we sometimes need to study the whole system. Many organs including kidney, brain, heart and blood vessels contribute to cardiovascular disease. Likewise different cell types are also involved in the complex cardiovascular system including the lining of blood vessels (endothelial cells), heart cells, inflammatory cells.

The heart functions as a pump and depends on the blood vessels bringing the blood to the system. Focusing on just the heart would be like a plumber looking at just the boiler without it connected to the radiators and piping system.

At present there are no lab-based models that can replicate the heart and vessels completely.

Which non-animal alternatives did you consider for use in this project?

3D multicellular in vitro organ on a chip models Human studies
In silico models

Why were they not suitable?

Organ on a-chip models have recently advanced lab based experiments by mimicking the organ function outside the body. Organ-on-a-chip have been characterised to mimic some of the functions of the vessel in a 3D system using human endothelial cells. However, these lab based experiments are unable to measure blood flow, vessel constriction and contain all of the different cell types. The 3D heart is not at a stage that can replace a functional heart in a lab base setting.

Human studies were considered. Human studies are limited to observational studies requiring large numbers to be able to control for certain conditions (age, ethnicity). It

is not always possible (or ethical) to conduct extensive physiological measurements on humans in a controlled fashion.

In silico (computer simulations) models can provide an alternative, however requires detailed physiological and molecular pathways to be inputted. Our research will be made available that will help the accumulate of data on the molecular pathways in the vessels for future computer simulations.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the numbers of animals based on our extensive prior experience studying cardiovascular disease using mouse models. In addition, we have information from prior studies and technical experience that help inform the amount of mice needed.

For the majority of the techniques the information from prior studies provides baseline measurements enabling us to plan the study and predict the appropriate number of mice that would enable us to test a hypothesis, understanding that we are expecting to see a change that would be clinically important.

This will minimise the number of mice needed for a study.

In studies that we do not have prior information we will either perform a small pilot study in a small number of mice to get an understanding of the numbers that would be required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

NC3Rs experimental design assistant is used to calculate the numbers, providing accurate power analysis, graphical representation of the study and defining the optimal control conditions. This ensures that we use the correct number of animals to get reliable results for any study.

Where possible the in vitro replacement model (placenta on a chip) will be used to assesses molecular pathways before conducting animal studies.

Non-invasive imaging will be used to allow sequential measurements in the same mouse. This makes the results more reliable because we can follow changes over time in individuals and it also means that we can use fewer animals. It also reduces the requirement to cull mice at a range of times points that would need to happen if

we used more invasive measurements.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Efficient breeding strategies will be used to reduce the number of animals bred for the experimental work. We regularly attend national seminars which can advise on best practice.

Statisticians will provide advice on experimental design before the study. Using more complex types of study design, such as random block design and sequential measurements, means that more information can be gained from fewer animals.

Pilot studies and previous data from our group or others in the division will be used in order to carry out power analysis.

In all experiments, once we have humanely killed the animals, a wide range of organs will be carefully collected, logged, and preserved for future use and sharing with other researchers, minimising the need to repeat experiments.

Data from our project will be clearly labelled and stored on data repositories so other scientists worldwide can use the data.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will take measurements in the mice using advanced technology that allows us to take measurements in small animals like what can be done in humans. These techniques e.g. ultrasound are non-invasive and either require the mouse to be restrained or lightly sedated. For example, we can use ultrasound to image the size of the moving mouse heart. This is a refinement as the alternative would be invasively open the mouse or kill the mouse to take measurements of the heart at multiple time points.

We use mouse specific anaesthetic equipment that allows automatic anaesthetic flow rates specific for mouse size.

To provide continuous blood pressure measurement in freely moving mice we will implant a telemeter. Although this involves a one-time surgery, this prevents invasive surgeries at multiple time points.

We will look at emerging technology or techniques to implement more refinement. Metabolic and cardiovascular phenotyping will take place using the most up-to-date technology as feasible. In most of the cases, we will use non-invasive techniques.

Where possible we mice will be restrained to take the measurements or lightly sedated.

The cardiovascular disease modelling such as aortic constriction, artery removal or angiotensin II infusion are regarding by the American Heart Association and European Cardiology Society as the best standardised models. This allows our data to be used by other researchers.

Why can't you use animals that are less sentient?

The mouse is one of the least sentient animals which has the best match for human physiology, with appropriate imaging tools to make similar clinical measurements.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

I will continue to seek funding to improve refinement and replacement of animal models and work with tech-companies to advance these.

We will use new technology (where feasible) such as anaesthetic machine which delivers low flow rates specifically for mice.

Study plans are in place and monitored by the NVS to ensure the use of techniques with minimal severity to enable the best scientific outcome. For new studies, pilot studies are designed to provide the knowledge necessary to be carried out in a larger cohort. Mice that are taking part in a study are regularly monitored and animal resource staff provide a wealth of experience to carefully monitor the mice.
For surgical procedures, animals will be provided with pain killers as required and recommended by the NVS to minimise the harms.

The resource unit regularly introduces new procedures to minimise welfare, such as increasing environment enrichment, handling (removing tailing) and behavioural conditioning.

Ways to conduct basic ultrasound without sedation will be explored.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice guidance for experiments will be acquired from discussions and attendance at webinars/workshops with local and national experts, NC3Rs, local user group, RSPCA, the Research Animal Training online programme, and other appropriate websites.

How will you stay informed about advances in the 3Rs, and implement these

advances effectively, during the project?

Our local animal users' group is kept updated with recent advances and organises a regular webinars and workshops from other research institutes.

We will continue to attend webinars and workshops organised by NC3Rs and RSPCA. We regularly review the NC3Rs monthly newsletter and articles to ensure we are aware of any advances.

I am in contact with sales and scientific representatives from various tech-companies that provide updates on technological advances that can have refinement benefits.

44. The Biological Function of RNA modifications

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Gene Expression, Cancer, T cells, Neurons, Infection

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how genes are regulated in healthy cells and how this alters during disease. Our major focus is understanding how immune cells develop and how they function to fight infection and cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Production of proteins directed by instructions provided in DNA is critical for cell health, development and function. Our research aims to provide new insight into how proteins are produced in healthy immune cells, how this helps immune cells fight infections and cancers, and how this may be damaged in diseases states. Our aim is for this work to provide new ideas and opportunities for the treatment of a range of human diseases and conditions.

What outputs do you think you will see at the end of this project?

At the end of this project, we should have a clear picture of how certain genes are regulated in immune cells and what their role is during development and in response to infection and cancer. We will understand how the different components of immune cell function are managed during an immune response and have some insight into what can be damaged in

immune cell disorders and cancers.
This work is likely to result in a series of discoveries which will be made public in scientific journals and at scientific meetings. Gene expression data and other data will be made public on open-access repositories so that other scientists can use it in their studies.

Who or what will benefit from these outputs, and how?

In the short-term, this research should be useful for the academic scientific community and be useful in drug discovery and development projects. Gene regulation mechanisms function to make protein in all human cells and therefore our research will have direct relevance in immunology, but also have impacts in all tissues. In the long-term, Gene regulation mechanisms are being investigated as therapeutic targets in immune disorders, neurodevelopmental disorders and in cancer. The research planned here is likely to aid drug discovery and development projects.

How will you look to maximise the outputs of this work?

I publicise our work widely so that it can be as useful as possible to the scientific community. I speak regularly at universities and conferences, where I can summarise our work and answer questions. We collaborate with scientists whose expertise is complementary to our own, which helps to get the most value from our work. I speak regularly with people in the pharmaceutical industry, which aids our research and informs their drug development programmes, particularly with target disease area decisions. We generate data from our samples which we deposit on open access databases. Our work is published in open access journals.

Species and numbers of animals expected to be used.

- Mice: 26000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice for our studies. Our focus is to answer questions about human health and disease. As mammals, mice have similar physiology and cellular architecture to humans. What we discover in mice is highly relevant to human biology. Mice are the animal model of choice for the majority of researchers who study health and disease and therefore many tools and much information are available about their physiology during development and in the adult. For the majority of our research, we study the development and function of immune cells, in 8-12 week old mice. This is an age sufficient for the development of immune cells.

Typically, what will be done to an animal used in your project?

The majority of mice will live healthy lives, will not experience any intervention and will be killed by humane methods. The mice will be housed, fed and cared for to consistently high standards by the animal facility team. We will study the cells in these mice to understand regulation of the immune cells. Mice used to breed more mice are typically killed at 6 months of ages, prior to the sporadic onset of age-related disorders. Mice used to understand immune cell function are typically killed at 8-12 weeks of age, when the immune cells have

developed sufficiently to study. A minority of mice (>5%) will receive injections of substances which allows us to see cells and cell functions better in the mouse.

These mice will feel minimal transient pain during the injection. Some mice (approximately 10%) will be infected with strains of bacteria, viruses or tumour cells which are weakened to minimise symptoms of disease. These procedures are expected to result in some symptoms of disease (weight loss, lethargy) and therefore we provide items like cold packs to provide relief from symptoms. We will frequently monitor these animals (daily or several times weekly), looking at their body weights, movement, feeding, and other clinical signs, to observe evidence of distress and humanely kill the animal experiencing unexpected or excessive symptoms. During infections, experiments typically last 2 weeks which allows immune responses to develop and immune cells to respond. Clinical signs of infection will typically last 3 days before subsiding. For mice with tumour cells adverse effects are not expected, and these experiments typically last 1-3 months.

What are the expected impacts and/or adverse effects for the animals during your project?

All mice will be housed and looked after according in facilities that comply with or exceed the current standards set in Home Office guidance and codes of practice. Approximately 90% of mice will experience no intervention and be killed by humane methods. Mice infected with bacteria or viruses are likely to experience transient weight loss (up to 10%) and reduced mobility over the first few days of infection and then recover. For subcutaneous tumours there should be no adverse effects behaviourally or clinically, however, the presence of a tumour will constitute a moderate severity procedure due to its size.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 90% of mice will experience mild or subthreshold severity when being breed or utilised in infection studies. In viral infection studies, less than 25% are likely to lose enough weight to be assigned as moderate severity. Following introduction of tumour cells in mice, approximately 90% will experience moderate severity due to the tumour size but are not expected to have other clinical signs. Overall approximately 5% of mice will experience moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our aim is to understand the process of gene expression in immune cell development and function. Cells continually respond to external signals by producing the proteins required for their function. This process of gene regulation is often inhibited or deregulated in disease

states. We use tissue culture and protein biochemistry when we can in the lab, but it is not possible to fully replicate the complexity of signalling pathways in the body that cells receive in order to develop or function. Therefore, we need to perform experiments on cells extracted from mice, including immune cells, which require a complex series of signals over time to develop. These cells are extracted from mice after they have been killed by humane methods. On limited occasions we need to observe cellular responses to signals in vivo, which requires carefully controlled experiments in live mice. We plan these experiments very carefully, consulting with experts, to minimise suffering to the mice and use as few as possible.

Which non-animal alternatives did you consider for use in this project?
The majority of work in my lab is carried out in cell lines and with isolated proteins. For example, we grow organoid cultures of intestines, derive neurons from immortalised pluripotent stem cells and work with immortalised immune cells. In such tissue culture experiments, we can learn a lot about how enzymes regulate gene expression in response to external signals. We perform as much experimental work as possible in these tissue culture systems. We only perform mouse work when we have exhausted all experimental data from these tissue culture systems.

Why were they not suitable?

Tissue culture systems are improving all the time to reflect the environment in which cells exist in the body. However, tissue culture cannot replicate the full development of cells in an animal. For example, naive T cells cannot develop in tissue culture and cannot be correctly activated in response to antigens. Outside of the mouse, we do not observe the complexity of T cell differentiation which we observe in a mouse body and therefore there is a limit to what we can learn about the immune system. We will continue to monitor the literature for the development of ever better tissue culture models, but currently we need to work in mice to fully understand the role of gene expression in biology.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We are experienced in most of the protocols used in this project. For those limited few protocols in which we have less experience, we have access to experts to help us with project design and estimations of number of mice required. We use the most efficient breeding protocols possible. For most mouse lines we have experience of the genotypes produced and their gender ratios, and we have our mouse numbers from previous years.

This has allowed us to predicted colony size requirements. For control mice we use litter mates where possible, or alternatively use age/sex match controls. We perform experiments as a team and therefore use the same mice to address different questions where possible, e.g. by harvesting several organs from the same mice or analysing as many surface markers on T cells as possible. Variability within a group of mice is minimised by the high standards of animal husbandry at the university. Studies are randomised and blinded. Most of our studies are performed on tissues and cells extracted from mice and analysed in tissue culture (outside of a mouse). These cultures and their analysis are well established and

predictable. For the infection protocols, pilot studies are used when analysing the response of mice with a genotype not studied previously. Typically 3-5 mice will be used in these scenarios, the data will be analysed for statistical significance, then if required studies will be performed with larger numbers of mice. The number of mice required to produce conclusive results will be determined based on these pilot studies and following consultation with experts in each assay.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have experience in the areas of research in which we have planned experiments and therefore have a good idea about how different protocols will progress. This allows us to plan experiments with the minimum numbers of animals required. When possible, we use the same animal for several different experiments and freeze tissues for further experiments. The N3CRs experimental design guidance and experimental design assistance is a useful resource for advice on statistical analysis methods and randomisation and blinding techniques. The scientists and the biological resource team at the university also provided useful advice when planning 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 work with the minimum numbers of mice to produce statistically significant and therefore useful data. Small scale pilot experiments are performed initially, with 3-4 mice of each type to give an indication of results. When necessary, we then use statistical analysis to determine the minimal numbers of mice needed to produce a significant result. By performing high quality experiments, we reduce the numbers of mice needed for repeat experiments. When possible, several experiments are performed on tissues or cells harvested from the same mouse, thereby reducing the numbers of mice used. For example, we can harvest T cells from spleens and lymph nodes and at the same time harvest and freeze other organs for future use. By using a careful breeding programme we keep mouse numbers as low as possible. Mouse sperm or embryos are frozen regularly to preserve lines that are not currently needed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We study the role of gene expression in immune cell function and neurons. Our aim is to increase our understanding of how humans develop, function and respond to disease.

Where we can, we investigate the role of gene expression in the lab with purified proteins or in tissue culture systems.

However, for some studies it is important to perform the experiments using mice. The mouse immune system and organs have been studied extensively and are very similar in humans. The mouse is the mammal of lowest sentience in which this project can be performed. Over

90% of the procedures which will be performed will be sub-threshold, utilising cells from mice being euthanised by humane methods. When we measure the response of T cells to infection, we will use the most refined and defined systems possible, consulting with experts in the fields to get the latest information on how they should be performed. Defined end points to these experiments are set as early as possible to gain meaningful results without causing undue suffering. We expect the great majority of mice to experience essentially normal welfare throughout their lives.

Why can't you use animals that are less sentient?

We need to use mice to study immune cells because they are close enough to humans to gain meaningful data which can be extrapolated to humans. We use mice at 8-12 weeks of age; any younger and the immune system has not developed sufficiently to provide valuable information relating to human immune cells. Most of our studies involve the culturing of T cells from humanely euthanised mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During experiments, live mice are monitored daily initially and then several times weekly when safe to do so. We have a series of criteria on which we base our assessment of animal health including monitoring of weight, movement, fur, diarrhoea. If animals are experience any unnecessary or unexpected clinical signs, they will be killed humanely.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs website has a comprehensive collection of the latest guidance on animal procedures and their refinement. In addition the PREPARE guidelines have been published to assist with animal research planning. We use such resources regularly to keep up with best practice. In addition we attend animal user forums at the institute 4 times per year and receive update information from the NC3R team by email. The British Society of Immunology meetings are an excellent source of advice on refinement.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The NC3R website is a very useful source of information about replacement, reduction and refinement. We frequently monitor this website and have automatic update emails from it. When we plan to perform experiments in live mice, we use this website to gain the latest advice and information. At the establishment, we have access to animal welfare specialists with whom to discuss plans. We also stay informed about the latest advances in refinement at the British Society of Immunology conferences.

45. Efficacy studies on metabolic and kidney disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Diabetes, ADPKD, acute and chronic Kidney disease, Metabolic disorders

Animal types	Life stages
Mice	adult, neonate, aged
Rats	adult, neonate, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate and develop possible animal models in relation to metabolic disorders and kidney diseases and also to provide services to clients.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We will provide a service for small companies that do not have in vivo research facilities or that do not have the specialization or experience to carry out the work

required. Such companies would find it incredibly difficult to develop a drug without evidence of efficacy in animal models and they rely on Contract Research Organisation’s (CRO’s) to provide them with preclinical data to develop their drugs. In addition, we will also provide services to pharmaceutical companies who rely increasingly on CRO’s to increase their pre-clinical in vivo output. Without these services available within small specialised CRO’s, new drugs to treat unmet medical needs would not be advanced to the clinic. As a CRO company we develop best possible models to undertake this work to provide service to the client and also to develop new treatment for metabolic disorders and kidney diseases.

What outputs do you think you will see at the end of this project?

We use animal models to investigate the efficacy of compounds for the treatment of certain diseases. These models would provide data on compound efficacy and safety, and improve our knowledge regarding their effect in these disease models. Data obtained in this project would also help in the development of structural analogues and in compound progression as well as helping to identify candidates for clinical evaluation. The data would also be useful for either publications or patents.

Who or what will benefit from these outputs, and how?

The benefit from the output from this project licence will cover the short term to long term development of novel therapeutics. The primary benefit will be to patients living with diabetes, obesity, kidney diseases and the associated complications that are associated with these conditions such as cardiovascular diseases. Metabolic diseases in particular obesity, diabetes, renal diseases are emerging as the epidemic of the 21st century.

The global prevalence of obesity has incresed year by year and 15% of the population are clinically obese. International Diabetes Federation (IDF) estimated the rise of diabetes to 552 million by 2030.

Non-alcoholic fatty liver disease, hyperlipidemia, and renal diseases are consequences of metabolic diseases. Staggering healthcare costs are associated with treating these diseases, limited pharmacotherapy intervention with limited efficacy, poor management of diabetes and renal diseases are the reasons to find new novel drug classes for the better management of metabolic and kidney diseases. This has resulted in several pharmaceutical companies entering in this research area to develop new novel drug classes.

Even though it is unlikely that in the timescale of this project licence that any compound for which we provide pre-clinical service work will be brought to market as a drug, our aim is still to help to develop the compounds that have the potential to be medicinal drugs of the future. Therefore the timescale for benefits to reach patients following work conducted under this license based on current pharmaceutical development would be approximately 10-12 years, most of which would be time spent in clinical development. Some compounds that have regulatory approval for the treatment of the diseases will have already gone though some relevant clinical phase development previously which will allow a quicker, approximately 5 years, timescale for benefits to reach the patient.

In the short to medium term, within the time frame of this licence, we will be assisting in the design and development of robust novel therapeutics as candidates by providing very high quality pre-clinical and informative data within our aim of the reduction, replacement and refinement of the animals that are utilised in drug discovery process. We will aim to generate data for the eventual use of new drugs in humans for example contribute to new medicines to treat impactful conditions such as metabolic kidney diseases.

In the long term these new drugs will contribute to treatment of patients, reduce healthcare costs, reduced negative impact of diseases on the economy, reduced limitations on work and other activities contributing to quality of life and increased longevity of the patients.

In addition to this the project may also expand scientific knowledge in the research community by identifying novel disease related mechanisms that help and direct current and future research and testing of new therapies in more relevant disease models.

How will you look to maximise the outputs of this work?

We successfully collaborate with academic institutions and other companies to address any needs and perform further analytical work where necessary. All scientific data would be published with the permission of the client. If a particular protocol is not successful, then this would be reported and this would not be used in any future work to avoid unnecessary time and animal welfare costs.

Species and numbers of animals expected to be used

- Mice: 3000
- Rats: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In all protocols either adult mice and rats will be used as these show a similar physiological response to humans. Neonate animals are used in obesity model as Monosodium glutamate induced obesity is effective in neonates only. Aged animals will be used in diabetes model as diabetes development is age dependent in many models of diabetes, and it is important to account for this during experimental planning.

Typically, what will be done to an animal used in your project?

In typical studies animals are exposed or treated with non-lethal doses of substances to elicit response in relevant disease models. The drugs and stimulants are administered through various routes and dose regimen was selected based on

published research articles and client needs. The experiments would not last more than 52 weeks in any of the protocols. Animals will not experience any adverse effect from these substances however if any animal is seen to be unwell then they will be closely monitored and humane end points applied as appropriate.

Blood samples, urine and faeces samples will be collected from the animals to assess the impact of the compounds in alleviating the symptoms by looking at glucose level, insulin level and other biomarkers in serum, plasma and/or urine in obesity, diabetes and kindey disease models. Animals will be weighed regularly to assess the changes in the body weight in obesity disease model and also to check normal health condition in the animals. Animals will be single housed, in metabolic cages, to collect urine and faeces. During glucose and insulin tolerance test in the obesity and diabetes animal models, animal will be fasted for maximum of 6 hours and necessary care will be taken to prevent the animals suffering to either hyperglycaemia or hypoglycaemia during these tests and also under other fasting and non fasting conditions. Surgeries will be carried out in animals under general anesthesia and pain relieving agents will be given pre and post operative surgery to reduce the pain. Animals will be single housed post surgery for the minimum period of time.

It will be relevant to look at the effect of efficacious test agents on prevention of cyst formation in the kidney and this can be assessed by collecting kidneys at the terminal end point and also assess the urine output, urine collection using single housed metabolic cages, in kidney disease models.

Animals will be humanely killed at the end of the procedures, terminal samples, tissues will be collected to assess biomarkers and necroscopy, histology analysis will be carried out.

What are the expected impacts and/or adverse effects for the animals during your project?

In our experimental protocols, it is expected that the animals will not experience more than moderate severity. All the animals are monitored on a daily basis and the adverse effects will be controlled with special care measures and humane endpoints. Animals which undergo surgery may experience some pain, but this will be controlled by providing pain relief before, during and after surgery. No major problems are associated with disease induction.

The diseases can interfere with the normal responses of animals which may result in weight loss and the weight loss will be managed by providing nutrition supplements.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

In most of the specified disease models the animals would not experience more than moderate severity as 30% animals would experience moderate and 70% animals

would experience mild severity and this severity categories will be dependent on control, induced and drug treatment groups.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

During preliminary research we use animals rather than humans as the principles of anatomy and physiology of animals were similar to humans as the certain strains of animals get the same diseases as human therefore the animal models are used in this project to understanding the diseases and developing appropriate treatments for the diseases at controlled environment and also free from other contamination.

The animals are selected based on various scientific research publications and the data obtained from using these animals would help to conduct clinical studies in humans.

Which non-animal alternatives did you consider for use in this project?

We have considered in vitro and ex-in vivo techniques to replicate or produce the results which can be obtained in vivo using animals, however it is difficult to find an alternative method to investigate the biological pathways in each of the specified diseases

Throughout the project non-animal alternatives will be reviewed and considered.

Why were they not suitable?

In-vitro techniques can provide basic information, however they do not offer the complexity for studying and modifying a disease caused by metabolic changes in the whole animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In this project, we will mostly use mice rather than rats for addressing client needs. However, where appropriate we will use rats to validate the scientific data, and for any client requirements. The numbers are estimated based on running experiments on each specified disease every year.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Based on Experimental Design Assistant various steps will be considered such as variability, use of different strains to obtain reproducible data to utilize the animal to its full potential and use of appropriate statistics will be looked at to reduce the number of animals used in the experiment. The proven experimental design, which are previously published, will be followed to minimise any research- associated errors.

By accounting for the influence of variables and addressing sources of bias, an adequately designed experiment will yield robust and reproducible data, ensuring that the data from every animal is utilised to its full potential.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To minimise the variability, we will use animals with a defined genetic background. Randomized pilot studies will be run with the small number of animals to ensure that the phenomenon could be observed. Various end point analysis will be achieved from the same animal as we will obtain multiple readouts from the same animal as specified in the protocol and this significantly reduces the total number of animals that would otherwise be used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use mice as a choice of animal in all our studies as it is comparable to human and small animals are easy to handle for the initial stages of any investigation. Where necessary, rats will be used to obtain further information on diseases based on published literature. Most of the biological readouts will be undertaken under terminal anaesthesia. The dose which will be used to elicit or treat any diseases would be kept optimum based on published literature or in vitro data to minimise those animals suffering. When surgery is required for the collection of blood or removal of organs the use of preoperative analgesia will be the norm. In the case when animals are treated with novel substances, and when no information is

available concerning toxicity, then we will begin with a low dosing strategy to minimise animal suffering.

Why can't you use animals that are less sentient?

The animals are used to mimic the human physiological condition for the treatment of diseases. All our protocols are designed to use minimum number of animals and minimise any welfare costs to the animals, we use animals which are relevant to the development of pharmaceutical drugs and design studies that address unmet clinical needs. The data we generate would help to provide opportunities for treating the human diseases.

Animals of a lower sentience would not provide translatable data to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All the procedures are vigorously followed to minimise the sufferings of animals such monitoring them at regular intervals and giving analgesic treatment in the case of any surgical procedures. As the protocols are designed to use minimum number of animals this would reduce animal welfare costs.

Mice will be cup handled where possible to reduce a handling stress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The best practice guidance produced by NC3Rs clarifies the responsibility in the use of animals in bioscience research in the most refined way.

The guidance sets out some general principles for good practice on the use of animals in scientific procedures including experimental design and statistical analysis, species selection, genetic alteration, dosing and sampling, anaesthesia, analgesia, welfare assessment, humane endpoints, and euthanasia.

Identifying the animal by the ringing, tagging or marking of an animal, should not cause more than momentary pain or distress and no lasting harm and trained people should undertake humane killing of animals under Schedule 1 as guided in ASPA.

The administration of any substance or article to an animal should be carried out for research purposes as certified by the Veterinary Medicines Regulations 2011 (http://www.procedureswithcare.org.uk) and provides guidance on dosing mice and rats. The NC3R's website also provides guidance on blood sampling in common laboratory animal species (http://www.nc3rs.org.uk/our-resources/blood- sampling), as well as for handling and restraint (http://www.nc3rs.org.uk/ handling-and-restraint) and training (http://www.nc3rs.org.uk/training-animals).

Aseptic techniques should always be used during surgical procedures to reduce the risk of post- surgical wound infection (http://www.procedureswithcare.org.uk) and follow LASA guiding principles.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our NACWO provides regular e-mails regarding any changes in relation to animal care/use and updates us with available refinements. We have a regular weekly meeting to discuss the procedures carried out in the animal facility. We also refer to websites such as: https://nc3rs.org.uk/who-we-are/3rs other resources such as from academics and pharmaceutical companies to update us time to time. We always work towards reducing and minimising the suffering of animals and we implement all necessary steps to achieve this. Keeping up to date with publications on relevant disease models.

During the project, if any scientifically advanced methods are available to address any of the specified diseases through various research publications, we would implement it to reduce the use of animals in the project. Regular updating on NC3R's website and would help to get any advances in 3R's immediately.

46. Investigating developmental neural stem cell development to better understand congenital disorders

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Nervous system, Organ development, Cell biology, Stem cells

Animal types	Life stages
Mice	adult, neonate, juvenile, pregnant, embryo
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand how cells organise to form tissues of the central nervous system, particularly the eye and spinal cord, during fetal development. We will focus on how neural stem cells balance decisions between proliferation to allow growth of the organ, and specialisation to generate the mature cell-types of the adult organ, e.g nerve cells.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Neural stem cells are cells in the developing nervous system (brain, eyes and spinal cord) while the baby is in the womb. They have two important properties; firstly, they multiply and increase in number allowing growth of the developing nervous system.

Secondly, they can transform and specialise into all the different kinds of nerve cells found in the adult brain, eyes and spinal cord. These properties must be balanced to generate a fully developed and functional nervous system. This is controlled by signals from the surroundings of neural stem cells and also the genes inside them. Sometimes, issues arise during pregnancy, disrupting the proper multiplication and specialization of neural stem cells, which can lead to disorders of the central nervous system. For instance, some inherited eye diseases, like microphthalmia (patients have small eyes) and coloboma (patients have under-developed eyes) may arise from a lack of neural stem cell growth. These conditions affect about 1 in every 10,000 births and are a major cause of childhood blindness. Although we know about 100 genes that can cause these issues, we don't fully understand how they work during eye development. So, it's crucial to study how neural stem cells balance growth and specialisation during normal central nervous system growth and how things can go wrong in these developmental disorders. Our research aims to uncover:

1. Important behaviours of neural stem cells and signals that guide central nervous system development.

2. The genes and processes that don't work properly during development, leading to congenital eye disorders.

What outputs do you think you will see at the end of this project?

The outputs of our study include new information and publications on:

1. How neural stem cells balance growth and specialisation during organ development.

2. Understanding the reasons behind the congenital eye disorders microphthalmia (patients have small eyes) and coloboma (patients have under-developed eyes). These are the most common cause of childhood blindness.

We will generate new animal models of congenital eye disorders. These will contribute knowledge of genes that are candidates for human inherited eye diseases and a better understanding of the processes that these genes control.

Who or what will benefit from these outputs, and how?

We will provide important information on stem cell decisions and tissue development. In the short-term this will benefit other researchers in the field. Although it is not directly translational, it may be of clinical use in the future, beyond the length of the project, in stem cell treatment/replacement therapies in degenerative diseases or damage in the central nervous system.

Our research in to congenital eye disorders will be relevant to clinical research scientists and clinical geneticists. We aim to test genes linked to eye disorders but also novel genes that have been predicted to be involved from whole-genome sequencing of patients. As such, we will expand the number of genes associated with congenital eye disorders providing new genes for clinicians to screen their patients for mutations.

How will you look to maximise the outputs of this work?

We will present our research at national and international conferences and as invited speakers at Universities and research institutes. This allows direct exchange of information, including best practices and meeting new researchers to expand our collaborative network. We currently collaborate nationally and internationally with other scientists working on neural development and have direct collaborations with clinicians working on eye diseases. This will help bring maximum impact of our work.

Species and numbers of animals expected to be used

- Mice: 3550
- Zebra fish (Danio rerio): 6650

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using two vertebrate species, zebrafish and mouse, to model the development of the central nervous system. We use immature and embryonic stages, both in zebrafish and mouse, which reduces suffering as embryos do not have a mature nervous system.

Refinement is central to our project as we use zebrafish as the least sentient model animal that shows a similar eye structure, organisation and cell composition to human, thereby reducing mammal use.

Zebrafish can also have coloboma and microphthalmia, the disorders of interest in the study. We use zebrafish to screen for new genes associated with human eye disorders. This is because zebrafish lay multiple eggs externally and we can simply collect them from their home tank, without harming the female.

We use mouse to model all regions of central nervous system development, particularly the eye and spinal cord. We follow up on the genes identified in zebrafish and test their function in higher vertebrates, but also explore more well established pathways and genes that are known to be important for central nervous system development. Mice are an important experimental system for neural development because they;

1. Share the same developmental brain regions as humans

2. Share similar gene patterns during neural stem cell maturation and specification of major nerve cell types.

Years of study of mouse neural development has generated a plethora of genetically altered mice, to a degree not available in other species. For instance, we will make use of mice with key neural development genes tagged with fluorescent proteins,

allowing us to monitor and probe developing nerve cells under the microscope, a key step in our objectives.

Typically, what will be done to an animal used in your project?

We study development of the central nervous system so the vast majority of our work will be on embryonic or larval stages in zebrafish and mouse. We will use genetically altered animals for natural mating to generate eggs (zebrafish), larva (zebrafish) or embryonic tissue (mouse) to be used in vitro research or to maintain the stock of animals.

Zebrafish

Typically, we will mate genetically altered zebrafish to obtain eggs and larva. The genetic alterations include reporter animals to aid detection of specific cell types, mainly neural stem cells, under the microscope. No phenotype is expected in these animals. Additionally, we will use animals with knockout and gene changes (mutations) in genes found in human eye disorder patients. We expect the severity of these animals to be mild as we expect some visual impairment, but not severe enough to affect feeding. Genetic alterations will be induced using microinjection of wild-type zebrafish eggs.

Mouse

We propose to use two types of genetically altered animals.

1. Most of these genetic alterations (90%) are to aid detection of neural stem cells and our proteins of interest under the microscope. No phenotype is expected in these animals. Genotyping will generally be undertaken using surplus material from ear notching for identification. Typically, we will mate genetically altered mice and obtain embryos or tissues after humane killing of the pregnant female mice. The embryos or tissue will then be used for in-vitro experiments.

2. For some strains (<10% of breedings), we may induce gene expression changes in the embryos by administering an inducing agent such as Tamoxifen or Tetracycline to the pregnant female, wait typically 1-4 days and then isolate embryos after Schedule 1 killing of the female. The downstream gene chances will occur in the embryos and we will administer between E8.5-E12.5 at unprotected stages. Most (85%) embryos will be typically collected at unprotected stages, but a subset of embryos, around 15% will be taken to protected stages up to E17.5 and then subject to schedule 1 cull.

The gene changes induced by Tamoxifen or Tetracycline will cause either a) activation of a fluorescent protein in neural stem cells in the embryos to help identification of these cells under the microscope or

b) knockout of a gene of interest in neural stem cells in the embryos. The genes of interest will affect how neural stem cells communicate with each other.

Tamoxifen is a drug frequently used to treat breast cancer in humans and Tetracycline is an antibiotic commonly used in humans for acne. They will be

administered by the most appropriate method. Where possible through the diet, but when we need a greater control on the timing of administration by injection into the body cavity (intra-peritoneal injection) of the pregnant female or gavage (oral administration).

What are the expected impacts and/or adverse effects for the animals during your project?

Mouse

We will use two types of genetically altered mice;

1. Reporter mice - mice containing genetic reporters to aid detection of specific cell types (mainly neural stem cells) and proteins under the microscope. No phenotype is expected in these animals because the addition of the reporter does not affect the function of the protein or cell.

2. Mice containing Tamoxifen or Tetracycline responsive genes. The transgenes and gene changes induced by Tamoxifen or Tetracycline will cause either a) activation of a fluorescent protein in neural stem cells in the embryos to help identification of these cells under the microscope or b) knockout of a gene of interest in neural stem cells in the embryos. The genes of interest to be knocked out will affect how neural stem cells communicate with each other. Most (85%) embryos will be typically collected at unprotected stages, but a subset of embryos, around 15% will be taken to protected stages up to E17.5 and then subject to schedule 1 cull. We do not expect an impact from the embryonic gene changes on the pregnant mothers. For the embryos taken to protected stages, there may be some alteration in the number or position of nerve cells generated, but we do not expect any adverse effects more than mild.

The routes of administration of inducing agents such as Tamoxifen and Tetracycline to mice are associated with a minor discomfort and animals may experience stress due to restraint and handling (up to 100%). Tamoxifen can be harmful to the pregnant mouse if administered too early during development (less than 7.5 days after conception). Therefore, we will administer Tamoxifen after this point to reduce harm to the pregnant female.

Zebrafish

We will use two types of genetically altered zebrafish; 1. Zebrafish containing reporter transgenes to aid detection of specific cell types, mainly neural stem cells, under the microscope. No phenotype is expected in these animals as the addition of the reporter does not affect the function of the cell. 2.

Zebrafish with knockout and gene changes (mutations) in genes found in human eye disorder patients.

We expect these animals to have smaller and underdeveloped eyes in either 1 or both eyes, which may lead to slight visual impairment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mouse - Mild - (100%)

- Reporter mice - No phenotype is expected in the Reporter mice and embryos as the addition of the reporter does not affect the function of the cell or protein.

- Pregnant female mice that will be induced to alter gene expression are expected to experience mild severity due transitory pain associated with administration of Tamoxifen or Tetracycline into the body cavity (intra-peritoneal injection) or mouth (oral gavage). For the embryos with altered gene expression due to Tamoxifen or Tetracycline and taken to protected stages, there may be some alteration in the number or position of nerve cells generated, but we do not expect any adverse effects more than mild.

Zebrafish - Mild 100%

- Reporter zebrafish - no phenotype is expected in these animals as the addition of the reporter does not affect the function of the cells.

- Zebrafish with knockout and gene changes (mutations) to genes found in human eye disorder patients. We expect these animals to have smaller and underdeveloped eyes in either 1 or both eyes and we expect the severity of these animals to be mild as we expect some visual impairment, but not severe enough to affect feeding.

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our studies investigate the complex interactions between cells and their patterning and organisation into tissues and organs during fetal development. There are replacement approaches such as in vitro organoid models of neural development and mathematical modelling. In vitro organoids are clumps of cells that resemble an organ and are grown from stem cells in a petri dish. Indeed, we use human retinal organoids and mathematical modelling in the lab. However, these approaches do not completely model the complexity of human or other vertebrate eye development.

Which non-animal alternatives did you consider for use in this project?

We considered using human retinal organoids and mouse embryonic stem cell protocols to study neural development and found relevant protocols through literature searching.

Why were they not suitable?

Human retinal organoids and mouse embryonic stem cell protocols do not re-capitulate the complexity of cell and tissue types found in the developing vertebrate nervous system or the 3D morphology and tissue shape changes that occur during central nervous system development.

For example, current protocols for human retinal organoids generate the neural, light-sensitive tissue but not together with other eye tissues e.g lens, retinal pigmented epithelium and surrounding mesenchyme which are known to influence eye development. Human eye disorders can arise from problems in multiple tissue types, not just the retina. So to fully understand the processes that lead to eye disorders, vertebrate animals are required. Furthermore, patterning of the tissue, such as patterns of gene activity, and patterning of cell types is crucial to generating a functional and structured organ. However, it is currently missing in in vitro organoids. For example, human eyes have a central macular, an area of increased light sensitivity, which is missing in retinal organoids. Therefore, in vitro alternatives cannot be used to study patterning.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the numbers of animals that will be used based on 1.Requirements for strain maintenance and 2. The number of breeding pairs required to generate the embryonic tissue for invitro studies:

Zebrafish

- the number of tanks needed for breeding to maintain a strain (based on 40 zebrafish per tank) per year, per strain. We plan to use 5 zebrafish strains.

-the number of tanks needed for generating a genetically modified zebrafish line. We plan to generate 3 out of the 5 strains.

-the number of breeding pairs required to generate enough embryos and larva for our in-vitro studies. We have based this on our previous experience experiments to determine how many zebrafish crosses are required per experiment.

Mice

-the number of breeding pairs needed to maintain a strain, per year, per strain. We plan to use 5-6 strains, with 2 breeding pairs set up every 4-5 months.

-the number of breeding pairs required to generate enough embryonic tissue for our in-vitro studies. We have based this on our similar published experiments to determine how many embryonic mouse litters are required per experiment. We have additionally taken into account the frequency of false pregnancies (mouse) we have observed over the last 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used the NC3R's Experimental Design Assistant website and ARRIVE 2.0 guidelines for advice on randomisation and blinding, sample size calculations and appropriate statistical analysis methods.

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 several strategies to reduce numbers of animals. While producing genetically altered animals, we will use breeding schemes that maximize the number of animals with the correct hereditary traits that are produced, this will ensure that as few as possible genetically altered animals are born unnecessarily. Colony numbers will be managed efficiently, in mice we will generally use 2 breeding pairs every 4-5 months to keep numbers low. To minimise the use of mice and mouse embryos for preliminary studies we use embryos of zebrafish, a lower vertebrate.

We will maximise use of tissue by freezing and banking it within our lab and also share it wider in the Establishment's tissue sharing resource.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are using two species, zebrafish and mouse, to model the development of the central nervous system. We will collect embryonic tissue after natural mating in both models. Refinement is central to our project as we use zebrafish as the least sentient model animal that shows a similar eye structure, organisation and cell composition to human, thereby reducing mammal use. Furthermore, we use embryonic stages, both in mouse and zebrafish, when embryos do not have a mature nervous system and so suffering and distress is reduced.

In generating embryonic mouse tissue we will occasionally (~10% of matings) need to induce gene activity in the embryos by administration of an agent. We will use the most appropriate and refined method, including by diet, intra-peritoneal injection or oral gavage. When we want to study processes that happen within a short time window during development, then oral gavage or injection are necessary because they induce a rapid change in gene expression.

Why can't you use animals that are less sentient?

We already use embryonic stages that are less sentient. Zebrafish embryos will be used replace and reduce mouse wherever possible; they will be used to screen genes associated with human congenital eye disorders because they provide a high-throughput approach with a reduced welfare impact compared to the mouse. We can't use less sentient experimental models to investigate human congenital eye disorders as C. elegans have no eye structures and Drosophila have compound eyes which have very different structure to humans. Therefore they do not represent human eye disorders.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Historically administration of inducing agents to pregnant females has been done by injection into the body cavity of the female (intra-peritoneal), we will refine this by testing and implementing alternative less invasive routes, e.g by diet and oral administration.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the best practice guidance from the Home Office on Animal Testing and Research from the Home Office, LASA, RSPCA, local AWERB guidelines, ARRIVE 2.0 guidelines of the NC3Rs and the PREPARE guidelines to ensure our experiments are high quality and the most refined.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are committed to seek and review advances that can improve our experiments throughout the project duration. We will keep abreast of reduction and refinement advances by attending workshops and seminars run by the Establishment as well as signing up to NC3Rs newsletter and IAT bulletin. We collaborate with groups that aim to improve organoid and stem cell models of neural development. By meeting with these researchers and attending conferences we will keep up-to-date on replacement approaches that avoid the use of animals.

47. Mechanisms of tumourigenesis in the nervous system

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Therapies, Nervous system, Tumour, Pre-clinical testing

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We wish to understand the mechanisms that drive several kinds of tumours in the nervous system. With the pre-clinical models we will use, we will also test new therapies for the treatments of these important tumour types.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Brain tumours kill more children and adults under 40 than any other cancer type. While we understand some of the drivers of brain tumours, there is a great need to further advance our understanding of these tumours and to trial new therapies in appropriate mouse models before they can proceed into clinical trials. Work in my

previous licence focussed, successfully, upon schwannoma and meningioma tumours, but we now wish to widen the scope of the work to include glioblastoma and ependymoma tumours, which share many of the same dysregulated signalling pathways. There is an urgent need to identify and test new and specific chemotherapies for all these tumour types. Currently surgery is a mainstay of their treatment with a significant risk to surrounding nervous system tissue and consequent effects.

What outputs do you think you will see at the end of this project?

We expect to generate publications in peer-reviewed journals and to trial new compounds in our pre- clinical tumour models as a prelude to clinical trials in human patients. We will continue to share our data, often prior to publication, with collaborators and at research conferences.

Who or what will benefit from these outputs, and how?

Work is currently under way to plan a clinical trial with one drug we have tested in our schwannoma model in our current license. In the longer term, we have signed an agreement with the Children's Tumor Foundation to trial new therapies in our mouse schwannoma and meningioma models. We have already screened one monoclonal antibody treatment and fully expect other trials to follow in the next 5 years and beyond. Our work with the research charities, who fund our work, means that we are able to share details of our work and its implications with patient groups during tours of our laboratory.

How will you look to maximise the outputs of this work?

We will continue to present our work as soon as possible, frequently prior to publication at research conferences and to discuss with patients groups. We will also publish our work in open access journals to make it widely available to all scientists and interested individuals. We will collaborate as widely as is possible and share our genetically modified animals with other research groups working in the same research areas.

Species and numbers of animals expected to be used

- Mice: 10000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Genetically modified mice are currently the animal of choice for the understanding of tumour formation and trialling of new treatments before they can progress through to clinical trials. Mice may be bred on different genetic backgrounds depending upon the analysis required. For the breeding and maintenance protocols, animals may be

bred and samples taken at different life stages i.e. embryo, neonate, juvenile, adult and pregnant animals for analysis of cell fate and possible tumour formation. For surgical procedures, then these will be performed in mostly adult animals, depending on the requirements for the experiment.

Typically, what will be done to an animal used in your project?

Animals bred on this license may be subject to differing procedures. Genetically modified animals in which spontaneous tumour formation takes place may administered compounds/medicines to reduce tumour growth, either by e.g. injection or into the stomach using a small tube placed into the mouth (gavage), normally daily, though this would depend upon the substances used. For such experiments, the duration of dosing would normally be 21 days or less. Cell labelling, to mark eg. dividing cells, may be subsequently be performed by injection of substances to animals (eg EdU). For animals undergoing surgical procedures, then they would undergo a single surgery followed by an analysis of tumour growth (eg. imaging techniques, usually weekly), before receiving compounds/medicines by e.g. injection or by gavage. For animals that have undergone surgery, these animals will experience some discomfort and some mild to moderate pain, which will be treated with appropriate pain relief. For such experiments, the time post-surgery before treatment starts would depend upon the tumour model used and may be between e.g. 7 days to several months. For such experiments the dosing may last up to several months, depending upon the mouse model, the compound(s) used and the experimental design.

What are the expected impacts and/or adverse effects for the animals during your project?

Some mice (approximately 50-60%) bred may show no impacts of the genetic modification upon animal health.

For some mice bred in this work, there will be spontaneous tumour formation (eg. schwannoma). To date with this model, we have not observed any significant signs of weight loss, pain or abnormal behaviours in tumour-bearing animals.

For protocols involving surgery, animals are given appropriate painkillers to minimise any pain and post-surgery checks monitor any weight loss and adverse effects of the surgery; animals would be killed should any adverse effects occur. For experiments to examine the relationship between injury and tumour formation, the sciatic nerve in the back of the leg will be injured to examine these effects, meaning that animals will lose function, usually temporarily, in that one limb. For experiments involving transfer of tumour cells and subsequent tumour formation in the brain, again animals are given painkiller drugs and are carefully monitored post-surgery for signs of weight loss or any abnormal behaviour. We use a technique called bioluminescent imaging in such experiments and this allows us to scan and measure tumour growth in the living animal and take any necessary actions before animal health is significantly impacted.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For the experiments with mice in this project, we would expect approximately 60% mild and 40% moderate.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animal models in this project in order to understand the mechanisms of tumour formation and to trial new therapies for these tumours. Tumour formation involves a number of different cell types (eg cells of the immune system, blood vessel cells), together with the surrounding environment; this cannot be accurately modelled in vitro. For the new therapies we will trial in our work, we need to know that the drug can reach its target in a living animal and be well tolerated before any of these compounds can be considered for progression into clinical trials.

Which non-animal alternatives did you consider for use in this project?

We always use cell lines and human tumour cells in vitro alongside our in vivo work. In addition to conventional cell culture work, our in vitro work also involves the use of 3D tumour cell spheroids for compound testing. Other in vitro systems such as brain organoids or 'brain on a chip' could be considered at some point in the future, but are not yet at a point in their development that they are appropriate for this work.

Only if modifying a pathway, either by using a drug or genetic change, shows promise in these in vitro models first will we progress to in vivo work to test their efficacy.

Other in vivo models, such as the fruit fly or nematode do not allow this analysis in a mammalian system, which is absolutely necessary for our work.

Why were they not suitable?

The in vitro modelling can only take us so far. Currently, the use of brain organoids or 'brain on a chip' technologies does not allow for the re-constitution of the correct tumour composition or an are effective measure of how the drug will target tumour tissue in a living organism.

Therefore, to accurately model tumour development, cell-cell interactions and the effects of genetic change and use of new therapies, we need to use an in vivo mammalian system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For both breeding protocols, we estimate breeding approximately 8000 mice in total over the course of the 5 year project. This number is based upon the numbers used in our current project license. A significant proportion of animals have complex genotypes, with 2 or 3 alleles being bred together (eg CRE recombinase, conditional alleles and GFP reporter alleles, thereby requiring these numbers to be bred on the license to secure animals with the correct genotype for our work. From these animals, many of these will be used for tissue collection and analysis, but a substantial number will then be used on other protocols such as surgery and then, potentially, for the administration of modulating substances protocols. While the majority of animals used on the project will be bred here, we may also bring other mouse strains into our facility (either from commercial sources or from other PPL holders with authority to supply animals of a type authorised on this project.)

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For all mouse breeding, appropriate genotypes are carefully selected to minimise both the numbers of breeding steps and also the generation of animals that cannot be used. For conditional knockout studies, wherever possible, animals are kept in a homozygous (fl/fl) genotype so that control and null animals are in the same litter and are appropriate controls for each other. For some other mouse lines, the genetics are complex and may require several generations to achieve the correct genotype of animal. Controls selected will be matched for age and sex to provide accurate data. Equal numbers of male and female animals will be used whenever possible. For animal models where there is spontaneous tumour formation, tissue may be taken from multiple sites to maximise the amount of tissue and data from a single animal. For experimental design and analysis, we will consult and use the NC3R's Experimental Design Assistant (EDA) in our work. For experiments involving trialling new compounds, where possible, we will use previously determined dosing protocols and administration routes to minimise any variability from previous work. Group sizes will be set from our previous findings, from published work and from discussions with collaborators. University statisticians may also be consulted for assistance on study design. Animals may be randomly assigned to treatment and control groups using the NC3R's EDA online tool.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For all mouse breeding, appropriate genotypes are carefully selected to minimise both the numbers of breeding steps and also the generation of animals that cannot be used. For conditional knockout studies, wherever possible, animals are kept in a homozygous (fl/fl) genotype so that control and null animals are in the same litter and are appropriate controls for each other. If appropriate, tissue from animals will be shared between research groups to maximise the use of each animal. For new models or drug treatments, pilot studies may be performed to test their efficacy before further experiments are performed. For the work in protocol #4, this will include the use of bioluminescent imaging equipment for the animals. This allows us to measure tumour size in an animal over a period of time. Before this, we needed to include as many groups of animals as there were timepoints. This now allows us not only to reduce the numbers of animals required for the work, but be able to monitor whether the tumour may be likely to impact the animal's health in the near future and appropriate action (eg. closer monitoring or killing) can be taken to prevent adverse effects to the animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For the mouse breeding that we do, wherever possible, we will use a conditonal knockout model. This means that that the protein of interest will be lost only in a defined cell type (eg. Schwann cells). This acts both to minimise any adverse effects, but also to increase the specificity of our data. For models in which there is spontaneous tumour formation, if there is data available or from our own results, we will endeavour to maintain animals for the minimum time period if adverse effects occur in older animals. For surgical procedures, we will consult with both NACWO and NVS to plan work with regards to the best pain relief available for the animal. Animals are monitored regularly and any adverse effects picked up quickly; the decision is then made quickly as to take remedial action or, if necessary, to kill the animal. The models of cell transplantation we will use have been widely used and refined to shorten surgery times and minimise suffering of animals. Experiments are planned and run for the minimum time necessary to generate robust and publishable data to share with the scientific community and patient groups.

Why can’t you use animals that are less sentient?

For tissue collection from bred animals, this will be taken at the appropriate time for the effect; this may be at an immature life stage or adult. For some tumour models, full tumour formation may not occur until the animal is an adult. The processes of development and tumour formation, with all the cell types and environment, cannot be modelled accurately in a non-mammalian system and the mouse is the animal of

choice as the genetically modified strains are available for use. For the surgical procedures, these will be performed at the appropriate age of animal. Some tumour types may start to develop in immature life stages, for other types of tumour, then surgery in an adult mouse is appropriate.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During my current project license, we have refined our methods for our work. This has included the purchase of bioluminescent imaging equipment that allows us to measure tumour size in an animal over a period of time; we will continue to do this in the new project license. This allows us not only to reduce the numbers of animals required for the work, but to use monitor whether the tumour may be likely to impact the animal's health in the near future and appropriate action (eg. closer monitoring or killing) can be taken to prevent adverse effects to the animal. For pain management following surgery, we have and will continue to review the pain relief given to animals peri- and post-operatively to minimise any suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have read and will apply the PREPARE guidelines in our experimental planning. We will consult with our NACWO, and through either the NACWO forum or directly, we can consult with other group leaders and their groups as to the best way to conduct the procedures we perform. For surgical procedures, we will consult the LASA guidance on 'Guiding principles for preparing for and undertaking aseptic surgery.'

For experiments with new potential therapies and dosing, we will consult published guidelines and use published or known pharmacodynamic/pharmacokinetic data to design our experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly monitor the 3Rs website and consult with both the NVS and NACWO to implement improvements in the 3Rs during the project. In addition, we will look at any possible improvements to our procedures that are published in the scientific literature and implement these in consultation with the NVS and NACWO.

48. Modulation of wound healing and scarring

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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
wounds, burns, pigmentation, chronic, scarring

Animal types	Life stages
Pigs	adult
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To assess treatment(s) that will prevent/reduce wound infection, inflammatory response and/or accelerate wound healing.

To assess treatment(s) that will reduce/prevent scarring following wound healing and investigate the mechanisms of all aspects of scarring including altered pigmentation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Wounds are a major healthcare burden and in particular chronic wounds and burns/thermal injury are a global problem. Wounds are a leading cause of loss of

hours of work and a drain on all healthcare systems around the world. One of the dangers of open wounds such as chronic wounds and burns, is the susceptibility to infection which can delay wound healing, increase risk of gangrene or lead to sepsis with a risk of death.Scarring following injury affects not only the cosmetic and psychological well being of the patient, but more importantly, growth and function, particularly in burn injured children. Current management of wounds relies largely on a large number of dressing products designed for mass therapy and lacks knowledge that can provide personalised care. Wound healing is a complex process and we need to understand the mechanism of why wounds do not heal or heal slowly and scar so that we can develop smart treatments that will speed up healing and prevent/control infection and reduce/prevent scarring. This project aims to address these challenges and advance our knowledge of wound healing and scarring.

What outputs do you think you will see at the end of this project?

We will test new substances/treatments designed to speed healing and reduce scarring as well as new treatments that fight wound infection. We will first study how these treatments change wound healing and scarring in animals before we test them in humans. In parallel, we will investigate how and what effects any new treatments of wound healing/scarring may have including side effects. We predict that these studies will provide us with more information into the mechanisms of wound healing and scarring that can be investigated for further development of new targets for personalised care. These studies will provide a safer route into clinical wound and scar management. The studies will specifically focus on the current challenges in wound healing such as improving speed of healing, preventing/reducing infection including targeting multi-resistant organisms and prevention/reduction of scarring.

The new information will be shared widely in the scientific and clinical communities.

Who or what will benefit from these outputs, and how?

The ultimate beneficiaries will be humans and will aid the healthcare professionals of various disciplines in managing patients with wounds and scars. Some of the outcomes of these studies may well benefit animals with wounds and veterinarians as well. The scientific community will definitely benefit from the basic science knowledge gained from these investigations.

In the short term, testing of new substances will allow us to identify the potential new treatments from those that need more work or are not suitable for use in human wound healing and scarring.

Development of non-invasive technologies to study state of healing/early detection of infection for timely intervention will be of great benefit to children in particular, as it will address the issues of pain and anxiety related to handling of wounds.

In the longer term, detailed scientific studies (molecular and genetic investigations) on tissue samples obtained from these studies will help to better understand the mechanism of action. The knowledge will assist scientists to develop even better target candidate treatments for rapid wound healing, prevent/reduce wound

infections, prevent scarring, assist with regaining normal skin pigmentation and texture of the scar as well as prevent deformities/limitations in mobility.

How will you look to maximise the outputs of this work?

The establishment has a large collaborative network in wound healing. We will maximise outputs by peer reviewed publications in the scientific literature and where appropriate, we will consider media engagement. We will collaborate with other researchers in various fields as we have developed expertise in various wound healing models and will have an extensive library of tissues from different types of wounds at various stages of healing/scarring. By wider collaborative working, we will be able to harness a wide range of expertise and be able to investigate different approaches and thereby accelerate the pathway into preclinical testing of such therapies.

Species and numbers of animals expected to be used

- Pigs: 75
- Mice: 210

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodents have been selected to test the efficacy of the novel therapeutics in accelerating wound healing/reducing inflammation and infection and in particular for mechanistic studies. However, as explained below, pigs will be used for pre-clinical studies and for studies involving wound healing, wound infection, scarring and altered pigmentation.

The pig has been chosen as its skin has similar anatomy and physiology to human skin. Pigmentation in the pig skin, unlike mice, is akin to the human skin in that the pigment cells are present in the hair follicles and in the epidermis (top layer of skin) and both play a role in pigmentation. Mice are loose- skinned with a panniculus carnosus (skin muscle) and heal mainly by contraction that is much more rapid than by re-epithelialisation (cells migrating over the surface of the wounds to form top layer of new skin) seen in ‘tight-skinned’ mammals such as humans and pigs. Our previous studies on human thermal/burn wound samples have confirmed similarities with the pig thermal/burn wounds.

Typically, what will be done to an animal used in your project?

Under general anaesthesia, thermal and/or open wounds on the backs of the animals will be created (maximum 12 wounds measuring 2.5cmx2.5cm on the backs of pigs and maximum two wounds no larger than 1cmx1cm, on backs of mice). The size of the wounds are well tolerated by the animals and are known to heal spontaneously. Some of the wounds will be infected with bacteria to produce local

wound infection. The wounds will be treated with novel treatments/dressings which will be applied either topically or by injection into the wound margins. The wounds will be dressed and animals allowed to recover. Wound healing, reduction/prevention of wound infection and “new skin” formation including the pigmentation and scarring will be monitored by imaging and measurements over various time points up to 10 weeks post-injury. At varying time points, wounds will be removed with the animal under terminal anaesthesia. After taking out the wounds, the animal will be humanely killed. The wounds will be processed and subjected to in depth analyses to study the mechanisms and pathways of wound healing/scarring using cellular and molecular techniques as well as effect of infection on wound healing.

In addition, novel non-invasive methods for checking progression of wound healing/wound infection through the dressings will be investigated at regular intervals. These methods have previously been tested in non-living animal experiments and shown to capture images through the dressings. We will perform tests to ensure it is safe and represents the stages of wound healing before the methods are used in humans.

What are the expected impacts and/or adverse effects for the animals during your project?

The procedure can cause post-surgical pain of mild to moderate severity which will be controlled with pain killers. These effects are well controlled once the wounds have been dressed and the pain relieving drugs are administered in a timely manner before the animal wakes up. The animals will be monitored closely for signs of pain and distress and treated promptly with further pain killers as required. The welfare of the animals will be closely monitored to check on any side effects of the novel therapies. With smaller animals weight loss can be a measure of animal distress and will be carefully monitored.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity of pain is estimated to be between mild and moderate and animals will be monitored very closely and appropriate pain relieving drugs administered as required. From experience, once the wounds are dressed and animals have had their pain medicine before waking from anaesthesia, they are comfortable and do not show any signs of pain and distress. Wounds can also become colonised or rarely infected with microbes but will be managed or treated within the moderate severity range.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Open or thermal wounds require cells from the circulating blood to interact with the local cells of the skin to allow complete healing of the wound and formation of the "new skin". We can study some aspects of wound healing such as re-epithelialisation (healing of the top layer of the skin) in ex-vivo models (culturing whole skin in petri dishes and wounding the skin) which we are currently developing for thermal and open wounds. However, in order to study the full science of wound healing i.e. development of the "new skin" after open/thermal wounds, requires animal studies with blood circulation and there are no alternative models available to us at present. Furthermore for testing new substances to improve healing and scarring, we need wounds on animals with circulating blood as ex- vivo studies are not suitable.

Which non-animal alternatives did you consider for use in this project?

1. We have considered collaborating with colleagues who can keep pig body parts from the abattoir "alive" by perfusing with artificial blood.

2. We are optimising an ex-vivo burn wound model of human and pig skin (surgical/abattoir waste) to study the effect on re-epithelialising (healing of the top layer of skin).

Why were they not suitable?

1. Use of perfused body parts for wounding and studying mechanisms are not suitable because the perfusion can only be maintained for 24 hours maximum to date and we need to study the long term effects of wound healing and scarring.

2. With ex-vivo experimental wounds or burn model, there is no circulating blood which contributes to the healing process. Ex-vivo burn wound model can only be kept alive for short period of time. However, we are currently working on optimising culture conditions in order to study some elements of ex-vivo thermal/open wound healing and intend to compare the in vivo thermal/open wounds with ex- vivo thermal wounds.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Data from previous studies have informed the estimated number of animals to be used. However, we will continually review the data and update the validity of the sample size calculations and number of animals required.

In this project, we aim to test up to 5 treatments. The generic design of the experiments is step wise where the pilot experiments will inform the design of the experiment i.e. comparison of treatment versus standard of care in same animal or between different animals dependant on whether the test treatment diffuses into the surrounding skin and gets absorbed or remains within the wound bed to avoid confounding the results. The pilot studies will also inform the optimum route of administration and doses to be tested for efficacy studies. Therefore the efficacy studies will have the benefit of data from which to recalculate the exact numbers required as mentioned above.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The design of the experiment was discussed with the establishment's statistician who analysed some endpoint data from our previous experiments. We also discussed with the NC3R team.

The experiments are designed such that the treatments and controls are within a block or incomplete block (depending on the number of treatments analysed within an experiment) and this allows to reduce the actual number of animals used per experiment. Furthermore, by using smaller pilot experiments initially, we can optimise the final experiments and recalculate the sample size at each stage.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For each treatment to be studied, pilot experiments will be used to optimise the design of the experiment, route of administration and doses to be tested for efficacy studies. Data will inform the validity of the sample sizes used. Previous experimental data has also been used to inform the current sample size. Observations from each of the dose response and efficacy studies will add to the data.

Our previous studies on pigs have provided data to combine re-epithelialisation (top layer of new skin) and early inflammatory infiltrate (blood cells which help with wound healing) studies to day 10; ongoing inflammatory response and myofibroblast phenotypes (cells which lay down the collagen in the new skin)in wound bed (early marker of scarring) to day 21 and long term effects on re-pigmentation, vascularity (blood vessels) and texture of scars to day 70 post-wounding. These aligned time points will reduce the number of animals studied.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Incisional/excisional wounds will be made in mice and incisional/excisional and thermal wounds of varying depths will be made on backs of pigs. These are standard wound models to study wound healing. In addition, we will study antimicrobial treatments on infected wounds in these animals.

The animals will be under the effect of general anaesthesia when the wounding is performed so they will NOT feel any pain or discomfort. The wounds will be well padded and covered. From previous and clinical experience working with patients, once the wounds are dressed and well covered, the pain is minimised.

Wound infection is unlikely as wound management/dressings are applied aseptically after washing with antiseptic solutions and sterile dressings are used.

The animals will be given pain relief at the start of the procedure to ensure minimal or no pain is felt after recovering from the anaesthetic. They will be observed closely thereafter and further pain relief administered as necessary.

From our previous studies, we have now refined the technique of surgical procedure and application of dressings and refined the dressings used, thereby reducing the frequency and the duration of general anaesthesia for the animals. The animals will be closely observed post-wounding for any signs of distress or pain and prompt management initiated as appropriate. After wounding, the animals will have robust dressings to avoid self-interference with the dressings and will be singally housed, but will be adjacent to other animals to allow social interaction.

Why can’t you use animals that are less sentient?

Following initial ex-vivo studies, we will initially check effects of test treatments on mice before we study the effect on porcine wounds (considered akin to human wound healing/scarring/re- pigmentation). The pig has been chosen as its skin has similar anatomy and physiology to human skin. Pigmentation in the pig skin, unlike mice, is akin to the human skin in that the pigment cells are present in the hair follicles and in the epidermis and both play a role in pigmentation. Our previous studies on human wound healing have confirmed similarities with the pig wound healing.

The pig has been selected as the model of choice for our more developed treatments down the translational pathway that have already been validated in small animal studies. For our early discovery pipeline treatments we will be using less sentient animals such as the mouse. Despite their lack of similarity to human skin, the detailed characterisation, genetic tractability and convenience of experimentation due to most of the literature being based on rodents, make mice ideal for earlier phase discovery studies such as initial trialling of novel compounds and mechanistic studies where specific pathways can be identified for more targeted therapy.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animals will be monitored very closely by the specialist animal care staff who are conversant with handling and welfare of mice and pigs. The animals will have time to acclimatise, for at least 7 days. Only staff competent in animal handling and assessment of animal welfare and surgery will be performing these procedures. They will interact with the animals during their acclimatisation and build up a rapport to minimise distress.

Following the procedure, enrichment will be provided such as bedding and toys.

Animals will be weighed periodically and their food and water intake monitored as well as their bowel movements along with the health of the skin and eyes to ensure they are healthy and not distressed throughout the period of the experiment. Animals will be assessed for pain and pain medication given timely. General appearance, body function, environment, status of the dressings and behaviour of the animals will be closely observed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

ARRIVE guidelines and guidelines by joint working group on refinement

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By attending the NC3R workshops, keeping abreast of the literature and regular communication with the Establishment 3Rs manager. If there are new advances in procedures and welfare of animals, we will seek amendments to refine our techniques and implement changes after discussion with the named veterinary surgeon.

49. The circuit mechanisms of sensory detection and discrimination

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

vision, learning, attention, cognition, neurodevelopmental disorders

Animal types	Life stages
Mice	adult, juvenile, pregnant, embryo, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aim is to understand how the brain performs two tasks that are very important for making correct decisions: detecting and discriminating information from our senses, including our eyes. We will investigate how we see, learn and pay attention (including eye movements) during detection and discrimination tasks in the normal brain and in mouse models with cognitive impairments such as problems with learning, attention and decisions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Detection and discrimination of information from our senses is very important to make good decisions that help us survive (for example, to obtain food and avoid threats based on what we see). The ability to detect and discriminate depends on the ability of animals to see, and on learning from experience, and paying attention to relevant information. Problems in seeing, learning and attention are associated with different mental health conditions, including neurodevelopmental disorders such as autism and schizophrenia. However, there is currently no effective treatment available for these problems, even though they have a major impact on the quality of life of patients, negatively affecting for example education and employment.

In the UK approximately 17% of adults suffer from a mental health condition and the associated annual cost in England has been estimated at £105 billion. Our research is particularly relevant for perceptual, learning, attention and decision-making problems in neurodevelopmental disorders such as schizophrenia and autism (affecting approximately 2% of the population or 1.3 million people in England alone). It is also relevant for other neurodevelopmental disorders including ADHD (affecting approximately 2% or 1.3 million people in England), other common mental health disorders such as depression (affecting approximately 4% or 2.6 million people in England), and learning and attention problems without mental health disorder diagnosis. For example, less conservative estimates suggest ADHD affects up to 17% of people. Problems with learning, attention and decisions are also seen in brain injury, dementia, and normal aging.

We will study detection and discrimination in both regular mice and mouse models with problems in learning and attention and investigate methods to treat them. This can help improve our understanding, diagnosis and treatment of mental health conditions such as autism and schizophrenia.

What outputs do you think you will see at the end of this project?

This project will generate behavioural data and detailed measurements of brain activity. We will use analysis techniques to measure the relation between behaviour and brain activity, and computer models that predict brain activity. We will describe the results in scientific publications and ensure open and free access to the results. We will present the results in national and international conferences.

Who or what will benefit from these outputs, and how?

The project will give new insights into brain activity during two fundamental sensory tasks that form the basis of sensory selection: visual detection and discrimination. We will use methods established in mice including optogenetic brain stimulation with light and genetic mouse models to study brain circuits. We will determine the involvement of low-level and higher-level visual brain regions, and the role of different cell types and projections within these brain regions. In the long-term, these findings can be translated to humans using related research approaches and help develop treatment of abnormal brain function with medication and brain stimulation methods in humans.

How will you look to maximise the outputs of this work?

We will ensure our research outputs are available open-access. We will also share data and newly developed research tools with our collaborators and others upon publication. Unsuccessful approaches will also be shared either as part of these publications or on other open platforms such as Biorxiv or F1000.

Species and numbers of animals expected to be used

- Mice: We expect to use approximately 8920 mice over a 5 year period.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice in this project since they are the lowest species in which this research is possible. Mice have a brain comparable to humans, they are capable of behaviours that are similar to important aspects of human behaviour, and there are unique methods available in mice such as methods to genetically alter mice that allow us to model mental illness and measure brain activity with great detail.

To generate genetically modified animals for experiments, we will breed and maintain mice across different life stages up to and including adulthood.

Generally, mice will only be used for other experimental procedure after they reach adulthood and we will only use adult mice to measure or modify neural activity and study behaviour. The only exception is that we use neonate mice if we need to measure or modify neural activity across multiple brain regions requiring injection in neonates. These animals will not undergo other experimental procedures during the neonate stage but only after reaching adulthood.

Typically, what will be done to an animal used in your project?

We use procedures to generate genetically-altered mice using standard protocols. This includes animals in which genetic modifications enable us to measure or modify neural activity in specific cell types and animal models of cognitive impairment (including learning and attentional deficits).

The experimental protocol enables us to measure and modify neural activity and study behaviour. We need to measure how activity in brain cells is linked to behaviour and therefore need to perform surgeries to enable neural recordings. During surgery, while the mouse is under anaesthesia, we will implant a head post, which is a small piece of metal connected to the skull that can be connected during behavioural testing to an external holder to fix the position of the head. Using a head post is the best way to stably fix the head without causing pain to the animal compared to directly clamping the head of an animal. Fixing the head is necessary in the majority of experiments to be able to control the sensory information that the mouse receives. Fixing the head is also necessary to allow for activity

measurements in brain cells using microscopy. Mice are gradually accustomed and habituated to head fixation with the use of rewards.

During surgery, we additionally make small openings in the skull to gain access to the brain to allow for measurements of activity in brain cells. In the opening we can insert electrodes in the brain to measure electrical activity of cells or inject substances that label brain cells enabling us to optically measure cell activity using microscopy in later experiments. In some mice we inject a substance to make some brain cells sensitive to a specific light, or we implant a small tube in the brain. This allows us in a later experiment to use either light stimulation or add a substance in the brain via the tube to temporarily increase or decrease activity in some brain cells to test what function those cells have in selecting sensory information. These methods that we use to measure or modify activity in brain cells (once the animal has recovered from the surgery) are not painful or stressful for the animal. At the end of the surgery, the openings in the skull are sealed with either a transparent glass window to allow for microscopy or with a special type of non-transparent cement that adheres to the skull. We typically complete the above procedures in one or two surgeries.

After mice are fully recovered, we will train them in behavioural tasks. Mice will participate in experiments where they learn new associations between sensory features and rewards. In some experiments, we need to fix the head of the mouse using the head post. During head fixation, we also place the mouse on a treadmill. The treadmill allows the mouse to move more freely (apart from the head which is fixed) because the mouse can choose to either sit still or walk (the treadmill only moves when the mouse decides to move) instead of being forced to sit still without a treadmill. Mice become accustomed to head fixation within a few days, and the treadmill helps mice to get used to head fixation more quickly than without a treadmill. Mice will then be presented with sensory features, including visual objects on a screen, sounds from a speaker, or smells coming from a tube.

Sometimes we need to restrict the food of the mouse to motivate the animal to learn a new task. In some of our experiments, head fixation is not needed and animals learn the associations between sensory features and food rewards while freely moving inside a training cage in which visual features, sounds and smells are presented to the animal. In these experiments we usually attach temporarily lightweight sensors to the head post in order to be able to measure the behaviour of the mouse (for example, the position of the animal in the training cage and the eye position of the mouse by using a miniature camera). We found that mice are not bothered by these lightweight sensors, for example, we find that the sensors do not change how mice move around. We also study natural behaviours that do not require training or food deprivation such as exploration of new environments. After all experiments are completed, mice will be killed by a humane method and brains will be studied to obtain additional details about the brain cells that were recorded including their location and cell type.

What are the expected impacts and/or adverse effects for the animals during your project?

The surgery is expected to cause moderate discomfort. This is visible for example, by a detectable temporary weight loss or reduction of activity levels. Mice recover within a few days and they will be given painkillers and post-operative care (for example, they receive special food that requires minimal chewing). During recovery, mice are closely monitored. For example, we compare the animal's weight to the weight before surgery and we check whether a mouse is eating and drinking and moving around the home cage normally.

The sensory features and behavioural testing procedures are not painful or stressful, and mice will learn within a number of days which features are associated with food rewards. For example, mice learn that when they see one type of visual pattern (e.g. vertical stripes) they can get a food reward when they lick a spout, and that they cannot get a food reward when they see another visual pattern (e.g. horizontal stripes).

Mice are expected to only experience mild discomfort during periods with food restriction where they have restricted amounts of food. This is necessary to control their weight to ensure they are motivated to perform the behaviour tasks for food rewards. The mice will be in good health and they are fed daily. We closely monitor the mice and check for example their weight to ensure they stay healthy.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild: 80% of mice

Moderate: 20% of mice

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of this project is to understand the brain circuits that underlie the detection and discrimination of sensory information. This requires study of neural circuits in an intact behaving animal.

Alternative experimentally-valid in-vitro or ex-vivo approaches (studies of isolated parts of brain circuits in an artificial environment outside of a live organism) to answer this question are currently not available. However, where possible we will take advantage of ex-vivo techniques that help to interpret our data. For example,

after completing experiments in-vivo (in a live animal) we will characterize electrode tracks and the cell type of imaged cells ex-vivo (outside of a live organism).

There is also currently no data available that is detailed enough to generate comprehensive and realistic computer models of how the neurons in the different brain areas function, how the circuit changes with sensory experience, and how different brain areas contribute to behaviour. However, our research generates data that we also use to develop simplified computational models of mechanisms of sensory processing and we collaborate and share our experimental data with computational collaborators.

There are also currently no techniques available that allow us to study neural circuits in humans with the required cellular resolution and that allow for establishing causal relations within the circuit.

However, where possible we perform experiments with human participants (e.g. studying eye movement and visual detection and discrimination behaviour) and we closely interact with human neuroscience labs to seek translation of results between mice and humans (for example by testing hypotheses suggested by work in humans and primates, and by generating hypotheses that can be tested with more large-scale methods in humans).

Which non-animal alternatives did you consider for use in this project?

In our research, we use computer models, advanced data analysis, and experiments with human participants to complement our animal experiments.

The computer models enable us to test candidate computational mechanisms based on our experimental data. Advanced data analysis enables us to determine the relation between different aspects of behaviour and neural activity. Experiments in human participants help establish translatability of observed results in mice and humans (for example, characterizing visual detection and discrimination in humans, and eye movement patterns).

Why were they not suitable?

The above approaches complement but do not replace animal experiments since they do not have the required cellular resolution and do not allow for establishing causal relations within neural circuits.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of animals is based on our previous experiments and statistical estimation of the number required to obtain reliable scientific results. We plan our experiments in accordance with best practice of experimental design (for example, the previously published PREPARE guidelines).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We are committed to using the minimum number of animals required to obtain reliable scientific results. We use statistics and data from our previous experiments to estimate the number of animals required. We also design our experiments to maximize the results obtained from each animal (by using long- term measurement to collect multiple data points from the same animals) to reduce animal numbers.

We use systematic literature searches (e.g. with combined keyword searches in online databases such as PubMed) to review relevant previous work to determine which experiments are necessary to answer our hypotheses.

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 long-term neural measurement techniques to record responses of large numbers of cells in different cortical areas, different time points, and different behavioural tasks within the same animal. This approach limits the number of animals needed and enables statistical within-animal comparisons (increasing statistical power). We will use the minimal number of animals per experiment that gives sufficient statistical power to detect experimental effects and make scientifically valid conclusions.

Based on our previous experiments, a typical experimental group size is about 10 mice.

Before each experiment we use systematic literature searches (e.g. with combined keyword searches in online databases such as PubMed) to review relevant previous work to ensure our experiments are necessary to answer our hypotheses.

Raw data and associated metadata, as well as analysis scripts are stored on harddrives and backed up on Network-Attached Storage (NAS) servers to avoid any loss of data and enable data reuse (data will be shared where possible). We usually start experiments in a smaller set of animals, enabling us to check the quality of the data (comparing the data to previously collected data and ensuring all relevant information for analysis is being stored correctly). This may also enable us to sometimes reduce the number of experimental conditions (e.g. if we find that effects of two pharmacological dosages are comparable).

We further optimize the number of animals in multiple ways. We maximize the yield from each animal, for example by using methods to increase the number of cells imaged simultaneously. We standardize experimental procedures to reduce measurement variability. We use statistical testing that takes into account the variability that is not related to the experimental manipulations, for example by using paired comparisons and multilevel analysis.

Experiments are planned in accordance with the PREPARE guidelines, and results will be reported in accordance with the ARRIVE guideline (to maximize the information from the research and minimize unnecessary studies).

In order to avoid unnecessary use of animals for breeding, we will closely monitor our breeding colonies (and reduce the number of breeding pairs if possible) to avoid producing more animals than needed for experiments and colony maintenance.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use mice including mice with genetic modifications. Animal suffering will be minimized by working in accordance with the 3Rs: reduction (reducing the number of animals to the minimal number required for obtaining scientifically meaningful data), replacement (only using animals if no other alternatives are available), and refinement (see details below in the section on how we refine the procedures to minimise the welfare costs for the animals).

Mice are capable of relatively complex behaviours that mirror important aspects of human and primate behaviour. Specifically, we developed visual detection and discrimination tasks that mice can reliably learn, using food rewards to train the animals. This makes it possible to use mice instead of primates. We use a head post to stably fix the head which is necessary for some neural recording methods and control of sensory input, in particular visual input. We use refined methods to enable head-fixed mice to move on a running treadmill (or sit still if they prefer). In some experiments, dependent on the neural recording method and task, we can study freely moving mice without head fixation.

Why can’t you use animals that are less sentient?

The mouse is currently a prominent model in sensory neuroscience and best suited for the proposed experiments for the following reasons. First, the anatomical and functional organization of the mouse sensory system is relatively similar to humans and primates. Second, there are unique methods available in mice that allow detailed measurement and manipulation of cortical circuits. In addition, the size of the mouse cortex is relatively small. This enables simultaneous measurement from multiple areas, which is not possible in larger mammals. Finally, mice are capable of complex behaviours that mirror important aspects of human and primate behaviour (e.g. visual detection and discrimination tasks). The combination of these factors

makes it possible to use the mouse instead of higher mammals to address our experimental questions while this is not currently feasible in lower animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To refine the animal's lives, we provide for example cage enrichment (e.g. running wheels). We house animals together where possible. We also house our mice in a reversed light-dark cycle, which means that the mice are in their active phase during experiments and undisturbed during their inactive phase. In general, we first perform experiments in a small subset of mice (typically 2-3 mice) and will refine experimental testing procedures if possible or necessary (e.g. adjust doses of pharmacological agents) before testing the other mice in that experimental condition.

We take the welfare of our animals very seriously: our mice take part in long-term experiments in which they typically learn during behavioural tests associations between sensory inputs such as specific visual objects and food rewards, and it is therefore necessary that the animals are not stressed and in good health. To reduce stress, animals are acclimatised to experiments by handling and giving food rewards. We have found that mice particularly like soy milk flavoured with strawberry taste and use this as food reward.

We use a head post to stably fix the head without causing pain to the animal (compared to directly clamping the head of an animal) which is necessary for some neural recording methods and control of sensory input. We use refined methods to enable head-fixed mice to move on a running treadmill (or sit still if they prefer). In some experiments, dependent on the neural recording method and task, we can study freely moving mice without head fixation.

Animals are monitored closely and if we observe any unexpected adverse effects we will consult specially trained staff and vets.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We design and carry out our research in accordance with the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence), the ARRIVE (Animal Research: Reporting In Vivo Experiments) and the Laboratory Animal Science Association (LASA) guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive regular updates from our animal facility staff and named information officer. I receive additional information from colleagues, and social media (e.g. the NC3Rs twitter account and newsletter). We review 3R advances with all lab members to ensure implementation. Through training and assessment and standardized protocols we further ensure best practice in our experiments.

50. Using zebrafish to understand the function of genes involved in protein clearance pathways 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

Zebrafish, Neurodegeneration, Dementia, Autophagy, protein clearance

Animal types	Life stages
Zebra fish (Danio rerio)	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

When nerve cells in the brain die, this causes dementia. This is often caused by too much protein building up in the nerve cells. We want to understand the processes that control how cells get rid of unwanted proteins as this will help us understand the causes of dementia and will help us to discover possible treatments.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There are over 900,000 people in the UK who have dementia and this number is increasing because people are living longer. At present, there are no known treatments that slow down or reduce the loss of nerve cells in the brain (which is the main cause of dementia). Our work will help to understand why proteins build up causing nerve cells to die. We will use this to understand who is at greater risk of getting dementia and also to find possible medicines that can be used to treat the disease. This work will help other scientists understand the causes of dementia so that they can diagnose the disease and also to design (small molecules which are like the active ingredients in medicines) to treat patients.

What outputs do you think you will see at the end of this project?

Our work will help to understand why proteins build up causing nerve cells to die. We will generate new scientific information that will allow scientists and doctors to understand who is at greater risk of getting dementia. In this work, we will also find compounds that reduce the number of nerve cells which die in zebrafish models of disease. These compounds can then be tested in other animal models and maybe developed to be used in patients. We will share this knowledge with other scientists and medical professionals in peer-reviewed publications and at seminars and conferences.

Where our work generates large amounts of data that is useful to other scientists, we will put this on websites which they can access for free. We will also make zebrafish with genetic alterations which may be useful to other researchers. We will share these with other research scientists.

Who or what will benefit from these outputs, and how?

In the short-term, our work will generate new data to help us understand why proteins build-up and cause nerve cells to die. We will identify new compounds which improve the disease read-outs that we observe in our zebrafish models. The next step will be for researchers to test whether these compounds work in other animal models and whether they are safe to use in man. In the longer term, researchers in pharmaceutical companies will use this information to develop these, or similar compounds and test whether they can prevent or slow the diseases which cause dementia in patients. These diseases are often referred to as neurodegeneration or neurodegenerative diseases. This term means that it is a disease where nerve cells (neurons) die.

We are particularly interested in how cells of the immune systems (the cells in the body which normally fight disease) may play different roles in neurodegeneration. There is already evidence that sometimes they might make the disease better and sometimes they might make it worse. In the short- term, our work will help scientists understand how immune cells might make neurodegeneration worse or better. In the longer term, this will help doctors know when to treat patients with different medicines so that they can be sure that the treatments are effective and will not accelerate or worsen the disease.

There are many different factors that increase or decrease a person's likelihood of developing dementia. The genetic work that we do will help identify the genetic

factors which alter someone's risk of developing these diseases. In the long term, this genetic information will help doctors tailor the correct treatment to the patient.

How will you look to maximise the outputs of this work?

We will publish our work in free-to-view journals and will present our findings at seminars and conferences. We will ensure that our work will reach a wider audience as we will also talk to patient groups and schools and give media interviews.

Where we have tested compounds and they are not successful or if genetic alterations do not cause the expected changes in our disease models, we will share our results with the research community to prevent others from adopting these approaches.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 46,600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will use zebrafish. To study neurodegeneration and identify potential treatments, we need to study this in a vertebrate animal with all the cell type that are present in man. We estimate that we will use approximately 46,600 zebrafish over the 5 years of this project (approximately 9,320 per year). Most of the animals used in this project will be embryos or larvae (juveniles). We use this stage of animals as this allows us to perform protocols of lower severity than at older stages. It also allows us to use microscopes to visualise disease changes without the need to perform invasive procedures.

Typically, what will be done to an animal used in your project?

Most of the procedures carried out in this work are unlikely to cause any pain or suffering (these are termed “mild” procedures). There are some procedures which involve the use of genetically altered strains of fish which develop the early stages of disease (termed “moderate” procedures). The experiments on these fish are typically performed on unlicenced stages (i.e. within the first 5 days after fertilisation) but there will be some occasions where we need to observe them for longer. The second type of moderate procedure is used to investigate whether drugs (compounds which are like medicines) or genetic alterations affect the progression of the disease. In some cases, the drug or genetic alteration might make the disease worse. Drugs are typically administered by adding them to the water in which the fish live. Any animals showing a worsening of the disease will be culled by a humane method. For drug treatments, we find out the amount of drug that it is safe to give without causing harmful effects using unlicenced stages. Occasionally, when treating older stages, some will show signs of toxicity, such as increased heart rate or failure to swim in the

correct position. We expect 90% of these experiments will be performed at larval/juvenile stages (up to approximately 20 days post-fertilisation, d.p.f.). Fish will be killed by a humane method as soon as any toxic effect is observed.

When zebrafish larvae are being reared to adulthood, food is given from 5 d.p.f. Zebrafish eggs are laid with a supply of yolk and this lasts for longer than 5 days, but food is provided from 5 d.p.f. to help the larvae transition to eating. For some experiments where we use larvae from 5 d.p.f. – 10 d.p.f., we will not give food. Previous work has shown that larvae are viable and develop normally without food to 10 d.p.f. It is necessary to withhold food in some of our studies of disease, where feeding could cause size and growth differences and would therefore introduce bias. In addition, we will not provide food in drug treatment studies on larvae between 5 - 10 d.p.f. For such studies, larvae are typically kept small dishes with between 1 - 10 ml of liquid. Food is not given for the duration of the experiment (i.e. for up to 5 days) as it would cause a build-up of waste in the liquid which would affect the water quality and therefore affect survival. Importantly, larvae will be culled at the end of the period for which food has been withheld (at no later than 10 d.p.f.).

All animals will be humanely killed at the end of procedures.

What are the expected impacts and/or adverse effects for the animals during your project?

Since we are investigating neurodegenerative disease, in some cases, the animals will develop the early signs of disease. We expect these signs to be changes in body shape, neurodegeneration, behavioural changes. For mild procedures, fish will be killed by a humane method at the first signs of these disease changes. For moderate procedures, fish will be killed at the point when any of the clinical signs listed above result in a failure to thrive (e.g. the defects observed reach a point that they affect swimming or feeding ability).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Zebrafish; Mild procedures: 61 %
Zebrafish; Moderate procedures: 39%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To investigate dementia, the disease must be studied in neurons surrounded by the other cell types that the brain needs to function normally. These studies need to be performed in a vertebrate animal so that the animal model has all the cell types and connections that are present in the human brain. We need to study possible treatments in living animals so that we can be sure that the drugs reach the blood circulation and make it into the brain.

Which non-animal alternatives did you consider for use in this project?

The majority of the work in our lab is performed in vitro (i.e. typically about 20 scientists work on cells grown in incubators whereas 6 scientists work on animal models). In our previous project licence, we replaced compound testing in zebrafish, instead performing these experiments on cells grown in the lab. The work in this new PPL application will focus on the validation of targets that have already undergone extensive validation in cells grown in the lab. These cells are often grown in dishes where every cell is identical (these are called cell lines). These have benefits because they are all identical and all behave the same way, but they are quite different from neurons. Therefore, our group have established technologies to make and grow neurons in the lab (these are made from cells which are capable of turning into every different cell type in the body) and have developed collaborations to perform studies in mixtures of cells which can be grown in the lab in small clumps (organoids). These organoids contain lots of different cell types and so are more similar to the tissues in our bodies.

Scientists hope that these organoids can be used to replace some animal studies and so we are investigating this in our lab.

Why were they not suitable?

Many of these approaches have been suitable. In our previous project licence, we used 20-25,000 less animals than anticipated by replacing zebrafish assays with cell-based assays. However, we still need to perform some animal studies. When we carry out experiments in cells, we can see whether they are alive or dead and whether proteins build up or not. However, we also need to test genetic alterations and compound treatments in living animals to see if they are able to prevent or slow disease. We need to check whether they change the speed at which disease symptoms appear and whether they can stop the severe parts of the disease from happening. We cannot measure these things in cells.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of animals for collection of gametes or for the generation, breeding and maintenance and generation of genetically altered (GA) lines is based on the different GA lines that are needed to achieve the project aims. We have 20 years of experience of using zebrafish and know how many adult animals we will need to maintain for each GA line in order to generate the number of experimental offspring for these experiments.

Although the aims of this project licence are different from those of our previous one, many of the techniques that we will use are ones that we have used previously and have been optimised to use the smallest number of animals to achieve meaningful and statistically significant results. In our previous licence, we used approximately 20,000 less animals than we originally anticipated as we were able to replace animal experiments with cell experiments. We have taken this into account in planning the number of animals needed in this application.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where appropriate, we have used the NC3R's Experimental Design Assistant to design experiments. We regularly perform analysis on both living animals and on their tissue after they are humanely killed to reduce the number of animals that are used. For example, the brain can be imaged using fluorescent microscopy in living animals and/or behavioural analysis can be performed. We can then look at markers of disease in tissue samples from the same animals after they have been humanely killed.

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 control groups in all of our experiments. These are animals that are normal and do not undergo any procedures or experimental steps, so that they are the normal animals that we compare our experimental animals with. The control group are siblings from the same batch of fertilised eggs as the experimental group. Experimental groups are blinded for analysis. Pilot studies are performed for new analysis methods to determine the effect size (this is how big the changes are that we expect to see in our experiments). Once we know the effect size, we can work out how many animals to use in an experiment (the sample size). This allows us to work out how many animals we will need to use to be able to see whether the disease has been improved or rescued.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically altered (GA) zebrafish for this project. Zebrafish are small tropical fish that have many advantages as an animal model for this work. For example, one pair of adults produces 100-200 fertilised eggs per breeding and young fish are transparent allowing internal organs such as the brain to be seen without surgery. Also, there is a high level of similarity between the genes and tissues in man and other vertebrates. We have carefully developed genetically modified zebrafish which have aspects of human disease but in which we have limited the severity of the disease, e.g. by expressing the disease-causing gene in only one cell type in the eye (in the rod cells, which are light receptors in the eye). When these cells degenerate, only one type of cell dies (causing night blindness) whereas if this disease-causing gene were expressed throughout the brain, the animal would have ill health and reduced lifespan. We also use a special technique so that parent lines do not express disease- causing proteins and do not have any signs of disease. The disease protein is only expressed when two fish with different genetic alterations are mated together. Following mating, only the offspring produce the disease protein and show disease pathology. Using such lines, we only generate offspring for experimental purposes and the adult animals that are kept to maintain a breeding colony are viable and healthy, with no sign of disease. Many of our existing GA lines and any new transgenic lines that are generated in this project will carry a fluorescent marker in a cell type that is not needed for our work (e.g. green fluorescent protein expressed in the pancreas or red fluorescent protein expressed in the heart). This allows us to identify the GA fish by fluorescence microscopy in 1- or 2-day old embryos and means that we do not need to take tissue biopsies from adults to identify the fish carrying the genetic alteration.

Why can’t you use animals that are less sentient?

We have refined many of our experiments to use GA zebrafish where the disease manifests at unlicenced stages (i.e. prior to 5 days post-fertilisation). We will use these models in preference to those that require us to perform licenced procedures in all instances where they allow us to meet our scientific aims.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We use record sheets to monitor animals and regularly assess whether these need to be modified to see whether we should increase or alter the monitoring intervals to ensure we identify any adverse events at the earliest opportunity.

Zebrafish are a social species - we have refined our protocols to reduce the number of fish which are kept in single tanks (for example, when identifying transgenic founders) by housing these with wildtype fish with a different pigmentation pattern (e.g. if transgenic fish is “stripy”, it can be housed with “spotty” wildtype fish).

Our animal facility staff are testing different types of enrichment (such as using plastic plants and artificial grass) to encourage females to breed and to improve husbandry.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We use the following guidelines:

ARRIVE guidelines for reporting of your experimental data, https://arriveguidelines.org/,

NC3Rs for zebrafish welfare https://www.nc3rs.org.uk/3rs-resources/zebrafish-welfare and reproducibility https://www.nc3rs.org.uk/3rs-resources/zebrafish-welfare.

PREPARE guidelines, https://norecopa.no/prepare

NORECOPA guidance on classification of severity of procedures (10.1258/la.2011.010181) FELASA guidance on pain management in zebrafish (https://doi.org/10.1177/00236772231198733)

and are awaiting the publications from FELASA working groups on "Severity classification in zebrafish and their larvae",

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive regular emails from the Named Information Officer at our institution. These contain details about advances in the 3Rs and are sent to all personal licence holders. In addition, we have regular meetings with our animal care technicians and vet who are part of additional information networks. We share best practice with other zebrafish research groups within our institution and with the wider zebrafish community using researcher forums such as ZFIN - the zebrafish information network (www.zfin.org) and ZHA - zebrafish husbandry association (https://zhaonline.org/). We discuss any advances at our weekly lab meetings and implement trials of improved husbandry and new experimental methods, where appropriate.

51. Therapeutic interventions in inflammatory kidney 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
  - 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

kidney, inflammation, therapy, autoimmunity

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Inflammatory kidney diseases are a common cause of kidney failure, ultimately necessitating kidney replacement treatments with dialysis or transplantation. It would be preferable to be able to treat these conditions before they reach this severe endpoint. Current therapies are significantly limited by toxicities. As a result of better understanding of the basis of inflammatory kidney diseases we are in the position to test new ways of treating them using novel agents or repurposed medicines. This project seeks to test new means of alleviating such kidney diseases with better more targeted strategies that may be associated with fewer adverse effects.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite much work investigating the nature of inflammatory kidney diseases we have made little progress translating this to new therapies for patients. One example however stands out, which is the recent introduction of Avacopan, a drug that blocks the inflammatory complement pathway, for the treatment of Anti neutrophil antibody associated vasculitis, a common cause of kidney disease, which was as a direct result of animal work demonstrating its effectiveness in a mouse model of disease. We have, as a community of clinicians, relied on a limited number of drugs for treatment of these diseases

- namely steroids and chemotherapeutic drugs such as cyclophosphamide, which are associated with significant side effects and may now account for a greater mortality to patients than the diseases they are used to treat.

What outputs do you think you will see at the end of this project?

We will have shown in pre-clinical experimental models potential new therapeutic strategies that could be translated into changes in clinical practice. For example, we are testing new sugar-based compounds (glycopolymers) that target an important molecule on white blood cells, called mannose receptor, already shown in knock out mice given experimental nephrotoxic nephritis, to be critical in mediating kidney inflammation. These glycoploymers would be readily upscaled for human use. Other examples would include novel complement inhibitors that can act locally in the kidney and block the impact of complement activation at the sites where they are deposited. Complement mediated damage or amplification of damage is now understood to underly a number of the inflammatory kidney conditions, and therefore such therapeutics, would be ideal for those conditions that are mediated by, or aggravated by, complement activation. Finally, we have understood more about the white blood cells that are mediating the damage - and the molecules they utilise, such as myeloperoxidase. We have shown previously that myeloperoxidase inhibition in our model can attenuate disease, we wish to test if it also attenuates the cardiovascular disease that accompanies kidney damage. This would be of benefit as current treatments with steroids, actually aggravate atherosclerosis and increase the patients' risk of cardiovascular events.

We would anticipate potential new patents, products to be brought to the clinical arena and clinical trials in inflammatory kidney diseases being initiated after we publish our findings.

Who or what will benefit from these outputs, and how?

Patients with inflammatory kidney disease who currently have limited therapeutic options- based on steroids and one or two immunosuppressants, all of which are non-specific, and are associated with significant adverse effects, would potentially benefit from novel strategies especially if they were less immunosuppressive, or

worked on synergistic pathways, allowing lower less toxic doses of treatment to be used. Inflammatory kidney diseases are one of the top three causes of end stage kidney disease and inflammation is a significant contributor to progressive chronic kidney disease, meaning that methods of inhibiting the inflammation without additional risk would be of significant clinical benefit to the kidney community. We have already patented the mannose receptor inhibition as a potential therapeutic and successful pre-clinical work in our mouse models would enable us to pursue this as a new drug for testing in patients within a 5 year period.

How will you look to maximise the outputs of this work?

We are always looking at publishing our work which is an essential part of academic medicine, however, we are also keen to translate our work for real patient benefit by partnering with biomedical or pharmaceutical companies or considering spinout companies that can progress the pre-clinical to clinical arena.

Species and numbers of animals expected to be used.

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will only use adults and these will only be mice. The adult mice have a fully developed kidney that has similar physiology to humans, reflecting the complexity that is not seen in lower sentient animals such as fish. In addition, rodents have readily inducible kidney disease that resemble the human conditions, and that their immune responses are similar to humans. These species are sufficiently close to human physiology, but yet sufficiently far down the sentient scale, to allow relevant conclusions to be drawn regarding what may be happening in patients with varied forms of kidney inflammation, while still avoiding use of higher animals such as non-human primates. We have extensive experience in using these animals for these renal models and so this is building on already established methodology. The explosion in the availability of genetically modified mice now allows us to dissect in detail disease mechanisms that were hitherto impossible. For example we can test ApoE knockout mice, which are susceptible to accelerated atherosclerosis, to allow us to investigate the impact of our therapeutics on the combined cardio-renal outcomes that affect our patients, who suffer from accelerated cardiovascular disease related to kidney impairment and its treatment.

Typically, what will be done to an animal used in your project?

Animals will have kidney inflammation induced through a variety of methods- nephrotoxic serum, drugs or other nephrotoxins. These will be introduced through injection (intravenous or intraperitoneal). The experiments last a variable amount of time depending on the induction agent and the outcome measure. In some cases

between 3-12 days for short term models of nephritis, whilst in others looking at cardiovascular outcomes (specifically finding alternatives to steroids that can reduce both renal and cardiac damage) they may last up to 12 weeks, which is sufficient time for atherosclerosis to develop. However, in these cases the kidney injury is typically less severe. Following induction of nephritis animal cohorts will have a variety of therapeutic interventions, such as conventional corticosteroids- (administered in the diet, by gavage or through injection), and this will be compared with novel agents such as myeloperoxidase inhibitors (delivered in the same fashion) or mannose receptor binding glycopolymers. Further experiments with novel agents may also be planned depending on their availability/promise following in vitro work. During the experiments the animals may have blood sampling (rarely and only if required for a specific experimental question) but all will have urine sampling at least once – collected while in metabolic cages. Overall, we expect to use 1000 or less animals in a year including breeding colonies.

What are the expected impacts and/or adverse effects for the animals during your project?

The development of kidney damage is generally without symptoms. The models we use do not induce severe kidney failure but modest kidney inflammation, which does not induce symptoms and therefore is not noticeable to the animals. Rarely there may be some animals with more severe kidney injury(if for example they started with less kidney function for whatever reason), and they may develop outward manifestations such as fluid retention, hunched appearance and raised fur. Transient symptoms of hunching around the time of disease induction may also rarely occur, due to the nature of the nephrotoxic serum, but these generally last only a few hours. The induction of various forms of kidney inflammation requires administration of substances by injection, but these are performed by experienced operators and suffering is kept to a minimum. At the end of the protocol animals are humanely killed and the extent of their kidney inflammation assessed by histology, biochemistry and immune monitoring.

Expected severity categories and the 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 induction of nephritis is generally only moderate in severity and this will be for the majority of animals used experimentally (40% of total animals used), with the exception of those which are kept for breeding only to maintain colonies, for which there is only mild (10%) or subthrehold severity (remaining 50%). The current transgenic breeding colonies we use show no harmful phenotype.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The complexities of resolving inflammation in a kidney are not easily modelled in vitro and we cannot always be certain that all the relevant mediators are present in such systems, whereas in vivo all the possible inflammatory circuits can be active. In addition, we have carried out extensive in vitro testing of potential new anti-inflammatory compounds, but now need to understand if they also work in a more complex in vivo setting before proceeding to plan human clinical trials.

We have therefore refined our use of mice to model various aspects of renal inflammation that can not be readily modelled, in vitro.

Which non-animal alternatives did you consider for use in this project?

We have used a number of in vitro models to examine if the pathways and the molecules we are interested in are active - for example in vitro models of kidney tubular injury, induced by lack of oxygen and nutrient deprivation followed by normal levels of both oxygen and nutrients, mimics ischaemia- reperfusion injury induced by blocking a blood supply to the kidney, for example in kidney transplantation or following renal artery occlusion. In addition, we have tested our mannose receptor and myeloperoxidase inhibitors in cell based assays modelling white-blood cell activation and their interactions with blood vessels (as may be found in patients with severe forms of kidney inflammation- called rapidly progressive glomerulonephritis). We have therefore gone almost as far as we can with our in vitro models but need evidence that in vivo these therapeutic strategies may be efficacious.

Why were they not suitable?

They were suitable but as stated above they are limited to perhaps a two or three cell co culture system which cannot adequately model the in vivo situation. There are complexities of in vivo immune regulation which can only assessed in a whole live animal. Therefore we feel that we had performed a number of in vitro validations of our pathways and molecules of interest but need to show translation to a preclinical system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This has been based on the last two years of animal work and our projection of what we will do each year for the next five years. Overall, we are doing less animal work

and more in vitro work but as previously stated we need to provide preclinical proof of effect but this will be in a limited number of animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have significant experience in performing these models. We have taken into account the variability inherent to each model and considered group sizes that would be sufficient to find differences in outcome. For example the nephrotic nephritis model tends to have some 15% of animals as outliers with less severe disease, meaning that we have to use 8-10 mice per group to achieve statistical differences. Experimental design (helped by use of the experimental design assistant -EDA) will make use of previous results to calculate sample group sizes and ensure a higher likelihood of successful differentiation between control and experimental groups. We minimise variability by using animals of similar age and gender, from similar breeding groups, or litter mates in the cases of genetically altered animals, where possible. We randomly allocate animals to experimental groups, to prevent bias, and where possible two experimenters are involved to allow us to treat animals in the respective groups and collect /analyse the data without being aware of the allocated group, by blinding samples. Such an approach for example allows us to score histological changes in kidney sections, by blinding the sections and having two individuals score the sections, followed by an averaging of the individual scores.

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 attempt to maximise the use of control groups by carrying out as many of the various experimental groups within the same experiment, reducing the need to replicate controls with each variable. Within the groups, experiments are planned to maximise the information obtained from each animal, by ensuring as many of the potential assays/outcomes that are possible to be performed are carried out at the end of the experiment. To this end we collect blood, to isolate serum, urine, and renal tissue for RNA extraction and immunohistochemistry, as well as renal draining lymph nodes and /or spleens for isolation of leucocytes for in vitro assays. In special circumstances, if the outcome measures warrant it, we isolate bone marrow from animals within the experimental groups. Therefore, the animals are utilised to their maximal potential. We will use robust statistical methods to compare groups, (two groups Mann Whitney U test, three or more groups, one way ANOVA test).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use models of inflammatory kidney diseases, mediated by injection of antibodies, drugs or toxins with or without dietary manipulation. The animals do not suffer with development of kidney disease which is silent.

Why can't you use animals that are less sentient?

Adult rodents have similar kidney physiology, structure and immunology to humans and therefore represent the best animal group to perform these experiments in. Lower sentient animals may lack complex kidney structures or have vastly different physiology making them less suitable. We have already performed a lot of work in vitro to avoid excessive animal use.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have been performing similar experiments for a number of years and have established a simple set of procedures that minimises distress or harm to the animals and yet provides us with the required experimental conditions and allows us to perform high quality modelling of human diseases. Newer techniques that may become available will always be incorporated into our protocols if they result in better animal welfare. Animal suffering is kept to a minimum through use of these tried and tested protocols that are humane and known to be well tolerated, while careful monitoring of the animals and use of modern animal husbandry methodology allows us to maintain the animals in a comfortable environment. Limits are carefully observed and animals that appear to be in distress are identified early and humanely killed. We are not using any severe protocols.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow our local Biological Services guidelines for conducting all of our animal work. Importantly, we have access to NVS, NACWOs and animal technicians with clear procedures in place in the case of animal concerns. We regularly provide updated protocols for each experiment which is reviewed by the NACWOS and for which we are provided with feedback if required. We are constantly looking at ways to improve our experimental models to ensure that we are minimising any harm to the animals. All animals are routinely monitored for adverse effects, analgesics and anaesthetics will be used where appropriate on advice of the NVS.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will be regularly updated by our institutional 3Rs newsfeed- supplied by the Biological services unit as well as the interactions we have with the NACWOS at our animal facility. Additionally, we will make use of the NC3R website and information from AWERB.

52. Ecology and management of badgers in human-dominated landscapes

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

  - Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Bovine tuberculosis, Forestry, Zoo, Wildlife health, Animal health

Animal types	Life stages
European badger	adult, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To develop sustainable ways for people in the UK to coexist with European badgers.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

As the UK's largest remaining terrestrial carnivore, European badgers play an important ecological role, even in ecosystems which are highly managed by people.

However, there are challenges to living with badgers. Ideal badger habitat is a mosaic of pasture (where they forage for earthworms and other invertebrate prey), and woodland (where they prefer to dig their setts – large, complex dens with many interlinking underground tunnels). In cattle pasture, badgers can become infected with *Mycobacterium bovis*, the pathogen which causes bovine tuberculosis (bTB), and can transmit the infection to one another and back to cattle, helping to maintain a disease which has a major economic and social impact in the UK's farming communities. In woodland managed for forestry, operating heavy machinery close to badger setts (where the ground may be unstable due to underground tunnels) risks damaging both the machinery and the sett. Badgers accustomed to entering gardens and farm buildings may also enter zoo premises, where they can potentially transmit bTB to zoo animals just as they transmit to cattle. All of these challenges require humane, effective, and sustainable management tools. Badger culling has been widely used to try to limit bTB transmission, and could potentially address other challenges to living with badgers, but nonlethal approaches would be more sustainable. This project aims to explore two such approaches. First, it evaluates badger vaccination (Objective 1) as a nonlethal way to limit bTB transmission to protect farm and zoo animals. Second, it uses high- resolution above- and below-ground tracking of badgers' movement behaviour on farms (Objective 2), in zoos (Objective 3), and in managed forests (Objective 4) to pinpoint high-risk areas for bTB transmission (e.g., water troughs, feed stores) or forest machinery damage (e.g., land within 20m of a visible sett entrances), and identify ways to optimise coexistence with badgers.

What outputs do you think you will see at the end of this project?

We expect this project to generate new evidence to help farmers, foresters, and zoo managers to coexist successfully with badgers. Specifically, by the end of the project we expect to have:

New information on the extent to which vaccination reduces the numbers of badgers testing positive for bovine tuberculosis (bTB), which can be used to inform policy development for badger management. In particular, building on previous work on trends in *M. bovis* infection prevalence on land not exposed to culling, this project will expand onto land where badgers were recently culled, helping to inform policies which envision a transition from culling to vaccination as part of the government's 25-year bTB eradication strategy.

New information on where in the farm environment badgers and cattle may infect one another with bovine tuberculosis, which can be used to improve farm management guidelines. This work builds upon our previous work showing that most infectious contact between badgers and cattle is likely to occur in cattle pasture, and should help to provide more refined biosecurity advice to farmers.

New information on whether, and how, badgers may use the environment in zoos, which can be used to develop zoo management guidelines. This work extends our previous work characterising badger contact with cattle to a new (but similar) environment, and should ultimately help zoos to balance their statutory requirements to both educate the public about wildlife conservation and protect the animals in their care from disease.

New information on how forestry operations impact (and are impacted by) badgers, which will be used to inform new guidance to foresters. This work mobilises new technologies to measure impacts on badgers underground which previously could only be estimated based on expert judgement.

Who or what will benefit from these outputs, and how?

These outputs should benefit farmers, foresters, and zoo managers, all of whom should ultimately find their work less impacted by badgers. The outputs should also benefit policymakers, by providing information which can be used to inform the development of policies, which currently envision a transition from badger culling to vaccination over the coming years. Additionally, they should benefit badger populations, which are currently subjected to large-scale culling for bTB control; more successful coexistence with people and human activities should mean less badger culling. A reduction or cessation of badger culling should have broader conservation benefits (as badgers play an important role in the UK's ecological communities). Furthermore, improved guidelines to foresters should lead to less disturbance of badger setts in woodland.

How will you look to maximise the outputs of this work?

This work will be published initially in the peer-reviewed scientific literature and then disseminated using print, broadcast, and social media. Evidence relating to bTB management will be shared with Defra and the National Farmers Union to maximise its impact on policy and practice. Evidence relating to zoo management will be shared through zoo networks such as the European Association of Zoos and Aquaria. Evidence relating to forestry will be shared with Forestry England and Forestry & Land Scotland.

Species and numbers of animals expected to be used.

- : 2590

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Because this project concerns the coexistence of people and badgers, it can only be conducted using free-roaming wild badgers living in environments dominated by human activities (farms, zoos, managed forests).

Most of the work will only involve adult badgers. But, our work on vaccinating badgers against bovine tuberculosis also includes cubs, because animals (like people) are best vaccinated when they are young, before they have had the opportunity to encounter the bacteria which cause bovine tuberculosis.

Typically, what will be done to an animal used in your project?
Traps are set in the late afternoon and checked at first light the next morning, to minimise the time each badger spends confined in a trap. A scientist will quietly approach any trapped badger and inject it with an anesthetic, so that it starts to become sleepy. Once the anaesthetic has taken effect, the badger will be taken out of its trap, laid on a blanket, and quickly checked to see roughly how old it is, whether it is a male or female, and whether it is reproductively active (e.g., lactating). It will be scanned to check whether it has a microchip and, if no microchip is detected, one will be injected just under the skin, just as it would be for a dog or cat.

At the same time, the black tips of the hairs on part of its back will be snipped off with scissors, exposing the white underfur and making a temporary mark which will disappear as soon it moults. This fur clip helps to avoid accidental re-use of the same animal as, if the animal is captured again, scientists can recognise it as a badger which has been captured recently, and immediately release it.

Our project mostly works in parts of Britain where badgers risk catching bovine tuberculosis. In these areas, the badger will be given an injection of vaccine, designed to protect it from this disease. A small sample of blood will also be collected from one of its veins, to help us test if the vaccines are working. If it has any wounds or abscesses, these will be sampled with a sterile swab (which looks like a cotton bud) to check if they are infected with bovine tuberculosis bacteria. Finally, another swab will be inserted a short distance inside the badger's anus to collect some faeces, again to test for the same bacteria.

For some adult badgers, a tracking collar will now be fitted. This collar allows the team to track very precisely where the animal goes and how it behaves. This means we can identify places (such as troughs) where badgers might encounter cattle or zoo animals, allowing bTB to be transmitted between species. We can even track badgers underground so we can see whether and how they respond when forestry operations are in progress nearby. The collars have been carefully designed to make them as light, compact, and comfortable as possible for the badger, and we fit them very carefully to make sure they are not too tight, and will not become too tight if the badgers gains fat. The collars are not designed to drop off, because drop-off mechanisms need extra batteries and make the collars too heavy.

The badger is then returned to the same trap where it was caught, where it can recover from the general anaesthetic. Typically it takes less than an hour for them to be back on their feet and ready to be released. As badgers are often caught close to the setts (underground dens) where they live, they usually run straight back down one of their burrows as soon as the cage is opened.

Where we are studying the effects of badger vaccination, all of this will be repeated once a year. Many badgers which were caught in one year are recaptured the following year; they will be recognised from their microchips, and vaccinated and sampled again.

If a badger was collared, a few months later traps will again be placed in the area where it lives. When it is captured, it will be anaesthetised in the just the same way,

so that its collar can be removed. On this second capture, it will be sampled again before it is released. No badger will be collared more than twice. In this way, we have previously managed to retrieve approximately three-quarters of the collars we fit. Since, in undisturbed badgers populations, about one-quarter of adult badgers die each year, recapturing about three-quarters of collared badgers suggest that few badgers are left wearing collars for longer than planned. Occasionally, we have re-trapped a badger several years after it was originally collared; in these cases we have seen no signs of collar injury.

What are the expected impacts and/or adverse effects for the animals during your project?

Probably the most unpleasant part of this procedure for the badger is being confined in a cage trap - this is likely to be frightening and stressful for a wild animal. Badgers' natural response to danger is to try to dig, and when they try to do this inside a metal cage, they can scrape their paws and foreheads. Most badgers show no sign of such injury, however, and those which are injured are likely to heal within very few days.

Vaccination can cause a small swelling at the injection site, which in captivity lasted up to 48 days. This small swelling appears not to be uncomfortable for the badger and there is no sign that they rub or scratch at it.

Places where needles penetrated the badger's skin to give it anaesthetic, vaccine, and a microchip may remain sore for a day or two, but they are unlikely to be very painful and any discomfort should end rapidly.

Tracking collars have the ability to cause harm if they are fitted too tightly, or are too heavy, bulky, or wide. For this reason we are extremely careful to make sure that we use only light, compact, narrow collars which are just tight enough that the badger cannot pull them off, but no tighter. We have fitted many such collars in previous projects without causing any injury, and we have never seen a badger scratching or rubbing at its collar.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect all animals to experience only mild severity.

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project is about whether, and how, badgers can coexist with human activities such as farming, forestry, and zoo management. The topics are important and serious, as they impact farmers' livelihoods, zoos' ability to protect animals in their care, and the safety and efficiency of forestry work. As a result, people feel very strongly about the answers to our research questions, and they deserve scientific results which have been tested in the real world. This means that our research can only be done with truly wild badgers living on real-world farms, zoos, and managed forests.

Which non-animal alternatives did you consider for use in this project?

Ma models have been used to predict the answers to some of the research questions we are asking.

Why were they not suitable?

Although mathematical models have been used to predict the answers to some of the research questions we are asking, their predictions are not reliable enough to inform management.

Policymakers, veterinarians, farmers, zookeepers, and foresters need to be able to make decisions based on data collected in the real world, and that means working with real animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of badgers that we will use in two ways. First, we estimated how many badgers we would need to capture to answer each of our research questions, and added them together. Second, we considered how many farm, zoo, and forestry settings we would need to study for farmers, zoo managers, and foresters to feel confident that our answers to research questions could be applied to many settings, and would not just represent the specific few places that we worked. Fortunately, both of these approaches gave us the same approximate answer.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We designed our studies of badger behaviour to gain as much information as possible from each collared badger, especially by comparing badgers across the different types of setting (farm, zoo, forest) to help make sense of our results. For our study of badger vaccination, we used statistical power calculation to estimate the number of badgers that would need to be blood sampled in order to detect a conservative reduction in the proportion of badgers testing positive for bovine tuberculosis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have based our study design on pilot studies conduted under previous licences. We have also sought to optimise animal numbers by allowing collared animals to contribute to studies of vaccination (and vice versa) and by allowing animals collared in one setting to act as comparison groups for those in other settings.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are using wild badgers - the animal of management concern - rather than animal models. We have chosen to use wild badgers because they allow us to monitor the effects of real-world management, to inform management guidance and policy.

Why can’t you use animals that are less sentient?

We cannot use animals less sentient than badgers, because the management problems which we aim to address concern the unique behaviour and ecology of badgers specifically.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We seek to continually refine our procedures to minimise welfare costs to animals. Through two previous licences wer have refined our trapping and sampling procedures to a point where further refinements may not be possible. However, new collar technologies have become available since we last deployed collars on badgers, and we plan to exploit these new technologies to collect more data with less impact on animals. We plan to build a small number of collars at first, and then refine their design in the light of experience with deploying them.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All of our badger trapping is conducted in accordance with government best practice: Natural England (2021) Cage-trapping and marking of badgers under licence (to enable vaccination) to prevent the spread of bovine TB - Best practice guide. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/998 280/cage-trapping-for-badger-vaccination.pdf

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As a project, we seek to continually improve our treatment of animals. We will seek to stay informed about relevant advances through (i) networking with colleagues working in the same or related field; (ii) the AWERB, NVS, Home office Liaison Officer, etc, at our parent institution; (iii) engagement with agenceies such as APHA, Natural England, Forest Research, etc.

53. Mammalian Erythrocyte Micronucleus Test

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

- 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

Erythrocyte, Micronuclei, Genotoxic, DNA, Reticulocyte

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to conduct the Mammalian Erythrocyte Micronucleus Test (micronucleus test) using either rats or mice so that it can be performed in accordance with regulatory guidelines to understand whether or not test chemicals can cause damage to DNA and are said to be genotoxic.

The micronucleus test can be conducted using the bone marrow method or peripheral blood method.

Potential benefits likely to derive from the project, for example how science

might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Conducting this test will assess whether test chemicals can cause damage to DNA. Assessing the risk of DNA damage is important as it could potentially lead to very serious illnesses such as cancer. This test will therefore help to assess if a chemical is considered safe for use in humans and/or exposure to the environment.

What outputs do you think you will see at the end of this project?

There are two outputs for this project:
The first is to determine a maximum tolerated dose (MTD) for a test chemical by conducting a small dose range finding (DRF) study from which appropriate and scientifically relevant dose levels for the micronucleus test can be set. The MTD is defined as the highest dose that will be tolerated without evidence of study-limiting toxicity, relative to the duration of the study, but not death or evidence of pain, suffering or distress necessitating humane killing.

The second will be evidence of whether a test chemical is able to cause damage to the DNA of stem cells in the bone marrow which mature in to red blood cells and an understanding of whether this could occur in humans. This knowledge is gained by carrying out the micronucleus test using at least 3 dose levels covering a range from the pre-determined highest dose (MTD) down to a low dose in which little or no toxicity will be observed.

This test design also allows for another genetic toxicity investigation to be conducted in the same animals as used in the micronucleus test, which means two markers of DNA damage can be obtained from the conduct of one test.

Who or what will benefit from these outputs, and how?

The conduct of this test is a requirement by law and all new test chemicals must be tested to ensure that they are not toxic to DNA and are safe to be taken by humans in the case of new medicines, or consumed in food for example food additives and flavours or not toxic to the environment, in the case of weed killers and fertilizers. Short-term, conducting the micronucleus test enables an accurate assessment of whether test chemicals have the potential to cause DNA damage in a living animal and whether it could potentially cause the same effects in humans.

Long-term, if a test chemical is found to cause DNA damage, it may not be given approval to be sold and its development halted. This is to protect humans and the environment from test chemicals which are potentially harmful to human health, which may lead to the development of cancer, or be highly toxic in ecosystems.
No testing will be conducted on cosmetic products or chemicals that are exclusively intended to be used as ingredients in cosmetic products.

How will you look to maximise the outputs of this work?

As this test will be conducted as a service, the ownership of the data will belong to the Study Sponsor. Data generated will be sent back to the Study Sponsors by way of a formal study report, which they will use as part of their regulatory package of work to be submitted to the regulatory authorities. Regulatory bodies will then make a decision as to whether the test chemical is safe for approval for use.

Any work that may be conducted on non-Sponsor owned test chemicals will be shared with the wider scientific community through publication.

Species and numbers of animals expected to be used

- Mice: 2400
- Rats: 7200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rats and mice have been used historically in safety evaluations and genotoxicity studies and are recommended by regulatory agencies.

Regulatory guidelines specify that rats and mice need to be healthy young adults aged between 6 and 10 weeks old at start the of dosing, though slightly older animals are also acceptable.

Typically, what will be done to an animal used in your project?

In the DRF phase, small groups of animals will be treated once daily for 2 days, with a test chemical in order to establish the highest dose to be used in the micronucleus test. An alternative dosing regimen is twice daily, also referred to as BID (*bis in die* meaning twice a day) for 2 days which is administered as a split daily dose with an interval of several hours in between to facilitate larger volumes. The test chemical will be given either orally or by injection in to the blood stream. Blood samples may also be taken from these animals to measure, for example, the amount of chemical in the blood. Animals will be killed humanely at the end of the DRF phase.

In the micronucleus test, animals will be treated, using the same route of administration as used in the DRF phase, with the test chemical once daily (or twice if BID) for 2 days. At the end of the study animals will be killed humanely and the femur or blood taken for analysis.

On occasions, a third dose on Day 3, 2-6 hours prior to humane killing may be required if additional genetic toxicity endpoints are assessed.

What are the expected impacts and/or adverse effects for the animals during

your project?
It is expected that up to 60% of animals in the DRF phase will experience adverse effects following dosing. These could include changes to breathing (increased or decreased), body weight loss (up to 19%), occasional changes in normal appearance (e.g. fur may stand up, animals may have discharge from the eyes and nose, or animals may hunch up), changes in normal behaviour (e.g. animals may not interact with their cage mates) and moderate pain or discomfort.

Animals may be restrained for the purpose of dosing or blood sampling into/from the tail vein, which may cause momentary distress. Minor pain or discomfort may be felt when the blood is sampled or test chemical injected.

In the main test approximately 80% of animals should not experience any adverse effects, but some in the highest dose group may experience, at worst, moderate adverse effects for a short duration as described above. Any adverse effects will be closely monitored and should not exceed a moderate degree of suffering.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

mild: 60%
moderate: 40%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is necessary to carry out the micronucleus test in animals in order to fully assess the true potential of a test chemical to cause DNA damage before giving it to humans or exposing it to the environment. It is only when a test chemical is administered to an animal that it is possible to assess how that test chemical is altered by a living body and incorporated into and handled by an animal's organs and tissues.
At the moment there is no non animal test available that the regulators will accept for this evaluation.

Which non-animal alternatives did you consider for use in this project?

Prior to animal testing, computer programs are used to assess if the test chemical will interact with DNA and could potentially cause DNA damage. Cell-based tests are

also performed to assess the test chemicals potential to cause DNA damage. These tests are very useful and provide a good indicator of the potential for a test chemical to cause DNA damage.

Why were they not suitable?

Computer programmes and cell-based tests are not able to fully represent how a test chemical is altered in a live animal system in terms of absorption, distribution, metabolism and excretion by the animal's organs and tissues.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals is based on the number of studies expected to be conducted over the 5 year period, the number of animals required in each treatment group and the number of treatment groups required to enable a statistically significant increase in DNA damage to be detected. It also allows for additional animals that may be required for blood sampling as blood sampling cannot be performed on the main study animals as it may affect the red blood cells.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experiments will be designed in accordance with the regulatory test guideline adopted in 1983 and updated in 1997 and 2016. The guideline includes the latest advancements in technology and ethical practices to ensure the tests are being conducted properly, using the least number of animals. The number of animals per study group has been shown, using statistical methods, to be the minimum number required to detect a doubling in DNA damage between non-dosed and dosed animals.

The integration of additional genotoxicity end points e.g. Alkaline Comet Assay, into the micronucleus test allows two genetic toxicology endpoints to be assessed in one model, thus replaces the need for two separate studies and reduces the total number of animals required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Dose range finding work will be carried out in small groups of animals so that a dose that can be safely administered for 2 days can correctly be selected for the micronucleus test. This will ensure that the risk of having to humanely kill animals

during the conduct of the micronucleus test, and potentially having to repeat work in extra animals, is kept to a minimum.

A thorough due diligence process will be undertaken to ensure work being requested is not a direct repeat of a study already conducted.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 micronucleus test is an established animal model used in genotoxicity testing and is routinely performed in rodents. It will be conducted in the Han Wistar rat or the CD1 mouse as these rodent species and strains are widely used in the safety assessment of chemicals.

Micronuclei can be assessed in the rat in bone marrow or in blood. Both methods require animals to be given 2 daily doses of test chemical 24 hours apart (or 3 daily doses in the case of Comet Assay integration). If the micronucleus test is conducted using the bone marrow method, animals are humanely killed after a defined period of time and bone marrow collected from the femurs. If the micronucleus test is conducted using the blood method, animals are humanely killed after a defined period of time and blood samples collected from anaesthetised animals which are then humanely killed.

The collection of bone marrow from animals that have been humanely killed ensures that animals experience the minimum amount of distress before collecting any tissues. Collecting blood from anaesthetised animals is a more refined method as it reduces any pain, suffering or distress potentially experienced by the animal without compromising the ability to process the blood sample collected.

Blood micro-sampling for determining blood levels of the test chemical will be conducted where possible in the dose range finding animals in preference to using additional groups of animals in the micronucleus test for this sole purpose.

Although the experimental design is highly prescribed in the guideline, a literature review will be conducted annually in order to identify and refine any procedures or processes which could be improved up on.

Why can’t you use animals that are less sentient?

The life stage of the animals to be used in this test is specified in the regulatory

guideline. Animals are required to be young adults that are ideally 6 to 10 weeks old at start of treatment.
Rats or mice are the preferred rodent species for regulatory toxicity testing and conducting the micronucleus test in as rodents are considered the least sentient model that will allow for the data obtained to be correlated with other regulatory toxicity studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be allowed a minimum of 7 days to acclimatise to their new environment following transfer. Animals will be housed in groups and provided with environmental enrichment items as agreed with the Named Animal Care and Welfare Officer (NACWO). Animals will be regularly monitored for health and welfare and routinely handled by fully trained and competent staff.

During the DRF phase and micronucleus test, animals will be carefully monitored to assess for the onset of any potentially adverse clinical signs. Particular attention will be paid to the animals around the expected peak exposure time to the test chemical. Any observations will be closely monitored until they have subsided, or if necessary, animals will be humanely killed if the humane endpoints are reached.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Experiments will be conducted in accordance with The Organisation for Economic Co-operation and Development (OECD) regulatory guideline. In addition, guidelines issued by the Laboratory Animal Science Association (LASA) and guidance from the NC3Rs on best practice in rodent studies will be followed, as appropriate. In addition, the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines will be followed, where applicable.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Although the experimental design is highly prescribed in the regulatory guideline and literature, regular reviews of the latest scientific publications in the field and the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) and Norway's National Consensus Platform for the Advancement of 'The 3R's' (NORECOPA) websites will be conducted in order to identify and refine any procedures or processes which could be improved upon.

54. Pharmacokinetics, Metabolism and Biomarkers

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Metabolism, Pharmacokinetics, Biomarkers, DMPK, ADME

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Assess the pharmacokinetic and metabolic properties, and/or the effect on biomarkers, of substances with the potential of being used for the treatment and/or modification of ill-health and physiological conditions in man, animals, or plants.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Suitable pharmacokinetic properties are essential to the selection of effective clinical candidates and their successful progression through clinical evaluation and into man, animals, or plants. Analysis of the major reasons for withdrawal of potential therapies from development in the 1980s showed that 39% of failures could be attributed to inappropriate pharmacokinetics. Between 1991 and 2000, the rate of failure of potential therapies in clinical trials that could be attributed to poor pharmacokinetics/bioavailability fell from 40% to less than 10%. This reduction remains consistent. The latest data 2010 – 2017 suggests 10-15% of failures can be attributed to poor drug-like properties. The consistency in the reduction of failures can be linked to the incorporation of pharmacokinetic (PK) assessments during the preclinical therapeutic discovery phase. Consequently, evaluating pharmacokinetic properties and identification and/or measurement of biomarkers very early in the drug discovery process is now considered vital. Early PK ensures that test items going into further development have appropriate in-life profiles in species that are likely to be used for safety assessment/toxicology studies. This use of early assessment reduces the use of animals in the later stages of drug development projects by identifying unsuitable test items at an earlier stage of the process.

Suitable pharmacokinetic properties are also essential for the selection of safe foodstuffs, feedstuffs or other substances or products which help prevent or treat ill-health in man, animals, or plants or for those test items used to regulate or modify physiology in the above or substances that will enter the human food chain either directly as food supplements or indirectly via animals or plants.

Biomarkers that translate from early discovery animal models to the clinical setting produce several benefits. Disease models with greater relevance may be designed that will translate directly to the disease/disorder situation; thus, reducing the number of animals used for projects and potentially reducing the amount of animal suffering.

Biomarkers may direct and focus research so that new and improved therapies can be brought to the clinic earlier. The discovery and application of Biomarkers in research may also lead to new diagnostic tools in the clinic, possibly replacing more invasive methods reducing pain and discomfort, and potentially leading to a more accurate diagnosis.

Without the services provided under this authority, new substances to treat unmet clinical needs are less likely to advance to the clinic; potentially denying improved treatments and ground breaking therapies to modify ill-health and physiological conditions in man, animals, or plants. In the case of biomarkers this may lead to earlier less invasive diagnosis.

	Target Discovery	Target Validation	Lead Compound Identification	Lead Compound Optimization	Preclinical Development	Clinical Trials
Average Length	1-3 years				1.5 years	6-7 years
Average Cost	$196 million				$122 million	$1-2.5 billion*
Goal(s)	Identification of a molecule involved in a disease ▸ Identify the target: a molecule integral to gene regulation or intracellular signaling ▸ Ensure the target is "druggable" and its actifity can be modulated by another compound	▸ Validate initial hypothesis through gene knockdowns ▸ Test antibody interactions ▸ Modulate the drug's affinity to target by changing molecular structure	Generation of molecule(s) that can interact with the target previously identified ▸ Test drug mechanism of action ▸ Initial safety tests conducted in cell culture ▸ Test pharmacokinetics and pharmacodynamics	Compound modifications for increased effectiveness and safety ▸ Alter design of molecule to prevent off-target effects ▸ Optimize dosage and introduction route (oral, injection) ▸ Conduct tests for drug's uptake by 3D cell culture systems	Drug testing *in vivo* for side effects and safety ▸ Test drug in alternate cell lines, and *in vivo*: most commonly mouse and rat research models ▸ Plan for either small- or large-scale production if approved ▸ Document and mediate side effects	New drug approval by the FDA or EMA ▸ File IND to begin trials ▸ Includes three phases of human testing ▸ FDA conducts reviews and approvals after phase III ▸ Continued monitoring for dosage and safety

What outputs do you think you will see at the end of this project?

The provision of studies under this licence allows customers to carry out an essential step in the discovery process, enabling timely decisions on the future of test items or projects. Studies may also be part of a proof-of-concept portfolio, where further discovery activities hinge on the data obtained.

We will provide high quality biological samples for customers to enable them to make enlightened decisions on further efficacy studies.

We will assist customers in identifying and measuring biomarkers which can translate directly into a clinical setting or indirectly for use in more specific disease/disorder related models.

We will enable customers to accurately assess the levels of a test item where it is destined to enter the human food chain either directly or indirectly via animals or plants.

We will, by carrying out the above, be helping to improve ill-health and physiological conditions in man, animals, or plants.

We will, by carrying out the above, reduce the number of animals used for testing by identifying higher quality test items or biomarkers earlier in the discovery process.

Who or what will benefit from these outputs, and how?

Short-term. Our customers will initially benefit from the outputs generated. Data produced will enable informed decisions on test items and/or biomarkers that may have potential utility across their respective areas of interest. The data will also help reduce the number of inappropriate test items progressing into more detailed, larger scale animal studies, therefore reducing the number of animals used going forward. Long-term. Following on from the initial screening the best test item candidates may

eventually provide novel treatments for the modification of ill-health and physiological conditions in man, animals, or plants. Alternatively, the identification and/or modulation of biomarkers may provide earlier and/or more accurate diagnosis in disease/disorder states.

How will you look to maximise the outputs of this work?

Although our work is customer confidential, we have a commitment to the dissemination of information into the scientific community and findings will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings where prior agreement has been received from the customer.

Our customers will use the knowledge and data produced under this authority to inform project progression. This may involve dissemination of information to investors, developmental partners, and publication where appropriate. The outputs will also be used to guide further development within projects, both in-house and externally, that are focused on the customers respective areas of interest.

Species and numbers of animals expected to be used

- Mice: 14400
- Rats: 8350

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 Absorption, Distribution, Metabolism and Excretion (ADME) profile for a given test item is multifactorial. In vivo studies need to be conducted to determine the actual disposition of a new test item in the body and/or the presence or absence of biomarkers which may be directly or indirectly affected. Although not identical, rats and mice have a similar physiology, systems and biomarker profiles to Man. This similarity across multifactorial processes enables these species to be used to characterise test items and biomarkers.

The decision of rat or mouse for each study will be based upon an understanding of the target, previous data if available, the potential pharmacodynamic model and the potential toxicology species.

Selection of species may also be based on sample type and volume required for bioanalysis. Where genetically altered animals are used in studies the majority will be mice.

Most studies will be carried out in male animals to reduce potential variation in data

and the number of animals required per study. However, where there is specific requirement from a customer female animals will be used.
Adult animals will be used under this authority as their systems are fully formed.

Typically, what will be done to an animal used in your project?

Tolerance (Pilot) studies.

These studies can be used to assess the potential of an unknown test item, a new class of test item, a new chemical series, or a dose of test item or challenge agent. These studies are used to assess the safety of the desired dose and potential adverse effects. Animals will be dosed in a staggered manner to enable monitoring to occur and ensure the minimum number of animals are exposed to potential adverse effects. If there are no signs of adverse effects at a pre-determined check point a further animal will be dosed. This will be repeated until all required animals have been dosed. Increasing doses may be given to subsequent groups until the maximum dose to be investigated is reached or adverse events occur. Group numbers are generally small, n=2- 3.

Likely dose routes are intravenous (i.v.), oral (p.o.), subcutaneous (s.c.), intraperitoneal (i.p.), intracerebral (i.c.v.), intranasal (i.n.), intramuscular (i.m), intratracheal/oropharyngeal (o.p.), intrathecal (i.t.), intradermal (i.d.), topical (tp), drinking water, added to diet or alternative diet (e.g. human food items, high fat diet etc).

Following dosing animals may have blood samples collected from superficial veins, or via a temporary (rat only) or indwelling cannula.

Rectal temperatures may be recorded. Body weight may be monitored. Animals may be placed into metabowls for a maximum of 96 hours to facilitate the collection of faeces and urine to measure excretion and/or biomarkers for both long and short acting test items. Terminal samples may be collected from killed or non-recovery animals.

Maximum study duration - 35 days after initial dose. Pharmacokinetics/Biomarker – single dose route studies.

Pharmacokinetic and/or biomarker analysis after either single or multiple doses, a single cassette dose, dosed immediately after each other (co-dosed), or dosed with a predetermined pre-treatment time between administrations given via a single dose route. Following dosing animals may have blood samples collected from superficial veins, or via a temporary (rat only) or indwelling cannula. Likely dose routes are intravenous bolus (i.v.), intravenous infusion (i.f), oral (p.o.), subcutaneous (s.c.), intraperitoneal (i.p.), intracerebral (i.c.v.), intranasal (i.n.), intramuscular (i.m), intratracheal/oropharyngeal (o.p.), intrathecal (i.t.), intradermal (i.d.), topical (tp), subcutaneous implant (e.g.minipump under recovery anaesthesia), subcapsular (under recovery anaesthesia) injection of a slow release material, drinking water, added to diet or alternative diet (e.g. human food items, high fat diet etc).

Rectal temperatures may be recorded. Body weight may be monitored. Animals may

be placed into metabowls for a maximum of 96 hours to facilitate the collection of faeces and urine to measure excretion and/or biomarkers for both long and short acting test items. Animals may undergo repeat anaesthesia to allow sequential imaging. Terminal samples may be collected from killed or non- recovery animals.

Maximum of 180 days following the final dose. Pharmacokinetics/Biomarker – multiple dose routes in a single animal.

Pharmacokinetic and/or biomarker analysis after a maximum of 3 routes of administration per animal. Each study will permit for example 2 test items or a challenge and test item dosed via 2 separate routes in a single or repeat dose study or a single test item dosed via up to 3 routes e.g. initial bolus dose i.v., followed by a second dose, which may be a single or multiple doses (p.o.) or an i.v. infusion/implant and a final single dose via a different route or the initial route may be repeated to complete the study. Repeated dosing may occur e.g. dosed immediately after each other (co-dosed) or dosed with a predetermined pre-treatment time between administrations. Likely dose routes are intravenous bolus (i.v.), intravenous infusion (i.f), oral (p.o.), subcutaneous (s.c.), intraperitoneal (i.p.), intracerebral (i.c.v.), intranasal (i.n.), intramuscular (i.m), intratracheal/oropharyngeal (o.p.), intrathecal (i.t.), intradermal (i.d.), topical (t.p), subcutaneous implant (e.g.minipump under recovery anaesthesia), subcapsular (under recovery anaesthesia), injection of a slow release material, drinking water, added to diet or alternative diet (e.g. human food items, high fat diet etc).

Following dosing animals may have blood samples collected from superficial veins, or via a temporary (rat only) or indwelling cannula.

Rectal temperatures may be recorded Rectal temperatures may be recorded. Body weight may be monitored. Animals may be placed into metabowls for a maximum of 96 hours to facilitate the collection of faeces and urine to measure excretion and/or biomarkers for both long and short acting test items. Animals may be anaesthetised on several occasions and undergo imaging. Terminal samples may be collected from killed or non-recovery animals.

Maximum duration of 180 days following the final dose. Microdialysis or cerebral Open Flow Microperfusion (cOFM).

Following implantation of a microdialysis probe, unbound analytes are continuously sampled, e.g. endogenous molecules (neurotransmitters, hormones, glucose) or exogenous test items are sampled to determine their distribution within the brain.

The collected dialysate sample will contain a representative portion of the extracellular molecules. Microdialysis is minimally invasive once the probe has been implanted and can allow simultaneous measurement of many analytes in one sample, subject to having high sensitivity analysis systems in place, thus the number of animals used in one study can be reduced e.g. 3 analytes plus test item levels could be measured in one sample.

Cerebral open flow microperfusion (cOFM) is a more novel tissue-specific sampling technique that builds on the strengths of microdialysis. OFM probes collect unfiltered

CSF and thus the samples generated contain all substances found in the extracellular space regardless of their physicochemical properties or molecular size. cOFM can also be used to deliver large molecules such as peptides or antibodies.

Likely dose routes are intravenous bolus (i.v.), oral (p.o.), subcutaneous (s.c.), intraperitoneal (i.p.), intracerebral (i.c.v.). Following dosing animals may have blood samples collected from superficial veins, or via a temporary (rat only) or indwelling cannula.

Probes may be removed and replaced with a sterile stylet and the rat or mouse returned to its home cage. Rats and mice may be used in this procedure on up to 5 different occasions (6 uses in total) with a wash-out period of at least 6 days between drug treatments. Rats and mice will only be re-used if they have been implanted with indwelling guide cannula(s) or cOFM probe(s).

Maximum duration, including potential re-use, 60 days following surgical implantation of probe/guide cannula.

What are the expected impacts and/or adverse effects for the animals during your project?

All expected impacts or adverse effects will be monitored carefully by a licensed member of our team.

The maximum severity limit in this licence is moderate. Possible adverse effects include irritation from local injection, piloerection, hunched posture, changes in respiration and body temperature, weight loss or mobility problems. Test items may also result in CNS-mediated behaviours following their administration (e.g., increased/decreased locomotion, head shakes, head twitch, sedation, excitability) but this should be transient (e.g., 1 to 2 hours). Any animal showing deviation from normal behaviour as judged by; body weight, body condition, general and coat appearance, gait or behaviour will be monitored. Mash may be given to encourage eating and prevent dehydration. We do also use temporary anaesthesia that puts the animals to sleep for a few minutes - this can be done if injection will occur in a sensitive part of the skin such as the foot or temporary restraint is required.

Therefore, we minimise any pain or discomfort that may be generated from the injection.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate - 70% for rats and mice. Mild - 30% for rats mice.

What will happen to animals at the end of this project?

- Killed

- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In silico and in vitro approaches are becoming increasingly powerful tools in the design of drug molecules with optimal drug metabolism and pharmacokinetics (DMPK) properties and are being used extensively throughout the Industry and Academia. These approaches can help in understanding various isolated aspects of a molecules ADME profile and these methods are used to screen out test items that clearly do not have the desired properties. However, because the ADME profile for a given test item is multifactorial, in vivo studies need to be conducted to determine the actual disposition of a new molecule in the body or changes in biomarkers.

There is a legislative requirement for pharmacokinetic characterisation of a medicinal product under ICH guideline M3(R2) which states “In vitro metabolic and plasma protein binding data for animals and humans and systemic exposure data (ICH S3A, Ref. 7) in the species used for repeated-dose toxicity studies generally should be evaluated before initiating human clinical trials”.

Which non-animal alternatives did you consider for use in this project?

Receptor or enzyme-based assays are used to screen compounds for binding affinity or inhibitory effects. These assays can also predict target selectivity in different species and inform the relevant choice of animal species for a programme. In vitro studies in cell-based assays to assess efficacy, potency and affinity will precede the use of animal studies and ensure the correct choice of compounds for ADME screens. Computer models may be applied to these compounds and are used by drug discovery scientists to estimate physicochemical properties, predict isolated ADME properties and to raise toxicological alerts. Lipinski’s rule of 5 (4) and calculated logP values are used to inform absorption/permeability screens in artificial membranes (PAMPA) and biological membranes (Caco-2). In vitro methods are used to determine species differences in rates and routes of oxidative metabolism (hepatocytes and microsomes). The integration of in silico and in vitro techniques into discovery programmes ensures that only selected compounds are progressed to in vivo studies for more complete characterisation. These in silico and in vitro approaches will be used where applicable and allow us to continue to reduce the number of in vivo studies performed.

Why were they not suitable?

The simple in vitro aspects mentioned above all provide excellent data and information on aspects of ADME however they model either individual or small aspects of the whole system. Whilst it is possible to draw conclusions on these specific aspects they are not definitive as they work in isolation rather than within the

full complexity of a whole body.

Pulling the data together does provide a guide to the metabolism and breakdown of a test item, however without screening in the body system in all its complexity it is not possible to provide definitive data or identify biomarkers. There may be downstream or off target consequences that are not visible or measurable within the isolated in vitro screens.

No suitable in vitro alternative which mimics the complexity of the entire system can be found in the scientific literature or animal welfare website searches (ATLWEB, Go3R).

At the current time, animal DMPK studies remain indispensable in the selection of novel drug candidates and remain an essential element in the design and interpretation of pharmacology, biomarker and safety studies in animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers are based on previous use and the likely requirement of customers over the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Due to the nature of PK studies the group size is not calculated using a power analysis formula. This function is utilised where an estimated potential effect size is used to establish how many animals are needed to observe that effect. PK studies are generally far more simplistic and follow a general industry standard within drug discovery research of 3 – 5 animals per dose group. This group size readily provides the following information. Is the test item(s) present? Yes/No. How much is present? Where is it present? An n=2 does not provide enough information to allow for standard deviation of the quantitative readouts to be calculated. Another issue with such a low number is miss-dosing. This would adversely skew the data. An n = 3-5 animals per group takes into account the factors of individual variability in the parameters being measured and reduces the effect of an animal being miss- dosed.

For example if two animals have equivalent levels of test item(s) or it’s metabolites then the third animal whose data are skewed may be removed from the analysis.

For biomarker and studies involving imaging, we will use the minimum number of animals consistent with achieving meaningful results. Group sizes will be the lowest

possible based on close discussion with a statistician, dependent on the readouts of interest. PK samples may be collected from the same animals, thereby reducing the number of animals required to produce a complete picture of the test item or biomarker.
For micro-dialysis we will use the minimum number of animals consistent with achieving meaningful results. Group sizes will be the lowest possible based on close discussion with a statistician, dependent on the analytes of interest and the effect size required. Typically group sizes are 6-8 thus for a simple study 12-16 animals would be required, generating up to 30 data points per animal. This is a significant reduction below the 90 animals that might be required should each data point have been generated by one single animal at n=3 per treatment. Given that microdialysis and cOFM samples can be scrutinised for more than one analyte this allows for increased and more robust data using these in vivo methods.

When appropriate, the dosing of more than one test item at a time (cassette dosing) will be recommended, reducing the number of animals needed per test item.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Increased assay sensitivity has led, in some cases, to serial sampling in mice and rats where full profiles are generated in single animals, rather than the multi animal, terminal sampling approach utilising 3 animals per time point. Where the sample size required from a mouse is slightly larger, a composite study design will be proposed.

The design of the composite study will be led by our statisticians to reduce the influence of any possible outlier.

Where vehicle or control animals are used for plasma/blood, tissues that are not required will be offered to other parts of the company for use in in vitro assays.

In vivo studies may be part of a full pharmacokinetic programme for a customer and as such, test items will have been through in vitro tests (highlighted above) before being tested in vivo. For single contract studies a rationale for the study will be identified via discussions with the customer. Results, if available will be reviewed internally by the group carrying out the sample analysis to determine whether larger (or smaller) group sizes are required to ensure meaningful data are generated. PK samples may also be used to obtain translatable biomarkers that enable exposure/effect models to be generated.

Where a new challenge agent or a novel test item is to be dosed a pilot study may be carried out to assess the optimal dose level for reproducible response and/or to find a dose that minimises the side effect profile.

Some surgically prepared animals may undergo re-use which will lead to a reduction in the total number of animals used across the lifetime of the licence. Blood or tissue samples will be collected from animals that have undergone surgery, where appropriate, to be use in further analyses to reduce the total number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The models to be used under this project licence are Industry recognised standard models utilising rats and mice as their systems can provide meaningful pharmacokinetic and/or biomarker data.

Rats and mice are dosed with the test item(s) and/or challenge agent of interest.

This occurs either as a single or multiple exposures over a number of days. Dosing may occur at set time points, sequential samples of blood, urine, faeces, CSF and/or tissues are taken to be analysed for the presence or absence of the test item(s) and/or its metabolites and/or biomarkers. During study design, there is close liaison between the Customer and Study Director to ensure that the correct questions are answered, e.g., metabolism or bioavailability. For example, if a test item with prolonged retention is the objective, then there is little point in having a number of sampling time points immediately following dosing. This way it is possible to screen for the required test item(s)/biomarker profile using the minimum number of animals and sampling time points. Where test items need to be dosed in the presence of further substances/molecules/markers to ensure that a pharmacologically relevant readout is achievable, combinations may be used.

Understanding the kinetics of biomarkers of interest can be used to aid design of further PK/PD studies and aid in endpoint identification.

Metabolite identification in vitro assays are performed when applicable to ensure that the correct species is chosen for any in vivo studies. The confidence with which data from pharmacokinetic and/or biomarker studies can be extrapolated to humans depends upon knowing whether humans are exposed to the same chemical entities (i.e., test items(s) and/or its metabolites) as the laboratory animals used in a study.

Why can't you use animals that are less sentient?

Mice and rats are the lowest vertebrate group on which well characterised and minimal severity pharmacokinetic models have been developed. Their mammalian bodies and systems are similar in complexity to those in man in many respects and are generally a good basis for predicting how, for example, a novel test item will react inside the human body or how a biological process will respond via biomarker measurement.

The decision of rat or mouse will be based upon an understanding of the target, previous data if available for the test item or chemical series, the potential pharmacodynamic model and possibly the potential toxicology species. Selection of species may also be based on sample type volume required for bioanalysis (e.g. blood, plasma, CSF etc) and and/or biomarker/tissue analysis.

Most studies will be carried out in male animals to reduce potential variation in data and the number of animals required per study. However, where there is specific requirement from a customer female animals will be used.

There is a legislative requirement for pharmacokinetic characterisation of a medicinal product under ICH guideline M3(R2) which states “In vitro metabolic and plasma protein binding data for animals and humans and systemic exposure data (ICH S3A, Ref. 7) in the species used for repeated-dose toxicity studies generally should be evaluated before initiating human clinical trials”.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where there is a choice of routes of administration, the most refined (whilst still being scientifically valid) will be undertaken. Challenge, vehicle or test items will be administered as a single or repeated administration by routes as detailed in the experimental protocols. Maximum daily dose volumes will be in accordance with guidelines as detailed below (as used under previous project licences held).

Test item/Challenge Administration

Vehicle, test items and challenge agents will be administered by the least severe route and administration will be performed by highly skilled staff, using appropriate dosing techniques, dose volumes and solutions (e.g., sterile solutions, appropriate pHs) to minimise any stress and discomfort to the animals. Appropriately sized needles will be used, and a separate needle will be used for each animal for systemic injection to maximise welfare and reduce the chance of inter-animal infections.

Oral administration to rats will normally be by gavage using flexible catheters to minimise oesophageal trauma. Short oral dosing needles are typically used for mice which are likely to bite the flexible tubing. Refinements to oral administration of drugs such as training the animals to consume material voluntarily from a syringe have been considered but would not be practical as we could not guarantee the full dose had been swallowed leading to the potential for greater variability in data and requirement for study repeats. Due to the novelty of the test items requiring dosing we could not be sure of their palatability and any potential for adverse influence on absorption.

Where prior knowledge exists regarding administration of the test item in vivo this will be used to optimise the choice of doses and route of administration wherever possible.

Within the context of this authority "item/substance" covers:

Test items/substances for which a pharmacokinetic/biomarker evaluation is required.

These may be substances with potential as therapeutic agents for the treatment of disease/disorder or those which may enter the human food chain via alternative routes such as diet.

Reference item/substances are those of a known pharmacokinetic profile. They may be substances that are currently available as a treatment or well established pharmacological tools.

Vehicles. The carrier substance for test, reference or challenge agents.

Dyes such as Evans blue.

Within the context of this authority "challenge agent" covers:
Challenge agents are those from which a specific response is expected in order to screen substances/measure biomarkers in an activated system. Challenge agents, such as Glucose, Lipopolysaccharide (LPS), insulin or dDAVP may be used.

Surgery
We constantly strive to improve the techniques used to reduce the likelihood of suffering in the animals and to improve our surgical and dosing/sampling techniques to minimise the experimental failure rate. Aseptic technique will be applied at all times to reduce the risk of infection, based on the latest Home Office guidance and in keeping with the Laboratory Animal Science Association's (LASA) latest guidelines.

To reduce stress in the animals prior to them undergoing surgery, they will be allowed to acclimatise to their environment for at least 7 days prior to surgery being undertaken. They will usually be weighed and handled for at least three days prior to surgery to familiarise them to handling and to provide information on their growth curve and general welfare.

The veterinary surgeon both observes our staff performing surgery and inspects the animals post- surgery, regularly.

Analgesia
Careful consideration has been given to the need for pre/post-operative analgesia in conjunction with the veterinary surgeon . The surgical sites are made as small as possible to allow access for implantation of minipumps, catheters or access to organs for direct injection. For all experiments animals will be treated pre/post-operatively following consultation with the veterinary surgeon (e.g., with a non-steroidal anti-inflammatory drug such as carprofen). The time course of the analgesia will be considered to ensure that it is beneficial from the time the animal may first begin to experience pain. An animal will not be used in an experiment until it is in a suitable condition, e.g., not displaying signs of experiencing pain, stress or discomfort. Animals are monitored for behavioural signs of pain by our staff who have extensive experience of post-surgical animals and by the veterinary surgeon.

Observations are recorded on individual post-surgical records by our staff. The Grimace Scales to recognise pain and assess its severity in post-operative animals

(https://nc3rs.org.uk/grimacescales) will be used unless animals have undergone implantation of an ICV cannula or microdialysis probe as it has been understood previously that in the case of animals that have undergone cranial implants, the facial features can appear altered due to the implant in the skull, unlike a non-surgical animal, making this scoring system less appropriate to adopt.

Housing
Animals will be group housed unless there is a scientific or welfare reason to single house an animal. Where animals have been surgically prepared it may be necessary to single house due to possible damage a cage mate may cause to the surgical site or the implant itself. Fighting in male mice is well recognised as an issue and where fighting is seen in group housed animals, cages will be split down to prevent injury or death from fighting. Mice specifically will be kept in their delivery groups to reduce the likelihood of fighting. Where multiple test items are being investigated animals may be single housed to prevent cross contamination of biological samples or where delivery groups don't breakdown into pre- determined test groups. Food and water will be available at all times and animals will be given increased environmental enrichment beyond the standard enrichment supplied (e.g., extra toys to play with). When animals are placed in metabowl cages enrichment will be included and where possible mice will be group housed.

Blood sampling
The taking of blood samples (anticipated to be primarily from the tail vein) is likely to be a frequent procedure undertaken. Heated chambers may be used to aid blood sampling. Restraint in a suitable chamber or manually may be used to facilitate the procedure. The use of local topical anaesthetic to reduce any pain and discomfort during blood sampling will be considered. Limits on volume of blood samples will be taken from the guidelines listed below.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidance outlined in the PREPARE and ARRIVE guidelines for experimental planning and publication of data produced during this project. We will also follow guidance issued by the Home Office, LASA, NC3Rs and RSPCA on animal re-use.
https://www.nc3rs.org.uk/news/re-use-needles-indicator-culture-care. NC3R's - Species specific Grimace scale for pain.
Refining procedures for the administration of substances. Morton et al. Lab Animals (2001) 35, 1-4

Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider. Turner et Al. J Am Assoc Lab Animal Sci (2011), 50(5) 600-613
A good practice guide to the administration of substances and the removal of blood including routes and volumes. Diehl, et al J Applied Tox (2001) 21, 15-23
https://www.rspca.org.uk/webContent/staticImages/Downloads/AdministrationOfSubstances.pdf and http://www.procedureswithcare.org.uk/ASMS2012.pdf), aseptic technique (http://www.lasa.co.uk/wp- content/uploads/2017/04/Aseptic-surgery-final.pdf and https://researchanimaltraining.com/article- categories/aseptic-technique/), and housing (https://www.nc3rs.org.uk/3rs-resources/housing-and-

husbandry/rodents).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep abreast of the NC3Rs website for any updates as well as following any guidance or information shared across the research community. Alongside this we will attend any relevant seminars and webinars produced by groups such as NC3Rs and Responsible Research in Practice. We will also keep up to date with the literature of advancements in the methods outlined in this licence and implement improvements to our working practices where practicable.

55. Understanding the role of FOXA1 in cancer development and metastasis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Metastasis, Nuclear Receptors, Therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand the role of a key protein called FOXA1 and its protein partners or downstream effectors in the development and metastasis of various cancer types, and how these affect response to treatment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

FOXA1 is a pioneer factor (one of the first proteins to contact DNA) that will influence the recruitment of other drivers of cell proliferation to DNA. While there are several factors that influence cancer resistance to standard of care therapeutics, most nuclear receptors will still use FOXA1 as their key pioneer factor. FOXA1 has proven

to be a difficult protein to work with because it's structure is so poorly characterized. Therefore it is of paramount importance that we focus on known protein interactors and regulators of FOXA1 and evaluate their role in FOXA1 dependant cancers, especially in terms of proliferation, drug resistance and metastasis.

What outputs do you think you will see at the end of this project?

This project will provide a deep insight into how FOXA1 promotes cancer development and the key primary outputs would involve the identification of FOXA1 regulators/interactors that have the most profound impact on cancer growth and metastasis. Then other outputs would be to assess the drug targeting capability of these regulators/interactors in vivo. Finally our lab has a track record of publishing in high end open access journals, and therefore, all results and ex-vivo subsequent material generated from these projects will be made freely available to the wider scientific community.

Who or what will benefit from these outputs, and how?

Our group (This work will be published in open-access, peer-reviewed journals and communicated at conferences and seminars).

Other academic and industry cancer research groups will benefit greatly from publication of the interactome of key transcription and pioneer factors. Previous protein interaction studies on tissue was published by our group in 2018, therefore many other groups (who may not have access to the required equipment) will benefit from us sharing this data in journal articles throughout the 5-year project period.

Pharmaceutical Industry (as we will identify potential novel therapeutic targets and publish these findings).

Patients (This research programme has already led to 1 currently ongoing clinical trial for breast cancer (PIONEER) and is expected to lead to more during the 5-year project period.)

How will you look to maximise the outputs of this work?

Open Access Journals:
Our lab has a track record of publishing in open access journals and we will continue to prioritize this. Open access publications ensure that research findings are freely accessible to the global scientific community, maximizing the visibility and impact of the work.

Active Participation in International Meetings:
We actively participate in international meetings to present the data and findings.

This not only contributes to the dissemination of knowledge but also provides opportunities for networking, collaboration, and receiving feedback from experts in the field.

Promote Collaboration and Resource Sharing:

We emphasize collaboration by making all biological and computational resources generated from the research available to researchers and collaborators worldwide.

This can be done through established platforms for sharing data and resources.
Utilize Social Media and Science Communication:
We actively engage in science communication through social media platforms, blogs, and other accessible channels. This not only disseminates information widely but also helps in connecting with diverse audiences, including fellow researchers, students, and the broader public.

Seek Collaborative Opportunities:
We actively seek opportunities for collaboration with other research institutions, industry partners, or organizations with aligned interests. Collaborative projects can amplify the impact of the research and open up new avenues for exploration.

Species and numbers of animals expected to be used.

- Mice: 3900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are being used in this project because we have a good understanding of their biology and the models we currently have to study FOXA1 dependant disease have been optimised in these models.

All animals used in this protocol will be at the young adult stage (approximately 6 - 8 weeks old) so as to maintain a steady state of circulating hormones for the study of FOXA1 dependant cancers (hormone dependant).

Typically, what will be done to an animal used in your project?

EXPERIMENTS ON TUMOUR BEARING MICE

Tumours will be generated by direct introduction of cells either into the mammary ducts (MIND), or by injection into the mammary fat pad or under the skin, or into the pancreas. Injection intro the pancreas may be either via a surgical incision in the abdomen or by use of ultrasound guidance to inject through the abdominal wall.

Tumour growth will be monitored at a maximum frequency of twice weekly for the duration of the experiments.

In some cases tumours will be genetically modified through ultrasound-guided intratumoral injection on no more than two occasions.

We may dose animals, through a maximum of two routes, at a maximum frequency of twice a day, for total period not exceeding 40 days.

Drinking water of animals may be supplemented with hormones or harmless labelling compounds for the duration of the experiment.

Tumours and their associated microenvironment may be imaged up to 10 times over the duration of the experiment.

At any point during the experiment, blood may be sampled using a superficial vein.

All animals will be killed via a Schedule1 method at the end of the experiment.

DRUG TOLERABILITY ASSESSMENT

Animals will be administered with novel therapeutic agents alone or rarely, in combination with a conventional compound through a maximum of two administration routes.

At any point during the experiment, blood may be sampled using a superficial vein.

At any stage or at the end of the experiment, all animals will be killed via a Schedule 1 approved method.

What are the expected impacts and/or adverse effects for the animals during your project?

In most instances animals are expected to make a speedy recovery from most procedures.

DRUG TREATMENT

Animals receiving new drugs may show signs of toxicity. Any such animals showing signs of toxicity for 48 hours will immediately be killed by a Schedule 1 approved method.

TUMOUR DEVELOPMENT

Near the experimental endpoint tumour growth might impede the normal movement of animals. Any animals found with impeded movement upon daily inspection will immediately be killed via a Schedule 1 approved method.

ANIMALS WITH METASTASIS

Animals with metastases can exhibit rapid health deterioration, pain and unusual behaviour. Any animals found exhibiting any of the signs upon daily inspection will immediately be killed via a Schedule1 approved method.

PANCREATIC TUMOUR DEVELOPMENT

Due to the very aggressive nature of pancreatic cancer, mice can suddenly start developing signs of ill health. These animals will be monitored daily and will be killed via a Schedule 1 approved method immediately upon detection of any of these 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)?

Protocol Number	Expected Mild Severity (%)	Expected Moderate Severity (%)
1, 2, and 3	90	10
4 and 5	20	80

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Tumours and the way they develop and respond to treatments are greatly influenced by the surrounding environment. Currently, there is not the technology to accurately mimic the human environment and the complex interplay between bodily systems without using an animal. We are using mice for our experiments as we require a mammalian host species to be a good representation of the human body.

Which non-animal alternatives did you consider for use in this project?

We have developed an in-vitro co-culture system to mimic the tumour microenvironment by cultivating tumour cells with some human environment derived cells. Furthermore, in collaboration with another laboratory in Australia, we have developed a human tissue explant assay where we can keep human tissue alive for up to 5 days post surgical resection to study different parameters such as drug response. In addition we are trying to generate in-vitro 3D breast organoids.

Why were they not suitable?

None of these models faithfully replicate the tumour micro-environment setting. Drug treatment and their responses in these alternatives do not faithfully recapitulate human biology in terms of drug uptake and bioavailability. Furthermore, the animal setting provides a crucial insight into fatal metastatic disease, something that is not

achievable in alternative non-animal models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For each protocol, we have defined a number of FOXA1 regulators, associated factors and target amino acids we are likely to pursue. Then, for each experimental setting, the AWERB statistician has carried out sample size calculations to achieve statistically powered results using the minimal number of animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All our experiments are designed, size sampled, randomised and ultimately analysed in an unbiased manner by the AWERB statistician, who has a significant amount of expertise in animal work.

Furthermore, we are up to date with all relevant literature in our field to reduce unnecessary duplication of work already done by other research groups.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Wherever possible we plan to obtain tissue from collaborators instead of unnecessarily duplicating work, and we will in return make all tissue from our experimental procedures available to collaborators. Our results will be made available to the wider research community through dissemination at international conferences, social media and publication in open access journals.

Our group has taken a major step forward in reduction, particularly while importing the intraductal method in our institute. We developed a method to reduce tumour measurement variability to a minimum which resulted in decreasing the number of animals we use per study by almost 50% (confirmed by the AWERB statistician)
In the case of novel therapeutics, our group plans to conduct small scale dose finding studies before proceeding with statistically powered experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during

the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

To implant a patient's tumour, we need to use an animal that has a greatly weakened immune system, or the tumour would be rejected. NSG mice are readily available animal models which lack a strong immune system. Care of NSG mice is well established at our facility and others worldwide. The techniques used are well supported by colleagues internally and externally. We are strictly limiting the size of tumours in the animals to 14mm mean diameter. When required, mice will be anaesthetised or given pain relief to prevent or minimise discomfort.

Most mice will receive tumour engraftment in a non-invasive way. In the case of pancreatic tumours, in this licence, we will try to refine our engraftment procedure which currently involves abdominal surgery by using a less invasive ultrasound guided injection method.

In addition, we will be trialling an alternative to surgical resection of a tumour, which consists of a non- invasive, light-based induction of primary tumour cell death. If successful, this will bring about a complete change in the way surgical resections of primary tumours in animals are currently done.

Surgical resection will be replaced by simple illumination of tumours.

Why can't you use animals that are less sentient?

Tumours and the way they develop and respond to treatments are greatly influenced by the surrounding environment. Currently, there is not the technology to accurately mimic the human environment and the complex interplay between bodily systems without using an animal. We are using mice for our experiments as we require a mammalian host species to be a good representation of the human body.

Furthermore, mice provide, especially in the case of hormone dependant cancers, a physiologically relevant amount of circulating hormones, recapitulating human biology.

After tumour implantation, we require that these animals stay alive for some time to allow for tumour growth and metastatic dissemination.

Subsequently we need drug treatment to be carried out in a physiologically relevant context, where bioavailability and pharmaco-kinetics play a major role and cannot be recapitulated using other non- animal based models

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The majority of mice are slated to undergo tumour engraftment through a non-intrusive approach. Specifically addressing pancreatic tumours, we aim to enhance

our engraftment technique, currently reliant on abdominal surgery, by adopting a less invasive method guided by ultrasound injections.

Moreover, we plan to explore an alternative to surgical resection, employing a non-invasive, light- induced method to trigger primary tumour cell death. A successful outcome could revolutionise the current approach to surgical resections of primary tumours in animals, replacing the need for surgery with a straightforward illumination of tumours.

All animals enrolled in any of our protocols will be monitored daily, and any animal showing signs of a welfare issue will promptly be dealt with.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

RSPCA , Categorising the severity of scientific procedures on animals, J.A. Smith and M. Jennings, Editors. 2004, RSPCA Research Animals Department. p. 45.
RSPCA and LASA, 2015, Guiding Principles on Good Practice for Animal Welfare and Ethical Review Bodies. A report by the RSPCA Research Animals Department and LASA Education, Training and Ethics Section. (M. Jennings ed.)
Workman, P., Aboagye, E., Balkwill, F. et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102, 1555–1577 (2010). https://doi.org/10.1038/sj.bjc.6605642
Hendriksen, C., K. Cussler, and D. Morton, Use of humane endpoints to minimise suffering, in The COST manual of laboratory animal care and use, B. Howard, T. Nevalainen, and G. Peratta, Editors. 2010, CRC Press: Boca Raton, Florida, USA. p. 439.
Wells DJ, Playle LC, Enser WEJ et al. (2006) Assessing the welfare of genetically altered mice. Lab Anim. 40: 111.
ILAR, DELS, National Research Council (2008) Recognition and alleviation of distress in laboratory animals, The National Academies Press.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The NC3R website remains our best source of information to stay informed about advances in the 3R's.

Furthermore, we are subscribed to the NC3R newsletter, and we are on the UBS-3R-Enquiery mailing list.

56. Understanding visual processing in freely moving animals

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Vision, Neuroscience, Movements, Behaviour, Vestibular System

Animal types	Life stages
Mice	adult, pregnant, neonate, juvenile, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Human and other animals have the extraordinary ability of using vision to control balance, perform complex movements, manipulate objects and navigate their natural environments – we call this ability visual-motor integration. The aim of the project is to understand the cellular basis of visual-motor integration under natural conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Vision is one of our most valued senses and large parts of our brains are dedicated to interpreting the signals from our eyes to enable us to "see" and perform the actions required by our daily life. We have a good understanding of how nerve cells in the eye respond to light, but we know comparatively little about how these responses are combined with internal information about our own movements. Filling

this gap is key to advance our understanding of how the brain controls behaviour – a fundamental question in neuroscience and psychology.

What outputs do you think you will see at the end of this project?

This project will advance our understanding on how the brain controls behaviour.

These advances will be communicated to the scientific community via scientific publications in international open access journals. To engage with the general public our science will be presented in accessible format at science festivals. The new material produced (new source codes to analyse data, new set-ups and assays for studying animal behaviour) will be uploaded on public repositories for the wider scientific community and anyone interested in using it.

Who or what will benefit from these outputs, and how?

In the short term the international scientific community interested in vision and behaviour. Since we are developing new techniques to study vision and behaviour in freely moving animals, such techniques will also have the potential to reduce suffering and stress compared with traditional approaches in which animals are physically constrained (thereby advancing 3R's Refinement). In the longer term some of our discoveries could provide benefits to the wider community by finding translational applications for vision restoration and movement disorders.

The outcomes of this project will also provide better understanding of how the brain integrates information from visual and vestibular systems. This is particularly relevant for patients affected by vestibular disorders such as Meniere's Disease (MD) and Persistent Postural Perceptual Dizziness (PPPD). We know that MD and PPPD affect our ability to see and maintain steady balance, especially during eye and body movements, but it is unclear how such deficits in balance and visual perception arise. This poses a challenge in developing effective rehabilitation therapies. Our project, by providing an improved understanding of the cellular basis of visual-vestibular integration, will lead the way to developing more effective rehabilitation strategies.

How will you look to maximise the outputs of this work?

Dissemination of knowledge will be maximised via scientific publications in international open access journals, talks at scientific conferences and public engagement activities. Data and software we develop for our project will be made freely available on public repositories to maximise uptake and data re-usage. Unsuccessful approaches will also be documented in publications and public repositories.

Species and numbers of animals expected to be used

- Mice: 931

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures. Explain why you are using these types of animals and your choice of life stages.

We are interested in how the visual system functions and how it controls behaviours. Although non-mammalian vertebrates have retinas that are rather similar to those of humans, their visual and motor systems differ from our own in other key respects. Firstly, they have a wide variety of light sensitive cells outside of the eye. Secondly, the functional and anatomical organisation of their cortical regions, the brain regions that changed the most during mammalian evolution and that contain fundamental stations for integrating visual and behavioural information, is substantially different from mammals and humans. For these reasons, we have no other alternative but to work with mammals.

We use laboratory mice because we can build upon a wealth of existing information about the visual system in this species, and because we have access to animals carrying naturally occurring mutations or engineered genetic modifications that are very useful for our objectives.

We use adult animals since vision and behaviours develop at this life stage.

Typically, what will be done to an animal used in your project?

The most common experiment will consist in presenting an animal with visual stimuli and recording eye/head/body movements and changes in electrical activity in the brain. To do that animals need to previously undergo recovery surgery to implant brain recording electrodes and a small attachment piece to secure miniaturised cameras used for eye tracking.

What are the expected impacts and/or adverse effects for the animals during your project?

Surgery will cause pain during the recovery period. This will be treated with pain killers, and we do not expect it to last for more than a few hours. There will also be transient stress associated with handling and with being placed in open behavioural arenas.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We are using mice for all protocols. Expected proportions of severities are:

- Moderate: 47%
- Mild: 32%
- Non-recovery: 21%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Vision and behaviours are emergent properties of the retina and the brain and, as such, can only be studied using humans or animals. We can measure movements in humans, but the range of techniques suitable for measuring and manipulating brain activity is far smaller in humans than laboratory animals. Thus, to access electrical brain activity from single and multiple nerve cells we can only use animals.

Which non-animal alternatives did you consider for use in this project?

We have considered using computer simulations and experiments in humans to address our scientific purposes.

Why were they not suitable?

We are a long way from being able to recreate in-silico such complex systems as retina, multiple brain regions and body movements working together. Therefore, computer simulations cannot address our scientific objectives at this stage. Experiments in humans cannot provide invasive measurements of nerve cell activity that are required for our scientific objectives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate is based on our yearly usage of animals in previous projects using a comparable approach.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Whenever possible we compare behavioural and neuronal responses to different

experimental conditions (typically different visual stimuli) within each animal, thus removing inter-animal variability. Presentation of the experimental conditions is performed by using randomised block designs to further reduce the number of animals required for our statistical analyses. When performing new protocols small pilot experiments are used to optimise experimental conditions before running larger scale experiments. Whenever possible to exclude bias in the results, pilot data recorded under the same experimental conditions are included to further reduce animal numbers.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We use advanced 3D pose analysis - advanced methods developed in our group to obtain 3D reconstruction of animal head, body and eye positions. These methods provide automated and objective analysis of mouse behaviour, maximising the amount of information available from each experiment and avoiding the potential for observer bias in scoring behaviour. In the same spirit, we employ the highest throughput electrodes available that enable us to obtain high quality data from the largest number of nerve cells from individual animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use laboratory mice because we can build upon a wealth of existing information about the visual system in this species, and because we have access to animals carrying naturally occurring mutations or engineered genetic modifications that are very useful for our objectives.

We are interested in how vision controls behaviours. Whenever possible we will focus on innate behaviours (e.g. spontaneous exploration, threat avoidance). These do not require training that would typically involve water / food deprivation adding additional stress to the animals.

It is fundamental for our objectives to study awake behaving animals. Whenever possible we will rely on experiments in freely moving animals. Compared with awake head-fixed experiments, freely moving is a more natural approach, it is better tolerated by the animals and does not require additional experimental sessions to habituate the animal to head-fixation.

We will record brain activity by implanting brain electrodes. The size, weight and wiring of these implants will be optimised before implantation in awake animals to

reduce burden to the animal.

Why can’t you use animals that are less sentient?

We use laboratory mice because we can build upon a wealth of existing information about the visual system in this species, and because we have access to animals carrying naturally occurring mutations or engineered genetic modifications that are very useful for our objectives.

Rodent visual systems develop after birth, meaning that we cannot address our questions at a more immature life stage. The core aim of our project is to study the interactions between vision and behaviour - therefore these experiments can only be performed in awake behaving animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Surgery will cause transient pain however we will expect this not to last for more than few hours. Appropriate analgesics will be provided to the animal before and after the surgical procedure. During recovery from surgery animals will be monitored daily for signs of pain and distress. In the rare event in which animals will be found to approach humane endpoints (e.g. maximum weight loss, piloerection, eye/face expressions) they will be promptly and humanely killed according to best established practices.

We will use specialised cages to facilitate feeding and drinking in surgically operated mice.

Animals will be gradually habituated to these new cages before the surgery to minimise post-surgical stress. We use advanced 3D pose analysis methods developed in the group to provide automated and objective analysis of mouse behaviour, maximising the amount of information available from each experiment and avoiding the potential for observer bias in scoring behaviour.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We consult protocols, training resources and guidelines on best practices in animal experiments available through the NC3Rs website (https://www.nc3rs.org.uk/3rs-resources) and will adhere to them whenever relevant. Topics covered include handling and restraint, euthanasia, humane endpoints, welfare assessment, anaesthesia, and analgesia.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are actively engaged with NC3Rs events (www.ncrs3.org.uk). Having developed new refined methods for studying mouse behaviour over the last 5 years, we have regularly given talks to NC3Rs events. We keep ourselves updated with 3Rs innovations by regularly visiting the NC3Rs website and attending 3Rs related

events. now receive regular 3Rs updates through the animal unit and our local 3Rs manager.

57. Assessment of the CNS activity of drugs: novel targets, efficacy, and safety

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Drugs, CNS, Efficacy, Side Effects

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The major aim of this project is to provide specialised, high quality, preclinical data to support the drug discovery projects of our customers. Customers may be seeking to treat CNS disorders, evaluate CNS side effects (including from non-CNS projects) and understand the impact of their target molecules on the CNS.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

CNS disorders encompass a wide range of psychological (e.g., depression, anxiety, schizophrenia, attention/behaviour disorders, autism, Down's syndrome, sleep disorders and drug abuse) and neurological (e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, dementia and epilepsy) conditions that negatively impact mood, behaviour, brain functioning, cognition, sensory or motor function. These disorders are highly prevalent worldwide affecting hundreds of millions of people in both developed and developing countries. For example, approximately 280 million people in the world suffer from depression (http://www.who.int/news-room/fact-sheets/detail/depression); 24 million from schizophrenia (http://www.who.int/news-room/fact-sheets/detail/schizophrenia), 50 million from epilepsy (http://www.who.int/news-room/fact-sheets/detail/epilepsy), 55 million from dementia (http://www.who.int/news-room/fact-sheets/detail/dementia).

Such disorders produce short- or long-term impairments and disabilities and therefore are an emotional and financial burden to patients, their friends and families and society. Furthermore, there is a strong link between psychiatric conditions, such as depression and schizophrenia, and suicide. Also, those with mental illness are at high risk for developing other chronic conditions such as cardiovascular disease, respiratory disease and diabetes. In response to the low levels of investment in these noncommunicable diseases (NCDs) globally, even though they cause three quarters of deaths worldwide, the World Health Organization has launched the Global NCD Compact 2020-2030 (https://www.who.int/initiatives/global-noncommunicable-diseases-compact-2020-2030). The aim is to accelerate progress on prevention and control of NCDs by ensuring that member states adopt policies and programmes that improve outcomes and save the lives of patients living with NCDs.

Pharmaceutical and Biotech companies both big and small recognise that despite increasing knowledge of neurobiology and improvement in treatments, there remains significant need for new medicines which offer higher levels of efficacy and reduced potential for side effects. The hope is to positively transform the lives of people living with brain disorders through drug discovery.

What outputs do you think you will see at the end of this project?

Data output from the project are of 3 types and expected to provide the following benefit to clients/customers:
Efficacy Data: To allow our customers (principally from the pharmaceutical industry) to make decisions regarding their candidate substance. Should the compound be progressed towards evaluation in clinical studies? If efficacy is poor/absent alternative chemical structures may need to be synthesised/tested? The predictive nature of in vitro tests, or the pharmacokinetic properties, may need to be re-assessed in vivo (screening cascade). The short-term benefit of such efficacy data is to allow medicinal chemists to improve understanding regarding the structure-activity relationship of molecules. Accordingly, molecules with improved efficacy may be synthesised. The medium benefit is the discovery of compounds with suitable efficacy and the long-term benefit may be a clinically effective drug (since regulatory agencies expect a drug's sponsor to have screened the new molecule for pharmacological activity in animal models, prior to assessing its therapeutic potential in humans).

Side Effect Data: Experiments may be performed to investigate whether candidate substances for CNS or non-CNS disorders have centrally mediated side effects or unwanted central activity. Such data provide our clients (principally from the pharmaceutical industry) an understanding of the side effect profile of their compound. Such information may lead to a compound's progression being stopped or if it had low propensity to induce side effects such data may lead to further progression of the molecule. Where a client has a known side effect issue with a particular project, multiple compounds may be assessed over several experiments so that the side effect can be screened out. The short-term benefit to the client is using these data so that compounds of different structures can be designed and an understanding as to how chemical structure affects side effect activity is gained. The medium benefit is the discovery (screening out) of compounds without that side effect and the long- term benefit (likely to be after completion of the licence) may be an efficacious drug with a low side effect profile.

PK/PD data: Pharmacokinetics (PK) refers to the fate of a drug within the body and a customer may wish to administer the test compound and measure tissue (e.g., blood, plasma, brain) levels of that compound over time after administration. Pharmacodynamic (PD) studies investigate the effect of a drug on the body (e.g., receptor occupancy of a compound in the brain). Such data are typically used by a client to determine whether a candidate substance is likely to have suitable characteristics for subsequent efficacy testing (e.g., suitable plasma exposure or receptor occupancy to interact with a target) as detailed above. Hence, the immediate benefit is to enable a client to decide whether efficacy testing is warranted and, if not, then compounds with suitable characteristics can be synthesised by medicinal chemists. This can then lead to suitable compounds being synthesised and tested for efficacy and the incidence of side effects (medium-term benefit). The long-term potential benefit is the development of clinically effective drug treatments for the treatment of CNS disorders.

Who or what will benefit from these outputs, and how?

In the short term our clients whose compounds we test will benefit in terms of providing useful data to determine whether a compound can progress to clinical trials. In the long term this work may lead to the approval of new treatments for the type of conditions outlined in the Project Plan section.

Achieving such benefits is realistic since this licence is similar in nature to previous licences held by the company. If the work leads to the registration of a new compound(s) for the treatment of a CNS disorder, which is ultimately what we hope for, then the benefit is potentially huge. Recently, three tested compounds, from three different customers, reached the clinical trials stage, and are potential treatment options to counteract treatment-resistant depression, schizophrenia, and major depression.

Since we perform studies for numerous different clients, often in the same assay/model, there are potential welfare and scientific benefits in these studies being performed centrally in purpose-built facilities by a skilled group of experienced licence holders and scientists with relevant expertise rather than in separate client laboratories under varying conditions and differing skill levels.

How will you look to maximise the outputs of this work?

Although work under this licence is typically customer confidential, we have a commitment to the dissemination of information into the scientific community and findings will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings where possible. Indeed, by such routes the company can get exposure to new potential customers. In addition, it is often the case that customers will attend conferences and/or prepare papers using experimental data that has been generated under the present licence, e.g., the following articles contain data obtained from the predecessor of this project:
Poulter S, et al. (2023) The Identification of GPR52 Agonist HTL0041178, a Potential Therapy for Schizophrenia and Related Psychiatric Disorders. ACS Med Chem Lett. 2023 Mar 14;14(4):499-505.

Hurley S, et al (Dec. 2021) The effects of psilocybin on binge-like feeding behaviour in rats. Poster Presented by Compass Pathways at the ACNP, USA. Poster M41: https://www.nature.com/articles/s41386-020-00890-7

Species and numbers of animals expected to be used

- Mice: 10,000
- Rats: 11,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project objectives each require investigation of candidate substances to be tested in an integrated behavioural/physiological/pharmacological model that requires the whole animal. Rats and mice are believed to be the lowest sentient animals that can be used to assess such effects in a manner that is translatable to humans. We will be using adult animals as they are most relevant to the diseases under investigation with suitably developed central nervous systems. Choice of species may be dictated by the model, the customer, or the nature of project. For example, some but not all models are only run in particular species; a customer might have extensive data in one species already and wish to use that species as the tox species; pharmacology or DMPK characteristics in one species might translate better to what is believed to be the case in humans. When using genetically altered animals, this will be in mice since this is the available species.

Typically, what will be done to an animal used in your project?

In a typical experiment animals will be dosed (most commonly once) and the effect of the drug dosed is then assessed in a behavioural assay. Injection routes used will

depend upon the study, but oral administration is expected to be the most prevalent since that typically translates to the preferred route in man. Relatively few animals are expected to undergo surgery but implantation of osmotic/iprecio mini-pumps is possible as is intracranial administration via stereotaxically implanted cannula.
The behavioural assays used are often sub-threshold in nature with animals able to exhibit natural investigative behaviours in non-harmful circumstances (e.g., novel object recognition, locomotor activity assessment and binge eating).

In some instances, animals are dosed (most likely once) with tissues (plasma, CSF, brain etc) taken after humane killing to determine compound exposure, receptor occupancy or changes in relevant biomarkers at pre-determined times post dosing.

What are the expected impacts and/or adverse effects for the animals during your project?

The impacts and, where present, adverse effects experienced by the animals during the project will likely be related to dosing and the pharmacological action of test compounds used in the project on the CNS (e.g., increased activity in a cage, increased behaviours such as the head twitch response indicative of activation of certain serotonergic receptor pathways, changes in plasma/brain biomarkers etc). In studies involving typical antipsychotics (e.g haloperidol), ataxia and sedation may be observed. Some compounds may also cause hyperactivity, headshakes, jumping, prostration, ptosis, diarrhoea, dyspnoea, hunched appearance, piloerection chromodacryorrhea reduction in food and water intake and weight loss. Most of these adverse effects are expected to be present in circa of 1% of the total animals used in this project.

The administration of compounds for testing will be undertaken using a combination of volumes, routes, and frequencies that of themselves will result in no more than transient discomfort and no lasting harm (e.g., an insertion of a needle with a transient prick). Based on experience with previous similar projects any compound mediated effects following administration are expected to be transient (e.g., lasting typically 1-2 hours only).

As a few animals are expected to undergo stereotaxic surgery, transient harms related to that are expected, such as, mild pain, stress and discomfort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild: 80% for both rats and mice Moderate: 20% for both rats and mice

What will happen to animals at the end of this project?

- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project aim requires investigation of candidate substances to be tested in an integrated behavioural/physiological/pharmacological model that requires the whole animal. Prior to testing in the present project, it is expected that candidate substances will be selected based on in silico and in vitro experiments. Indeed, some of these studies may be performed within the company which has extensive capabilities in drug discovery outside of in vivo pharmacology. Importantly, although providing clients with important information regarding the discovery of candidate substances, such data relate to discrete parts of the animal and are not suitable for replacing ex vivo or in vivo tests in whole animals. A large number of ex vivo binding studies are predicted to be performed during the lifetime of this licence. Whilst such studies do involve drug dosing to animals, they are generally brief and mild in nature, involving a small number of animals.

Which non-animal alternatives did you consider for use in this project?

It is not possible to use non-animal alternatives for this project due to the nature of the data acquired as outlined above. In silico and in vitro approaches are routinely used in drug discovery (and in this company) and will be used to select substances assessed in this project.

Why were they not suitable?

This is because there are no in silico or in vitro model systems that can adequately model the living brain and body as a whole to be able to acquire the type of data we propose to be collecting in this project. Also, regulatory authorities expect a drug’s sponsor to have screened new molecules for pharmacological activity and acute toxicity potential in animals, prior to assessing its diagnostic or therapeutic potential in humans. The data produced by this project for our clients helps fulfil that expectation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used in the project over its duration and the group sizes of animals used in the individual studies is based on extensive experience from running

such projects and experiments. The numbers are based on the previous project but will be affected by customer demand for the services which may differ over the next 5-year period. This is the fourth project that the company has undertaken with a view to assess customer compounds in specific in vivo assays. Sample sizes for our experiments are estimated from past experiments with input from statisticians within the company.

A typical study consists of 50 rats or mice (e.g., control group, 3 drug treatment groups and a positive comparator with 10 animals per group). Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of 10 to achieve the quality of results we need. An average of 46 rat studies and 40 mice studies per year are carried out, thus 11,500 rats and 10,000 mice during the 5-year lifetime of this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the expertise of our statisticians who can perform power calculations to ensure that studies are suitably powered to detect changes. Similarly, the statisticians advise on experimental design to improve statistical power and reduce animal usage. Use of the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) Experimental Design Assistant will also be considered.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Multiple tissues can be taken at the completion of studies, and these can be used for ex vivo analyses. Indeed, we will be measuring multiple parameters in the same animal where possible (e.g., locomotor activity studies may involve the collection of brains for neurochemical determination or for ex vivo binding).

In past projects, such samples have been transferred to other providers, including overseas, for analysis. This potentially avoids the running of multiple in vivo studies at different vendors to gain the same answer. Although rare in this project, animals may undergo re-use which will lead to a reduction in the total number of animals in used across the lifetime of the project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rats and mice will be used since their central nervous systems are well documented and they are the lowest form of mammal that can provide meaningful neurochemical, behavioural and pharmacokinetic data translatable to humans. Occasionally, genetically altered animals may be used to model specific CNS disorders and the majority of these are likely to be mouse models. Animals with harmful phenotypes will not be tested and it is expected that the mutations will be described in detail in the available scientific literature.

A range of methods will be used in the project. In many cases, experiments will not try and model specific diseases (or aspects of them) but be simple in nature with little done to the animal: e.g. dosing test compounds and/or challenge substances and taking tissue samples (e.g. plasma/brain/CSF) for later analysis of key analytes; or dosing of test compounds and/or challenge substances and monitoring behaviour (e.g. pupil diameter, activity, head twitches, body temperature, instances of catalepsy, general behaviour).

Some methods used in the project (e.g., novel object recognition, marble burying, open field activity) allow animals to exhibit their natural behaviours and can be classed as sub-threshold in terms of pain suffering, distress, or lasting harm. For example, the novel object test assesses an animal’s memory for previously investigated objects; the marble burying test is a model of anxiety/OCD and involves assessing an animal’s natural behaviour to bury objects into the sawdust; open field is an assessment of how an animal explores a novel space potentially offering insight into anxiety and how a drug treatment might affect that. Our binge eating model involves irregular access to powdered chocolate which the animals clearly enjoy as illustrated by the bias of ingestive behaviour to the chocolate from chow on these occasions.

Occasionally, genetically altered animals may be used to model specific CNS disorders. Animals with harmful phenotypes will not be tested and it is expected that the mutations will be well known.

Examples of mice that may be used in the project include, TDP-43, rTg4510, APP/PS1, Tg2576, 5xFAD, SAMP-8 and APP23 models, our recommendation to clients will be for these studies to be run with animals of both sexes whenever feasible). Such mice (especially males which are not social animals; Kappel et al 2017) may be singly housed throughout the duration of studies. This is to ensure no effects of aggression or social stress (potential phenotypes under study) are evident in cage mates necessitating humane killing of animals or affecting data. The above-mentioned models are well described in the literature and will be used before the expression of age-related harmful phenotypes. Animals will be group housed except whether there is a scientific or welfare reason to singly house.

Why can’t you use animals that are less sentient?

Rats and mice will be used as their anatomy, physiology, behaviour, and genetics has been well documented and they are the lowest form of mammal that can provide meaningful results that are translatable to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Drug Administration:
Animals are acclimatised to the facility for at least one week prior to the initiation of any procedures. A handling process may be introduced in advance of some studies (e.g., where measuring biomarkers which may be affected by stress, such as prolactin) or where longer-term dosing may be required. We have found on previous PPLs, that this often helps provide a stable baseline and will be used where it is considered appropriate and a refinement.

Vehicle and candidate compounds will be administered by the most appropriate route and administration will be performed by highly skilled staff, using appropriate dosing techniques, dose volumes and solutions (e.g., solutions/suspensions, appropriate pHs, possible crystallization of agents) to minimise any stress and discomfort to the animals. Appropriately sized needles will be used, and a separate needle will be used for each animal for systemic injection to maximise welfare and reduce the chance of inter-animal infections and reduction of pain. Oral administration to rats will normally be by gavage using flexible catheters to minimise oesophageal trauma. Short oral dosing needles are typically used for mice which are likely to bite the flexible tubing. Refinements to oral administration of drugs such as training the animals to consume material voluntarily from a syringe have been considered and cannot be used as drugs have to be soluble in small dose volumes and palatable in sucrose or saccharin solution, which is not the case for most drugs aimed at treating CNS disorders therefore making this refinement impractical. Where prior knowledge exists regarding administration of the test compound in vivo this will be used to optimise the choice of doses and route of administration wherever possible.

Surgery and analgesia:
Relatively few animals are expected to undergo surgery but implantation of osmotic/iprecio mini-pumps is possible as is intracranial administration either via an implanted cannula or injected directly into the brain under anaesthesia using stereotaxic surgery. The team within the company has extensive surgical skills in both mini-pump surgery and more complex surgeries (e.g., stereotaxic intracranial surgery both for injection and implantation). Accordingly, we are constantly striving to improve the technique to reduce the likelihood of suffering in the animals and to improve our surgical and assay techniques. Aseptic technique will be always applied to reduce the risk of infection.

For all experiments involving surgery, animals will be treated pre/post-operatively with an analgesic following consultation with the Named Veterinary Surgeon (NVS) (e.g., with a non-steroidal anti- inflammatory drug such as carprofen). The time course of the analgesia will be considered to ensure that it is beneficial from the time the animal may first begin to experience pain. Animals are monitored for behavioural signs of pain by our staff who have extensive experience of post-surgical animals, by the NVS and by the Named Animal Care and Welfare Officers (NACWOs). They will also be carefully monitored post-surgery until fully recovered from the anaesthesia (as done in human surgeries) and will be provided with heat lamps and wet mash, where appropriate, to aid recovery.

Blood sampling:
Controlled heated chambers may be used to aid blood sampling from the tail vein. The use of a topical local anaesthetic to reduce any pain and discomfort during blood sampling will be considered.

Temporary cannulation will typically be used in rats to avoid multiple needle entries over short periods of time. Limits on frequency of blood samples will be taken from guidelines. Specifically, up to 10% of the total blood volume (TBV) can be taken on a single occasion from a normal, healthy animal; this volume may be repeated after three to four weeks. Where blood is sampled repeatedly (e.g., weekly), the limit is 15% TBV over 28 days. Daily blood sampling would be a maximum of 10% TBV.

Although there are variations in TBV across strains and size of animal, TBV has been calculated as Mouse 72 ml/kg and Rat 65 ml/kg based on the literature and globally used values e.g., https://www.jax.org/news- and-insights/2005/october/how-much-blood-can-i-take-from-a-mouse-without-endangering-its-health; Diehl et al. 2021 (https://pubmed.ncbi.nlm.nih.gov/11180276/.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidance outlined in the PREPARE and ARRIVE guidelines for experimental planning and publication of data produced during this project.
We will also follow guidance issued by the Home Office, LASA, NC3Rs and RSPCA on animal re-use (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/470 008/Use_Keeping_Alive_and_Re-use_Advice_Note.pdf), blood sampling (https://www.nc3rs.org.uk/microsampling and https://www.nc3rs.org.uk/general-principles), substance administration and needle use (https://www.nc3rs.org.uk/news/re-use-needles-indicator-culture-care, https://www.rspca.org.uk/webContent/staticImages/Downloads/AdministrationOfSubstances.pdf and http://www.procedureswithcare.org.uk/ASMS2012.pdf), aseptic technique (http://www.lasa.co.uk/wp- content/uploads/2017/04/Aseptic-surgery-final.pdf and https://researchanimaltraining.com/article- categories/aseptic-technique/), and housing (https://www.nc3rs.org.uk/3rs-resources/housing-and-husbandry/rodents).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep abreast of the NC3Rs website for any updates as well as following any guidance or information passed on by our Named Information Officer (NIO). Alongside this, we will attend any relevant seminars and webinars produced by groups such as NC3Rs and Responsible Research in Practice. We will also keep up to date with the literature of advancements in the methods outlined in this licence and implement improvements to our working practices where practicable.

58. The identity and function of sensory-motor networks underlying behaviour

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Sleep, Decision making, Behaviour, Sensory, Computation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to gain a fundamental understanding of how the brain processes, stores and recalls information from its environment and past experiences to make decisions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Learning how the rodent brain is able to coordinate incoming information and use that information to make decisions is an important step in knowing how any brain performs this function, as these processes are evolutionarily conserved from flies to humans. In order to be able to treat neurological disorders we need to understand

how the brain normally functions first. We need to know how these processes work to be able to treat abnormalities at the root cause in the future and not just to treat symptoms.

What outputs do you think you will see at the end of this project?

The outputs of this project include the discovery of new information and contribution to current knowledge. Any tools, methods, and data we collect will be published where possible, as well as shared through other means.

Who or what will benefit from these outputs, and how?

This information and data obtained from this project will be of interest not just to experimental neuroscientists, but to scientists in other areas of research including theoretical neuroscience and bioinformatics.

Researchers and other professionals with an interest in animal welfare will benefit from the knowledge gained on mouse behaviour, as well as refinements and animal management applied during the course of the project.

The general population will indirectly benefit from the knowledge gained from this project, by contributing to the understanding of the mechanisms by which the brain converts sensory input into motor behaviours and the systems underlying these behaviours. This information may benefit future studies into key brain functions such as vision, hearing, or talking.

How will you look to maximise the outputs of this work?

Research findings will be made available through publication in peer-reviewed journals and presentations at scientific conferences and meetings, as well as through local channels such as retrospective review meetings and project presentations.

Species and numbers of animals expected to be used

- Mice: 16000
- Rats: 7000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The experiments in this project will use mice and rats. The ability to use transgene technologies established for these species, allowing highly refined experimental design and detailed analysis of neuronal networks with molecular, synaptic, cellular and circuit level resolution. Rats offer additional advantages in terms of their size and

ability to learn highly complex sequences. This permits the implantation of more electrodes to record more data from the same animal, however transgenic rats are underdeveloped where mouse transgenics is the cutting-edge model for mammalian life science research.

Rodents are the phylogenetically lowest for which direct comparisons can be made with the structure and functioning of the human brain, and mice are the species of choice in most areas of biomedical research because of the ease of access to a vast library of existing genetically modified strains, which can be used for targeting specific brain areas of interest to this project. This direct comparison as well as this accessibility and availability of information makes rodents the most appropriate species to study the neural circuits and behaviours targeting in this project.

We will breed specific transgenic rodents to enable us to target genes or brain regions of interest within our experiments - this encompasses the use of egg & embryotic, neonate, juvenile, and pregnant animals. The majority of experiments will be conducted in adult animals aged 2-12 months old, as animals at this age are competent to withstand the surgical and behavioural testing required.

Occasionally, aged animals >15 months old will be used - typically this will be in longitudinal studies where we seek to understand the effects of aging on specific behavioural outcomes.

Typically, what will be done to an animal used in your project?

In a typical experiment, an animal will be acquired or bred under standard laboratory conditions until the relevant developmental stage is reached (typically adulthood). At any stage of the study, animals may undergo several environmental or social manipulations, such as increasing or decreasing the number of animals in a social group, or altering the light-dark cycle of the room in which they are kept. Subsequent testing will allow us to measure the neural changes and responses to these varying environmental and social dynamics.

At any stage of the study, animals may undergo administration of substances to express genes, enable cellular visualisation, or to administer a therapeutic agent. Animals may also have their blood sampled in non-harmful volume.

The animal will be submitted onto one of three main pathways of the project:

<u>1 - Free-moving behavioural testing</u>
The animal will undergo surgery under deep anaesthesia to provide access to the brain, enabling several procedural types to be carried out to facilitate the manipulation and monitoring of neural activity. Major surgical types include:

- Implantation of cranial devices, such as mini-microscopes or other recording devices, to enable neural activity to be chronically monitored.

- Injection of substances into the brain, to induce genetic expression or modulate brain activity, or to facilitate visualisation of specific tissues.

Most animals will undergo only one cranial surgery, but others may experience up to a maximum of 3 surgical procedures when it is not possible to accommodate all required procedures within one surgical window, or where time is required between surgeries to allow a particular gene to express or other time-sensitive change to take place.
After recovery from surgery, the animal may be trained to perform a behavioural task over several weeks to several months, depending on the experimental requirements and task complexity. For instance, animals may be trained to discriminate different sensory stimuli, or to associate a specific sensory stimulus with a reward. Animals may be food or water restricted during their behavioural training to motivate their learning and task performance - animals will earn supplementary food/water rewards on successful completion of the behavioural task. The animal will be monitored during its performance of the behavioural task, and may have different physiological measurements taken during its behaviour.

Specific parts of the brain may be recorded, or the activity of specialised types of brain cells measured across one or multiple recording sessions.

2 - Head-fixed testing and imaging
The animal will undergo surgery under deep anaesthesia to provide access to the brain, and to have apparatus installed to the skull that will facilitate head-fixed testing and imaging. These may include head-bars and plates, and cranial windows. Substances or light may be additionally administered to the brain to induce chemical or optogenetic changes to the cells or mechanisms.

Animals will be habituated to head-restraint through a pre-testing period of acclimatisation to the head- fixation rig. Once suitably habituated, animals will undergo head-fixed imaging to visualise and monitor deep brain structures and specific cell types that cannot otherwise be accessed by non-fixed methods. Additional sensory stimuli may be applied to the animal as it undergoes imaging, to investigate the neural changes triggered by this stimuli.

3 - Free moving behavioural testing, and head-fixed testing/imaging
To establish robust causal relationships between neural elements and their function (e.g. between the activity of specific neuronal populations and defensive behaviour) a small subset of animals will experience both head-fixed imaging and testing, and free-moving behavioural testing within their lifetime.
Animals will undergo surgery under deep anaesthesia to provide access to the brain, enabling several procedural types to be carried out to facilitate the manipulation and monitoring of neural activity, notably:

- Implantation of cranial devices, such as mini-microscopes or other recording devices, to enable neural activity to be chronically monitored.

- Injection of substances into the brain, to induce genetic expression or modulate brain activity, or to facilitate visualisation of specific tissues.

- Installation of cranial windows, or head-bars and plates for head-fixed imaging.

Most animals will undergo only one or two cranial surgeries, but others may

experience up to a maximum of 3 surgical procedures when it is not possible to accommodate all required procedures within one surgical window, or where modifications are required to the installed devices to permit different types of testing.

Animals will undergo both free-moving behavioural testing tasks, and head-fixed imaging sessions, utilising the methods described previously. Animals may undergo up to 3 ‘blocks’ of each type of testing - these may rotate between testing types, or be continuous. To reduce any stress caused by the different types of procedures, animals will be (re)habituated to the imaging rigs and/or appropriately trained on the behavioural task required when switching between testing blocks.

Animals may be maintained post-procedure up to a maximum age of 24 months when required for longitudinal study.

In some cases, recordings in terminally anesthetised animals will be carried out to quantify the physiological function of neuronal networks independent of the behavioural or motivational state of the animal. Beyond baseline characterisation, this is also essential where recordings from deep brain regions that prevent recovery are required.

At the end of each experiment, the animal will be killed humanely and brain or other tissues will be collected for histology or other ex-vivo testing.

What are the expected impacts and/or adverse effects for the animals during your project?

Some animals on this project will experience adverse effects such as impaired mobility and increased susceptibility to seizure activity due to their genetic alteration.

Instances of ataxia or mobility problems typically onset at a pre-weaning age - any animals experiencing difficulty with normal movement will be provided with food upon the cage floor, and accessible water sources, to ensure that they are able to feed and drink normally. Animals susceptible to seizures will be monitored closely; if seizures are repeated and/or causing significant adverse health consequences, the animals will be humanely killed if amelioration of the effects is not possible.

Most substances applied to animals, for the purposes of gene induction, therapeutic intervention or the visualisation of cells or neural structures, will be innocuous and cause no adverse effects. Where animals are dosed with tamoxifen to induce genetic recombination (eg. Cre/lox mouse models) minor adverse effects due to the tamoxifen administration may be experienced, such as transient scrotal swelling and abdominal discomfort.

The main procedures used in the project require a surgical procedure to gain access to the brain, and adverse effects will mostly result from post-operative complications (such as scabbing at the surgical site), which in the majority of cases can be ameliorated or avoided using appropriate post-operative care. Adverse effects after surgery may reach moderate severity levels for a short period of time because of post-operative pain, but all animals receive pain relief and are closely monitored until they recover completely, and usually recover fully after 24 to 48 hours.

Many procedures undertaken in this project involve studying free-moving behaviours in which the animals are expected to experience no or minor adverse effects. In some experiments, the animals have restricted access to food and water to motivate them to perform tasks and earn food reward; however, they are carefully monitored to ensure these restrictions do not affect their welfare. Some minor weight loss may be experienced in the first instance but this will be maintained at an appropriate level as not to impact animal welfare.

In some experiments, the animals will be head-restrained in order to record brain activity and might initially experience some transient stress from the head-restraint.

However, through a robust habituation period prior to head-fixed imaging or testing, animals will be allowed to slowly get used to the experimental conditions such that stress and discomfort will be minimised.

In a small number of experiments, animals will undergo both head-restrained imaging and testing, and free-moving behavioural testing, in up to 3 experimental 'blocks' of each testing type. The expected adverse effects of these experiments encompass the harms described above, as animals may need to be alternatively diet or water restricted to motivate behaviour, and habituated to imaging rigs prior to imaging testing. To minimise the cumulative effect of any stressors, all animals will be monitored closely, and only moved to the next experimental 'block' if they appear healthy and to be experiencing no detrimental effects due to the previous testing type. Prior to commencing any new 'block' of testing, the animal will be (re)habituated to the task, to alleviate any acute stressors that may be experienced by the different task type.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities for animals undergoing procedures on this license range from:
Non-recovery - estimated 5% of animals. These animals will experience no procedural harms or harms related to their genetic modifications. These animals will experience a terminal procedure only (eg. Protocol 6)

Sub-threshold - estimated 5% of animals. These animals will experience no procedural harms or harms related to their genetic modification - eg. animals used for breeding that do not display an adverse phenotype (eg. Protocol 1)
Mild - estimated 10% of animals. These animals will experience only mild procedural harms or stress, such as those induced in behavioural tasks. (eg. Protocol 3)
Moderate - estimated 80% of animals. Most animals on this license will undergo surgical preparation for future behavioural and/or imaging studies, and will experience moderate severity levels for a short period of time due to the surgical intervention. (eg. Protocol 2, 3, 4)

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project involves the study of dynamic properties of synapses, neurons, neuronal networks and their behavioural output in response to sensory stimulation and during motor output. Studying these processes in live brain tissue is therefore essential for this fundamental biological research, and the use of animals is unavoidable for the important scientific questions we would like to address.

Which non-animal alternatives did you consider for use in this project?

This project involves the study of dynamic properties of synapses, neurons, neuronal networks and their behavioural output in response to sensory stimulation and during motor output. Studying these processes in live brain tissue, and within an entire living organism, is therefore essential for this fundamental biological research.

The use of non-regulated animals for these experiments (eg. flies, flatworms) was considered, but due to the complex learning tasks required and the resultant behavioural phenotypes that we wish to interrogate, these animals are unsuitable for the study. We consider the use of animals to be unavoidable for the important scientific questions we would like to address.

Our lab has and will continue to use theoretical methods to understand the physiological properties of cells and networks. We frequently use computer modelling at the synaptic, cellular and network levels to test possible scenarios before experiments are carried out. This informed approach allows us to reduce animal usage by designing better experimental protocols that more precisely target our brain regions or cellular networks of interest, especially within complex non-predictable systems such as network dynamics.

Moreover, we will use sophisticated data analysis to extract the maximal amount of information from a particular experiment. This combination of experimental and theoretical approaches lies at the core of our Establishment's scientific philosophy. Our close ties with units providing computational and digital expertise within the building further ensures that alternative computational approaches are adopted wherever possible.

Why were they not suitable?

As we learn more about neurons and synapses under investigation, we will be able

to use mathematical modelling more extensively, but for such approaches to be useful they will need to be tightly constrained with biological measurements taken in live animals.
Computational and in-vitro models are currently not suitable to complete replace animals in this area of research.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The main source of these estimates is data from projects carried out under the current and previous licences, using identical or very similar experimental methodologies. In contrast to previous work, modern tools offer the opportunity to reduce numbers, by enabling data collection via more than a single modality (e.g. physiology and anatomy, or anatomy and imaging, for example).

Also, many of the physiological experiments under this licence involve long-term training and recording from individual animals. From twenty years' experience using these approaches, we find that an individual scientist can work on no more than 6 experimental animals per batch; more typical would be three or four animals, depending on the complexity of the experiment and time commitment required for each mouse. The duration of time that an individual animal from within a batch may stay under investigation can range from two to fifty-two weeks. While it is not possible to accurately estimate the maximum number of experiments a particular mouse line (or rat) may be used for, it is possible to know the minimum. Factors influencing this number include the train-ability of an individual and the number of units that can be isolated.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Wherever possible, we aim to test multiple hypotheses and parameters within the same animal. For example, in an experiment where we use viral infection to express molecules for optogenetic stimulation and behavioural recording, the data gathered from this experiment will additionally be used to investigate the anatomy of the neural structures involved. At the end of this experiment, the brain can be collected and then used for microscopy, producing a detailed map of the brain's structures that have contributed to the recorded activity and behaviour By using the data gathered from one experiment in multiple different ways, we significantly reduce the amount of animals that are required to undergo procedures.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will employ very recent methods that maximise the amount of data collected from each animal. For example, with two-photon calcium imaging, one of the core methods of the proposed research, we can record up to several hundred neurons at the same time, thereby reducing the number of animals required to address the key questions to how neurons encode sensory information (approx. 10-fold reduction compared to other techniques).

We will use computer modelling at the synaptic, cellular and network levels to test possible scenarios before experiments are carried out.

We will use sophisticated data analysis to extract the maximal amount of information from a particular experiment.

We will aim to record repeatedly from the same animals in longitudinal studies, we can obtain more valuable data about the dynamics of neuronal processes from individuals, thereby again reducing the number of animals needed, compared to single time-point experiments.

We will use efficient breeding practices to avoid wastage. This may include using the minimum of necessary breeding pairs to maintain the lines, and maintaining transgenic lines as homozygous to reduce the need for genotyping or animals of an unusable genotype.

Any animals no longer required from the breeding colony will be used for histology or tissue samples to avoid wastage.

Brain tissue from an animal will be shared and/or move through other experimental analysis, for example whole-brain imaging of connectivity in fixed tissue following in vivo recordings in order to reduce the number of animals used in total.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice and rats for this project that are sufficiently close to humans to reveal principles of information processing in the brain. Our primary experimental approaches include stimulation and recording from a slice preparation ex vivo, and from different regions of mammalian brain in vivo.

We will apply combined in vivo electrophysiology and circuit tracing methods that

have been developed in our laboratory. These are the most refined tools that can be used for the study of information processing and connectivity in genetically and anatomically defined neural networks.

Why can't you use animals that are less sentient?
Mice are the photogenically-lowest species for which direct comparisons can be made with the structure and functioning of the human brain. Furthermore, rodents have been a long-standing useful model for behavioural studies of learning and memory, which enables us to build upon a large body of research already carried out, and to relate our findings to previous results.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

When performing regulated procedures under anaesthesia we will ensure that the animals are sufficiently anaesthetised using standard procedures (e.g. pedal withdrawal, pinch reflexes, rate, depth and pattern of respiration), as well as use appropriate pre or peri-operative analgesia.

We will use appropriate post-operative monitoring, including analgesia for a minimum of 48hrs post- surgery, or as required.
Animals will be habituated to behavioural experiments, and removed from tasks if they are found to not be tolerating or learning the task within the expected time-frame for that particular experiment (this may vary based on factors such as task complexity).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

- Refinements to rodent head fixation and fluid/food control for neuroscience. Barkus C et al., J Neurosci Methods. 2022. Nov 1; 381:109795
- LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. A report by the LASA Education, Training and Ethics section. (E Lilley and M. Berdoy eds.).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep up to date with 3Rs developments through the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs), including recommendations for best practices in neuroscience rodent experiments.

We will further be informed by the local 3Rs group at the establishment as well as working closely with animal facility staff and Named Persons, to continuously implement 3Rs advances.

59. Development of biochemical indicators to infer growth and nutritional condition of wild juvenile fishes

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Marine fish, Ecophysiology, Growth, Nutrition, Temperature

Animal types	Life stages
Dicentrarchus labrax	juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop RNA-based indices for estimation of growth and nutritional condition in wild juvenile fish.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

To complete their life cycle, many fishes have critical requirements for coastal habitats during juvenile stages. Identifying the habitats required by these populations is necessary to prioritize conservation measures and understand population dynamics in support of fish biodiversity and fisheries sustainability. Full assessment of the quality of a habitat for juvenile fish requires knowledge of how it supports growth and development, but few suitable tools are available to take these

measurements in wild juvenile fish. Development of an RNA-based index of juvenile growth in this project will help to understand which habitats are of the highest quality and therefore facilitate the efficient allocation of management and conservation resources.

What outputs do you think you will see at the end of this project?

This project will develop RNA-based indices to infer the growth and condition of wild-caught juvenile fish based on the biochemical composition of their muscle tissue. Findings will be reported in scientific papers directly and will form key chapters of two PhD theses. These papers and theses will be a platform for numerous future research outputs investigating habitat requirements of juvenile fish.

Collectively, results will inform conservation and fisheries management practice.

Who or what will benefit from these outputs, and how?

In the immediate term the outputs will benefit the field of fish ecology and conservation by providing new, biochemical proxies for growth and condition with which to map juvenile habitat quality for wild populations (i.e. food limitation, growth and nutritional condition). In turn, this information will benefit management organisations and policy makers by filling evidence needs on habitat requirements necessary to achieve nature conservation and sustainable fisheries. Effective management of coastal ecosystems will benefit society by supporting sustainable fishing, biodiversity and a wide range of other ecosystem services.

How will you look to maximise the outputs of this work?

We will maximise communication of outputs through multiple publications in the scientific literature and oral communications at scientific and management meetings. The ICES Working Group on the Value of Coastal Habitats for Exploited Species will act as a forum for disseminating results to the international community. Samples from the experiment will be saved and made available to researchers working on related fields for added scientific outputs.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The common sole *Solea solea* and the European seabass *Dicentrarchus labrax* will be used in this project because:

They are not endangered species (least concern for sole and bass).

They are species of high commercial and recreational value and therefore of interest to the fisheries sector.
They have a strong reliance on shallow, inshore habitats as juveniles, so would benefit greatly from a better understanding of habitat requirements through the development of biochemical tools. This has been identified as a key DEFRA evidence need and is associated with very specific and relevant policy drivers (e.g. Bass Nursery Areas, Fisheries Management Plan, impacts of Hinckley C and other large energy infrastructure)

They are available locally.

Their life-cycle and physiology is well known.

They have been used in several experiments before and are aquaculture species.

Therefore, we have an understanding of the acceptable and optimal rearing conditions.

Juvenile individuals are used because the project is focused on juvenile fish and using another life stage would not be relevant. Studying the needs and responses of juveniles to environmental changes is of particular importance because early life stages can be very sensitive to environmental conditions and represent critical stages determining population dynamics.

Typically, what will be done to an animal used in your project?

All fish will be captured in wild environments, transported to the lab and acclimatised for at least 1 week.

A sample of fish will be taken from the experimental population in the lab and euthanised to take baseline measurements.

Fish will then be grown under different temperatures (range = 12-24 deg C), salinities (15-35ppt) and food rations (no food, maintenance ration, moderate ration, *ad libitum*), representing the conditions they are exposed to naturally in the wild, for a period up to three weeks. This will include a treatment where food is withheld for up to three weeks.

During experimental treatments, *S. solea* will be housed individually in tanks lined with sand to allow natural burrowing behaviour. *D. labrax* will be kept in groups to maintain natural schooling behaviour.

On one occasion during experimental exposures, fish will be weighed, measured and marked individually using a minimally invasive method: this will be undertaken under general anaesthesia where appropriate. Prior to weighing and measuring, fish will be starved for <48hrs to evacuate gut contents.

At different points during experimental exposures, individuals will be humanely

euthanised, weighed, measured, then frozen for biochemical analysis of tissues.

What are the expected impacts and/or adverse effects for the animals during your project?

All fish will be caught in nets from the wild during a short sampling bout. Some individuals may experience mild damage or stress from the netting.

All fish will be transported to the establishment: some may experience mild damage or stress from handling, movement and interaction with conspecifics during transport.

Some individuals may experience mild damage or stress as a legacy from capture and transport, or moderate stress as a result of cumulative effects of holding conditions and / or food removal (up to 3 weeks fasting).

Some fish may experience delayed recovery from anaesthesia, during weighing, measuring, marking and anaesthesia.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

D. labrax: moderate, 100%
S. solea: moderate, 100%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The focus of the research is on better understanding aspects of the biochemistry of selected species to support the development of research tools supporting management. At present the tools must be ‘calibrated’ for each species studied, and this has not yet been conducted for *S. solea* or *D. labrax*.

While there have been attempts in the literature to develop general indices, these are not yet sufficiently robust for application. Through the use of a small number of animals, we can provide research tools to support sustainable management at the population scale.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives are not available to address this research question.

Why were they not suitable?

We are working in non-model systems, on a question which requires empirical measurement.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of animals is the product of the number of treatments and the number or replicates within each treatment. *D. labrax* must be held in groups so we have added tank mates to ensure that all fish are in groups of at least six individuals.

Number of replicates:Data from our previous work suggests that eight fish per group is suitable replication.

Number of treatment groups:

Temperature: Previous work also suggests that responses to temperature may be non-linear so at least 4 treatment groups are required to characterise temperature coefficients.

Food ration: For objective 2, at least three time points are required to understand how quickly RNA begins to change after food removal and the point at which it stabilises. For objective 3, our previous work suggests that four food rations produces a suitable range of growth rates for calibrating RNA- based indices.
Salinity: at this stage we will simply test the hypothesis that RNA-based indices differ among experimental salinities so two treatment groups will be required.

Tank mates:In *D. labrax* it is necessary to hold fish in groups so that they can exhibit normal schooling behaviour. In the case of the time course experiment, the minimal number of animals will be achieved by removing individuals from the same, larger group. Groups must be sufficiently large to maintain minimal densities across repeat sampling. We will ensure that controls are in place to account for reductions in density.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experimental design builds on my experience conducting and publishing RNA-

based growth indices and my knowledge of published studies in the wider literature. We met on several occasions as a research team to plan the experiment. We critically evaluated how the number of animals could be reduced to the minimum necessary to meet the scientific objectives. The experimental plan was independently reviewed by the NACWO, NVS, AWERB committee and scientists within the department who frequently undertake 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 use a sequential experimental approach to strengthen the design. This mainly focuses on refining the experimental duration, but it will also permit adjustments to numbers of replicates. Existing power analyses, developed for other species in previous work, will be updated with estimates of effect sizes in initial experiments to optimise the number of animals used in later stages.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The project will focus on European seabass *Dicentrarchus labrax* and common sole *Solea solea*. Rather than a model system approach, we are directly interested in generating data for these species in order to fill evidence needs for the conservation and management of wild populations. Our efforts will develop tools to infer juvenile habitat quality: this is important for these species because they spend prolonged periods in shallow, inshore areas which are vulnerable to human impacts.

In order to interpret biochemical indicators of growth and nutritional status under the conditions to which these species are natural exposed, it is necessary to grow them under a range of different temperatures, salinities and food rations: all these will match those routinely encountered by populations in the wild. Unless our study incorporates this range, we risk that applications will not be transferable to the natural environment.

Weighing, measuring and marking of individuals is necessary to calculate individual growth rates.

Why can't you use animals that are less sentient?

The goal in this case is to develop tools that can be applied in specific species of juvenile fish. The index is sensitive to the species and life stage studied and

universal indices are not available. A less sentient substitute cannot be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Netting methods will be adapted, based on catch rates, to minimize the duration of deployments and therefore the stresses associated with capture. Regular checks will be made after capture and during transport to ensure that individuals experiencing pain, suffering, distress or lasting harm are humanely euthanised by schedule 1.

Laboratory steps chiefly involve holding fish under natural conditions, so a key refinement will be the clear recognition of humane endpoints to enable termination before animals experience pain, suffering, distress or lasting harm. All individual fish will be observed in water at least twice daily and scored against a welfare scoring matrix for laboratory experiments once daily. According to this welfare scoring, a fish considered of urgent concern or remaining of concern for an unacceptable period will be euthanized.
Moreover, we have designed the experimental plan and divided it into three experiments in order to best (i) reduce the number of individuals subjected to the longest fasting period, and (ii) be able to stop the experiment when humane endpoints are approached, without compromising scientific outcomes.

General anaesthesia will be used, where appropriate, to mitigate stresses of handling, weighing and marking.

Individual recognition will be necessary in order to calculate individual growth rates. In *S. solea*, marking will be avoided by holding fish in individual tanks. In *D. labrax* individual holding could deprive fish of social contact so individual marking and group holding is considered a more refined approach. Individual marking will be performed using minimally invasive methods: subcutaneous injection of dye is considered the recommended approach for small fish and will be adopted as a preference. A small fin clip is also considered to cause only minor, transitory discomfort and will be used as an alternative in case of issues with subcutaneous tagging (e.g. tag retention;

ASRU 2014; Sandford et al. 2020).

Individual marking will be conducted under anaesthesia to minimize handling stress.

References:
Sandford M, Castillo G, Hung TC (2020) A review of fish identification methods applied on small fish. Reviews in Aquaculture 12 (2):542-554
ASRU (2014) Code of practice for the housing and care of animals bred, supplied or used for scientific purposes. p 183

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

PREPARE Guidelines (Smith et al 2018) will be discussed with members of the project team, the animal facility managers and the NACWO in order to ensure that the experiments are conducted in the most refined way.

Refinement in our experiment is strongly linked to understanding the welfare status of the animals to ensure that they are humanely euthanised before they experience substantial pain, suffering, distress or lasting harm. We are following best practice for identifying severity limits and welfare scoring systems to ensure humane endpoints that sit within these. Severity limits are based on Hawkins et al (2011) while welfare indicators follow reviews by Martins et al (2012), Barreto et al (2022) and Browning (2023). Our framework for assessing welfare is also advised by models / systems including MyFishCheck (Tschirren et al 2021) and SWIM (adapted for *D. labrax* by Yildiz et al 2021).

We have followed the recommendations and decision tree from Sandford et al (2020) and guidelines from ASRU (2014) to identify subcutaneous dye injection and fin clipping as the most refined method for individual identification.
Individual marking will be conducted under DOPS, to the satisfaction of the University of Plymouth NTCO.

References
ASRU (2014) Code of practice for the housing and care of animals bred, supplied or used for scientific purposes. p 183
Barreto MO, Planellas SR, Yang YF, Phillips C, Descovich K (2022) Emerging indicators of fish welfare in aquaculture. Reviews in Aquaculture 14 (1):343-361
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Yildiz HY, Chatzifotis S, Anastasiadis P, Parisi G, Papandroulakis N (2021) Testing of the Salmon Welfare Index Model (SWIM 1.0) as a computational welfare assessment for sea-caged European sea bass. Italian Journal of Animal Science 20 (1):1423-1430

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The project team has followed the best available information about anaesthesia practices, individual marking and welfare scoring to manage humane endpoints. We will undertake regular searches of the literature and the resources on NORECOPA in order to learn of developments in these areas. The project team will meet regularly

with the NACWO and NTCO to share information.

60. The effect of Medicinal products on the Gastrointestinal System

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Gastrointestinal, Pharmaceutical, Safety, Efficacy

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to examine the effects of medicinal products (e.g. pharmaceuticals) on the Gastrointestinal system. This may be to assess efficacy (if they can affect the Gastrointestinal system) to see if they are better than existing drugs, or to identify undesirable side effects (including if they are safe to administer to humans).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Work performed under this licence may identify more effective drugs for example to those already on market, with fewer side effects and that work better than existing drugs.

Some studies will be required by regulators to help them decide whether potential drugs both work in their chosen indication, and are likely to be safe in humans.

What outputs do you think you will see at the end of this project?

The principal benefit of the project is the provision of data to facilitate sound decisions on safe/effective product development and appropriate regulatory decisions on clinical trial approval or marketing authorisation for new medicines to which humans will be exposed, thus contributing to their protection and safety.

The potential benefits of this project would include the discovery of new treatments for gastrointestinal diseases, and the confirmation of the safety of new treatments for gastrointestinal diseases prior to first administration in man.

Work under this Licence will also show which compounds are not suitable to move forward into patients due to them not being able to moderate the clinical condition examined, or they are not safe to go into humans, for example.

Work performed under this licence may identify more effective drugs to those already on market, with fewer side effects and that work better than existing drugs.

Who or what will benefit from these outputs, and how?

Patients will benefit from these studies as this work will contribute to the development of new drugs that help alleviate human gastrointestinal conditions. These new drugs may work better in the clinic, relieve or cure diseases and have better side effect profiles. We may, by our work, also contribute to better knowledge and understanding of these types of drugs, and that knowledge may be used to develop further new drugs.

One of the key benefits is the production of data that is required by regulatory authorities, to ensure medicines have their desired therapeutic effect in humans (e.g. stop episodes of sickness in the specific model). The drugs that will be tested are for a variety of conditions, in some cases where there is an unmet clinical need to treat such conditions.

In addition, the models on this project may be used to assess the safety or other in life properties of a new drug, and find a dose that causes no adverse effect. This is important when planning future trials in humans, to make sure any starting dose in a clinical trial is safe for the patients taking it.

Our customers will also benefit, as the data we generate will allow them to progress their new drugs into clinical trial, or otherwise if they are found to have adverse side effects.

How will you look to maximise the outputs of this work?

The work will be shared with customers who will use it to determine their future strategy, or for submission in documents required by regulatory authorities. Whilst we have no direct control over what happens to the data after we have shared it, we trust from information given to us that it is used to support candidate selection or to support drugs progressing to clinical trials). Where appropriate, we collaborate with our customers to share data we have produced in the form of scientific publications that are in the public domain.

We regularly advise our customers on which studies are required in their development programme and on suitable study designs, based on our experience and on knowledge gained from previous feedback from customers and/or regulators, leading to focused and effective studies.

It is difficult to predict how the benefits of any work done on this project will be seen in the future due to confidentiality issues. However, this work will contribute to the efficacy and safety of pharmaceuticals that can be administered to humans, either by informing on safety and allowing to progress to clinical trials or preventing pharmaceuticals reaching the market due to safety issues), which in itself reduces the overall number of animals used, by preventing further testing.

Species and numbers of animals expected to be used

- Mice: 500
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use adult rats and mice on this project.

Rats and mice are used because they respond to gastrointestinal agents in a similar manner to humans and the data produced will help model gastrointestinal conditions that occur in humans, predict how well the potential medicines will work in humans, and predict the potential side effects of medicines in humans.

Typically, what will be done to an animal used in your project?

The vast majority of experiments performed under this licence will be short term (much less than 24h) using rats and mice, and will involve animals being dosed with a drug once, usually by using a tube into the stomach, or an injection into a vein or by an injection under the skin for example. In some cases, we may also use a marker to see how test substances affect the passage of an inert substance like charcoal down the intestines for example. This would also usually be dosed by a tube into the stomach.

On some occasions we may have to take blood samples to measure drug levels or biomarkers. These will be taken using accessible veins like the jugular vein in the neck, with strict limits on frequency and blood volumes we can take.

What are the expected impacts and/or adverse effects for the animals during your project?

Most procedures would cause nothing more than transient discomfort to the animals (e.g. dosing with a tube into the stomach or giving an injection in a vein or under the skin). When dosing an animal by injection or taking blood, the degree of pain or discomfort an animal feels is similar to what a patient would feel having an injection done or blood taken by a doctor.

Many of the experimental measures we look for are carried out after animals have been humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

On the last project, 100% of animals were classified as having experienced mild severity. We would expect nothing more than mild to moderate severity occurring on this project, with an expectation of no more than 10% of animals experiencing moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The gastrointestinal system is a complex organ system performing a wide variety of functions essential to life, such as secretion, digestion, absorption, excretion and defence. There is no adequate model to replace the whole animal experimental model, as the complex mechanisms under investigation cannot be adequately modelled in non-animal preparations.

Specifically, it is not possible to model the effects of pharmaceuticals on gastrointestinal transit in in vitro systems.

Some of the tests performed are required to support regulatory submissions, for administration to humans, for which non-animal alternatives are not accepted.

Which non-animal alternatives did you consider for use in this project?

Before any animal experiments are carried out, drugs are tested in test tubes to assess things like toxicity, metabolism, and how well they bind to their target receptors, for example. Many of these compounds don't go forward into animal testing, because they are simply not good enough to become drugs based on the results of tests like these.

Experimental designs are constantly reviewed and alternative cell assays considered as technology improves, however due to the complex nature of the gastrointestinal tract there are no current alternatives but to use animals. Similarly, the regulators who decide whether potential new drugs are safe to be tested in man, will not accept tests solely using non animal methods.

Why were they not suitable?

Although there are in vitro tests that can model aspects of the gastrointestinal tract, and some tests showing how drugs get into our bodies, and how our body deals with them, and can identify undesirable effects, for example, there is no series of in vitro tests that brings all these complex events together, as in the whole human organism. It would be impossible to assess gastrointestinal motility for example, without using a whole animal, as it involves multiple processes and other organ systems.

That is why we need to test new drugs in animals, as they have similar physiology and processes as humans, and that testing gives us a good idea what may happen if they are subsequently used in humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received in the past. It is, however, impossible to accurately predict the number of studies that may be performed, in the circumstances.

The numbers of animals used in each study are in some cases specified in the regulatory guidelines; where not specified, numbers are based on established minimum regulatory expectation, or on scientific estimates of the minimum numbers required to meet study objectives.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All experiments will be designed in order to achieve the scientific objectives using the minimum numbers of animals. For study types that are less well established and for

which historical data may not be available, the literature (scientific publications) will normally be consulted to help decide the group size.

Statisticians are often consulted particularly where the study type is not routine, as they can use calculations to estimate the correct number of animals needed to get a meaningful result.

Where possible, common control groups will be used in order to minimise the numbers of animals used.

A preliminary study may be conducted in which smaller numbers of animals may be used to generate data in order to ensure that the experiment operates as we would expect and to generate some data which may be used to get a better study design. From such pilot studies, the variability of the measurements is used by statisticians to determine the required number of animals per group required to identify whether the test substance actually has an effect in a main study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will try to get as many outputs as we can from a single animal where possible, without adversely affecting its welfare. So if we need to take several different samples, for example, we will often do that in the same animal, rather than using separate ones, when possible. .

We will try to get as many outputs as we can from a single animal where possible, without adversely affecting its welfare. So, if we need to take several different samples, for example, we will often do that in the same animal, rather than using separate ones, when possible.

The experiments we perform are only permitted on the condition we use the least number of animals possible to get a meaningful result to assess safety or whether a drug has the desired effect. These numbers are sometimes based on a number set by a regulator or based on our own experience. We regularly consult with statisticians when new study types are performed. They do a special calculation (power calculation) which takes into account the size of likely effect we will see to help determine the number of animals we use. Although we do use the least number of animals possible, it is important to use enough animals to get a meaningful result, otherwise we would end up using more animals overall than we needed to.

Where we can we only use one control group per study (effectively dosed with the drug formulation without the drug in it) which acts as a baseline to compare any effect of the drug itself.

Studies are also carefully designed to combine the different aspects needed to evaluate the safety or whether a drug has its effect combined, and again this reduces the overall numbers of animals used.

Variables that may affect the study are kept constant wherever possible to make sure the experiments stay the same time after time. This actually means the data is more reliable and meaningful, and easier to make assumptions about.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

On this project we will use adult rats and mice. Rats and mice are the standard species used as they are of the lowest sentience. Dosing and sampling procedures will be undertaken using a combination of dose volumes, routes and frequencies that of themselves will result in no more than low levels of discomfort and no lasting harm and will be the minimum consistent with the scientific objectives. Many of the procedures carried out produce only minor levels of discomfort, due to the nature of the procedure, and the skill of the person performing it. For example, an animal having a blood sample taken would feel the same level of discomfort as a patient in a doctor's surgery having a blood sample taken.

Most of the experimental outputs in the charcoal propulsion model are taken after the animal has been humanely killed. The inert tracer agent (e.g. charcoal) is introduced by a tube into the animal's stomach.

Food and water withdrawal will be kept to a minimum. This is often required so as a gastrointestinal tract full of food does not disrupt the outcomes of the experiment.

Why can't you use animals that are less sentient?

There is a scientific and regulatory requirement for safety/efficacy data in rodents (mice, rats), and they are considered the species of lowest sentience that give a valid comparison to human physiology.

Adult animals are used as we are modelling conditions that are present mainly in adults.

Many of the conditions we are modelling would be affected by the use of anaesthesia, therefore it is not practical to keep animals terminally anaesthetised.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During dosing and restraint, animals are constantly and closely watched for signs of distress.

If we have to repeatedly inject animals or withdraw blood using a needle and syringe, we would choose different sites to do this where possible to minimise local adverse

effects. Where appropriate we place temporary cannulas in blood vessels to reduce the number of needle punctures necessary. If we can take blood samples when an animal is deeply unconscious then we do so. All personnel performing these procedures are trained to a high standard to minimise adverse effects.

All procedures are subject to ongoing assessment and technique improvement, and we participate in cross-company working parties on best practice. Animals are regularly reviewed for general health and veterinary staff are on call at all times to assess any adverse events and provide supportive care and treatment as appropriate.

Refinements to improve the animals experience include but are not limited to group housing, environmental enrichment, including shelters for rodents, gnawing materials, extra bedding, human interaction, acclimatisation and training to procedures.

We have dedicated working groups on animal welfare for each species (in this case a rodent specific group) with a permanent brief to identify potential measures to improve animal welfare, and to trial such measures and make recommendations for adoption.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

ICH Guideline S7A. Anon 2000. Committee for Proprietary Medicinal Products (CPMP). Safety Pharmacology studies for human pharmaceuticals. The European Agency for the evaluation of medicinal products. London, November 16, 2000. Reference CPMP/ICH/539/00

A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes, Journal of Applied Toxicology, 21, 15-23 (2001).

Prior, H., Ewart, L., Bright, J. and Valetin J-P. (2012) Refinement of the Charcoal Meal Study by Reduction of the Fasting Period. ATLA. Vol. 40, 99-107.

First report of the BVA/FRAME/RSPCA/UFAW joint working group on refinement (1993), Laboratory Animals, 27, 1-22.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This will be achieved by discussions with our Named Information Officer, colleagues in Animal Technology, and by attending appropriate training courses and conferences, or getting feedback from such events.

61. Assessing tick-borne disease risk to livestock

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

ticks, livestock, risk, land-use change, climate

Animal types	Life stages
Cattle	juvenile, adult, pregnant, aged
Sheep	juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how ecological, environmental and management factors affect ticks and tickborne disease risks to livestock and people in farmland habitats.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Risks from tick-borne zoonotic diseases in agricultural landscapes are increasing rapidly in temperate regions, including the UK, and could be increased by climate change and large scale policy-driven changes to increase woodland and biodiversity on farmland. To mitigate these risks we need to better understand the ecological conditions allowing tick-borne pathogens to establish and spread and how these are modified by land-use and climate change. This will facilitate the design of more

effective, better contextualised disease prevention strategies. Tick-borne diseases transmitted by the same tick vector Ixodes ricinus, which also transmits Lyme disease and Tick-borne encephalitis virus are emerging as significant threats to livestock farming in grassland and moorland habitats in the UK and can cause human disease. Firstly, bovine babesiosis or redwater in cattle, caused by Babesia divergens (Bd) can result in high mortality in naïve herds; secondly, Louping Ill a febrile illness in sheep, cattle and grouse, caused by louping ill virus (LIV) can lead to encephalitis and severe mortality; thirdly, tick-borne fever in sheep and cattle, caused by Anaplasma phagocytophilum (Ap) which decreases host immune function and increases effects of other infections including LIV. These under-recorded livestock tickborne diseases rank highly amongst farmer priorities and are defined as "high impact, low prevalence, priority diseases" with severe long-term impacts on production in tick suitable areas, that will affect new areas and pose a public health threat as tick habitats change. While the human health impacts of Bd, Ap and LIV are poorly defined, Lyme disease (LD), caused by Borrelia burgdorferi (Bbsl), is a significant emerging Public Health threat in open habitats, since livestock can contribute to enzootic cycles.

What outputs do you think you will see at the end of this project?

The outputs will be new information on the factors which influence risks from ticks and tickborne diseases to livestock and humans in lowland and upland farmed areas of the UK.

Who or what will benefit from these outputs, and how?

By the end of the project we hope to understand the factors which influence risks from ticks and tickborne diseases in lowland and upland areas of the UK. This information can be used to help farmers and vets manage and best mitigate risks from tickborne diseases, and inform policymakers on the impacts of policies to increase biodiversity on farmland. This will benefit animal welfare, livestock production and animal and human health, and sustainability of farming systems. We will produce publications and present our findings at conferences, and share knowledge with stakeholders including vets, farmers, general public and policymakers.

How will you look to maximise the outputs of this work?

The findings of the project will be communicated to farmers and veterinarians through open access scientific publications, open access data sets and knowledge transfer materials and webinars.

Policymakers will be engaged through stakeholder workshops and meetings.

Species and numbers of animals expected to be used

- Cattle: 1800
- Sheep: 1200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are studying cattle and sheep of all life stages (apart from neonatal) as the diseases can affect animals of any life stage from juvenile onwards. By studying animals on farms we will ensure our findings will be relevant to the farm situation.

Typically, what will be done to an animal used in your project?

The sheep or cattle will have a blood sample and attached ticks collected. This procedure will typically take 15-20 minutes. Apparently unaffected and clinically affected animals will be included in the sampling.

What are the expected impacts and/or adverse effects for the animals during your project?

The cattle and sheep will experience brief pain or discomfort during the sampling which will last 15-20 minutes. After the animals have been examined, any clinically affected animals will be given relevant treatment for their condition as prescribed by their own veterinary surgeon

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severity is mild for all animals

What will happen to animals at the end of this project?

- Rehomed
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We wish to take blood tests samples from farm animals exposed to ticks and tick-borne pathogens and also sample ticks from grazed habitats. It is important to do this in naturally exposed sheep and cattle and in the farm environment so that the findings of the study are relevant to the real world situation

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives to this.

Why were they not suitable?

There are no non-animal alternatives to this.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals used in the study are based on advice from a statistician and experience gained from previous published studies on tick-borne pathogen prevalence and seroprevalence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used online sample size calculators (www.open epi.com) and advice from a statistician. We also referred to previous published research and our experience of the different disease presentations we see on farms.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continually monitor the study as it progress to ensure that only the required number of samples are collected.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 study disease in naturally occurring affected sheep and cattle. We will collect the minimum number of samples and minimally invasive samples from these sheep and cattle. Sampling will be brief (approximately 15-20 minutes). All diseased animals will receive appropriate treatment for the disease. All animals will remain on the farm of origin.

Why can’t you use animals that are less sentient?

There are no animal models for this disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will study disease in naturally occurring affected sheep and cattle. We will collect the minimum number of samples and minimally invasive samples from these sheep and cattle. Sampling will be brief (approximately 15-20 minutes). All diseased animals will receive appropriate treatment for the disease. All animals will remain on the farm of origin. The study will be supervised at all times by an experienced farm animal veterinary surgeon. All staff involved in farm sampling will be experienced in handling animals and trained personal licence holders. Where appropriate, sampling will be linked with handling for other husbandry procedures. If an animal becomes overly distressed by the handling and blood sampling procedure, the procedure will be stopped and the animal returned to the herd/flock.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Blood sampling will have minimal adverse impact on animals. ARRIVE guidelines will be used for study design and reporting https://arriveguidelines.org/.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The project licence holder will stay informed about advances in 3R's through engagement with the National Centre for Replacement Reduction and Refinement of Animals in Research Website and through seminars and information disseminated through the research institution where the project licence is held.

62. Developing therapeutic intervention strategies using sheep to model human neurological disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Neurodegeneration, Battens, Sheep

Animal types	Life stages
Sheep	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to build on previous work to determine the efficacy of two different therapeutic approaches for Battens disease, specifically the use of viral vectors or enzyme replacement therapy (delivered into the brain and spinal cord).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 will serve to characterise changes in disease progression following intervention in sheep models of human neurodegenerative conditions. The ability to examine disease processes and scale up therapeutic intervention strategies including routes of administration will help to bridge the translational gap between rodent derived

therapeutics and something which is likely to be effective in the clinic. This work is critical for shedding light onto the potential use of these therapeutic approaches for human patients, and provides essential samples for biomarker (a biological factor which can be used as a read out or prediction of disease status/health) discovery investigations. Using existing samples banked from sheep we have carried out some preliminary proteomic analysis and hope to publish some of these findings later in 2024.

What outputs do you think you will see at the end of this project?

The outputs will be information shared via public engagement, archived samples for access, open access publications, progress towards understanding neurodegenerative disease and therapeutic approaches.

The initial outputs from a sheep model of infantile NCL were observational, with homozygous founder animals closely monitored to document physiological disease development over time. Our initial characterisation has been published, comparing the ovine phenotype with what is known about human clinical disease resulting from the same PPT1 mutation, thereby benefiting the research community.

Under our previous license we extended our understanding of the model and working with international collaborators have published a recommended approach for neurological clinical scoring in large animal models. Our initial assessment of therapeutic intervention via repeated infusions of recombinant PPT1 protein demonstrated the potential utility of this approach in a manner comparable to human surgical delivery. This work has now been published, thereby benefitting the research community. The therapeutic in question has now been provided to a child in Germany under compassionate use with some benefits reported. We are submitting applications to the NIH with our collaborators and a pharmaceutical company to investigate and optimise this therapeutic approach.

We also have a 5 year commitment for ongoing NIH funding in collaboration with academic partners in the USA to investigate the utility of viral delivered therapies and have been making excellent progress with this work.

We are working with international charities and companies under CDA for molecular biomarker discovery using samples archived from work under a previous licence. We will continue to bank samples (post mortem) under this project license and will provide access to this resource as appropriate.

Who or what will benefit from these outputs, and how?

The published outputs of the work noted above will benefit the academic research community immediately in terms of improving our understanding of these conditions.

The tissue bank will be on ongoing resource which can provide benefit immediately and for as long as the bank is maintained.

The proposed attempts to modulate disease progression by therapeutic intervention is unlikely to have immediate benefit (except with the potential to inform the

exceptional use of such an approach in a compassionate use basis). Any information around biodistribution could have immediate benefits to others in the context of informing on appropriate routes of administration. In the first 5 years following the conclusion of this license the information could benefit a case for either - further characterisation of any therpeutic assessed here and/or support a push towards clinical application. In 5-10 years the benefits could be community based with therapeutics making their way into clinic.

How will you look to maximise the outputs of this work?

We have an international collaborative network to help disseminate our research findings. We interact with patient charities and families of affected children and will share research progress and failures to them. We will seek to publish work at the conclusion of the studies proposed in open access journals providing a free flow of information to those who may be interested. Indeed, we will also seek to make public any approaches which are ineffective. This is especially important in the context of studies investigating routes of administration in order to help streamline other studies and inform our understanding of nervous system dynamics.

Species and numbers of animals expected to be used

- Sheep: 52

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 only use animals in our research when the questions posed cannot be answered using non-animal alternatives. We only use livestock in order to bridge the translational gap between high quality rodent studies and likely effectiveness on a human scale.

In this case, whilst a therapy may be effective on the scale of a rodent, it is not simply a case of multiplying the therapy by the difference in body mass and having an effective therapy. Rodent brains are smooth (lissencephalic) and less complex than a human (i.e. folded appearance of the cortex - gyrencephalic), and the composition of the brain is very different. For example the grey to white matter ratio in a human is approx. 50:50, in a mouse its 90:10. In a sheep it is 60:40. This is much closer to a human.

If we give a therapy into the fluid filled spaces of the brain of a mouse, it only has a few mm to travel in any direction for full coverage. In a sheep and human it is in the order of cm. The distribution of a therapy in the fluid filled spaces is governed in part by how the fluid moves through and around the structures of the brain (dynamics). The sheep is much more like the human than a mouse.

We would never scale up to sheep without having a strong rational from existing work (i.e. rodent model investigations) that an approach has promise. But such assessments of therapeutic effectiveness (i.e. scaling up, distribution and persistence) cannot be accurately modelled using computers or cell based systems.

They can be addressed in larger model systems and indeed that is what we seek to achieve here. We will use juvenile and/or young adult sheep for this approach. The benefits of using a younger sheep in the context of a therapeutic interventional approach is that for conditions such as neurodegenerative disease a therapy may well halt symptom progression, but is unlikely to reverse existing damage. As a result the earlier the intervention is attempted the more likely a long term favourable outcome becomes.

Typically, what will be done to an animal used in your project?

The sheep will be moved to the research facility and acclimatised to the environment. They will be observed and interacted with in a manner as close as possible to what would be practicable with children affected by this condition in a hospital context.

We use non invasive observational techniques for regular clinical scoring (how do they walk, how is their vision etc), and occasionally we seek to examine functional readouts of visual integrity such as ElectroRetinoGram (ERG) recordings. ERG will be performed under general anaesthesia and neuromuscular blocking agents (NMBA) may be required to control ocular positioning for examination. In such circumstances only staff with specialist training (PIL D) will facilitate this work. We also use imaging such as MRI and/or CT under general anaesthesia to visualise the disease progress in the brain. We employ surgical approaches as close as possible to what is utilised a human clinical setting. Anaesthesia teams and neurosurgeons will carry out the work. Gene therapy (GT) for Spinal muscular atrophy and enzyme replacement therapies (ERT) for CLN2 (another form of Batten disease) are in the clinic now and GT for CLN5 (a further form of Batten disease) has FDA (U.S. Food and Drug Administration) approval for clinical testing. The CLN5 GT has approval based on preclinical studies in sheep. It is estimated that such scale up pre clinical approaches improve success in efficacy and safety.

Gene therapies for this condition will be using viral delivery and are a one off dose of therapy delivered into the nervous system (in the first instance). This requires delivery into the brain (into the mass of the brain or the fluid filled spaces). For this, a sheep have a one off dose of the therapy under general anaesthesia. The earlier the better as such therapies may slow or halt disease but is unlikely to reverse existing damage. The sheep will recover and be observed with non invasive clinical observation scoring recorded monthly. The sheep will likely have three MRI (brain imaging) scans under general anaesthesia in its lifespan (before, after, and at a predefined endpoint) to track disease progression and the effect of the therapy. At the end (sheep will live for no more than 24 months), post mortem samples of as many tissues as possible will be taken for banking and analysis.

Enzyme replacement therapies use protein (the enzyme which is missing from the affected individual) produced in the lab and infused into the nervous system. This

infusate is used up by the brain and therefore requires. Different enzymes have specific properties such as functional activity/stability/turnover etc. So in the case of CLN2 topped up every 2 weeks. For CLN1 the enzyme appears much more stable and rather than every 2 weeks as with CLN2, we are aiming around every 4 for CLN1 (under general anaesthesia). The difference between ERT and viral therapy is that in order to infuse the therapy into the fluid filled spaces we will implant a port under the scalp which allows physicians it infuse the enzyme simply by injecting through the scalp into this port which then allows the enzyme to go into the brain via a catheter attached to the port. This is the approach currently used with kids for CLN2.

By keeping the processes as similar as possible to a human hospital we maximise the likelyhood of translation into the clinic and minimise the number of animals required to get to this stage.

What are the expected impacts and/or adverse effects for the animals during your project?

Many animals in the current license will experience no more than mild severity (wild type and heterozygous controls). Those that may progress to moderate severity may be as a consequence of disease progression or as a result of the surgical intervention. Adverse effects can be associated with surgery and general anaesthesia. The surgical technique involved is not anticipated to cause adverse effects beyond the immediate post-surgical period owing to the experience and skill of staff involved and careful pre and post-operative monitoring. Post-operatively, sheep could develop neurological signs related to complications would include (but not limited to) cranial osteomyelitis, meningitis, abscess formation, ataxia and/or seizures, abnormal reflexes, proprioception, impaired vision, change in behaviour, including increased aggression or increased/decreased sensitivity to sensory stimuli.

However, we have not observed any of these effects in sheep who have been utilised in work preceding this license.

Sheep cope well with anaesthesia and usually recover to standing within 15-20 minutes after cessation of anaesthesia. In addition, we take advice from specialist veterinary anaesthetists in devising our anaesthetic regime.

The degenerative processes in affected sheep become obvious at approx 12 months. This is due to visual deficits. As with affected children the degeneration progresses rapidly, and in the affected sheep there is a loss of approx 30% of brain mass (compared to controls) by 18 months. Although we are aware of the capacity of prey animals to mask external appearance of discomfort and weakness, at this point sheep do not appear to be in any pain and are still freely moving and feeding.

Hindlimb weakness has been noted at 18 months in some affected sheep as determined by our experienced in house staff. We would therefore seek to end the experiment prior to this for affected sheep as we would be able to determine the likely effectiveness of any therapeutic intervention by this point in time.

To monitor, control and limit any adverse effects, animals will be closely monitored throughout life by experienced animal technicians. 24-hour veterinary cover and

clinicians with experience of livestock will be available. We have established humane end points, but where an adverse phenotype is suspected (not reaching these set points) advice will be sought from the NVS in the first instance unless the animal is in distress, in which case it will be euthanised immediately. Where an adverse phenotype is present, other animals with the same or similar genetic alterations will be closely monitored and a system put in place with clearly defined endpoints relating to the clinical signs.

The interventions carried out under the previous project license have shown very few adverse effects. Importantly for therapy development and assessment, due in part to the high degree of conservation of these genes between human and sheep, there have been no adverse reactions to the delivery of the human form of the therapy.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Maximum severity on this license is moderate. Up to 70% of the sheep moved to this license will experience moderate severity. This includes severity as a consequence of surgical intervention (in wild type controls, heterozygous or homozygous for the disease causing gene edit) OR as a consequence of late stage disease progression.

The rest will not exceed mild severity (i.e. non surgical controls).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are no non animal alternatives for the research detailed here. We need to use a sheep model of the disease in question to determine if the potential therapies tested in simple organisms such as rodents, can be scaled up and given by an appropriate route of administration (RoA) to modulate or rescue disease processes in a complex nervous system. See section on expected harms. We discuss why it is necessary and important to use animals (specifically livestock) in this context. We only use animals in our research when the questions posed cannot be answered using non-animal alternatives. We only use livestock to bridge the translational gap between high quality rodent studies and likely effectiveness on a human scale.

In this case, whilst a therapy may be effective on the scale of a rodent, it is not simply a case of multiplying the therapy by the difference in body mass and having

an effective therapy. Rodent brains are smooth (lissencephalic) and less complex than a human (i.e. folded appearance of the cortex - gyrencephalic), and the composition of the brain is very different. For example the grey to white matter ratio in a human is approx. 50:50, in a mouse its 90:10. In a sheep (or a pig) it is 60:40.

This is much closer to a human.

If we give a therapy into the fluid filled spaces of the brain of a mouse, it only has a few mm to travel in any direction for full coverage. In a sheep and human it is in the order of cm.

The distribution of a therapy in the fluid filled spaces is governed in part by how the fluid moves through and around the structures of the brain (dynamics). The sheep is much more like the human than a mouse.

We would never scale up to sheep without having a strong rational from existing work (i.e. rodent model investigations) that an approach has promise. But such assessments of therapeutic effectiveness (i.e. scaling up, distribution and persistence) cannot be accurately modelled using computers or cell based systems.

Which non-animal alternatives did you consider for use in this project?

Where possible we do use alternatives to animals. For example, we can do a lot of work in cells to refine methods of genetic manipulation before they are used to create genetically altered animals. In modelling Batten disease, much earlier work has been done in cell culture in addition to mouse models. We even use drosophila for many experiments before moving to rodents. However, for testing a gene therapy, reliance on the mouse model alone is not appropriate, due to its size and physiology.

Why were they not suitable?

The limitations of non livestock systems and indeed rodent models (after a certain point of therapy development) are described above. We cannot accurately consider and account for the complexity of the nervous system and issues surrounding effectiveness at body size scaling in non animal systems. Indeed the multisystemic nature of such conditions requires consideration of multiple organ systems. Again, at an appropriate scale to the bridge translational gap to clinical efficacy.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?
On a separate project license for the generation and breeding of genetically altered livestock, we will breed a cohort of a maximum of 32 sheep that are homozygous

(hom) and 8 sheep that are heterozygous (het) for the PPT1 R151X mutation, over a period of 4 seasons, that will be moved to this licence. If necessary, up to 12 age matched wild type controls will be moved to this licence. For large animal work we have found that n of 4 is sufficient to draw conclusions within each experiment while also allowing us to account for potential gender balance i.e. 2M and 2F per group.

Such numbers would allow us to carry out up to 4 experimental interventions over 4 breeding seasons. Each ideally containing 4 treated and 4 untreated homs and 4 controls in each grouping if required. Het animals are included as we would wish to determine if we can elevate therapeutic levels over a reduced background PPT1 prior to an interventional assessment in Hom animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

From a purely pragmatic perspective, working with livestock is very expensive. Budgetary constraints require that we always seek to utilise the minimum numbers of animals that will yield a robust dataset, enable publication and thereby comply with ARRIVE guidelines.

Additionally, the minimal sample numbers proposed here are sufficient to address multiple aims as determined by our academic and industrial partners who have experience with preclinical trials for assessment of therapeutic efficacy in “larger” animal models. This was also the case with CLN2 interventions in children.The numbers described within the experimental groupings are consistent with those detailed for sheep model CLN5 gene therapy data which was sufficient to facilitate clinical trial following FDA approval.

Moreover, we are anticipating that therapeutic intervention will result in gross improvement to both morphological and behavioural alterations during the course of disease progression – if these changes are so subtle that they require statistics to demonstrate their efficacy then we will have demonstrated that this approach is unlikely to be suitable for clinical application. The homozygous animals in question will develop normally until approximately 9 months of age and then degenerative processes will result in a comparative difference in brain matter in excess of 30% loss relative to wild type controls. If the therapies in question cannot strikingly modify that process they will be of no use to children in the clinic.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our experience with the models and processes under our previous license demonstrate that this is an appropriate route of assessment for therapeutic efficacy.

The numbers of animals fit well within a proactive breeding strategy spanning 5 years.

Indeed a good breeding strategy would minimise the unnecessary production of excess sheep. Any sheep not required for interventional assessment will be used to provide post mortem tissue samples for our in house tissue bank for pathological and

molecular analysis. Such samples represent an important and valuable scientific resource and will be treated accordingly.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All animal experimentation raises ethical concerns. The last ten years have seen a rise in the use of sheep to model human neurological disorders and their acceptance by regulatory agencies for physiological relevance over NHPs and companion animals .

Sheep in particular have many advantages for nervous system centric research. Indeed we have generated the first CIRSPR edited ovine model of a childhood neurodegenerative condition and form of dementia (the CLN1 form of Batten disease). This is caused by deficiencies in fully functional PPT1 protein and our model incorporates the most common genetic variation seen in the human population r151x. This has provided us with a large model which is more representative of the human condition than the equivalent rodent systems.

We will use sheep as a platform to investigate the effectiveness of therapies for neurological disorders. Neurosurgical delivery of therapies will be performed under anaesthesia in a manner as close as possible to those used in a human hospital for children affected by these disorders. Following recovery the animals will be closely monitored for adverse reactions and measured against criteria which are present to safeguard against unnecessary discomfort.

Why can’t you use animals that are less sentient?

There are no non-animal alternatives that can provide bio distribution, efficacy and safety data on the use of treatments of neurodegenerative conditions. The information of significance for us results from the not only the route of surgical administration itself (which is a key consideration for getting therapies to where they need to have an effect), but also efficacy and duration of benefit. We only use animals in our research when the questions posed cannot be answered using non-animal alternatives. Rodents are important for discovery and proof of principle research in this context but their utility only goes so far in the body of evidence for route to clinical application. We only use livestock to bridge the translational gap between high quality rodent studies and likely effectiveness on a human scale.
How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We aim to minimise any stress or discomfort to experimental animals. Sheep will be housed in groups of more than 2 with respect to their treatment.

The initial experiments will focus on taking an incremental approach. We have in the previous license started with cadaver experiments to optimise the surgical procedures.

These procedures included therapeutic surgical delivery and assessment before progressing into performing surgeries for on live sheep under anaesthesia.

All sheep will receive peri-operatively analgesics, as part of a refined multimodal anaesthetic protocol, which will be continued for as long as needed in the post-operative period. Sheep will undergo daily checks and regular clinical and neurological examination.

Animals with any signs of distress or disease will be examined by a vet and treated as appropriate or euthanised. Humane endpoints will be based on moderate severity limits, with consideration of weight loss, body condition, appetite, respiration, behaviour and dehydration according to a clinical/neurological scoring system.

In treatment groups a post-operative MRI at set points throughout the disease will be regarded as go/no go points relating to therapeutic efficacy. Any euthanised animals will undergo post-mortem examination and brain tissue collection. Our previous work allowed us to establish monitoring protocols to effectively mitigate potential discomfort and/or risks.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have used the PREPARE guidelines for planning the animal experiments. To enable us to achieve our aims we are utilising methods which our group is extremely knowledgeable in and have been previously validated in our labs. We will use these methods to generate novel results which will inform on efficacy and unexpected effects with these approaches in our research environment. We have already carried out: Cadaver brain tissue experiments; brain surgery in sheep; clinical care of sheep having undergone a brain procedure; and histological assessment of post mortem tissues. Any new investigation with methodologies previously unused by our team will be appropriately triaged through a similar process.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All group members will regularly keep updated about 3R advances largely through online courses and research.

Online resources will include: Pubmed and Google searches, Altweb (global clearinghouse for information on the 3Rs; http://altweb.jhsph.edu/resources/links.html), Norecopa (https://norecopa.no/3r-guide-database), National Centre for the 3Rs (www.nc3rs.org.uk/informationportal),

Alt.Tox (advancing non-animal methods of toxicity testing through online discussion and information exchange; http://alttox.org), AnimAlt-ZEBET (https://www.bfr.bund.de/en/zebet_database_on_alternatives_to_animal_experiments_on_the_internet
_____animalt_zebet_-1508.html), DB-ALM ECVAM Database Service on Alternative Methods to Animal Experimentation (https://ecvam-dbalm.jrc.ec.europa.eu/), Fund for the Replacement of Animals in Medical Experiments (http://www.frame.org.uk)

Regular contact with the veterinary services at our Institutes will provide first contact for any 3R updates. Local courses run through these institutes will also be attended.

63. Neurobiology of inflammation-induced behaviour

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Neuroinflammation, Immunobiology, Neurobiology, Behaviour, Immune cells

Animal types	Life stages
Mice	adult, neonate, juvenile, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to study and understand how the brain responds to immune system activation and inflammation, and how these biological responses impact on neurobiology and behaviour over time.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Inflammation-induced behaviours comprise mood changes, fatigue, social

withdrawal, impaired memory and greater sensitivity to pain, resulting in an increased burden upon the individual, their family and socioeconomic community. Chronic inflammation is emerging as a key driver of multiple diseases including depression, other psychiatric disorders and the dementias. The specific mechanisms of how this happens remain unclear, but there is growing evidence that signalling molecules in the circulation that are produced as part of the inflammatory response have an effect on the brain. These can affects how the brain “wiring” operates and this can lead to symptoms such as depression.

What outputs do you think you will see at the end of this project?

This work will allow us to gain a better insight into how inflammation in the body can impact the brain and generate changes in behaviour. We will advance current knowledge and understanding of how inflammation in the body is linked to the brain by studying how immune cells from the bloodstream enter the brain, where they are active, and how the brain's own cells respond. Our research will be disseminated to the scientific and lay community by publication in relevant open-access peer-reviewed journals and presentation at conferences (UK and worldwide).

Who or what will benefit from these outputs, and how?

Initial studies that aim to uncover the mechanisms of inflammation-induced neurobiological and behavioural changes will allow our research group, other researchers in the field, and the pharmaceutical industry to identify points of modulation/intervention in order to improve the behaviours. The potential outcomes of this project are worthwhile as they will in the long-term benefit a range of disorders (from arthritis to depression) regarding choice of treatment and treatment efficiency.

Additionally, insight into why some patients show improvements in psychiatric symptoms in response to anti-inflammatories while others do not, will potentially allow more targeted therapies to be developed.

How will you look to maximise the outputs of this work?

Every year, we participate in conferences where we present our research (successful and unsuccessful studies) via posters or talks and participate in discussions related to our project, either in a local setting or with a worldwide audience at international seminars.

When finalised, we also publish our data as peer-reviewed scientific papers. Where appropriate we will collaborate with other groups and make our findings and data available by open-access publication and data repositories.

Species and numbers of animals expected to be used

- Mice: 8200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 these studies, we are proposing to use mice because we need to study the complex relationship between the mammalian nervous system and immune system. We aim to link the changes we see in the immune system with changes in the brain circuits and study how this leads to changes in behaviour. Other possible methods such as cell cultures do not allow the complex connections to be measured nor can they be linked to behavioural outcome. Mouse brain regions are similar to those in humans, and many models of human inflammatory diseases and nervous system diseases are already well-established in mice.

We will be using mainly adult mice, and in some cases, juvenile and embryonic animals will be used when we need to study the effect of early inflammation on the adult brain.

Typically, what will be done to an animal used in your project?

In this project, we will first have to trigger an inflammatory reaction in the animal. This can be done in different ways:

by delivery of compounds that will generate a systemic inflammatory response e.g. applying a cream on the skin for up to five days, or surgically implanting a mini-pump under the skin that leads to release of inflammatory molecules over time
by using transgenic mice that, when treated with a drug delivered orally or directly injected into the brain, will start producing an inflammatory molecule in the brain
by subjecting the mice to stress to induce inflammation; these protocols extend over several days (minimum=1 day, maximum= 28 days) during which the adult animals will be uncomfortable for periods of time (10min – an hour/day for the 4 weeks protocol) interspersed with recovery periods. Stress can also be induced by maternal separation of pups for a defined period of time daily until weaning.

Some of the animals will be assessed by behavioural testing at defined time points; if performance on several behavioural tests are to be assessed for the same animal, a period of recovery time will be introduced between each test. Some tests are designed to assess emotional changes to their normal behaviour and consist of placing the animal in an arena for them to explore. Other tests that evaluate cognitive function require training.

The sucrose preference test will be used to detect depressive-like behaviour.

For some animals, we will use compounds to modulate the immune and brain response. This will be done mostly by delivery either once (e.g. injection) or a single delivery over a few days (e.g. mini- pump).

Where necessary (e.g. to compare serum levels at baseline and post- therapeutic treatment) We will occasionally collect small blood samples.

We will occasionally administer dyes (intravenous injection) to some groups of animals (e.g. to monitor blood-brain barrier integrity) before they are humanely culled.

In summary, most animals will undergo between 1 to 5 procedures, spread over several weeks.

What are the expected impacts and/or adverse effects for the animals during your project?
There will be mild, transient pain/discomfort from injections/dosing.

Triggering inflammation can lead to acute fever, weight loss and diarrhoea or abnormal behaviour such as marked hunching. These effects can last a few hours (diarrhoea) to a few days (weight loss) before the animal recovers.

Mild and transient discomfort will occur when stressing the animals. The stressors used in this protocol are limited in time and animals have time (overnight) to recover between each stressor. The stress protocols will not prevent the animals from exhibiting normal behaviour once back in their home cage. However, as this is a protocol that spreads over several weeks, the mice might lose weight and possibly show other signs of ill-health such as reduced grooming.

Mild and transient discomfort (e.g hunger/thirst) will be generated when the animals are subjected to some behavioural tests for the time they are in the apparatus.

Mice undergoing surgery for under the skin mini-pump placement will suffer mild discomfort that will be reduced by pain relief treatment.

Mice undergoing surgery to deliver substances directly into the brain will suffer mild to moderate discomfort and will be carefully monitored and receive pain relief.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

100% of mice on the breeding and maintenance protocol (Protocol 1) will be classified as sub- threshold, while of those on Protocol 2-4, overall, we expect the proportion of mice classified as mild will be 70%, and moderate will be 30%.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of this project is to understand how inflammation and the brain interact to generate biological and behavioural changes. To explore these interactions we need to use animals that can demonstrate the behaviours associated with inflammation.

We also need to link these behaviours to the changes in different areas of the brain and over different time-points. This would not be possible in cell culture models.

Which non-animal alternatives did you consider for use in this project?
In essence we need to test our hypotheses in live animals both in terms of the effects of inflammation on the intact brain and on subsequent behaviours.

Although other non-protected invertebrate species such as worms (Caenorhabditis elegans) or fruit flies (Drosophila melanogaster) do contain neurons, they do not display appropriate behaviours, nor are their nervous systems similarly complex, to be a useful model of inflammatory diseases in humans. We will complement our animal work with non-animal cell culture models to first generate new hypotheses and pathway data. Wherever possible, testing of compounds that may have a therapeutic effect will be assessed in cell culture/primary cell culture in the first instance.

Why were they not suitable?

The inflammatory responses in the central nervous system develop over time and involve different types of brain cells (neurons, astrocytes, microglia), that respond to resident and recruited immune cells. Hence it is essential that we work at a three dimensional-network level, i.e. the entire animal, to assure the reliability of the results. Although other non-protected invertebrate species do contain neurons, they do not display appropriate behaviours, nor do their nervous systems contain the appropriate structures or complexity, to be a useful model of inflammatory diseases in humans.

'Simpler' vertebrates such as zebra fish do have more complex brains, but they are still not suitable to model the human brain systems and diseases of interest in this project.

Therefore, we will carry out our experiments in mice, as their key brain regions are similar to those in humans, and numerous models of human nervous system disease are already well-established in rodents. Mice also enable the use of the advanced genetic tools needed to dissect the cellular circuitry of the brain in a precise, cell-specific manner.

Reduction

Explain how the numbers of animals for this project were determined. Describe

steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

As we and our collaborators have over 15 years' experience of animal work in this area (behaviour, inflammation), we know how to calculate the mice needed per type of experiment; from this we have estimated how many animals we would use over a 5 year-period, with the support of the NC3Rs Experimental Design Assistant for the general design of the experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experiments will be designed to use the minimum number of animals possible, through:

Performing power calculations (when appropriate) using an estimate of the size of effect we expect from previous findings, and the variance present in the population, to establish the size of the experimental sample group required to produce publishable results.

Using the NC3Rs Experimental Design Assistant for the robust design of experiments to yield reliable and reproducible results, support regarding randomisation, blinding and sample size calculation.

Consulting colleagues and statistician to ensure that the correct statistical tests are used.

Keeping variability to a minimum by using the same strain of mice for experiments within a series.

Maximising the information gained from each animal with factorial statistical analysis where appropriate.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We and our collaborators have prior experience regarding the experiments within the project which give us an advantage for realistic and adequate planning and to avoid any overestimation of the numbers of animal needed.

We already share tissue between lab members and with other labs, when possible.

All experiments will be carried out in as controlled a manner as possible to reduce the animal-to-animal variability and thus reduce the number of animals used.

Variable controls include:

Wildtype littermate controls bred under the same conditions as transgenic models will be used as a control comparison wherever possible.

We will use animals of both sexes in most experiments. We will record the sex of animals used in our experiments to allow us to monitor whether or not sex differences are a confounding factor.

In order to maximise the amount of data gathered per animal, we will perform bilateral brain injections where possible to allow data to be gathered from both hemispheres.

The numbers of animals required for the experiments detailed have been peer reviewed both at the funding application/award stages and the publication stage.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

General: Animals will be housed to the highest welfare standards possible. The aim of our research is to better understand human brain physiology through use of animal models.

The models that we will use have been well-validated and are known to faithfully reflect specific aspects of human inflammatory disease, thus represent the most refined way of studying these disorders.

Mouse weight will be monitored, and animals will be provided wet food (e.g. baby food or wet mash) prior to the start of the procedure to mitigate weight loss. Non-aversive and gentle handling will be used, together with a quiet environment in order to avoid any unnecessary stress.

Genetically-altered animals will be used where possible to model the neuroinflammatory response. Genetically-modified mice allow us to selectively target specific types of cell, as opposed to initiating a response system-wide.

Subcutaneous mini-pumps: Some mice will be implanted with an osmotic mini-pump under the skin. This will allow us to study the effect of chronic (long-term) rather than acute (short) immune activation and the impact on brain physiology by continuous release of inflammatory mediators over time. The benefit for this methodology is that there is only one surgery and not daily injections and handling.
Stress models: Some mice will undergo stress models as this is a known initiator of

neuroinflammation. In maternal immune activation and early life adversity models, where possible mothers will receive known inflammatory stimulators rather than undergo physical stressors to reduce direct intensity of adverse effects on both dam and pups.

The chronic unpredictable stress model will be used to replicate real-life daily stressors rather than acute, severe stressors. Therefore, the stressors are less intense and there will be a shorter duration per trial.

Surgical delivery of compounds into the brain by injection: Some experiments will involve making one or more anatomically-guided injections of viral vectors to express a protein of interest before performing other experiments with the animal (for example behaviour, induction of inflammation). The amount of time needed for transgene expression to reach useful levels can vary by construct and brain region.

There are no known adverse effects due to long-term expression via viral transduction of the genetic tools needed in this project, so animals experience no additional harm from long incubation times. In our experience, these surgeries normally last around 30 to 45 minutes: to enable the speedy recovery of animal from anaesthesia, we will use a volatile anaesthetic such as isoflurane. Peri- operatively we will give animals analgesics such as ketoprofen and / or topical lidocaine at the incision site on the head, then we will provide additional fluids and analgesics such as buprenorphine and/or non-steroidal anti-inflammatory drugs (NSAIDs) as needed in the days following the surgery. Surgery will be performed on heated pads to reduce risk of hypothermia. Animals will be closely monitored daily after surgery to ensure that they gain appropriate weight after recovery and show no signs of postoperative infection. All surgeries will be carried out using aseptic technique, so we do not anticipate infection of the wound site. However, if any infection occurs, we will treat using topical and/or systemic antibiotics, under the supervision and guidance of the NVS or NACWO. If any animals show signs of distress and do not respond to treatment within 24 hours, they will be killed humanely using a schedule 1 method or perfusion-fixation under terminal anaesthesia.

Behaviour: All behavioural tests will be carried out with as little stress to the animals as possible; indeed, many of the behavioural tests promote behaviours that rodents would display in the wild so could be considered a form of environmental enrichment. Mice will be habituated to the apparatus and handler for several days to minimise stress and optimise results when data collection begins.

Why can't you use animals that are less sentient?

Our project is investigating the link between inflammation in the body and the brain and how it leads to neurobiological and behavioural changes. The inflammatory response in the central nervous system leads to alterations in brain cells (neurons, astrocytes, microglia), and the connection between these cells in various regions across the network.

Hence it is essential that we work at a three dimensional- network level, i.e. the entire animal (body and brain), to assure the reliability of the results. Although other non-protected invertebrate species such as worms and fruit flies do contain neurons,

they do not display appropriate behaviours, nor do their nervous systems contain the appropriate structures or complexity, to be a useful model of inflammatory diseases in humans.

‘Simpler’ vertebrates such as zebra fish do have more complex brains, but they are still not suitable to model the human brain systems and diseases of interest in this project.

Therefore, we will carry out our experiments in mice, as their brain structure and behavioural responses are the closest to those in humans, and numerous models of human neurological disease are already well-established in rodents. Mice also enable the use of the advanced genetic tools needed to dissect the cellular circuitry of the brain in a precise, neuron subtype-specific manner. For electrophysiological recordings, these will be performed under terminal anaesthesia so the mouse will not unduly suffer.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Most of the procedures we are using necessitate regular monitoring of the animals and usually involve post-operative care and pain management. Also, we will handle and/or train the animals on a regular basis in order to decrease their basal handling-related stress levels so that it does not interfere with our experiments and behavioural readings.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the NC3Rs advice and the resources and training material provided on their website for the refinement of our procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?
We attend local 3Rs and Culture of Care meetings and are in regular contact with the NVS, NACWO, NTCO and animal-facility staff for any suggestion that they might have. We keep updated with the literature on the subject with regular searches on Pubmed, the NC3Rs website and attendance of conferences and training related to animal welfare.

64. Opioid receptor signalling and behaviour

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Opioids, G protein-coupled receptors, Neurons, Addiction, Receptor signalling

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Opioid drugs such as morphine and heroin are effective analgesics but are also addictive and have the risk of fatal overdose. Opioid drugs work by activating opioid receptors and the aims of this project are to better understand how opioid receptors work potentially leading to better, safer opioid drugs.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Opioid receptors are present throughout the body, but particularly in the brain. They are activated by proteins in our body such as endorphins and are involved in a range

of behaviours such as modulation of pain, motivation, mood and addiction. Chemicals that also activate opioid receptors (opioid drugs such as morphine, codeine, oxycodone) are very effective analgesics and are widely used, but they are not without their problems. All current opioid analgesics are rewarding substances meaning that some people choose to take them recreationally and can develop addiction to them. Further, opioid drugs also cause respiratory depression which is why cause of fatal opioid overdoses occur, causing hundreds of thousands of preventable deaths every year.

This project is aimed at gaining a greater understanding of how opioid receptors work in the brain, what their functions are, and how opioid drugs interact with those receptors. Ultimately this research will help inform drug discovery to design better, safer opioid drugs.

What outputs do you think you will see at the end of this project?

This project is aimed at gaining a better understanding of opioid receptors, and how opioid drugs affect them.

There are several overlapping experimental objectives including:
- deciphering how different opioid drugs can cause opioid receptors to signal differently (so-called 'biased agonism').
- understanding how benzodiazepine drugs (eg. diazepam, aka Valium) interact with opioid receptors to increase the risk of fatal overdose.
- assessing how opioid receptors might modulate addiction-related learning.
- gain greater understanding of the expression and action of opioid receptors in different brain regions.

Who or what will benefit from these outputs, and how?

Short-term benefits: By the end of this project we will:
- provide fundamental insight into the neurochemistry of a brain region thought to be critical in mediating stress and motivation. Our findings will be of interest to the neuroscience research community, particularly those interested in mood, stress, motivation and addiction.
- provide fundamental insight into the ability of opioid receptors to exhibit 'biased signalling'. These findings will be of interest to other opioid researchers, but also to the broader G protein-coupled receptor research community. G protein-coupled receptors (GPCRs) are the largest family of cell surface receptors in mammals.

There are >800 different GPCRs playing critical roles in a wide range of biological processes. ~30% of current drug treatments target GPCRs. Gaining fundamental insight into biased signalling of GPCRs will advance knowledge in the GPCR research field.

Gain insight into how benzodiazepine drugs modulate the effects of opioid drugs.

This will be of interest to other opioid researchers, and to researchers and clinicians

working with drug addiction.

Gain insight into how kappa opioid receptors modulate motivation in an animal model of drug-seeking behaviour. This will be of interest to other researchers in the opioid field, and in the addiction field.

Medium-term benefits: By the end of this project we aim to suggest novel harm reduction strategies, based on our findings, that will reduce the risk and incidence of fatal overdoses in people who use drugs. We will disseminate this information to stakeholders: service users and service providers, and will aim to obtain further funding to evaluate the effectiveness of these strategies and further refine them.

Long-term benefits: Beyond the time-line of this project, the aims of the research are to provide fundamental insight into opioid receptor function such that better, safer, opioid analgesic drugs can be designed and future treatments for drug addiction can be designed.

How will you look to maximise the outputs of this work?

Findings from these experiments will be published in open-access academic journals as well as presented at conferences to maximise dissemination of our results to the research community. We will also work with the Institution's Press Office and Public Engagement teams to maximise dissemination to, and work with, the general public and stakeholders.

Further funding will be sought to develop on the findings from this project to enable design of safer opioid analgesics and novel treatments for drug addiction.

Species and numbers of animals expected to be used

- Mice: 2850
- Rats: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are studying complex brain anatomy, behavioural function, and receptor function in neurons. This type of research requires nervous systems that have similar structure and neurochemistry to humans in order to increase our understanding to develop novel therapies. Mice and rats are the least sentient animals that have brains that are sufficiently similar to humans.

There is also extensive scientific literature using these species in similar research that this project will build from.

The use of genetically-altered mice will permit the study of neuron-type diversity.

The data outputs will be from juvenile/adult animals so that the brain structure/neurochemistry has matured to the adult state and developmental changes aren't introduced as an experimental confound.

Typically, what will be done to an animal used in your project?

Most mice in our project will be of sub-threshold/non-recovery severity as the only procedure they will undergo will be killing while under terminal anaesthesia for the preparation of high-quality brain slices and/or they will be genetically-altered mice with a non-harmful phenotype. From the tissues obtained we can make electrical recordings from neurons.

Some mice will undergo procedures to determine the *in vivo* potencies of opioid drugs using nociceptive testing. These methods measure the animal's response to an acute thermal stimulus that is only mildly aversive. The animal can escape from the stimulus and limits are in place that prevent any lasting harm. During these experiments injections of compounds will be administered. In many cases this will happen twice to an individual animal, with two instances of nociceptive testing. In some experiments, an individual animal will be administered substance 4 times (once daily), with nociceptive testing taking place 8 times (twice daily).

Some mice will undergo behavioural testing using a non-invasive method of assessing motivation and drug-seeking behaviour that utilises the animal's innate exploratory behaviour and learning processes in a non-aversive environment. A typical experiment involves an individual animal receiving injections of compounds 6 times (over 5 days) and placed in a novel, non-aversive environment 13 times (once daily).

A minority of animals will undergo surgery to express neuronal tracers or viral vectors. These are inert substances that cause no harm to the animal other than the surgery itself. An animal will undergo a single surgery (<1 hour in duration) during its life, where they are administered a single substance by intracranial injection through a burr hole then are expected to make a full and unremarkable recovery.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals will experience no, or few, adverse effects and no lasting harm. Many of the studies involve only genetically-altered mice of a non-harmful phenotype or a 'non-recovery' procedure: killing while under terminal anaesthesia. These animals are not expected to experience any adverse effects or clinical signs.

In studies of analgesia and conditioned place preference, mice will experience several instances of substance administration, and/or low-intensity thermal nociceptive stimulation which are expected to cause only momentary discomfort.

To minimise the impact of surgery we have in place an established monitoring system, and protocol of pre-, peri- and post-operative care including administration of

analgesic and anti-inflammatory drugs.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-threshold: 25%
Non-recovery: 35%
Mild: 35%
Moderate: 5%
NB The protocol to permit breeding and maintenance of genetically-altered animals is a 'mild' protocol but we expect the actual severity of all genetically-altered animals to be 'sub-threshold'.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We continually read the relevant scientific literature, and attend scientific conferences, to look for relevant alternatives. At present the most promising area that could lead to replacement in this project is brain organoids. However, to date the technology is not advanced enough to recapitulate the organisational complexity of the mammalian brain, or the signalling profile of adult mammalian neurons.

Therefore, animal models are currently the only way to achieve our experimental objectives.

Which non-animal alternatives did you consider for use in this project?

We have considered brain organoids but the technology is not sufficiently advanced, at present, for them to allow us to achieve our experimental objectives.

However, a range of *in silico, in vitro* and *ex vivo* methods are an intrinsic part of the overall research plan. These include: computer modelling of ligand-receptor interactions; mathematical modelling of G protein-coupled receptor signalling; G protein-coupled signalling, ligand binding and receptor internalization using cultured cell lines. We will continue to use these approaches whenever appropriate.

Why were they not suitable?

We will continue to use non-animal and/or Schedule 1-only alternatives wherever possible. However, there is considerable evidence (from our lab and others) that opioid receptor function varies depending on the particular neuron in which it is expressed. Indeed, investigating and understanding this is one of the key aims of this project. Therefore, although alternative methods can provide proof-of-concept data it is only by using adult mammalian neurons that we can fully achieve our experimental aims.

Where the experimental objectives require behavioural experiments (antinociception, reward/motivation) it is currently not possible to recapitulate the complex behavioural responses by *in vitro* or modelling methods.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals we estimate using derives from power analyses based on our own experience with previous experiments of a similar type. These numbers are then scaled up based on the experimental objectives to be addressed and expectations of the number of researchers in my lab over the next five years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We plan all of our experiments using the ARRIVE guidelines and take care to ensure that as many variables as possible remain constant: eg. performing experiments at the same time of day, same experimenter performing each individual experiment, careful control of housing conditions both within an experiment and between experiments. All of these decrease variability and contribute to reducing animal numbers. For behavioural experiments, these will be fully controlled, randomised, and the experimenter will be blinded to treatment.

The power calculations we have performed give the minimum numbers required to achieve statistical significance, and so the minimum number of animals required to detect an effect. Following generation of new data sets these power calculations will be revised and, if necessary, the sample size will be changed.

The range of *in silico, in vitro* and *ex vivo* approaches that we (and our collaborators) use within these projects means that licenced procedures on animals are only used when absolutely necessary.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where animals are used to prepare brain slices, several slices can be prepared from a single animal, and they remain viable for 7-9 hours. Therefore, where possible we will obtain data from more than 1 slice per animal. Where this takes place different specific experiments will be performed on different slices so that they can be treated as independent samples.

Based on our experience, our method of killing to prepare brain slices (killing while under general anaesthesia) provides higher quality tissue, enabling us to generate more data per animal, but causing no more harm to the animal than Schedule 1 methods. Therefore, although this method increases the number of licenced procedures (rather than Schedule 1 killings), it leads to an overall reduction in animal use.

Where possible we will perform experiments in which each animal will act as its own control allowing comparison of pre-drug and post-drug administration parameters thus removing the requirement for a separate control group. This reduces the number of animals that we will use.

We will also strive to generate multiple data sets from a single animal (for example, record behavioural measurements then, after killing, use the brain tissue for subsequent immunohistochemistry or brain slice electrophysiology experiments).

The use of genetically-altered ‘reporter’ mice should lead to an overall reduction in animal numbers. In some brain regions neurons exhibit a high degree of heterogeneity. For example, if the primary goal of a specific experiment is to determine the effects of mu-opioid receptor agonists in a specific brain region, but only 30% of neurons in that brain region express the mu-opioid receptor, then transgenic 'reporter' mice can be used so that only recordings from mu-opioid receptor-positive neurons are attempted. This reduces the number of recording 'failures', ultimately leading to reduction in animal numbers required to achieve the scientific objectives.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 method of killing for preparing brain slices causes no more harm to the animal than Schedule 1 methods.

All of the genetically altered animals are of a non-harmful phenotype (sub-threshold severity) and therefore induce no more harm than experienced by a wildtype strain.

Where possible, with ‘transgenic reporter’ genetically altered animals, rather than tissue samples being taken for the purpose of genotyping, ‘GFP goggles’ can be used.

We will use brief, non-tissue-damaging tests of antinociception rather than inducing a pain model that would cause the animals to exhibit clinical signs of pain. These models apply an acute thermal stimulus that is only mildly aversive, and escapable. Cut-off times are used to prevent any lasting harm.

The model of motivational/rewarded behaviour is a non-harmful, non-invasive method of assessing motivational state and utilises the animal's innate exploratory behaviour and learning processes. The animal will experience no harm during this protocol beyond that caused by administration of substances.

Surgery will only be performed when absolutely necessary, to increase the specificity of electrical recordings from brain slices. In most brain regions neurons exhibit a high degree of heterogeneity: different neurons project to a range of different downstream brain regions, and receive input from a range of different upstream brain regions. In order to gain a full understanding of the neurochemistry of a certain brain region, neuronal tracing and optogenetics techniques need to be used. These procedures require surgery.

Why can’t you use animals that are less sentient?

Rodents are the least sentient species appropriate for this work. The anatomical distribution of opioid receptors in rodents is similar to man, as are the behavioural responses to opioid agonists. Similarly, the brain regions and neuronal network structures involved in nociception and motivation/reward are similar to in humans.

This is not generally the case for invertebrate species or other less sentient animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The majority of procedures in this project induce no lasting harm to the animal and no clinical signs are expected. However, we will routinely reflect on best practice in terms of basic husbandry and handling to minimise overall stress to the animals.

The small number of animals that undergo surgery may experience pain or discomfort following surgery. This will be minimised by an established protocol or pre-, peri- and post-operative administration of analgesia, and antibiotics and extensive monitoring and record-keeping post- operatively including general demeanour, weight and the ‘grimace scale’. These will be regularly reviewed with discussions with the NVS and NACWO and further optimised whenever necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

General guidance will be sought from the ARRIVE and NC3R guidelines, published

documents and reports from the Laboratory Animal Science Association (LASA).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will consult the scientific literature on the 3Rs. Our institution regularly sends out notices regarding the NC3R's newsletter and webinars which we will use.

Our institution, in collaboration with other nearby institutions, holds an annual 3Rs one-day conference that we will attend, and contribute to, where possible.

Our institution has an 'Animal Users' Forum' with 3 meetings per year each with a 'best practice' section regarding best practice and advances in the 3Rs.

65. Studies to investigate the development of immunity during the prenatal and neonatal period in the bovine calf

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Calf health, Immunity, Passive transfer

Animal types	Life stages
Cattle	adult, pregnant, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the interplay between calf and maternal factors during immune development. To investigate how these factors affect calf health, performance and survival. To improve the diagnosis of inadequate transfer of immunity (known as failure of passive transfer) after birth.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-

term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There is an urgent need to improve the health and welfare of calves reared from commercial dairy farm origins in the United Kingdom. The occurrence of disease in this population is a result of an interplay between their environment, exposure to pathogens, and their immunity. With regards to the latter, due to the structure of the bovine placenta, immune cells (including antibodies) are unable to pass from mother to calf during pregnancy. The calf is therefore born without a fully functional immune system, and relies upon absorbing immune components including antibodies within the first milk produced by its mother, known as colostrum. These immune components provide protection, or "passive immunity" until the calf's own immune system is fully functional. If this is insufficient, the calf will be at a greater risk of succumbing to disease until it is able to produce its own antibodies, from around 5-6 weeks of age. This was recently evidenced in a study of a large dairy herd located in America. The authors demonstrated that, when compared to calves with excellent levels of passive immunity, inferior values were associated with greater risk of diarrhoea and pneumonia. This is highly important since within UK dairy herds, inadequate transfer of passive immunity is highly prevalent, with over 20% of calves having poor transfer of passive immunity.

Many tests are available for the measurement of antibody concentrations in the calf. One of the cheapest and most accessible methods is called refractometry. A small portable device called a refractometer is used to estimate the protein concentration within the blood and the result is used to estimate antibody concentrations. Although it is used very commonly, this method is only accurate in calves aged between 1-7 days of age. In older calves, measurement is rarely performed. This represents a challenge for producers who buy calves over a week old, since they are unable to quickly assess if stock have sufficient antibody-derived immunity. There is an urgent need to improve cost effective, fast tools for the measurement of antibody concentrations in calves aged over a week old and to produce guidelines regarding "normal" concentrations and their association with risk of disease.

This would provide stakeholders with improved guidance regarding the purchase, management and subsequent rearing of their stock in ways that optimise antibody-derived immunity.

Calfhood immunity is not only provided by antibodies. In fact, the development of immunity begins during pregnancy and continues during the neonatal period. Gene expression analysis of neonatal calves has identified deficiencies in specific immune components. What is relatively unknown is the importance of these immune deficiencies on the development of disease. Does under- or over- expression of specific genes or other immune components result in a greater risk of disease or death, and how does the timing interact with disease risk? What are the impacts of colostrum management and maternal health during pregnancy on gene expression in circulating immune cells in neonatal calves, and the function of immune components, and how does this impact their health and growth outcomes?

What outputs do you think you will see at the end of this project?

- Results will provide data regarding antibody concentration measured during early life and the disease risk.
- Results will also provide information regarding the development of calf-derived immunity and its association with health and performance
- Results will inform the accuracy and precision of a unique test for the measurement of antibodies in calves prior to weaning. It is a lateral flow test (similar to many at-home COVID tests), whereby a drop of solution is added to the testing device and the appearance of red line(s) across the test window allow the interpretation of results. The test can be performed quickly and easily on-farm by the farmer, and results collected rapidly. Although lateral flow tests for the measurement of specific antibodies in calves already exist, this test is unique because unlike those currently available it is semi-quantitative. This means that the results provided are on a scale, rather than just "pass" or "fail", and this allows its' use in calves aged over a week old. If effective, this could provide farmers and vets with a low-cost tool to measure immunity in calves during this period.
- Publications: Results will be published in peer-reviewed literature as well as the farming press, presented at scientific and industry conferences nationally and internationally.
- Study outcomes will be discussed at internal seminar meetings of the institution.

Who or what will benefit from these outputs, and how?

The proposal should provide a net benefit towards promoting calf health and welfare. Animals: Diagnosis of low antibody concentrations could inform improved management of high-risk individuals, to reduce their risk of disease. Results will also inform calf producers about current standards of rearing, and if improvements are required. This feedback-loop will allow subsequent cohorts to be managed better, improving disease resilience and reducing the risk of poor health and mortality whilst improving calf performance (such as growth, production and reproduction parameters). The health outcomes in calves could also be improved by gaining a better understanding of how the health of the mother during pregnancy may alter both the development of the calf's own immune system and the quality of the antibodies the mother delivers via her colostrum.

Veterinary surgeons and Farmers: Results will provide information regarding thresholds for measurement of antibody concentrations in calves aged older than a week of age, and risk of disease. In clinical veterinary practice, this might be especially useful to veterinary surgeons both for the diagnosis of failed transfer of passive immunity at the individual and herd level. Understanding of the dynamics of antibody concentrations in older calves in response to disease is crucial: these calves may not produce an appreciable quantity of their own antibodies during the preweaning period, hence if they encounter disease are antibodies "used up"? If so, this will impact on how stakeholders interpret antibody concentrations when performed on older calves. An understanding of the dynamics of antibody concentrations will also inform the use of a calf-side lateral flow test for the measurement of immunoglobulins in calves prior to weaning. Pilot studies have already shown good accuracy when performed in calves aged between 1 and 14 days of age, but no data are currently available to show its usefulness in older

calves.

How will you look to maximise the outputs of this work?

Findings will be shared with the veterinary and farming communities via presentation at scientific meetings and veterinary clinical clubs, and publication in open-access journals. We will utilise the social media to disseminate key findings. This project represents a collaboration between academia and veterinary clinicians, which will be useful for the practical utilisation of the findings.

Species and numbers of animals expected to be used

- Cattle: 1100 (500 calves and 600 mothers (cows)

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We intend to use calves reared on commercial farms from birth until weaning, as well as their mothers. Our research specifically pertains to calf development during pregnancy and during the neonatal and preweaning periods. On a typical commercial farm weaning (defined as the transition to a 100% solid- food diet) occurs at around 8-10 weeks of age, although occasionally calves may be weaned slightly older, and it is from birth until this age range that we intend to study. The management of calves and their risk of disease is different based upon whether they are going to be kept on their farm of birth as a replacement dairy cow or sold onwards. This project seeks to follow both populations.

Initially, it will monitor a population of replacement female dairy calves on a single dairy farm. Thereafter, a population of calves born on a dairy farm but destined for beef production will be monitored.

Typically, what will be done to an animal used in your project?

This study will be performed on commercial farming facilities and on this basis animals enrolled will be kept according to normal commercial husbandry conditions. Two types of facility will be used, commercial dairy farms and commercial calf rearing organisations.

For each (pregnant) mother, blood will be collected at approximately two timepoints from either the tail vein or the neck vein: Around the time of pregnancy diagnosis and during mid gestation. Colostrum taken as part of routine herd management will also be collected at the first milking after calving.

Sampling of each calf will be performed at a maximum frequency of every 5 (+/- 2)

days, with a maximum of 1% blood volume collected at a single sampling (~27mls for a 45kg calf), for a maximum of 12 weeks, although we expect 8 samplings to be the most common. The volume of each sample will be considerably less than maximum permitted volume suggested by the NC3Rs guidance. On each occasion samples will be collected from the neck vein, whilst a subset of samples will additionally be collected via nose pricks. Nose prick samples involve the collection of a very small volume of blood a similar manner to the collection method performed in humans for the measurement of blood sugar concentrations in diabetics. This is the recommended sample collection route for the lateral flow device to be tested and is becoming a standard method of collection in clinical veterinary practice. At every sampling visit the health of the calf will be assessed using a published scoring system, which considers faecal consistency, discharge from the eyes and nose, temperature, ear and head position, and presence or absence of a cough.

Beef calves will be enrolled into the study upon arrival at a commercial calf rearing facility, aged between 1 and 4 weeks old. For some of these animals we expect to be able to gain information from prior diagnostics performed routinely on the farm of birth (under the Veterinary Surgeons Act). Where possible these will be used to minimise further sampling.

Repeated sampling will be performed approximately weekly from arrival until weaning. The first sample will be collected within 7 days of arrival. This is essential, since this is the period during which immune responses are likely to be crucially affected by transportation and mixing. Collection of this sample is therefore required to develop our understanding of how these management practices affect immunity and the occurrence of disease. Using this sampling strategy, the maximum number of blood samples collected from a single calf is 11, although we expect 5-7 samplings to be the most common.

What are the expected impacts and/or adverse effects for the animals during your project?

The only harm we expect during this project is that associated with the collection of blood.

In all cases of blood collection, we expect animals will experience a mild, transient discomfort associated with the passage of the needle or prick device through the skin.

Very rarely there is the potential for short lasting adverse effects (minutes to days) associated with the collection of blood which may include bruising under the skin, and even more rarely inflammation of the vein. In the event that these occur, and it is necessary to take further samples as part of the procedure, another sampling site will be used where possible. Thereafter, the animal will not be used again until an improvement is seen.

Animals may also encounter stress from handling. This is unavoidable, but will be minimised by ensuring that animals are handled by trained operators in a manner that avoids prolonged handling and minimises stress, where possible in their housing environment or appropriate handling facilities.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Calves: 100% mild
Cows: 100% mild

What will happen to animals at the end of this project?

- Kept alive
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal models are required for the study of immune development and response to disease in a commercial farming environment. It is not possible to replicate the complex interactions between the animal, the environment and disease pathogens within a test tube or using other laboratory techniques alone. Furthermore, by performing this study on animals within a commercial environment, the results of this project will be highly applicable to calf-rearers, which will maximise the impact of the study outcomes and their relevance.

Which non-animal alternatives did you consider for use in this project?

A literature search using the search engines Pubmed and Agricola were used following established guidelines (Fund for the Replacement of Animals in Medical Experiments, FRAME) to research the alternatives to animal models for the study of passive transfer. No suitable alternatives were found by this method.

Why were they not suitable?

The study of passive transfer from mother to calf cannot be replicated sufficiently through the use of either cell lines or organoids, especially given that the underlying physiology is multi-organ and not limited to a specific tissue or cell type. Study of maternal effects are not possible via these methods. Validation of a lateral flow device for antibody measurement requires animal disease models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to

minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We followed the PREPARE* guidelines regarding experimental design, which provide clarity on how to ensure this stage of our research is optimised.

In accordance with our study objectives and hypotheses we conducted three pilot studies to determine:

The agreement of the novel diagnostic test with a "gold standard", when performed on leftover blood from samples that had been collected for other reasons. This allowed us to estimate the expected accuracy of the novel test.

- The current levels of passive immunity within herds at present.
- The current levels of disease in calves between birth and weaning in UK dairy herds as reported in recent literature.
- This allowed us to produce an estimation of the minimal number of animals required to be enrolled in order to fulfil the study objectives.
- We considered and took into account the risk of potential losses-to-follow up, either due to departure from the farm or death as well as the clinically-relevant effect size we should seek to measure.
- We took into account our interest in considering two discretely managed populations exposed to different risk factors for naturally occurring disease: replacement dairy calves and calves destined for beef production reared at commercial facilities.

We considered recent, similar publications within the same field.

We sought advice from colleagues (including a biostatistician) experienced in the design of animal studies regarding sample size determination.

*Planning Research and Experimental Procedures on Animals: Recommendations for Excellence. https://norecopa.no/prepare

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimental design was refined using the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines as well as the NC3R's Experimental Design Assistant. These guidelines can be accessed using the following links: https://norecopa.no/prepare and https://eda.nc3rs.org.uk/

- Sample sizes were estimated using standard power analyses based upon our pilot data.
- Taking into account the results of a pilot study (unpublished) of 16 farms within Southern England, and through collaboration with a veterinary practice, we considered the effect of farm selection on risk of losses to follow up and prevalence of disease to ensure selection of an appropriate farm that meets the

requirements for this project.
- We will use residual blood samples wherever possible, already routinely collected under the Veterinary Surgeons Act (VSA) for the purpose of measuring mother metabolic and health status pre-calving, and for measurement of calf health and immune status (with permission from our Clinical Research and Ethics Review Board).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- Data previous research was used to produce a more accurate estimation of the minimum number of animals necessary.
- We sought and found collaboration from colleagues who could use samples collected from the same protocols outlined to avoid the requirement for future studies with similar design, and to ensure that wastage from collected samples is minimised.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.
Pregnant bovine dams will be enrolled and blood samples taken from either the tail or neck vein to study their health, metabolic and immune status. Optimisation of the sampling process will be through efficient, fast sampling by experienced operators in appropriate handling facilities to minimise the duration of possible distress.

In the calf population we will seek to minimise distress during blood collection by using animal-specific restraint such as a halter, locking yoke or with an assistant holding the animal.

During the study no specific treatments will be withheld and animals will be kept under the same animal husbandry conditions as calves on the premises that are not being studied.

Sampled animals will be monitored for signs of adverse effects during visits.

Why can't you use animals that are less sentient?

The study outcomes of the project are only relevant to cattle, and are specific to their immune development during pregnancy and during the neonatal period. Due to its complexity, animal models are the only available method and there are no less sentient species that can be used for this research.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be handled by trained operators in a manner that avoids prolonged handling and minimises stress, either in their housing environment or using appropriate handling facilities. Study animals will be monitored for signs of adverse effects associated with venepuncture during subsequent visits. Risks will be minimised by use of a sterile needle for each collection of the smallest practical gauge. In circumstances where the collection site is dirty it will be cleaned prior to sampling.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Published practical guidelines are available to us to ensure that the design, collection of data and reporting of our scientific findings are clear, repeatable, and legitimate. These guidelines are variable depending on the type of study which we report. Since this project licence covers a number of potential publications, there are various guidelines which we will follow. For each guideline mentioned, further information is accessible via the hyperlinks provided.

For example, during the experimental planning and design stage we have followed the PREPARE guidelines (provided by Norecopa), which provide clarity on how to ensure this stage of our research is optimised.

During the project reporting stage of the project, we will follow the ARRIVE 2.0 guidelines. We will also consider the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines and, for comparison of diagnostic tests, the Studies of Diagnostic Accuracy (STARD) guidelines when writing for publication to ensure transparent experimental design and clear reporting. Each one of these guidelines provides the authors with specific advice relevant to the study design which we intend to report.
https://norecopa.no/prepare/ https://arriveguidelines.org/arrive-guidelines
https://www.strobe-statement.org/
https://www.equator-network.org/reporting-guidelines/stard/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This institution is active in the dissemination of NC3Rs and 3Rs relevant news and training via a newsletter and regular group meetings, which I will participate in.

66. Diabetes mechanisms, biomarkers and treatments

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

diabetes, streptozotocin, neuropathic pain, neuroinflammation, therapy

Animal types	Life stages
Rats	adult
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project has two main aims. (i) Investigate novel mechanisms, biomarkers, and treatments for complications of diabetes mellitus using established rodent models of type 1 and type 2 diabetes (ii) Validate new endpoints of neuropathic pain and analgesia in rodent models of type 1 and 2 diabetes to improve forward translation drug discovery into clinical development and animal welfare.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Diabetes is a major metabolic disorder that affects 537 million people worldwide,

mainly because of an increase in obesity, and it is estimated that at least 60-70% will develop associated complications, with prevalence increasing with duration of diabetes and age. The majority of diabetic patients (~90%) suffer from type 2 diabetes and by 2030 it is predicted 690 million people worldwide will have type 2 diabetes. Despite differences in cause and disease prevalence, secondary complications occur in both type 1 and 2 diabetes mellitus and are often resistant to existing treatment causing considerable distress to the patient. Diabetes complications lead to a reduced quality of life and pose a huge economic burden to the health system and society. In 2011 in the UK, the NHS spent almost £24 billion on diabetes, 30% of which was spent on managing complications, such as neuropathic pain. By 2035, it is estimated that diabetes will cost the NHS approximately £40 billion, accounting for 17% of total health resource expenditure in UK. This project aims to identify new mechanisms, biomarkers, and treatments in rodent models of type 1 and 2 diabetes to establish proof of concept and open new avenues for clinical development through academic-pharmaceutical industry collaboration to benefit people with all forms of diabetes.

A contributing factor for the lack of development of new treatments for diabetic neuropathic pain is the mismatch of assessment endpoints in preclinical (animal) trials versus clinical (human) trials. Animals can't self-report and rate their level of pain like humans. Reflexive quantitative sensory testing (QST) remains the primary assessment endpoint required by regulators for neuropathic pain and analgesia in animals and can be argued is not clinically relevant due to the continued lack of forward translation and failures of treatments in the clinic. We have recently reviewed the importance of bidirectional research in helping to validate new endpoints such as burrowing and facial grimacing and electroencephalography together with currently used QST endpoints [Fisher et al., 2021]. This is a key aim of my current project and this project to create improved translational rodent research models of type 1 and 2 diabetes for proof of concept in this project, in the industry and ultimately drug discovery success for patients suffering with neuropathic pain.

What outputs do you think you will see at the end of this project?

The intended output of this project is to identify new mechanisms, biomarkers, and treatments for complications of type 1 and 2 diabetes. Further the project will look for an overlap in mechanisms across complications and look for potential biomarker predictors of neuropathic pain and pain relief. The project will generate data to support or refute the clinical development of novel therapies including active molecules from plant extracts that could lead to a controlled clinical studies and therapy. The project will generate data to validate translatable markers of chronic neuropathic pain and analgesia in animals that can be translated directly into and back from human clinical studies to increase success of drug discovery. The project will continue to refine rodent models of type 1 and 2 diabetes to inform current practice in research communities, including NC3Rs.

The outputs from this project will be original, and the short-term benefit over the lifetime of the project will be to disseminate the new information widely to both research and clinical communities in the form of national and international conference presentations at scientific meetings, publications in peer reviewed journals with our collaborators and support academic-industry partnership to optimize novel assets through investment potentially leading to intellectual property. Ultimately in the long term we expect outputs from this project to direct benefit to

diabetic patients since novel assets will treat those aspects of the disease that will assist in patients returning to a normal lifestyle. Further we aim to deliver new information and 3Rs benefits for animal welfare and new recommendations to current practice in the field of preclinical in vivo pain studies.

Who or what will benefit from these outputs, and how?

Collaborators and Researchers in the Scientific Field

In the short-term this project will benefit other researchers in the scientific field from collaborations, publications and presentations at conferences. This project will establish new collaborations to develop refined in vivo models for application in academic and pharmaceutical industrial research and Animal Health.

Pharmaceutical Industry

The medium-longer term benefit from this project is to use the new information as proof of concept and target validation for the pharmaceutical industry to collaborate to develop and optimise novel pharmacology targets and novel substances in the clinic.

Patients

In the long-term people with or at risk of type 1, type 2 and all other forms of diabetes will benefit from new treatments that manage their complication(s). Isolating and describing the active constituent compound(s) in plant extracts will inform controlled clinical studies measuring levels in patients currently taking the extracts as medication. Improvements in how plant extract is provided to patients, in concert with education, will have a positive social and economic impact by improving the health and wellbeing of people living with type 2 diabetes.

How will you look to maximise the outputs of this work?

I have a good track record of publishing work in abstract, manuscript, editorial, newsletter form and oral/poster presentations at key national and international scientific meetings, and 3Rs events so maximising outputs of this work is built into this project. New knowledge from this project will be disseminated in the form of conference presentations and high-impact journal publications in the field of diabetes, pain, complications, and we will aim to publish in open access journals.

Contingency is built into this project e.g., a null hypothesis will provide valuable new information on these mechanisms in diabetes still leading to new information disseminated in high quality scientific publication

To maximise collaboration and make our research more relevant and our findings more likely to benefit patients my research group will participate in the stakeholder focus groups and participatory workshops. My research group will gain valuable insights and develop a patient-centred approach. By learning from patients with lived experiences this will inform our pain assessment approaches and by involving patients in the research process will aim to ensure that the outcomes of this project will address their needs and concerns.

In my current project a PhD studentship project has maximised output beyond the original project objectives by validating the anti-depressant drug ketamine and psilocybin sleep-wake using EEG.

NC3Rs welfare impacts will be promoted through annual research conferences to our student/staff community and wider impacts to influence current practice through our dedicated 3Rs public webpages and NC3Rs conferences.

This project will provide a biobank stock of tissues for other researchers at our establishment (e.g., histopathology group studying nephropathy, neuropathy) and collaborators maximising outputs from this project.

Species and numbers of animals expected to be used

- Rats: 1200
- Mice: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult rodents are the most appropriate models of studying diabetes to meet the objectives of this project. It is not ethical to conduct experiments investigating novel mechanisms and test substances on humans or patients without prior preclinical efficacy and safety understanding, especially where those experiments require the removal of parts of the nervous system / vital tissues for ex-vivo investigations of complications e.g., neuropathy, blood vessels.

Adult rodents are the choice of animal for this project objectives since these are a well-established model for investigating type 1 and 2 diabetes complications and have been utilised in successful translational projects (in my laboratory and by collaborators) for proof of concept demonstrating face and predictive validity.

Typically, what will be done to an animal used in your project?

Animals will undergo administration of a chemical (streptozotocin) by injection intraperitoneally (injection into the gut cavity) to develop a type 1 diabetes high blood glucose. To develop high blood glucose and insulin resistance (type 2 diabetes) animals will consume a high-fat feeding diet followed by administration of streptozotocin intraperitoneally.

Animals will undergo of administration of various substances by injection peripherally. Animals will be subject to implantation of small pumps which administer substances (e.g., insulin) continuously without the need for repeated injections or administration of substances by oral gavage, intraperitoneal, subcutaneous (injection under the skin) or intramuscular (injection into the muscle).

Animals will undergo behavioural testing including electroencephalogram (EEG)

measurements where animals will undergo surgery to implant surface brain EEG electrodes and a radio transmitter placed in the peritoneal cavity.

In the case of surgical implantation of small pumps and EEG electrodes, anaesthesia followed by analgesia will be used.

What are the expected impacts and/or adverse effects for the animals during your project?

Approximately 80% of animals are likely to experience moderate levels of severity. This is because they will develop a sustained level of hyperglycaemia from approximately 4 days post-dose of STZ. The remaining 20% of animals that will receive a vehicle dose (or remain non-diabetic) are likely to experience mild severity as they will typically experience repeated blood sampling and dosing of substances. Our previous experience indicates that up to 10% of STZ dosed animals will fail to become diabetic and will be recorded as experiencing mild levels of severity.

STZ injection evokes typical symptoms of type 1 diabetes, including polydipsia, polyuria, glycosuria, hyperglycaemia, and gradual body weight loss between 5-10% with stabilisation within 4 to 5 days. Neuropathic pain is expected in approximately 80% of diabetic animals from approximately 7 days post-STZ dose and behavioural signs of spontaneous pain may include guarding of a hind paw and slight changes in gait, paw diameter and / or posture and have no behavioural impact on the animal (the animal moves freely around its home cage, it feeds and grooms normally).

Animals will usually be fasted for 6 hours or overnight in the same way a diabetic patient needs to fast before a blood test to establish glucose and insulin tolerance.

Anaesthesia, analgesia will be used to mitigate pain associated with surgery to implant a transmitter device under the skin or electrodes under scalp to record e.g., body temperature or brain signals (e.g., telemeter, mini-pump, EEG). Animals are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in hospital. No adverse effects are expected following injections of test / reference substances. There are limits to the number and frequency of injections, blood sampling and behavioural assessment that any one animal can experience. Some behavioural assessments will require separation from cage-mates. Separation will be as short as reasonably practicable.

At the end of the experiments the animals will be humanely killed, and tissues may be used for in vitro molecular / biochemical investigation / biobanking of samples.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Rat Moderate (80%) diabetic animals (protocol 2 and 3)

Rat Mild (20%) non-diabetic control animals; this proportion includes a group of non-diabetic control animals within the experimental design and up to 5-10% of animals that receive STZ and fail to develop diabetes (protocol 2 and 3)

Mouse / Rats Mild (40%), Moderate (60%) (protocol 1)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal models of diabetes are essential for the study of the disease pathogenesis and complications that are seen clinically. Diabetes is a chronic, inflammatory metabolic disorder that progresses to a range of complications, including neuropathic pain. The whole organism (i.e., intact nervous system) is required to model and measure these complications e.g., a painful response.

To address this project, it is essential to use animal models of disease with good face, construct, and predictive validity to investigate novel mechanisms, biomarkers, and treatments for diabetes complications to be translated from the preclinical stage to the clinic (patients).

A complimentary aim of this project is to refine these animal models of diabetes for improved translation directly into and back from the clinic (bidirectional) by studying ethological and objective markers of neuropathic pain and analgesia (e.g., EEG, burrowing) which we cannot achieved without animals.

Which non-animal alternatives did you consider for use in this project?

This project utilises non-animal in silico models (e.g., molecular modelling), in vitro cell culture models of hyperglycaemia as well as animal primary cell lines/tissues (some supplied from protocols in this project or collaborators).

We use molecular biology, biochemistry, pharmaceutics, and pharmacology experiments to isolate key constituents of plant extracts, measure predictive activity (e.g., molecular modelling, HPLC, mass spectrometry) and biological activity (e.g. in vitro cell/tissue models). We use in vitro studies to guide in vivo mechanistic and efficacy experiments in this project. Drugs to be tested in vivo in this project will have been first tested in silico and / or in vitro (non-animal and / or animal).

Why were they not suitable?

These non-animal systems cannot replicate the whole functioning organism e.g., hyperglycaemia drives complications via multiple mechanisms that can only be investigated in the whole organism. It is not ethical to conduct experiments of novel test substances on humans without prior preclinical efficacy and safety understanding, especially where those experiments require the removal of parts of the nervous system for ex-vivo investigations e.g., neuropathy. Therefore, these non-animal alternatives are complimentary and cannot entirely replace the use of a living animal that would allow the aims of this project to be met.

The integration of in silico, in vitro, ex vivo and in vivo data is essential to meet the specific objectives and benefits as outlined in this project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of animals have been estimated for each protocol (1,2,3) based upon the experimental design of the funded projects and projects seeking funding over the duration of this project.

When animals are administered test substances (molecules) they will usually be tested at 3 or 4 dose levels (dose-response), in some instances only one dose may be required e.g., when comparing several test substances in the same experiment. Experiments will generally include a group administered with vehicle and in some studies a group administered with other control substances (e.g., standard reference compounds) to allow statistical comparisons to be made. For some experiments test substances will not be administered to animals and the development of diabetic phenotype (often in comparison with non-diabetic group) will be measured and tissues collected for evaluation and in some instances, ex vivo test substance evaluation.

For most experiments (in vitro/in vivo), sample sizes have been set using power analysis, generally using a significance level of 5%, a power of 80-90%, to detect a difference between groups of 25% (e.g., G Power, Lanigan et al., 2023, appendix I). For most in vivo behavioural studies numbers of animals per group will be in the 8-12 range per treatment group. In addition, for in vitro /ex vivo experiments the numbers of animals per group will be at least 3, but normally between 3-6 animals per treatment group. Protocols 2 and 3 will be powered to take into consideration those animals that do not develop diabetes (~10%) and those that do not develop diabetes induced neuropathic pain (~20%). We will continue to monitor group sizes and modify as appropriate based on their analysis.

Before commencement of the protocols, each proposed study will undergo a rigorous evaluation process: 1) when ordering animals consideration of the 3Rs is made including whether the scientific purpose (objective) could be achieved using techniques not requiring the use of live animals, 2) in vitro and / or PK/PD data supports the dosing regimen (e.g. reference / test substances) to be used in the study (if applicable); 3) a formal study plan (Study of Home Office Procedures, SHOP) is agreed with NACWO, which includes the experimental treatments, the size of the experiment (number of groups, number of animals/group), and the experimental material; and an outline of the method of analysis of the results / outputs; 4) Completion of internal risk assessment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experimental designs steps of protocols 1, 2 and 3, have been based upon previous project licences and in conjunction with a Biostatistician group, and collaborators in line with NC3Rs ARRIVE guidelines.

Some experiments will involve parallel groups, and some will involve a cross-over (Latin square) group design. Animals will be balanced across groups using appropriate variables for the respective protocol, e.g., body weight, baseline behavioural assessment. Groups will then be randomly assigned to treatments and behavioural assessments will be taken by an observer that is blinded to the treatments, where appropriate, to minimise biases and variability.

Substances can be administered using a cross-over design, whereby each animal receives all treatments and acts as its own control e.g., where animals have been trained to perform a task, such as burrowing or surgically prepared for EEG. Within animal comparisons, are less variable than between animal comparisons, so this will allow the use of smaller groups of animals, to consistently achieve statistical significance.

70-80% of type 1 and type 2 diabetic rats develop a peripheral neuropathic pain demonstrated by allodynia (response to Von Frey hairs). The remaining 20-30% non-pain responder diabetic rats may be used as PK satellite animals, minimising unnecessary use of additional animals to determine the PK exposure of novel test substances in diabetes.

Raw data will be recorded in lab books and stored in standard electronic files such as Microsoft Word,

Excel, Powerpoint, GraphPad Prism files on lab computers. Statistical data will be stored e.g. in SPSS or GraphPad prism. All results obtained will be accompanied by clear and accurate records of the scientific procedures to enable sharing and rigorous integrity, in the form of progress reports, peer review publications or theses in accordance with the NC3Rs ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Multiple tissue usage within the experimental design integrates in vivo behavioural with in vitro and ex vivo molecular, biochemical, pharmacological data from each animal strengthening our understanding of the relationship between mechanisms and complications within this project.

Careful planning of each experiment under each protocol will optimise the number of animals needed under this project. Tissues from the same animals under each protocol in this project can be used for several types of experiments (e.g. correlation of complications to neuropathy and biomarkers) and beyond this project (e.g. university histology research group studying diabetic nephropathy, collaborator research group investigating the effects of neurovascular coupling in diabetes) maximising data output and optimising the numbers of animals we will use in this project.

Under previous licences efficient multiple tissue usage from type 1 diabetic animals for 'biobanking' and sharing has reduced animal use, maximised data output and enabled undergraduate and postgraduate research students to work with diabetic tissues ex-vivo (e.g., investigate a novel target mechanism on GI, bladder, vascular, pain complications all from same animal).

A collaborative QR funded postgraduate research project with a contract research organisation supplies surplus tissues from end of client contract research studies reducing numbers of animals used across projects. Target validation and mechanistic experiments will be determined in non-animal recombinant cell lines to inform the in vivo neuropathic pain behavioural/tissues studies optimising numbers of animals used in this project. A QR funded postgraduate project adopts a systematic review and human feasibility study in addition to the rodent's type 1 / 2 diabetes experiments optimising numbers of animals used within this project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In rodents' systemic administration of chemicals that are selectively toxic to the B-islet pancreatic cells such as Streptozotocin (STZ) and alloxan (ALX) induce a rapid and sustained diabetic state of hyperglycaemia and insulin deficiency and generate animal models of type 1 and 2 diabetes that generate diabetic complications, e.g., neuropathy, vascular dysfunction, neuropathic pain. Further, medicines with efficacy in the clinic e.g., pregabalin are efficacious in STZ diabetic neuropathic pain models thus increasing its translational predictivity. Studies undertaken in my drug discovery projects in industry identified distinct populations of animals that develop painful and non-painful diabetic neuropathy following STZ and led to delivery of 2 candidate molecules into clinical development for diabetic neuropathic pain.

For many researchers, STZ is the agent of choice due to its more reproducible induction of diabetes through greater stability than ALX at physiological pH. Transgenic or highly inbred animal strains have also been developed that spontaneously or conditionally develop diabetes (e.g., ob/ob, Zucker), although the use of these genetically determined models is unlike heterogeneity seen in humans.

The nicotinamide STZ rat, a model for non-insulin-dependent, insulin-deficient type 2 diabetes, is limited in not being insulin-resistant, a major feature of most human cases. The use of high-fat feeding to induce insulin resistance, followed by low-to-moderate doses of STZ to produce mild to moderate insulin deficiency, is currently the most useful of the type 2 diabetic models. The high-fat model is generally considered the best for characterizing many of the long-term complications (including insulin resistance) associated with human diabetes and evaluating potential anti-diabetic agents, with good face and predictive validity. The rodent STZ

models of diabetes are well-established in our laboratory, and in our collaborator laboratories are highly reproducible and lead to moderate severity. Within hours following STZ injection animals show typical symptoms of diabetes, such as polydipsia, polyuria, glycosuria, dyslipidaemia, and hyperglycaemia.

Why can't you use animals that are less sentient?

Less sentient animals such as Drosophila or Zebrafish are not suitable to meet the objectives in this project. Mammalian (rodent) models of diabetes are essential for the study of the disease pathogenesis, mechanisms and complications that are seen clinically and provide proof of concept (POC) that can be translated directly to patient (e.g., face and predictive validity). Diabetes is a chronic, inflammatory metabolic disorder that progresses to a range of complications, including neuropathic pain. The whole organism (i.e., intact nervous system) is required in order to model and measure some of these complications behaviourally e.g., a painful response via QST and ethological / objective endpoints e.g., EEG.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Increased monitoring

Based on previous experience we do not expect any of the diabetic rats to exceed the severity band limit 'moderate'. STZ injection evokes typical symptoms of diabetes, including polydipsia, polyuria, glycosuria, hyperglycaemia, and gradual body weight loss between 5-10% with stabilisation within 4 to 5 days. Therefore, animals will drink more than usual, and this will be taken into consideration during the husbandry care by providing extra drinking water and their home cage bedding changed more frequently due to polyuria. Post STZ injection, sucrose added to the drinking water with a choice of plain water and sweetened baby food may be provided for typically 48 hours to avoid the initial hypoglycaemia, and animals will typically stay pair/group housed to help maintain body temperatures.

Animals will be weighed regularly and closely monitored.

A low-moderate dose STZ (e.g., 55mgkg ip) will continue to be adopted to provide equivalent level of hyperglycaemia, neuropathic pain and analgesia compared to high standard dose STZ (>65mgkg ip) whilst improving animal wellbeing (reduced weight loss, increased burrowing). Should further refinements in dosing of STZ and / or feeding of high fat diet be published we would seek to incorporate these.

A humane endpoint non-invasive scoring method for monitoring animal welfare (Fur cleanliness, Faeces consistency, Urine Colour, Paw Changes, opacity changes, body condition score, BCS) (SilvaReis et al., 2023) will be adopted for all protocols. BCS will be useful for capturing fat deposition in type 2 diabetes model (protocol 3, Hickman & Swan, 2010)

Pain management

Given our type 1 and 2 diabetic models mimic many of the complications observed in the human condition some level of neuropathic pain is expected. However, experience from previous licences has shown us that the level of pain (indicated by

evoked hyperalgesia/allodynia) experienced by diabetic animals is not such as to cause any major changes in the welfare of the animals, compared to the diabetic animals unaffected by pain. Behavioural signs of spontaneous pain may include guarding of a hind paw and slight changes in gait, paw diameter and / or posture and have no behavioural impact on the animal (the animal moves freely around its home cage, it feeds and grooms normally). To minimise discomfort of spontaneous pain (allodynia, guarding) animals will be placed on suitable bedding and animals monitored daily.

Training of animals and Behavioural assessment

General behavioural assessments which are used in the clinic are used where possible. To measure neuropathic pain and analgesia we use reflexive endpoints (e.g., Von Frey hairs) and spontaneous behaviours/ non-invasive endpoints (e.g., QoL, affective / cognitive) in a home cage (where possible) or test environment, reducing external factors such as stress on behaviour. Testable reflexive allodynia and hyperalgesia is applied for the shortest time practicable the animal has direct control over the duration of the noxious and innocuous stimulus applied, by removal of foot.

Objective 2 of this project will continue to refine / develop these diabetes models characterising translatable non-invasive pain endpoints such as QoL, affective / cognitive, EEG power spectra (telemetry) and cutaneous blood flow (laser/thermal imaging) as translatable pharmacodynamic markers of central pain and analgesia. We have shown that type 1 STZ diabetic rats' benefit from social housing enrichment when burrowing pea shingle (QoL) compared to non-diabetic rats. We will continue to investigate the social housing welfare impact on both evoked and novel pain endpoints in our diabetic rats to maximise welfare. We recommend housing rats in social cage-pairs (instead of single) in test chambers for QST pain assessment, where possible for improved animal welfare.

Wheat grains or seeds may be offered daily or post behavioural assessment as an enrichment to promote foraging. Introduction of 'playtime' in a large group 'playpen' may be introduced for e.g., weekly during a non-behavioural assessment day as a social enrichment.

Glucose and insulin tolerance tests may be performed to determine whether type 1 and 2 diabetic animals have impaired glucose tolerance. Rats will be fasted for 3 to 4 hours for insulin tolerance tests and for up to a maximum of 16 hours (overnight) for glucose tolerance tests (with free access to water) to determine a basal blood glucose level, followed by a glucose or insulin challenge and monitoring of the ability of secreted insulin and glucagon to restore normal blood glucose levels.

Substance administration

Following substance e.g. STZ administration animals will be monitored for general health and adverse dosing effects for the first hour after dosing. Insulin (dose ranged IU/day) many be administered for reversal of hyperglycaemia for improved welfare control and improvement in painful phenotype.

Post-operative care

Telemetry devices may be implanted to measure physiological parameters such as body temperatures, EEG or mini pumps for continuous administration of substances e.g., insulin. We have monitored body temperature recordings in STZ type 1 diabetic rats using implantable subcutaneous microchip transponders enabling multiple recordings in a home cage social setting, allowing the continuous collection of data without the need for any manipulation. The benefit of the surgical implantation will improve the overall lifetime experience of the animal compared to repeated restraint procedures. A further refinement we will develop is recording body temperature using thermal imaging avoiding the need for surgery.

To monitor pain and discomfort, rodent grimace scale will be used. Peri-operative analgesia will be given and maintained after surgery for as long as is necessary to alleviate pain. To monitor pain and discomfort, mouse and rat grimace scales will be used https://www.nc3rs.org.uk/grimacescales

All recovery and long-term non-recovery surgery will be done using aseptic techniques in order to minimise the risk of infection surgery and will be carried out to HO Minimum Standards for Aseptic Surgery and/or LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery http://www.lasa.co.uk/pdf/lasa_guiding_principles_aseptic_surgery_2010.2.pdf).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

ARRIVE Where appropriate we will follow the NC3Rs format for experimental design (Percie du Sert et al., 2020).

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery

LASA guidelines on injection volumes

Grimace scale from NC3Rs

Best practice guidance is reported in the concordat on openness in animal research report to University AWERB.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As the University representative of the Concordat on Openness in Animal Research reporting to the AWERB I oversee the implementation of the commitments demonstrating the University is transparent in informing its own community and the public about advances in the 3Rs with the highest standards of rigour and integrity. This role requires up to date awareness of advances in the 3Rs and implementation of these for all teaching and research involving animals (including my project and PILs) at the University and submission of the university annual report to UAR.

As Chair the Concordat on Openness in Animal Research Steering Committee I lead on 3Rs activities at the annual research conference (survey researchers, 3Rs posters, interviews with animal researcher / technician), providing continuing professional development for staff and researchers including PILs, run ethical 3Rs

scientific debates for undergraduate bioscience students. All PILs must consider 3Rs on procurement of animals, demonstrating 3Rs awareness, implementation.

The NTCO shares NC3Rs news, events, and resources whilst senior media and public relations officer provides social media, media and sector news including 3Rs.

I coordinated University endorsement of the national British Pharmacological Society curriculum for undergraduate education in the use of animals for research and teaching in the UK.

67. Vitamin A, retinoids and other lipid signalling molecules in the central nervous system

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Retinoid, Cannabinoid, Receptor, Lipid, Neurodegeneration

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, pregnant
Rats	juvenile, adult, pregnant, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To study the action of vitamin A and its interaction with similar molecules that control function in the brain, particularly how it relates to neurodegenerative disorders such as Alzheimer's, Parkinson's, and motor neuron disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The function of vitamin A in the brain is via its active product, retinoic acid, and its

study is important from a basic science perspective, in particular to its vital role in learning and memory and control of body balance (homeostasis). The project will determine the pathways activated by retinoic acid, cannabinoids and interacting lipids. In addition, one arm of the research is drug discovery and developing therapeutics to treat neurodegenerative diseases of the brain. These therapeutics target the retinoic acid receptors. Presently there are no treatments that stop the advancement of neurodegenerative disease, several of which are on the increase with the ageing population, such as Alzheimer's disease. The research we plan to undertake will actively identify candidates to take towards clinical trials for therapy.

What outputs do you think you will see at the end of this project?

The outputs will include new discoveries on retinoic acid signalling in the brain, in particular its influence on a region of the brain called the hippocampus and how it supports learning and memory. Further outputs will be discoveries on the action of retinoic acid on another region of the brain called the hypothalamus, important for regulation of body homeostasis. These discoveries will be disseminated via publications and engagement with the public. The drug discovery aspect of the project will provide important stepping stones to potential drugs to be taken into future clinical trials.

Who or what will benefit from these outputs, and how?

Those benefiting from these outputs will include researchers studying the brain, in particular those working on learning and memory and control of body balance by the brain. The vitamin A research community will also benefit from the emphasis of the research on the brain as this is an understudied area and our research is making novel progress in several areas of brain function.

Further gain will arise from the work we are performing developing and testing new retinoid-based drugs. We will be testing drugs for efficacy in preclinical models. These are the essential steps necessary to take these drugs into clinical trials. If we are successful in clinical trials this will directly benefit the patients with neurodegenerative disease and provide clinicians with new approaches to treatment.

How will you look to maximise the outputs of this work?

The outputs of this work will be maximised through the scientific collaborations we have with groups in the UK, US and France. Our goal will be to publish our research in relevant journals of high impact to reach the highest number of scientists. All journals chosen will be open access. Publishing will include research that has revealed approaches or ideas that have not supported the original idea for the research to make researchers aware of those that are invalid. Before publication research will be presented at scientific meetings and, following publication, resources such as data will be made available to other researchers.

Species and numbers of animals expected to be used.

- Mice: 3600
- Rats: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodents (mice and rats) are chosen as a species to be able to understand how the mammalian brain functions, with the final goal of comprehending the human brain.

Rodents are very similar biologically to humans, belonging to the same class (mammals) with enormous parallels in the fundamentals of how all organ systems work, reflected in the 97.5% similarity in DNA (that is expressed as protein).

Rodents are essential for development of therapeutics and, for instance, the SOD1G93A mouse model is considered the gold standard for motor neuron disease drug screening and is an essential pre- clinical test required before progression to clinical trials Rodents allow some level of experimental manipulation, following appropriate welfare and ethical guidelines, that could not be performed on humans, but still providing essential information on the types of molecules that form the basis of mammalian brain function. Further, rodents are used as the initial (pre-clinical) steps of therapeutic drug testing to identify doses that are effective but do not cause adverse effects on the animals. In these studies mice are predominantly used. In some specific experiments though rats will be employed. Rats will be used for the vitamin A deficiency studies as rats, like humans, can be made vitamin A deficient by removal of dietary vitamin A over several months. Mice can store vitamin A in their liver to such a degree that they cannot be made completely vitamin A deficient over their lifetime (although mice deficient in liver retinoid binding proteins lack liver stores and so can be made deficient). For this reason of similarity to humans, rats will also be used in studies manipulating retinoid signalling genes in the hypothalamus to determine its influence on body vitamin A homeostasis. Mice will also be used in such studies to allow transgenic mice to be studied that are altered in retinoid signalling genes.

This will allow the role of these genes to be determined. In most cases adult animals will be used because our studies are on adult brain function. A series of studies however are planned to determine whether retinoic acid signalling in the postnatal animal contributes to adult disease and retinoic acid signalling will be disrupted in newborn and juvenile animals.

Also, for some studies, we will grow neuronal cells from mice or rats in a dish. In these cases, the neuronal cells from the younger animal grow better and will be taken from postnatal animals and allowed to mature to become more adult like in the dish.

Typically, what will be done to an animal used in your project?
Mice or rats will be injected with drugs that alter retinoic acid signalling pathways, or

other lipid signalling routes known to influence the brain. Injection will most be by oral or intraperitoneal route, the latter being injection through the peritoneum (the thin, transparent membrane that lines the walls of the abdominal cavity). In some cases animals will be studied for their behaviour (such as motor coordination or memorisation skills in mazes) or metabolism (food intake versus energy output). At the end of these treatments the animals will be killed and the molecular changes in the brain studied using a variety of sensitive techniques.

Infrequently drugs or other substances will be delivered directly into the brain or spinal cord requiring what is known as stereotaxic surgical techniques. These surgeries will involve either injection directly into the brain or brain tube implantation with a minipump under the skin or spinal cord injection. In the case of use of minipump this delivers a constant low flow of drug that avoids repeated injection.

General anaesthesia will be applied in these cases. These surgeries, performed under aseptic conditions, will last up to 2 hours under appropriate level of anaesthesia and wound closure These animals will experience some discomfort after surgery and some mild to moderate pain which will be treated with pain relief. Pain relief will already be provided prior to surgery,at the time of surgery and also post-surgically.

Also infrequently vitamin A levels in animals will manipulated through diet. This will be done through an alteration in dietary composition and the study will be complete before overt physiological signs are apparent.

What are the expected impacts and/or adverse effects for the animals during your project?

In some experiments potentially therapeutic drugs will be employed that have not been previously used on animals, for instance retinoids. The typical adverse effects of such drugs include weight and hair loss as well as drying of skin and eyes. These are carefully monitored and kept within limits that loss of weight is not beyond 10% or hair loss beyond 25%, and a monitoring sheet is used to follow the course of change in animals. If the adverse effects are kept within these bounds then these studies may continue for up to 3 months. Some experiments also include surgery in which pain will result but will be limited using appropriate pain relief post-surgery given post-surgical pain may occur but not expected to last longer than 3 days. Other studies include diet manipulation but these will be completed before significant adverse physiological effects are apparent.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The largest majority will be mild and no procedures will be severe. For mice 20% will be moderate and for rats 15% will be moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The principle aim of the proposed program of work is to understand how retinoic acid, cannabinoids and other interacting lipid signalling pathways regulate the function of the developing postnatal and adult brain. For certain important biological questions simple in-vitro systems (e.g. neural cell lines) cannot substitute for this highly complex biological system that involves interaction between multiple organ systems and the use of animals in the proposed project is therefore unavoidable.

The final goal of the project is to develop drugs to treat human disease and it is essential that we understand the pathways under examination in the mammalian system as they differ radically in the way they operate (or are missing entirely) in the invertebrate. Rodents provide a relatively “primitive” mammalian species on which there is a vast amount on information into which we can tap regarding brain function.

However we always search the literature for potential replacement and make use of platforms such as SyRF for meta-analyses of in vivo studies.

Which non-animal alternatives did you consider for use in this project?

In all cases possible, we replace animals with in-vitro studies and, for instance, when basic questions on cell signalling are investigated then cell lines are used to answer these questions. Only approximately 15% of the work in the laboratory makes use of animals.

For instance for the therapeutic action of retinoid related drugs we have performed very extensive studies with the SH-SY5Y cell line.

We are also making extensive use of human primary neuronal culture and will be using neuronal cells derived from iPSCs in the future. To search for alternatives we use sites such as the “Center for Alternatives to Animal Testing (CAAT) as well as Researching Alternatives to Animal Testing (FRAME).

Why were they not suitable?

As discussed earlier, animals are only used when the scientific questions we are asking require to take into account the interactions that take place within intact biological systems.

To understand how drugs will interact and regulate the brain requires the intact system of the CNS in which neurons interact correctly within their local environment

that includes the glial niche. Further, the neurons need to be correctly positioned between the cerebral spinal fluid (CSF) and blood supply which precisely regulate the nutrients provided via the blood brain barrier. There is a major problem of reproducibility of complex biological systems even in in-vitro cultured neural organoid systems which lack the controlled delivery and removal of compounds from the CSF and blood supply.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number calculated for each study is based on previous experience with the techniques we propose to use, as well as search of the literature to be able to, when changes occur with treatment, reach significance in comparison with control.

Typically, this is based on the variability we have with assays we use such as qPCR which is used to quantify mRNA as a measure of gene expression for which we use between 5-8 animals per group depending on genes to be examined. In some behavioural tests, such as the maze test, the variability is such that a group size 12 is needed. The sham surgical controls used are essential to include to account for the influence of the surgery independent of the action of the drug. Without this the influence of the drug may be incorrectly interpreted. These numbers, combined with the number of experiments we need to obtain our objectives, provides an estimate of the total number of animals needed.

We maximise the data output from the animals we use by, for instance, taking and storing multiple tissues and these are available for our scientific collaborators. For each specific protocol we describe below, in each section, how variability is minimised, group size set, when and how pilot studies are used and how studies are randomised and blinded.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

As part of good experimental design, we design our animal experiments to use the minimum number that is thorough to provide statistical significance. This is based on our own extensive experience on the protocols but through weekly searches of the scientific literature through pubmed etc. We also regularly review other appropriate resources detailing replacements e.g. NC3Rs; RSPCA; FRAME; CRACK IT etc. A wide range of in-vitro studies are performed in advance to reduce the number of animals used in the project. Many of the basic questions on cell signalling were answered using neuronal cell lines and these were used as much as possible until the point at which the signal needed to be studied in the complex system of the

animal. Similarly, many of the first steps to analyse the therapeutic action of retinoid related drugs were performed in neuronal cell lines. When using methods we have used previously (which is the majority of the studies) we will be able to accurately determine the minimum number of animals to use to determine statistical differences between treatments For new approaches we use pilot studies to determine effect size with the minimal animal use. As necessary for many of the journals we publish in, we follow ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

At the end of the majority of experiments, we will harvest as many tissues as possible at post-mortem. If we don't need to analyse the tissues immediately, we freeze these for later use and will also make them available to other researchers working on similar questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.
Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal models used for this study are chosen to result in the least pain, suffering, distress, or lasting harm to the animals. The models consist of the following:

Models in which retinoic acid/vitamin A, cannabinoids and other lipid signalling pathways are altered in the whole body or brain by chemical or genetic approaches.

The model and methods used involve the least pain and suffering to obtain the scientific objective because the manipulation of retinoic acid/vitamin A cannabinoids and other lipid signalling pathways will be at the minimum level to result in change and, when possible, directed to limited regions.

Anything that results in broad effects on the animal will interfere with the ability to reach the scientific objectives. The work continues on from studies of cells and tissues in-vitro and requires to look at the whole animal and so no other options are available. In these studies the animals are checked daily for signs of ill-health using a monitoring sheet designed for alteration of the lipid signalling pathway involved. The chosen end points of the studies are specifically designed for each region of interest such as the hypothalamus and are the earliest possible to result in a measurable effect.

Models of neurodegenerative disease including those for Alzheimer's and

Parkinson's disease as well as motor neuron disease and treatment of these model with drugs triggering the retinoic acid, lipid or cannabinoid signalling pathways as possible therapeutics.

The models used involve the least pain and suffering to obtain the scientific objective by choosing those in which these are kept at a minimum e.g. In the case of Alzheimer's disease the models chosen show only slow cognitive impairment and will come to a completion before any distress occurs. For models for Parkinson's and motor neuron disease all studies are stopped at an early stages of the disease to reduce harm but still allowing determination of whether a therapeutic effect occurs.

All drug treatments will be at a dose with minimal adverse effect – which is an obvious course of action as any adverse effects will interfere with any possible therapeutic action. These studies have developed from our earlier in-vitro experiments but to determine the potential of these drugs to have a therapeutic action in disease a disease model is essential and no other options are available. In these studies the animals are checked daily for signs of ill-health. As previously mentioned, the chosen end points are the earliest possible to result in a measurable therapeutic effect and to minimise the pain and suffering that may result from the Parkinson's and motor neuron disease models.

Why can't you use animals that are less sentient?

Our scientific questions that require the use of animals are ones directed to the function of retinoic acid and lipid signalling molecules in the adult brain and use of younger animals for these studies would provide incorrect answers. This is similarly the case for the drugs we plan to test based on the signalling systems which are directed to the neurodegenerative diseases of adulthood. The goals for the use of these drugs is to treat human disease and so the animal models requires to be the closest to this species while still keeping sentience as low as possible for ethical and moral reason and the avoidance of harm to animals of high sentience. Rodent species are the compromise reached.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For all animal use we use the refined method of tubing/cupping animals to move them around rather than, e.g. picking up by the tail. In studies in which animals are treated with drugs that alter retinoid, cannabinoid or other lipid signalling pathway, particularly when this is with a drug not used before, the animals are checked daily for signs of ill-health using a monitoring sheet based on the potential adverse effects of the lipid signalling pathway involved. Adverse effects might include weight loss, loss of fur or drying of skin and eyes. If these are observed the animals will be treated accordingly, and if animals develop severe effects they will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The various published guidelines will be read to assist with planning animal research

and testing, including the PREPARE guidelines together with associated information:
http://journals.sagepub.com/doi/full/10.1177/0023677217724823
https://norecopa.no/prepare

The internet will also be searched for the various other resources that are available including guidance and publications from the NC3Rs and Laboratory Animal Science Association.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I keep myself aware of the legal and ethical considerations relevant to the use of protected animals by regularly keeping up with the resources listed by e.g. the NC3Rs and receive their monthly newsletter. Further, I have frequent discussions with the Named People and attend the regular Licence Holder Refresher Training seminars provided by our organisation. Over the last several years we have developed alternative methods, such as brain slice culture, to reduce animal number, cell culture to replace animals and constantly consider new approaches to refine my work.

68. Application of rodent models of neurodegeneration for the development of novel therapeutics

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - 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

Neurodegeneration, Motor neuron disease, Frontotemporal dementia, Therapeutics, Disease mechanisms

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Motor neuron disease (MND also know as amyotrophic lateral sclerosis -ALS) is a condition where the nerve cells controlling muscles (motor neurons) gradually degenerate, leading to weakness and eventually affecting a person's ability to move, speak, and breathe. The aim of this project is to use mouse models to study what goes wrong in MND and related diseases and to test new treatments

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The diseases we work on need better therapeutic options to improve the quality of life of patients and relieve the burden on the, their families and other care givers and the NHS. We need to understand the disease mechanisms to be able to come up with new treatments and we need to test those treatments in the disease models to make sure we have the best chance that they will work in patients

What outputs do you think you will see at the end of this project?

We expect to generate data that gains insights into disease mechanisms and how to measure disease processes in motor neuron disease. We will also gather data that allows us to develop new therapies and indicates which are the most promising therapeutic approaches to take forward into human clinical trials and which should be discontinued. We also hope to develop new ways of measuring disease and produce data which helps with the clinical testing of these new therapies in humans.

Who or what will benefit from these outputs, and how?

In the short term (1-5 years) our research group and collaborators in Industry will benefit as well as the wider scientific community as it will help focus research in fruitful directions enable decision making or funding to be raised to take the best approaches further in preclinical and clinical development. On a longer timescale (3-7 years) clinicians will benefit as they will be able to test new therapeutics and diagnostics in platform trials such as EXPERTS ALS. Beyond this on a 5-10 year timescale, MND patients will benefit from new therapies and diagnostics coming into clinical trial and ultimately being approved.

How will you look to maximise the outputs of this work?

As an academic group we aim to publish all our work in the scientific literature, including negative studies. We also disseminate our findings at international and national conferences. In some cases we may need to patent protect our findings to ensure future commercial exploitation which is often a pre- requisite for new therapies reaching patients. We will work with our technology transfer team at the University to make sure these opportunities are captured enabling maximum impact.

Species and numbers of animals expected to be used

- Mice: 8700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice because these are currently the most amenable for genetic approaches which match the genetics of human disease. Mice are also the best validated models of neurodegenerative disease - in particular the G93A model of MND, which is the model of choice in MND groups across the world, although the TDP-43 models we and others have developed are recognised a key addition to the field.

We use mice at all developmental stages as for some studies delivery of gene therapy vectors is most efficient at early post-natal stages but disease phenotype emerge later in juvenile and adult mice..

Typically, what will be done to an animal used in your project?

Mice which carry mutations or human version of genes with mutations that cause motor neuron disease or frontotemporal dementia or a combination of both will be bred for use in experiments. Typically, these mice develop muscle wasting and paralysis. In the majority of experiments this may not progress to more than a severe waddling gait or slight dragging of the feet.

Some mice will be allowed to develop up to this stage and behavioural tests such as running on a rotarod once a week, or being put in a cage with marbles to see how well they explore the cage and bury the marbles will be conducted. Other typical tests include measuring the response of muscle to electrical stimulation. This is done under inhalational anaesthesia to prevent pain and allow the mouse to remain still. Typically, this test is done once a month and the mice recover fully relatively quickly (within 10 minutes). At the end of the study mice are killed and tissue collected for biochemical measurements or histological analysis.

Other groups of mice will have the same tests as above but be dosed with drugs typically once a day for up to 6 months but typically for 2-3 months and in many cases for shorter studies of up to one week, and these studies can be conducted in normal mice. This involves delivering the drug by oral dosing into the stomach with a dosing needle with a bulb on the end which causes mild and transient discomfort.

Other studies will involve groups of mice injected with gene therapy vectors or cell therapies into the blood stream by intravenous injection or directly into the ventricles or cisterna magna of the brain.

They can be injected twice in the brain or up to monthly intravenously

To test new diagnostic methods, mice will be evaluated as above and the new needle based Raman spectroscopy and/or electromyography (EMG) probes placed in different muscles under anaesthesia. Raman spectroscopy is a technique we have developed to study the molecular composition of muscle. EMG is a standard electrical test of muscles that can be used to look for evidence of disease

What are the expected impacts and/or adverse effects for the animals during your project?

The model itself causes motor or cognitive decline. The mice with cognitive decline generally are more apathetic and eat more, they put weight on, are less interested in their surroundings but otherwise do not show typical signs of distress. Mice with motor system decline shows abnormal gait, tremor, abnormal reflexes and partial limb paralysis which is progressive. These mice do not show typical signs of pain and distress until very late in the disease and can score low on distress scoring scales even with partial or near complete limb paralysis. The mice lose weight later in the disease course. The most advanced stage of disease is where mice can no longer right themselves when placed on their backs or they show significant weight loss, mice will not be taken to this stage in this license. The administration of therapies in general causes mild and transient distress. Some therapies of themselves cause weight loss. For surgical techniques conducted under anaesthesia the pain following recovery is controlled with analgesia. Sometimes surgical wounds require repair.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities include non-recovery, sub-threshold, mild, moderate and severe Mice 4% non-recovery, 28% sub-threshold, 17% mild, 51% moderate, 0% severe.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To characterise molecular mechanisms of neurological disease we must perform some experiments at the level of the whole organism. We use cells and tissue samples where it is possible to do so, but ultimately, we need to understand how neurons die in the in vivo context. Neurons are highly specialized cells, which interact with a wide variety of other cell types both inside and outside the brain and spinal cord. For example, a motor neuron in the lower spinal cord can send processes, over a metre long, out to muscles in the foot and in so doing makes unique and intimate interactions with at least four different cell types. Each interaction has its own complicated chemical and physical signals. Such complexity is impossible to replicate in culture systems.

Which non-animal alternatives did you consider for use in this project?

To achieve the objectives of this project we are using cultured cells to investigate molecular mechanisms and to develop therapeutic approaches. For example, we can use a primary human cell culture system to screen multiple compounds for the ability to limit astrocyte toxicity. The best hits from this cellular assay can be taken forward into preclinical development in mice and we have several examples of this. Other possible systems include the use of brain organoids and iPSC derived motor neurons or cortical neurons.

Why were they not suitable?

The research question we have involves the complex interplay of both the in vivo physiology of the motor and cognitive systems and their associated cellular and anatomical complexity. These systems also interact with other whole organism physiological systems, e.g the immune response. In the case of therapeutic testing, these complex whole organism systems also interact with the pharmacology and pharmacodynamics of drug action across the whole organism. This interplay cannot be replicated as yet with in silico or in vitro models

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For studies where we test the effects of interventions such as gene therapies we have used previous data to understand the variations seen in the measurements we take and had help from statisticians to perform sample size calculations. In some cases we have published these calculations along with a typical study design. This has allowed us to use a consistent approach so we understand the numbers needed. Typically 14 animals per group are needed based on the most variable readout that we measure which is rotarod performance and measurement of muscle physiology.

We always use a staged approach to new therapy development. For example we start by making sure the therapeutic can reach the target tissue. We can do this with small group sizes such as 3 per time point per dose tested if we are measuring drugs directly and 6 per group if we are measuring downstream effects such as changes in gene expression. When we are developing new readouts from the models or new types of therapy we may start with small pilot studies to make sure we are happy with the experimental setup and to identify any issues.

For breeding we have used our experience of running similar studies over the last 5 years to estimate the number of animals needed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Reducing variation in the measurements we make in the model is one of the best ways to reduce the number of animals used. In the SOD1G93A model we used a congenic background strain that was well defined, unlike the majority of other research groups who use a mixed genetic background. This allowed us to reduce the number of animals needed for each study. For example, in the mixed genetic background the recommended number of animals per group is 24, whereas we can use typically 14 per group. In addition we have taken this through to other models where we only use mice on defined genetic backgrounds

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When we collect tissue for histological analysis at different stages of disease we make sure that it is collected in a way that allows analysis across multiple experiments and can be shared for other studies and by other users.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We make refinement of our studies a high priority. Our previous funding from the NC3Rs to refine the G93A model of MND demonstrates this. The G93A mouse shows a progressive disease course, starting with a reduction in wheel running and rotarod performance, followed by altered hind limb splay and tremor, leading to hind-limb paralysis, forelimb paralysis and finally loss of righting reflex. Under this license we will mainly investigate mice in the early stages of disease, before substantial phenotypes manifest and move onto severe phenotypes when we have evidence that it is mechanistically valid.

We will continue to review our models and procedures regularly and take every opportunity to reduce the severity of our protocols whenever scientifically practicable.

Why can't you use animals that are less sentient?

We cannot use terminally anesthetised animals as we need to observe the effects of interventions and understand disease mechanisms across weeks and months. We have considered using drosophila and zebrafish models and we currently use these for some aspects of our work but the differences in genetics, neuronal architecture and physiology often makes it difficult or impossible to translate findings into humans or test the mechanisms or interventions we are interested in. Such systems can be useful for screening approaches. Finally an important aspect of the work is how the therapies interact with the organism, and we can only understand this well enough to enable translation to humans when we use mammals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use early endpoints where possible to obtain data on motor and cognitive degeneration, for example sensitive tests such as CMAP and marble buying show motor and cognitive changes at relatively early stages of disease progression where there are no obvious signs of distress in the mice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use relevant published guidance to ensure we use best practice including NC3Rs guidance: https://nc3rs.org.uk/3rs-resources

LASA guidance https://www.lasa.co.uk/current_publications/

PREPARE guidelines:
http://journals.sagepub.com/doi/full/10.1177/0023677217724823

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our Veterinary Services Unit sends regular updates on new guidance and information relating to the 3Rs. I have signed up to the NC3Rs newsletter and will attend relevant events or nominate project members to attend and report back.

69. Development of combination immunotherapy to treat cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, immunotherapy, cancer vaccine

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To develop new combinational approach focusing on immunotherapy for better cancer treatment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer immunotherapy is a type of cancer treatment that harnesses the body's own immune system to recognize and target cancer cells. Immunotherapy such as immune checkpoint blockade that aims to enhance the body's natural immune response against cancer cells, has shown promise in treating advanced or metastatic cancers that may not respond well to other treatments, though it may not

be effective when patients do not have immune responses to fight the cancer cells. However, cancer vaccines can induce tumour-specific response and can be combined with checkpoint blockade to enhance anti-tumour immunity. Moreover, immunotherapy can be used in combination with other cancer treatments, such as chemotherapy, radiation therapy, or targeted therapies, to enhance their effectiveness.

Tumours even within the same type exhibit significant diversity in character and content (heterogeneity). Combinatorial approaches can target multiple pathways or components of the tumour simultaneously, addressing this heterogeneity more effectively than single-agent therapies. Also, cancer cells often develop resistance to single treatments over time. Combining different treatments with distinct mechanisms of action can reduce the likelihood of resistance development, as it becomes more challenging for cancer cells to adapt to multiple simultaneous attacks.

Many cancer treatments have toxic side effects. By combining treatments, it may be possible to use lower doses of individual drugs, reducing the overall toxicity and improving the patient's quality of life. Moreover, some combinations of therapies can have a synergistic effect, meaning that their combined action is more potent than the sum of their individual effects.

We seek to find new combination therapies that can improve the standard of care treatments for cancer patients.

What outputs do you think you will see at the end of this project?

The information generated along the project will be used to justify specific clinical trial designs, such as optimal timing of different therapeutic approaches and relevance of specific combination therapies.

Some combination therapies may result in no synergistic effect, so preclinical data will be informative to avoid unnecessary and expensive therapeutic interventions in humans.

Combination therapies may involve 2 or more therapeutic interventions, and the order of therapies, dose and timing are important parameters to consider for optimal clinical outcomes. However, trying all the possible permutations in a clinical trial setting is virtually impossible, so the preclinical studies are informative as we can investigate the combination that is more likely to result in the best treatment outcome in patients.

In addition to provide preclinical evidence for optimal clinical trial design, this data will be published in scientific journals, so other research institutions can make use of this knowledge to follow-up on the work or base their clinical design on the published data.

The information generated in this project can also lead to generation of new products or methods for the generation of medicinal interventions that can have a direct impact on people's health and well- being.

Who or what will benefit from these outputs, and how?

The study of combination therapies in the context of cancer therapy has several implications for the translation of these therapies into humans.

The short-term benefits would be a better understanding of specific parameters for optimal therapeutic outcomes to be evaluated in clinical trials. Testing of therapies in preclinical models is an important step prior to testing in humans as we can study the intervention safety and efficacy with more flexibility of time and more combinations. This step can inform us on how to approach combination therapies in the clinic. Successful combination therapies have the potential to directly impact people who suffer from various types of cancer by decreasing the side effects due to the reduced dose of certain therapies and giving them a longer life expectancy after partial or complete tumour regression.

In addition, we aim to study mechanistically tumour formation, escape and relapse. As cancer is a set of a highly complex diseases, it is necessary to take into consideration the particularities of each one of them. Different tumour types may present distinct escape mechanisms that should be taken into consideration for combination therapies; thus, studying these mechanisms would be informative in the long term for the scientific community when developing therapies for specific tumours.

Adding mechanistic knowledge to the existing scientific literature on combination therapies is a valuable output as it benefits the current clinical approaches whilst supporting future research on how to develop novel and tailored interventions to tackle the obstacles cancer present to humanity.

How will you look to maximise the outputs of this work?

The results generated in the project will be published in international scientific journals, presented in oral and poster presentations in local and international scientific events.

To publish optimal combination therapies, the suboptimal combinations should also be presented, which can inform the scientific community about specific settings that may not result in the desired treatment outcomes.

Collaboration with groups will add valuable inputs so experiments are performed in such a way that we can obtain as much information as possible, so the generated knowledge will enrich and guide other projects for a faster generation of knowledge and therapies.

Species and numbers of animals expected to be used

- Mice: 31000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have been widely used as a model for combination therapies in multiple preclinical studies and have provided valuable information about therapies toxicity, pharmacodynamics, pharmacokinetics, distribution, clearance, and efficacy.

Mouse models are important models for the study of immunology and combination therapies due to a series of advantages:

- The murine immunology is very well studied in the context of health and disease, particularly cancer. Mice are mammals, so their anatomy and physiology can model better human physiology and immunology in comparison to fish, for example.

- There are numerous murine and tumour models that mimic several aspects of human tumour immunology when concerning specific anti-tumour effector cell (T cells) infiltration patterns in tumours (inflamed, immune desert and immune excluded). In addition, these models can replicate mechanisms by which the tumour microenvironment (TME) escape therapies, which is very informative when studying combination therapies for the treatment of cancer.

- There are a large range of well-established protocols that we can explore to answer different questions regarding to treatment outcome read-outs, therapy mechanisms of actions. In addition, we have highly trained personnel experienced in mouse models of disease and the experience to train newcomers.

- We have at our disposal numerous reagents that can be used to answer multiple questions in the same experiment. And as this is a worldwide used model, multiple studies with different methodologies are published, which can be used in our project to fasten the generation of meaningful results.

It is well established that the function of the immune system decline with age, so it is important to take into consideration the age of the mice in testing different therapeutic approaches. Indeed, young mice respond better to vaccination than geriatric mice. Importantly, this difference is also observed in humans, so the development of therapies that work in a broad range of ages could be informative for clinical trials set up.

In addition, cancer can be a chronic disease with late onset, meaning that therapeutic interventions could only be studied in certain models in mice at an older age. Importantly, after successful therapeutic outcome with complete resolution of tumours, for example, mice may need to be monitored for an extended period to ensure the therapy was efficacious in avoiding late tumour relapse.

Typically, what will be done to an animal used in your project?

We will use several genetically altered mouse models in this project. Immunodeficient mouse strains will be used to assess the impact of the absence of a particular arm of the immune system on tumour response. These animals may not show a harmful phenotype but are more susceptible to infections.

Genetically altered mice will be bred by conventional methods. Some strains of mice will have alterations in their T cells which will not cause them any harm or that are inactive until they are given a substance to activate the gene of interest. This substance is typically Tamoxifen or Doxycycline.

However, some mice will have mutations in cancer or immune related genes. In these strains the mice will be susceptible to growing tumours as they age. These mice may then grow tumours naturally because of gene activation. The tumours can take as little as ten days to develop or take up to 10 months.

Some mice will undergo tumour induction by subcutaneous injection of chemicals or cells. General anaesthesia may be applied for precision injection of the tumour cells. These tumours are not expected to cause the animal any systemic problems. These tumours can be measured using callipers which is non invasive and only requires the animal to briefly restrained.

Some mice will undergo tumour induction by the intravenous route which will give rise to systemic tumours.

Some mice will be given therapeutic agents to see how they affect the tumour growth. We will use commonly used routes at a frequency and duration which are not expected to have any welfare consequences.

To refine drug administration, we may surgically implant a small pump known as an osmotic minipump. Surgery will be carried out to at least the home office minimum guidelines and animals will be given pain relief as humans that have received surgery would do.

To monitor the reaction of the immune system to therapies in some animals, repeated small blood samples will be taken.

Tumour growth and progression may also be monitored by non-invasive imaging, such as MRI.

Some animals will be exposed to either a sublethal dose of irradiation to suppress the immune system or given carcinogenic substances to cause mutation or uncontrolled growth of tissues or cells.

To cause leukaemia some mice will be exposed to a lethal dose of irradiation to deplete their bone marrow before undergoing the administration of donor bone marrow cells to reconstitute their immune system. This process is like bone marrow transfusion in humans which is used to treat blood and immune systems diseases which affect the bone marrow.

Our studies can be as short as one week but as long as three months dependent on tumour growth or therapeutic intervention.

At the end of the experiment all animals will be humanely killed, and tissues harvested post mortem for analysis in the laboratory.

What are the expected impacts and/or adverse effects for the animals during your project?

Tamoxifen may cause transient weight loss of up to 15%, which is readily recovered from once the dosing regimen is completed.

Subcutaneous tumours may attach themselves to muscle making it uncomfortable for the animals to move. As these tumours are external, they may also ulcerate and need treatment or may dry up without intervention within 48 hours. Where these tumours progress to form a wet ulcer, we will allow 48 hours for it to dry. If the ulcer does not dry we will humanely kill the animal.

As with human patients, animals that have tumours can experience weight loss of up to 15% compared to their weight prior to the tumour being induced. We would support these animals with moist palatable food to ameliorate the weight loss as soon as their body weight drops to 10% from baseline. Typically, we would expect to see improvement within 24-48 hours of this intervention. If recovery of body weight is not observed and results in weight loss of more than 15%, the animal will be humanely killed.

Mice with internal tumours may be occasionally used. These mice will be under close observation to ensure they are not distressed. If they have signs or symptoms, they will be flagged for close observation, and if they develop further signs and symptoms of tumours such as hunching, weight loss or lethargy, they will be killed by schedule one methods.

Irradiation may result in some toxicity. The toxicity from this exposure will lead to weight loss, diarrhoea, partial hunched posture. There is also increased risk of infection, but this is mitigated by housing the animals in a bio secure environment. We would support these animals with moist palatable food to ameliorate the weight loss as soon as their body weight drops to 10% from baseline. Typically, we would expect to see improvement within 24-48 hours of this intervention.

Imaging techniques to measure and monitor tumour growth will be performed under general anaesthesia and is not expected to have any adverse effects. During any procedure involving general anaesthesia the animals will be kept warm and hydrated (and eye drops will be used as necessary) with a close monitoring programme incorporated during the recovery phase as you would do with the human patient.

Expected severity categories and the 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: 15% Subthreshold, 35% Mild, 50% Moderate

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To find better ways to treat cancer, we use animals to simulate tumours similar to those in cancer patients. We then test different treatments like immunotherapy and chemotherapy on these mice. However, these treatments can have side effects. We do these experiments in mice because it is not ethical or practical to do them in humans due to the differences among cancer patients. The variety among patients makes it hard for us to understand why new cancer treatments work the way they do. To study these treatments more thoroughly, we need mice that are similar to each other and can be exposed to the same conditions consistently, like mice kept in controlled environments.

Mice are the best model to study combination therapies, particularly immunotherapies, against tumours for a series of factors. Their physiology recapitulates important immunological processes that must be considered for therapies efficacy. The immune system is a complex network of cells and tissues that maintains homeostasis through several very well-regulated mechanisms. Even though this system has been studied for more than a century, we still do not know all the positive and negative regulators of the immunological processes that happen in multiple tissues throughout an organism's life. In addition, the immune system is highly compartmentalised, and no technology to date has been developed to recapitulate this compartmentalisation and the cellular and structural complexity to replace the use of animals for the applications we propose in this project.

Furthermore, mice can inform us about the safety of the combination therapies tested prior to translating into humans, and be used to study of direct tissue damage as well as immune-mediated pathologies. This is because combined therapies may exacerbate the effect of each other in an unexpected manner, so it is important to verify their safety in animal models.

Which non-animal alternatives did you consider for use in this project?

In vitro experiments can be informative for the investigation of how specific drugs can kill tumour cells or by providing information on dose response studies, and their impact on expression of specific genes. This will be done for proof-of-concept in some experiments, but ultimately, these data must be validated in animal studies as cells on a dish cannot recapitulate all the spatial and physiological parameters found in a living organism.

Organ-on-chip is a technology that has had great advances in recent years, but it is still lacking in terms of mimicking immunological responses. While organoid models have advanced significantly, they still cannot fully replicate the complexity of the entire immune system.

Why were they not suitable?

The non-animal alternatives are not well suited because these do not have a fully functional immune system. Also, there are limited options of non-animal alternatives, and the ones that exist are limited in their replication of human immunology and physiology.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers are based on our previous experience with experiments of these specific tumour models and design. A statistician helped us with calculations using typical variations from our own earlier experimentation to calculate minimum numbers of animals to be used whilst ensuring that the results are statistically significant. Calculations typically show that we need group sizes of 5-8 to achieve the quality of results we need. For experiments where we will assess the effects of a particular treatment on tumour growth, calculations typically show that we need group sizes of 8 to achieve the quality of results we need. For experiments where we are trying to understand how or why mice responded in a particular way, 5 mice per group is typically sufficient to achieve statistical significance. Key experiments will be repeated once (or twice if low consistency between replicates) to confirm reproducibility, with data presented as combined from experiments.

We have 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?

To reduce the number of animals being used in this project, we used scientific literature to decide on dose and treatment scheme, discussed with collaborators who have done similar experiments and can give us advice based on their previous experiences, developed a clear hypothesis, selected of the most suitable models, optimised experimental timepoints and read-outs with small number of animals.

Where possible all data analysis will be performed blind to the assigned treatment groups. Mouse numbers per group will continue to be chosen as stated above and following the 3Rs principles. We will continue to comply with the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines). NC3R's Experimental Design Assistant will be used.

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. 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. Where unavoidable excess animals become available from breeding protocols they are shared with other researchers where these may be of use. Where a strain is not required for a prolonged period it will be cryopreserved and breeding ceased.

Pilot studies will be performed to gather information to perform sample size calculation. Each experiment will be designed to maximise the data output generated per mouse used.

Where possible tissues from a single animal will be used to provide multiple readouts (for example histopathological, measures of gene expression, and cellular immunology).

Both sexes will be used for experiments, which ensures generalisability of findings and minimises inefficient breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this project we will use several genetically altered and wild type mouse models.

To evaluate the impact of the immune system on vaccine-induced responses, mouse strains with an intact immune system will be used. Immunodeficient mouse strains may also be used to assess the impact of the absence of a particular arm of the immune system may have on tumour response.

Appropriate housing conditions for each strain will be followed to minimise harm and the animals will be housed for the minimal amount of time possible.

For tumour studies we will implant tumours (or use genetically altered models that lead to spontaneous tumour development) and will monitor tumour growth following best practice guidance to minimise suffering, distress, or lasting harm.

Subcutaneous tumours usually do not invade locally or metastasise to healthy organs, with minimal damage to animal welfare.

Tumour induction will be performed under brief gaseous anaesthesia to allow better positioning of the tumour. We will also use analgesia prior to induction when

administering tumour cells into the brain (following surgery under anaesthesia). Additional analgesia will be given if signs of pain become apparent.

In efficacy studies, tumours will grow up to the minimum size (< 1200 mm3 in the preventive setting and < 1500 mm3 in the therapeutic setting) necessary to obtain relevant data in the preventive setting, in order to minimise clinical signs caused by tumour burden.

To minimise discomfort to animals during administration of therapeutics, whenever possible the least invasive route of administration will be chosen. If intravenous injection is needed for compound administration, both lateral tail veins will be used to minimise the risk of vein damage.

To minimise the risk of potential toxicity to animals where tolerable doses need to be established, a small number of animals will be used for establishing tolerated doses.

Bone marrow transplantation are used to generate mouse model of leukaemia to study different therapies. Irradiation will be performed to condition the mice for bone marrow transplantation. We will use the lowest possible dose of the irradiation to fit the purpose. We will ensure the irradiation time is short. Irradiation is the most refined means of bone marrow ablation as it is non-invasive and gives greater reproducibility than alternative methods involving cytotoxic drugs. The radiation exposure will be fractionated to reduce the impact of the high radiation dose on the mouse.

Why can’t you use animals that are less sentient?

To verify if a combination therapy against cancer is effective, the animals should be treated and monitored for a few months after they reach adult age. Therefore, the use of less sentient stages of life would not be appropriate to answer the scientific questions we have.

Furthermore, species that are less sentient, such as fish, do not replicate important physiological and immunological parameters of mammals, which should be taken into consideration when studying new combination therapies. Thus, mice models are a better choice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where we use routes of administration that are likely to cause pain, (e.g., intramuscular, and intra- tumoral injections, we will use general anaesthesia.

For procedures done without anaesthesia, such as intraperitoneal and intravenous injections, oral gavage, and blood collection, the animals will be handled with care while aiming to minimise the time the animal is immobilized.

For blood collection and intravenous injections, pressure will be applied to the site of the vein to stop any blood loss.Following procedures, the animals will be monitored for any physical manifestation of pain or suffering. If a new treatment or a new combination of treatments are being evaluated, the mouse will be weighed 2-3 times

a week to monitor for weight loss, which is a sign of the animal health state. Body condition scoring will also be used when suitable.

When transferring mice to a new cage or container, tubes or cup handling will be used to avoid handling the mice by the tail.

For tumour studies, we will follow published tumour model guidelines to ensure adequate pain relief is provided where necessary and severity is kept to a minimum.

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 Laboratory Animal Science Association (LASA): https://www.lasa.co.uk

NC3R: www.nc3rs.org.uk

Animal Research: Reporting of In Vivo Experiments (ARRIVE)

Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have signed up to the NC3Rs website and will review the information published here regularly to check for new updates. We will pay attention to best practice resources available and implicate recommended refinements as we become aware of them.

We will also attend regular internal 3Rs meetings, conferences, seminars and events focused on new techniques related to 3Rs.

70. Development of personalised anti-cancer strategies.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Biomarkers, Metastasis, Therapy

Animal types	Life stages
Mice	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The main objective of this project is to identify new drugs or combinations of drugs to treat cancer patients. We will use mouse models of cancer to achieve this. Research will also be carried out to better understand how cancer spreads in a well characterised mouse model, so that new drugs can be developed to stop the spread of cancer. Finally, research will be carried out on early disease generation, to see if we can find cells or molecules in the blood stream which allow doctors to detect the cancer sooner so the patient can be treated quicker. This will be done in both test tubes and in mouse models of cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The research carried out in the project will help us understand the complex biology of cancer which is likely to tell us whether new drugs will work in certain types of cancer. The outcome of this research would then allow doctors to have more confidence to test these drugs in patient clinical trials, with the ultimate aim of finding drugs that can be approved and used to treat patients on a regular basis as an approved drug treatment for cancer. This project license will also help us understand how and why cancer spreads throughout the body.

What outputs do you think you will see at the end of this project?

The research should allow us to better understand why some patient cancers respond to a drug whilst others do not. From this, further research could then identify new drugs that might work in those patients who do not respond to current drugs. The research will allow us to better understand how some cancer types spread throughout the body to other organs. Further research will identify new treatments for these patients with the ultimate aim of improving patient care.

Who or what will benefit from these outputs, and how?

Other members of the research group will benefit firstly from the outputs of this research. Mice are used to grow cancer models using cells taken from patient blood or tumour biopsies These models may be used for ex vivo research (e.g. in a petri dish) including drug screening and co-cultures (cell cultures containing two more more different types of cell). The use of ex vivo cell culture will also reduce the number of mice used in our research. Large scale drug testing using ex vivo methods can eliminate the need for certain models prior to drugs being tested in vivo, preventing mice being used unnecessarily.The work will also be shared with other groups within the establishment, academic groups worldwide and the pharmaceutical industry.

Secondly, the research should enable clinicians to select patients for whom specific drugs are more likely to work and in turn minimise any time a patient might be exposed to a therapy that is unlikely to work. In the longer term, we will identify new targets/therapeutics which may advance to clinical trials in the hope to improve patient care/survival.

How will you look to maximise the outputs of this work?

Our findings will be made available to other scientists through collaborations, publication in high profile peer-reviewed journals and presentations at scientific conferences and meetings. Our Establishment has a policy of ensuring that all publications generated are available on open access to all. In addition, our work has direct translational and clinical applications that we will investigate through collaborations with clinicians at the Establishment and other medical cancer centres worldwide. Data will also be shared with the general public through outreach activities within the local community, social media and other public engagement activities.

Species and numbers of animals expected to be used

- Mice: 17,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is an ideal organism in which to study cancer biology and treatment. The mouse genetic code is of a similar size to the human genome, with a great deal of genetic identity/similarity between the two species, and there is considerable similarity between gene expression profiles in the mouse and human immune system. This is particularly important as it allows us to investigate the role of the immune system in disease development and treatment. Mouse biology mirrors that of the human remarkably well. An extensive catalogue of genetic mutants exists, facilitating the exploration of functional responses including transgenic knock-in (genes are mutated) and knock-out (genes are deleted) models.Experiments demonstrating that new types of therapy work in mice have led to the development of successful new treatments for cancer patients. Mice are the lowest sentient species that have lungs to allow investigation of e.g. lung cancers

The majority of studies will utilise young adult mice (typically 8-12 weeks old), although some studies may use elderly mice (up to 18 months of age at the time when the experiment is started; equivalent to elderly humans, ~60-70y). Aged mice better represent the aged human population that typically develop cancer. This is important as the immune system is known to enter functional decline with age which may limit the immune response to cancer and treatment efficacy

Typically, what will be done to an animal used in your project?

Mice will be housed in individually ventilated cages with free access to food, water, nesting material and environmental enrichment (e.g. chew bars, tunnels and houses). Some mice may be given a drug, initially at a very low dose to test its tolerability, whilst others may have cancer cells implanted under the skin or directly into organs. Some mice will have cancer cells injected into their blood stream, brain or the heart chambers under recovery general anaesthesia (mice wake up after the procedure). Mice will be monitored regularly to assess cancer growth using either callipers (for superficial tumours/tumours on the back of the mouse) or the latest imaging methods for internal tumours. Imaging will be undertaken under inhaled general anaesthesia from which the mice will be allowed to recover. Some of the mice which have grown tumours will be given novel drugs, some irradiation treatment and some will receive conventional anti-cancer treatment. On occasions it may also be necessary to look at combinations of these three treatment options.

Typically, mice will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (into a vein, under the skin or into the abdomen). Mice may experience adverse effects from the drugs themselves, but this will be kept to a minimum and mice will be monitored regularly. Some mice will undergo surgical procedures under inhaled general anaesthesia to remove superficial tumours and allowed to recover and followed to see if secondary

cancers develop. They will be given pain relief during surgery, so any discomfort is only transient.

Mice will experience mild and transient discomfort from blood sampling from a tail vein.

At the end of the experiment, all mice will be humanely killed, and in some circumstances selected tissues and body fluids taken for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Tumours grown on the back of mice rarely cause adverse effects, however, cancer cells injected into the blood stream or by organ specific injection may cause internal tumours. Adverse effects of these tumours may include difficulty breathing, enlarged liver and in some instances restriction of movement.

There might be on occasions (up to 10%) where acute effects of novel agent administration could result in adverse effects. The expected adverse effects of procedures used in this research are mainly weight loss, a change in normal behaviour and a loss of condition of their fur. The mice will be closely monitored by trained and competent scientists to make sure they do not show symptoms of suffering such as breathing difficulties, pain, weight loss of >20%, loss of well-being or appearance or internal tumours. In the unexpected event that they do suffer more than this or adverse effects are seen for 48 hours they will be humanely killed by trained staff.

Some mice will have minor surgery, carried out by trained and competent scientists, to remove a superficial tumour from their back. They are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in hospital.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

20-25% mice will experience mild (mice undergoing simple, non-surgical subcutaneous implantation of tumours that do not grow beyond a certain size and do not ulcerate) symptoms. 75-80% mice moderate (mice bearing more invasive implantations and tumours that may impair vital functions or undergo surgical procedures). We aim to minimise the appearance of severe endpoints (animals found dead overnight) with careful monitoring, surveillance and care. The actual severity varies across specific studies so these values are averages based on the procedures contained within the protocols on the license and planned projects and collaborations. Annual returns data from previous license cannot be used as a predictor of overall severity for this license.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cancer is a complicated disease, with cancer cells interacting with lots of other cells in the body, e.g. blood vessels; cells of the immune system. Drugs also interact with other cells in the body – the best example of this is a cancer patient's hair falling out when they are given a drug. Therefore, to get a true reflection about how a cancer and the patient is going to respond to a drug it is important to carry out the research in an animal and not only in a test tube. By doing this research in mice, it gives the best chance that the finding will be relevant when the drug is used in patients. Before we do this, however, we are able to screen the best potential drugs by doing experiments in a test tube, so we know which ones might have a better chance of working and therefore not using mice unnecessarily.

Which non-animal alternatives did you consider for use in this project?

We will continue to assess new ex vivo model systems to complement the in vitro models. We will continue to test and develop these in vitro and ex vivo systems over the next few years to address how well they can effectively recapitulate mouse models. In particular, we will look into the use of microfluidic chips to simulate organ- and tissue-level physiology.

We will also consider the use of organoids or explants as an alternative approach to assess therapeutic response. A single tumour (from a single mouse) could be processed into single cells and grown in the laboratory and used to investigate a number of therapies. This in turn will reduce the number of mice involved in the study.

We have considered the use of Nematodes (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8929040/) and fruit flies (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9949803/) for our research however neither are suitable.

Why were they not suitable?

The study of cells in culture (in vitro) provides us with clues on the mechanisms of cellular processes or therapeutic response in a simple and valuable context allowing the establishment of hypotheses regarding the function of cells in a living animal. Whilst enormous progress has been made in the field of cancer research using in vitro models, there are a number of questions that can only be addressed using animal models of the disease. In particular, heterogeneity (differences in tumours), complexity and metastatic dissemination (how the tumours spread throughout the body) of disease cannot currently be modelled outside of a living organism. Thus, in order to investigate tumour heterogeneity, cancer progression and responses to

therapy, we need to perform studies in living organisms with systems (e.g. an immune system) that function similarly to that of humans.

Although in vitro models have not currently developed to the point where they are comparable to animal studies for metastatic dissemination of disease, we will, non-the-less follow the development of this field and consider alternative approaches where possible.

Nematodes are used in bioassays to detect cancer but not in models of cancer. Although they have digestive, excretory and nervous systems, they do not function in the same way as humans. Similarly, fruit flies, although have a broader use in cancer research, do not have lungs and a very different microenvironment from mammalian cancers.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used previous annual return of procedures data, generated whilst using the same mouse models of cancer, as well as the upcoming collaborations/study plans to estimate the number of mice that we will need to use for these studies.

The effect of anti-cancer drugs in mice with induced or implanted tumours will be tested at expected effective doses determined in protocol 1 (tolerability studies). The number of groups will be determined by the research question, for example if the compound is to be given as a monotherapy or in combination with another compound. If pilot studies are required, these will be carried out in small numbers of mice.

Controls are an essential feature of any good experimental design and need to experience all contributing variables in common with the treatment group(s). In the context of this project, potential confounding variables include age, genotype, diet, microbiota (e.g. gut bacteria), inflammation and stress from any procedure. Thus, controls will be required to undergo equivalent procedures as treatment groups. Control animals will be dosed with the vehicle for the compound (or combination of compounds) being tested.

The overall aim will be to generate models whereby a measurable effect e.g. reduction in tumour volume following inhibition of a target of interest can be determined using a minimal number of mice. In our experience 5 to 8 mice per group provide sufficient power to detect statistically significant differences. Advice will be sought where available from the literature and we enlist the help of a statistician from the Establishment. Additional resources may be used to aid experimental design

such as the NC3Rs experimental design assistant tool (https://www.nc3rs.org.uk/experimental-design- assistant-eda).

For our established models, we have determined the numbers of mice required to enable us to detect effects of 20-30% (the minimum therapeutic effect considered to be of value). For superficial or metastatic tumours, group sizes of 5-8 are typically sufficient.

The in vivo models are designed to provide maximum, statistically valid, information from the minimum number of mice . Wherever practicably possible, multiple parameters will be measured from each mouse to minimize use whilst considering the overall cumulative severity. For instance, the same mouse can experience therapeutic dosing, blood sampling, tumour volume measurement and tumour removal. Tumours can then be analysed by measuring physical and chemical characteristics (flow- cytometry), as well as histology, or molecular profiling by RNA or protein analysis.

Careful thought will be given to potential confounding factors which may contribute to variability. Experiments will be designed with adequate independent biological replicates and may be repeated/validated via an alternative follow-up experiment to minimise the likelihood of spurious, non- reproducible results.

Experiments will be carried out ensuring all mice are of the same strain, age and sex, dosing is carried out at approximately the same time each day and food, water and enrichment are the same in each cage.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use new technologies and techniques that will permit the minimum number of mice to be used. For example, new techniques in the laboratory allow us to digest a tumour using enzymes, leaving us with pure tumour cells. We can then inject these cells instead of using surgery to implant a tumour piece. Injecting cells instead of pieces means that we use fewer mice for each experiment; only one tumour (one mouse) is required for the initial implant rather than multiple tumours/mice, and the resulting cell suspension has less dead cells/necrosis therefore implanted tumours grow more successfully and in a more uniform manner. These cells can also be used for in vitro experiments so we can, for example, screen multiple compounds before testing in animals.

Experiments will be designed following the principles outlined in the experimental design tool on https://eda.nc3rs.org.uk/ (such as randomisation, power, blinding) and reported following the ARRIVE guidelines (https://arriveguidelines.org/). We will also discuss new experiments with Biostatasticians based at the institute.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- By doing as much preliminary work as possible in culture models in vitro and using computer analysis prior to engaging in in vivo studies

- By minimising variability in results through utilising inbred (related) strains and by housing them under identical conditions.

- Where necessary, performing pilot studies using small numbers of mice when information is lacking in the literature/from collaborators, so that the number of mice used in experiments is optimised.

- By running experiments in parallel so that they can share a single control group where possible

- By taking care to ensure that each experiment is appropriately analysed and the maximum amount of information is gathered thus reducing the need for experiments to be repeated.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are used in our research as they are more closely related to humans than other species such as flies or zebrafish, which, for example, don't have lungs.

The conditions under which experimental mice are kept are designed for the least possible disruption of natural behaviour and the highest possible quality of life. Continuous improvement in husbandry and experimental procedures minimise actual or potential pain, suffering, distress or lasting harm and/or improve animal welfare in situations where the use of mice is unavoidable.

For example, if a tumour is surgically removed, under recovery anaesthesia, from a mouse, the wound is either stitched closed or surgical glue is applied instead of using surgical clips. This causes less pain and/or irritation to the mouse and ensures the wound heals quickly. In addition, analgesia will be used whenever a surgical intervention is utilised. Further examples include single use of needles, non- stressful handling and environmental enrichment.

Why can't you use animals that are less sentient?

The cancer models we use very closely recapitulate human disease and thus allow us to understand the molecular and cellular events involved in cancer progression and response to therapy. The mouse is far more similar to humans than other less sentient animals and this is critical for increasing our understanding of cancer development/progression and developing therapies that can be translated to the clinic.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We constantly work to improve husbandry and procedures to minimise actual or potential pain, suffering, distress or lasting harm and/or improve animal welfare. Animals will be group housed wherever possible, however due to the nature of the project mice may be singly housed for periods of time . For example, at the end of a dosing study where some tumours have reached the study end point, one mouse may be left from the dosing group. All mice will be provided with enrichment and handled by either tunnel or cup-handling. Extra enrichment will be provided for singly housed animals. Every effort will be made to keep internal tumour growth at a level that minimises animal suffering.

Wherever possible this will be achieved by using non-invasive imaging modalities to monitor tumour growth and the development of metastatic disease. In addition, as detailed in individual protocols, steps will be taken to minimise the severity of the procedures.

Finally, we will ensure that all mice receive the highest standard of care and preventative medicine (including anaesthesia and analgesia where required) will be used.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Surgical procedures will be carried out according to the LASA Guiding Principles for Preparing and Undertaking Aseptic Surgery.

We will follow guidelines of good practice [Morton et al., Lab Animals, 35 (1):1-41(2001); Workman P, et al. British Journal of Cancer, 102:1555-77 (2010)].

Administration of substances and withdrawal of blood will be undertaken using a combination of volumes, routes and frequencies that themselves will result in no more than transient discomfort and no lasting harm.

We will consult the NC3Rs guidelines and monitor refinement where such practices are published (NC3Rs website and elsewhere).

Where we use aged mice, we will refer to Wilkinson (Laboratory Animals 2020, volume 5(34)), relating to the husbandry and care of aging mice

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By reading 3Rs literature and participating in 3Rs workshops locally and nationally. We will discuss refinements with our NACWO, NVS and will receive regular updates from the Establishment AWERB.

The whole team regularly attends and contributes to our Retrospective Review and Licensees meetings and the 3Rs poster session, all of which take place annually at our establishment

71. Diet composition and nutrient use efficiency of grazing animals

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

ruminant, nutrition, environment, grazing, livestock

Animal types	Life stages
Sheep	adult, juvenile
Alpacas	adult, juvenile
Goats	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this work is to increase our understanding of plant/animal interactions, and in particular the factors that influence diet composition and nutrient use efficiency by large herbivores.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 UK has legal obligations to reduce greenhouse gas emissions and support nature recovery. Within the context of grassland-based livestock farming, diversifying the pastures grazed and the species of grazing animal offers opportunities to simultaneously deliver production and environmental gains. This project will deliver an improved understanding of the interactions between pasture composition, diet and animal performance. The work will improve the efficiency of production of ruminant products and help reduce the environmental impact of ruminant agriculture. For example, more efficient use of forages for animal growth will reduce the amount of methane (a greenhouse gas) emitted per unit of product (meat, milk) produced. Simultaneously, an improved understanding of grazing behaviour and related impacts on different vegetation types will underpin the development of grazing guidelines which increase biodiversity and support habitat restoration.

What outputs do you think you will see at the end of this project?

The outputs of this project will be new information that will be disseminated to farmers, to other scientists, and to policy-makers in government. The information will be published as reports, information sheets, specialist periodicals, and in peer-reviewed papers in scientific journals.

Knowledge will also be transferred via stands at grassland events and agricultural shows, open days, presentations at scientific conferences, and as features on radio and television.

Who or what will benefit from these outputs, and how?

The agricultural community will benefit from new grassland knowledge and management guidelines which increase economic returns at a time when governmental agricultural support payments are under review. Beneficiaries of improved and more consistent grassland productivity and quality will also extend into the wider supply chains for red meat. This includes benefits for feed companies, food retailers, and the rural and bio economies.

Increases in nutrient use efficiency through sward and/or animal management and corresponding reductions in inputs and/or nutrient loss from the system will ensure progress towards national and global targets to reduce greenhouse gas emissions from agriculture in general, and livestock production in particular. In addition, the general public will benefit from improved traceability of ruminant products reared on home-grown feedstuffs. Meat and milk from forage-fed animals have also been proven to have a healthier fatty acid profile than those from animals fed predominantly on cereals. Reduced reliance on cereals for ruminant feed will free up high grade agricultural land for cropping for human consumption or bio-fuels, thereby further improving food security and sustainability.

Understanding species and breed differences in diet selectivity, and how these vary with season and resource availability, will ensure management prescriptions on semi-natural communities restore and support biodiversity across different taxa. This in turn will ensure the UK meets its international obligations as regards conservation of endangered habitats such as heather moorland. Restoring blanket bog and other peatland communities will safeguard the vast stocks of carbon stored in upland peat soils. Healthy peatland bogs also act as natural water filters, reducing the costs of

subsequent processing by water companies (75% of the UK's drinking water comes from upland areas). Stable upland vegetation communities also retain water more effectively and can help to reduce the risk of down-stream flooding following extreme rainfall events.

How will you look to maximise the outputs of this work?

We always aim to publish the results of work that we carry out in the peer-reviewed scientific literature, regardless of whether the work was deemed successful or otherwise. Some work is funded in collaboration with industrial partners, and even in those cases we aim to publish results. We aim to maximise the availability of publications by publishing open access articles. This means that the information is available to anyone, not just those individuals or organisations that subscribe to the journals that we publish in. Likewise, the original data will be made available to scientists and other interested parties. Much of the research is done in collaboration with experts in other fields as part of multi-disciplinary research programmes, further increasing the visibility and impact of the findings. The findings will also be passed to those developing agri-environment schemes and related prescriptions as well as those refining UK greenhouse gas reporting.

Species and numbers of animals expected to be used

- Sheep: 100
- Camelids: No answer provided
- Goats: 30

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 animal species being used are the subjects of the research (rather than models for any another species). To understand the benefits, or otherwise, of feeding different diets to animals, and to measure the effects on the production of meat, it is essential to use growing livestock. The way young animals are cared for in early life can have significant consequences on how they grow. Physiological status (growth, pregnancy, lactation) also strongly influences an animal's nutritional requirements, and in turn their foraging strategy and grazing behaviour at pasture. For this reason, it is important to be able to work with animals from just after birth until they reach maturity and are productive. At other times we will use mature animals that are representative of breeding flocks/herds, as well as wethers/castrates to have a consistent animal unaffected by hormonal cycles.

Typically, what will be done to an animal used in your project?

Prior to any experiment the animals are adapted to the diet and location over 2 - 3 weeks. With experiments conducted at pasture the animals are held within

experimental plots that may range in size from 0.1 ha to 100 ha. They may be dosed with inert markers to be able to estimate the amount of biomass they have eaten and the amount of methane they have emitted. They may also have faecal samples taken (usually daily for 5 days) to enable the botanical composition of the diet they have selected to be reconstructed, and blood samples taken to determine their related metabolic profile (once per measurement period). They may wear halter/collar mounted activity recorders or breath sampling equipment. Measurement periods may be repeated across different stages (early, mid, late) of the pasture growing season to track how grazing behaviour changes in response to changes in the availability and nutritional quality of different plants. These procedures carry a mild level of severity.

Indoor feeding experiments focus on quantifying nutrient use efficiency more precisely. Following an adaptation period the animals may be housed in methane chambers or metabolism crates and combinations of intake, in vivo digestibility, nitrogen use efficiency and methane emissions measured over short-term (3-5 day) periods. Because the animals are socially isolated while in the chambers and crates (they can see other animals but can't touch them), this procedure is classified as moderate.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals on a restricted diet (i.e. fed to maintenance; done to ensure they eat all of a mixed diet) may suffer some weight loss, but nothing that would compromise the welfare of the animal. Only mature, barren animals are used for this type of experiment and losses would be replaced quickly when the animal returns to the main flock/herd. Markers are given using dosing guns used in commercial farming. Their insertion into the mouth may cause temporary discomfort. Animals that are blood sampled may experience mild transient pain when the needle is inserted. The use of equipment to collected breath samples from the animals requires the use of head collars and/or neck collars to attach sampling tubes and collection cylinders; this may initially cause mild distress in some animals for a few minutes but the risk of this is minimised by training them to become used to the equipment before it is used for experimental collections. Animals that are socially isolated from others may become distressed and/or agitated. This generally eases within 15 minutes, and the risk is reduced by preparing the animals by housing them in individual pens with reduced contact with others for 3 -5 days before they enter the chambers.

At the end of the experiment the animals will be re-homed in the establishment’s herd or flock, sold to another farm, or will be sent to slaughter as part of the normal supply chain for human consumption.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Indoor experiments require fewer animals than experiments at pasture and around

25% of the animals studied will be used in these, which may carry a moderate severity if the animals are housed in methane chambers.

The greater variability associated with animal behaviour and measurements at pasture mean that more animals will be used as part of these experiments, which involve procedures with a mild severity.

What will happen to animals at the end of this project?

- Kept alive
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In the research covered by this licence the animals are the objects of study and are not being used as a model for any other species, and the conditions in which they are studied are designed to be as close as possible to those used in commercial livestock production systems.

In grazing and growth studies it is not currently feasible to gain the results satisfactorily from any method not entailing the use of protected animals. These studies integrate behavioural, ingestive and metabolic parameters and no suitable procedures exist to obtain the required results.

Which non-animal alternatives did you consider for use in this project?

We already use some laboratory methods for initial screening of feeds for, e.g., the potential to produce methane when fed to ruminant animals. These methods are helpful in being able to rapidly and cheaply assess large numbers of feed samples that are available only in small quantities and allow us to prioritise those to produce in larger amounts for further study, but they do not as yet accurately predict corresponding in vivo data. We have also considered computer prediction models.

Why were they not suitable?

Outputs from the alternatives above are limited and they do not reflect animal behaviour and performance in the real world. Data obtained during the course of this project will be made available to those modelling plant/animal interactions to improve the predictive power of related mathematical models. The availability of appropriate alternatives will therefore be monitored throughout the project in case new developments are made which would remove the need for animal experimentation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles

used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based on totals reported across previous years when carrying out similar experimental work. Experimental treatments will be defined based on the specific research question being posed. In comparative grazing behaviour studies sheep are generally used as a control to represent business-as-usual within upland farming systems, whereas in comparative forage use efficiency experiments ryegrass is frequently the control. The minimum number of animals required to detect statistical significance at the 95% confidence level is used in each experiment. The actual number required is determined by the variation associated with the measurements to be made within a given experiment, as estimated using previous results for similar animals kept in similar conditions. For example, fewer animal are required when determining the effect of treatment on a parameter such as digestibility, which has a co-efficient of variation of around 5% (n = 5 to find a statistically significant difference of 15% about a mean), than are required when measuring voluntary intake, which has a co- efficient of variation at least 10%.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will employ the NC3Rs' experimental design guidance and experimental design assistant to plan our experimental design, practical steps and statistical analysis.

Change-over (e.g., Latin square) experimental designs are particularly efficient because each animal receives each treatment, and therefore each animal effectively acts as its own control, and are used where possible. This approach works well for experiments with ensiled or dried feed where food chemistry is more consistent over time. However, changeover design experiments are not appropriate for some studies (e.g., growth studies), and therefore more animals are typically required per treatment in order to control variation. During the development of each experiment careful consideration is given to what data and statistical analysis methods will be used. This ensures that appropriate measurements are made and can be analysed in a statistically valid manner. Consultation with a statistician during the planning of an experiment is important, to make sure that animal numbers are appropriate, and to that the latest statistical methods can be employed during the data analysis part of the experimental process. Statistical blocking of animals during the experimental stage is used where possible to reduce variation caused by known factors, such as breed, age, season, etc. Careful consideration is also given to the choice of animals and these are grouped to reduce known variation.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

If possible similar experiments required to meet different research objectives are conducted concurrently, and a single set of control animals (e.g. those grazing ryegrass) used for multiple trials.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 research is targeted at understanding plant/animal interactions within mono- and co-species grassland-based production systems based on sheep, cattle, goats and alpacas. The methodologies have been carefully chosen to minimise the impact on the animals and thus ensure that the data generated is as representative as possible of grazers within typical farming and/or land management (e.g. conservation grazing) systems.

Why can't you use animals that are less sentient?

This work needs to be carried out on growing and mature productive animals, to assess the effects of treatments on growth and meat/fibre production.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals to be used on experiments undergo a programme of habituation to familiarise them with regular human contact, being housed, and being isolated from other animals. Any animals which do not adjust well are not used on experiment.

All stock are daily assessed for health and well-being, as determined by e.g. alertness, mobility, feed and water intake. Any sign of ill health will be reported to the NACWO or veterinary surgeon, with the animal being removed from trial if symptoms persist or are severe.

Where it is possible to identify the individual animal defecating, faecal samples are collected from the ground. Only when the amount of faeces is small (e.g. from sheep) and the surface challenging (e.g. rough pasture) are animals caught and rectal grab samples taken.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines (Smith et al., 2017) when formulating experiments, discussing the resources required, and ensuring quality control. In addition, we will follow the latest developments in best practice via the resource library developed by the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs). We will also adhere to the Code of

Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Discussions with NACWOs and other PPL holders is an important part of ensuring that the latest and most appropriate methods in animal research are understood and used where possible. The 3Rs is a standing item on the organisation's AWERB agenda, which allows an open discussion at each meeting to ensure new methods of good practice are shared among all named persons. The NC3Rs has a monthly newsletter that provides updates on funding opportunities and publications, some of which may be relevant to the work and animal species that will be carried out and used under the authority of this project.

72. Enabling Development of Therapeutic Drugs for Cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Therapy, Drug Discovery, Pharmacology, Tumour Models

Animal types	Life stages
Mice	adult, aged
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project licence is to develop our understanding of how mechanisms involved in cancer can be modified for therapeutic benefit and to understand the properties of new test agents we generate by investigating their tolerability, the molecular, biochemical, and physiologic effects of the test agent (pharmacodynamics) and how the test agent moves into, through and out of the body (pharmacokinetics).

We will identify and/or test an estimate of 50-60 new candidate drugs, or their surrogates, in non- tumour and tumour-bearing animals to support the development of new medicines and benefit cancer patients.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit -

these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.
Why is it important to undertake this work?

It is estimated that there were 18 million new cases of cancer worldwide in 2020. It is projected that by the year 2040 there will be 28 million new cases of cancer each year [https://www.cancerresearchuk.org/]. Cancer is a complex disease with more than 200 types described and the four most common cancers occurring worldwide are breast, lung, bowel, and prostate. These cancer types account for more than 4 in 10 of all cancers diagnosed worldwide. In the UK, someone is diagnosed with cancer every 2 minutes. Despite the recent advancements in treatment of cancer, there are still many types for which targeted therapies are not available. In other cases, therapies are available, but treatment is not effective for all patients and many patients who do respond to these targeted therapies will eventually develop resistance and their tumour grows back.
Of those patients diagnosed in the UK, only 50% will survive for 10 years or more following their diagnosis (source https://www.cancerresearchuk.org/).

Our research project will use animals to support our efforts to discover and develop novel therapies for cancer to benefit these patients. The use of animals enables us to mimic complex aspects of the disease and drug properties that is currently not possible in a laboratory. Using animal models, we test the activity of our new drug candidates in a relevant way in a live animal. It is important to undertake our programs of work to support the development of new drug formats and technologies for cancer therapy. This program of work is expected to enable us to generate data that will be used by our team to progress new cancer medicines through the drug development pipeline.

What outputs do you think you will see at the end of this project?

The work undertaken in this project will provide important data to enable initial testing of our experimental cancer drugs in relevant animal models. This work will allow us to understand how our drugs distribute in the body after dosing and how quickly they are eliminated from it. It will develop our insight into how our drug candidates work, to establish safe doses to use in animals, and will help discover specific responses that we can measure as biomarkers. These can then be taken forward into larger, more complex animal models of disease to evaluate their activity. If successful, eventually human clinical trials would be conducted with the new experimental drug. Work undertaken will also help us to identify the most appropriate drugs to combine and will guide clinical teams on finding the right dose level and schedule.

Importantly, results may also be used to determine which drugs should not be progressed further.

As well as building datasets for pre-clinical work packages on new experimental cancer drugs, we expect to publish our results and present out findings externally to scientific peers.

Who or what will benefit from these outputs, and how?

This program of work will enable us to progress new cancer medicines through the drug discovery pipeline. Ultimately, if successful, these medicines will be approved for use in the clinic to treat patients with cancer, who otherwise are likely to die from their disease. New experimental cancer drugs developed using this licence have the potential to benefit millions of cancer patients globally.

The work carried out under this project licence will contribute knowledge to the wider scientific community through the publication, presentations at external conference and through collaboration with experts in the field.

How will you look to maximise the outputs of this work?

Our team has an excellent track record of publishing our advance in drug discovery and animal modelling. We aim to publish both successful and unsuccessful experimental results in relevant scientific journals, and to share our data and learnings with collaborators to avoid other's repeating work. We present our research at international conferences for the benefit of the broader scientific community. This public dissemination of our results has the potential to lead to new collaboration and opportunities to develop innovative new experimental cancer drugs and associated biomarkers.

Species and numbers of animals expected to be used

- Mice: 13,500
- Rats: 400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and rats are the most common animals used for the type of work that we are doing. They have an immune system that resembles that of humans in some respects. This means that we can grow tumours in these animals to investigate our new experimental cancer drugs and their effect on the immune response to tumours. We will also use immunocompromised animals: these animals bear defects in one or more immune components. These mice or rats allow us to transplant human material into them without rejection. We will create models, which we termed 'humanised'; by injecting both human immune cells as well as human tumours to model aspects of the disease that we cannot do in mouse only systems.

In most experiments, we will use mice, rather than rats. This is because many more tumour models are available for mice, and a wealth of published data exists to help

guide our experimental plans and interpret our results. Occasionally we may use rats when a mouse tumour model is not available to us,
e.g. when the experimental cancer drug is active only in rates or when we need to compare against other data generated in rats. An example of this is safety data, where rats are often the preferred species.

Typically, we plan to use adult mice (6-12 weeks old) at the start of our studies as the immune system is considered immature in younger mice and would not represent the biology of the cancer patients we aim to treat. Occasionally we may use significantly older animals. This is especially relevant as cancer is generally a disease of middle to old age in human patients. For those studies we plan to use animals up to 24 months old, as our previous experience has shown that this age sufficiently demonstrates the changes in the immune system associated with age but before mice begin to suffer serious effects of aging.

Rats used will also be adult stage (approximately 6-12 weeks old) at the start of study. We do not intend at present to undertake studies with aged rats.

Typically, what will be done to an animal used in your project?

Animals are kept in high-quality facilities, free from pathogens (disease-causing organisms such as bacteria, viruses, and parasites) and with access to food, water, and environmental enrichment. Animal care staff are highly trained in rodent welfare and ensure animal suffering is minimised.

Animals are housed in groups except in exceptional circumstances, for example when aggressive behaviour puts the welfare of the animal at risk or when cage-mates have been removed for experimental reasons.

We will use both tumour-bearing and non-tumour bearing animals for our projects. It is not necessary to use animals bearing tumours, for example, in some cases to determine whether a drug is tolerated by the mice or to measure how much of a drug enters the bloodstream, or to understand the impact of our drugs on specific aspects of the immune system.

For those animals bearing tumours, these will be used to test the tolerability of drugs where the tumour microenvironment plays an essential role in the activity of the new experimental drug, or where the tumour is needed to determine the kinetics and/or mechanism of action of the new drug. We use cell lines derived from mouse and human sources, and patient-derived xenograft (PDX) models of many different tumour types to model human disease. PDX models are generated by transplanting human patient tumour material into host immunocompromised animals and are maintained by passaging tumour fragments from host to host. Both cell line and PDX derived tumours will result from the injection of tumour cells or tumour fragment implantation under the skin where they grow into a tumour at the site of injection. They are easy to monitor and measure their size with callipers, and additionally we also use a tumour condition scoring system to assess tumour condition. On rare occasions animals may be shipped to us after tumours have been induced in another laboratory and begun to grow.

These cases will be limited to superficial tumours only which can be easily monitored, and to those tumours that are expected to grow slowly. Animals may be shipped from within the UK or internationally. Shipment of mice bearing tumours is necessary so we can acquire tumour models that are slow growing and/or difficult to establish. This is because shipping PDX tumour fragments or vials of cells would likely result in the loss of important characteristics of the tumour and would also significantly delay the development of new treatments for cancer by as much as a year or more, during which time patients would not have access to new treatment options.

In some studies, we will use advanced imaging techniques (such as bioluminescence imaging) that allow us to track tumour growth or to follow distribution of a drug throughout the body. To undertake these imaging sessions the animal will be anaesthetised.

Blood samples may also be collected during some studies to measure levels of drug, or human immune cell components in humanised models. Blood samples are of a small volume and are taken from a vein while the study is ongoing, whereas larger blood samples are taken at the end of study if a greater volume is needed.

Our experimental cancer drugs are commonly injected into the peritoneal cavity (i.p.; into the abdomen), intravenously (i.v.; into the tail vein) or directly into the tumour. Occasionally drugs may be administered orally (p.o) or under the skin (s.c.; subcutaneously).

At times these substances may only need to be administered once, but more often they are administered according to a schedule that requires multiple administrations. For example, drugs injected into the peritoneal cavity are typically given two to three times per week, whereas drugs given orally would typically be administered once or twice a day, often for the duration of the study. In most cases our studies last approximately one to two months but on occasion when tumours grow slowly, they could last for 6 months.

To understand the impact of our work on the animals, here is an example of a typical study with an immunocompromised host animal:

1. implantation of a microchip under the skin (s.c.) for identification
2. injection of human immune cells into the tail vein (i.v.)
3. injection of tumour cells under the skin (s.c.)
4. administration of experimental therapies by one or more of the following routes: i.p. into the peritoneal cavity (typically this will be done twice a week for three weeks); intravenous i.v. into the tail vein (typically this will be done once or twice a week for 3-4 weeks); orally (by by gavage force-feeding; (typically this is done once or twice a day for the duration of the study)

5. collection of a blood sample
6. At the end of procedures, all animals will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

In our studies, the likeliest sources of adverse effects are from the size and condition of the tumour, from surgical procedures, from the drug treatment or in humanised models from graft versus host disease (GvHD). GvHD is a systemic disorder that occurs when the graft's immune cells recognize the host as foreign and attack the recipient's body cells. “Graft” here refers to transplanted human immune cells and “host” refers to the animal.
Animals will be classified according to a scoring system that is based on the degree and duration of clinical observations such as body weight, activity level, posture, and body condition.

We will humanely kill any animals that have developed large tumours to minimise unnecessary suffering using well-defined tumour burden limits. Generally, studies with subcutaneous tumours will reach these limits within 30 days of implantation if they are not treated with a drug, but tumours that grow more slowly could take 2-3 months before reaching the same limit. The slowest tumour type that we currently work with takes approximately 6 months to reach the same size without any drug treatments that might inhibit tumour growth. For some tumour models mice may develop a discoloration of the skin at the site of the tumour that does not have any welfare implications. Animals that have been implanted with tumours before shipping may experience mild stress due to the presence of the tumour in addition to the standard stresses associated with shipping. However, we only intend to ship animals that bear very slow-growing tumours which are not expected to reach the size limits mentioned above during transit, nor to interfere with the normal behaviour of the animal.

Animals undergoing surgical procedures are assumed to experience pain due to the surgery and so anaesthetic and pain relief (analgesia) will be provided when surgery is performed.

Treatment of animals with cancer therapies may also lead to unwanted effects like those experienced by human patients. While humans may experience fatigue or fever soon after receiving the therapy, we observe similar responses in rodents such as reduced mobility, hunched posture, and piloerection (bristling of fur). Most of these effects will be mild and of short duration; however, some animals may experience moderate effects. We expect that approximately 65% of animals will have moderate adverse reactions to drug substances whereas the remaining animals will not experience any adverse reactions.

Occasionally, the use of genetically modified mice is necessary for our work. We have recently begun using a new mouse strain that expresses human versions of two proteins important for controlling immune responses to tumours. This strain can display subtle jerky movements that appear as mild tremors or altered breathing similar to hiccups. These unusual movements are intermittent and occur for short durations (typically 10-15 seconds) most typically when mice are handled outside the cage, therefore they may be a response to stressful situations. Although the clinical signs may recur, they are very mild, do not worsen over time, and are not considered harmful. It is expected that the behaviour of the mice will return to normal once

returned to the home cage. Advice from the Named Veterinary Surgeon (NVS) or Named Animal Care and Welfare Officer (NACWO) would be sought if the movements/breathing patterns began to appear while in the home cage, if they became constant while out of the cage, if they significantly worsened after performing procedures, or additional clinical signs or weight loss were present that could not be explained by other factors.

An estimated 10-20% of mice of strain NSG (and their derivative strains, such as NSG-SGM3 and NSG-MHC I/II double knockout) may show swelling around the hocks. Any animals experiencing swelling around their hocks may be given an altered enrichment, pain relief and/or anti-inflammatories in consultation with the NACWO and/or NVS.

At the end of procedures, all animals will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For both mice and rats, we expect the following proportion of severities under this project licence (PPL):
Mild = 35%
Moderate = 65%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although we do many experiments in the laboratory using cells, molecular biology, and computer modelling, it is still necessary to use some animals for research so that we can more accurately assess the interaction of cancer cells with other cells and organs within the body. Isolated cells and organs do not reproduce the complex nature of in vivo (in a live animal) biology. The use of animals also allows us to understand cancer in the organ of origin or as it spreads throughout the body. An important aspect of our work is to understand how the immune system can be harnessed to attack tumours, and it is not possible to fully recreate these complex interactions outside of a living animal.

Which non-animal alternatives did you consider for use in this project?

Our organization regularly uses a range of in vitro methods. These experiments take

place in a test tube or laboratory dish, outside a living animal . Such methods may include experiments containing a single cell type or multiple cell types grown together, which allows both direct interaction and indirect communication between cells. These types of studies are well-established, 2-dimensional experiments that are useful to understand the specific ways that our experimental drugs affect cellular function.
More recently, we also have developed a more complex 3D experimental systems, such as the tumour slice culture system and patient-derived organoids. These methods preserve the 3D structure of a patient tumour and are expected to be more representative of the biology of the whole tumour compared with the 2D experiments mentioned above.
Most commonly, new experimental cancer drugs will undergo analytical tests such as stability and to detect impurities and will include where possible potency testing in a relevant assay. However, due to the wide range of different types of drug substances and modalities that we test, it is not possible to provide specific go/no-go criteria that have broad applicability across our portfolio. Animal studies will only commence once lead candidate drugs have been validated extensively in the laboratory to identify a small panel of lead candidates (typically 3-6 drugs).

Why were they not suitable?

Cell-based methods are useful to gain an understanding of the way that our experimental drugs impact the function of different cell types outside the body, but do not adequately test whether the drugs remain stable after they enter the body, can reach the site of the tumour, or whether they are capable of inhibiting tumour growth in a live animal.
The 3D assay systems such as tumour slice or organoid cultures are a valuable addition to our experimental toolbox, however they can have a short lifespan and can show modification of cellular function but do not demonstrate that this effect can shrink a tumour.
None of the alternatives investigated can demonstrate how our experimental therapies are broken down by the body, nor can they provide any information about how to schedule dose regimens. Unlike experiments using animals, they also do not permit identification of specific signals produced in the body which can be linked to tumour growth inhibition.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers were calculated based on our usage over the last several years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our organization includes a team of statisticians who have implemented a Good Statistical Practice program where animal experiments are formally reviewed before they start. This review is conducted by a researcher and a supporting statistician team to ensure the goal, experimental design, and data analysis align. This process ensures the design is robust and the minimum animals are used that are needed to answer the scientific question.
In addition, the following guidelines and online tools are also used to influence the design of our animal studies:

- The PREPARE Guidelines, found at https://norecopa.no/prepare
- The NC3Rs Experimental Design Assistant, found at https://www.nc3rs.org.uk/experimentaldesign- assistant-eda.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We ensure small scale pilot studies are carried out for new tumour types or experimental methods which enables us to design our studies using the minimum number of animals needed to achieve our scientific objectives. We also have implemented innovative study designs to reduce animal numbers where possible. Using data analyses from past projects, we have analysed the tumour tissues of the most frequent types of tumours that we grow in mice to define their cellular, molecular, and genetic characteristics. This characterisation has been important for reducing animal use since it ensures we can select the most appropriate tumour type for each experimental question, thereby reducing the overall number of experiments and allowing us to maximise the benefit gained from each mouse. A large amount of data has been generated from our characterisation work and is archived for use by all our scientists to guide the design of future animal studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The most common type of tumour that we use involves injection of tumour cells under the skin resulting in tumour growth at the site of injection. This is the simplest method available to grow tumours in rodents and carries the least welfare risks since the tumours are not located in vital organs. They are also the easiest to monitor since they can be easily observed and measured under the skin. This method is

preferred except in specific cases when we need to understand more complex questions.
For example, we may need to understand the spread of cancer from one site to another, the influence that specific cell types and organs have on tumour growth, and the responses of tumours to our therapies in these varied settings. In these cases, we will use more complex experimental methods in which tumours often develop inside the body cavity. These more complex studies also may involve surgical implantation of tumours. Where possible, non-surgical methods will be used for implantation and imaging methods used to monitor tumour burden.

Why can't you use animals that are less sentient?

Rodents are the lowest species of mammal (meaning they are the least sentient, or least aware of feelings and sensations) that allow us to adequately study the complexity of human cancer and immune system biology. Because many of our experimental therapies are designed to impact the immune system, it is essential that we use adult animals with mature immune systems in our research. Our studies also monitor the growth of a tumour over a period of weeks to months therefore it is essential that the animals are conscious as the use of anaesthesia (an agent that induces a state of unconsciousness) would not be possible for such an extended period. In addition, the behaviour of conscious animals also often alerts us to adverse reactions to our therapies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are committed to refining our procedures to minimise harm to the animals and have a track record of doing so. We ensure small-scale pilot or tolerability studies are carried out for new tumour types, experimental methods, or therapies. We carefully monitor tumour burden including the use of whole- body imaging techniques when possible. We also use tumour-free mice in some cases when tumours are not essential. We have implemented innovative study designs to reduce animal numbers and enhanced health checks to minimize suffering. General welfare of the animals is assessed by checking body weight and watching for the development of clinical signs such as activity levels, appearance of the coat, posture, and body condition. To this end for our humanised models, we have developed a GvHD (graft versus host disease) Scoring System that we are constantly refining to ensure welfare and business need are balanced. When two or more drugs are scheduled to be injected at the same time, we will also combine them into the same syringe where possible to minimize the number of injections administered to the animals. In addition, a non-surgical method of tumour implantation in the mammary fat pad has been developed and is used in preference to surgical methods wherever possible.
Additionally we are constantly refining our surgical implantation techniques including that for patient- derived xenograft (PDX) tumour fragment passage. When unexpected events occur, these are thoroughly investigated to find out what happened so action can be taken to prevent a reoccurrence.
When shipping mice that have previously been implanted with a tumour, we will ensure the following conditions will be met prior to transport:

Animals will have recovered from implantation procedure with healed wounds.

- Tumours will be confirmed as palpable and measurable before transport (around 200mm3 volume).

- Tumours will be expected to be slow growing and will not reach tumour size limits during transport and acclimatisation period. The standard acclimatisation period for national or international shipments set by the establishment will apply to these animals. Currently these are set at 7 days for national shipments or 14 days for international (overseas) shipments.

- Animals will have no other clinical signs and deemed fit to travel by responsible person.

Any animals experiencing swelling around their hocks may be given an altered enrichment, pain relief and/or anti-inflammatories in consultation with the NACWO and/or NVS.

Aged mice up to 24 months old will be used only when necessary for studies requiring certain aspects of the biology of aging. We note that animal younger than 15 months old are considered middle aged, rather than old, so when studying immune responses in old age studies may require aging animals up to 24 months old.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our practices follow the ARRIVE guidelines developed by NC3Rs for publication of our work in peer- reviewed journals (ARRIVE Guidelines version 2.0 released in July, 2020 can be found at https://arriveguidelines.org/).
Our practices incorporate many of the guiding principles of the PREPARE guidelines (Smith et al., PREPARE: guidelines for planning animal research and testing. 2017. Laboratory Animals). LASA (Laboratory Animal Science Association) also has a range of published guidance documents with principles that can be applied to our animal studies which are found at https://www.lasa.co.uk/current_publications/
Our team also is aware of advances in 3Rs through the NC3Rs and establishment websites and via participation in conferences and events sponsored by organizations such as LASA (Laboratory Animal Science Association, IAT (Institute of Animal Technology) or NC3Rs

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our organisation is committed to the 3Rs and continues to follow advances in the community. We routinely hold Symposia and invite external speakers to talk on these topics and we have a dedicated 3Rs contact who regularly disseminates information related to 3Rs.
Our team is actively involved in promoting the 3Rs and participates in global 3Rs activities including an annual poster competition. This award was given to individuals working on the development of alternative models e.g. a team working on an ex vivo (taken directly from a living organism) tissue slice culture system in 2017.

Our team also is aware of advances in 3Rs through the NC3Rs and establishment websites and via participation in conferences and events sponsored by organizations such as LASA (Laboratory Animal Science Association, IAT (Institute of Animal Technology) or NC3Rs.

73. Influence of radiation-induced inflammation on Clonal Haematopoiesis and Acute Myeloid Leukaemia development

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Ionising Radiation, Leukaemia, Risk Modifiers, Clonal Haematopoiesis, Inflammation

Animal types	Life stages
Mice	juvenile, adult, aged, pregnant, embryo, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to determine how radiation exposure effects the frequency of cells carrying leukaemia- associated mutations in the blood of a specialised mouse model and how this may influence Leukaemia development. An additional aim is to provide specialised mouse models of radiation associated Leukaemias as frozen embryos to contribute to other radiation research studies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Humans are exposed to ionising radiation (IR) from both environmental and man-made sources e.g., medical imaging (X-rays, CT scans) and radiological accidents (e.g., Chernobyl). IR can cause cancers such as leukaemia, with radiation-induced Acute Myeloid Leukaemia (rAML) being the most common.

rAML originates from the bone marrow (BM), which is highly sensitive to IR exposure and is the principal site of blood cell production. Primitive BM cells divide to produce immature daughter cells which then further divide, eventually producing mature blood cells. In AML, a block occurs in this process, causing collapse of mature blood cell production and an accumulation of abnormal immature blood cells called leukaemic blasts.

rAML development (known as leukaemogenesis) is a complex multi-step process where primitive BM cells become leukaemic blast cells by accumulation of mutations in specific genes. Research has shown that this is influenced by multiple factors, such as genetics, diet, and inflammation. More research in these areas is needed to develop accurate estimates of leukaemia risk for IR exposed populations and new approaches for rAML prevention or detection.

Studying rAML in humans is difficult because samples where the exact radiation dose and conditions are known are rare and cannot be used to study early pre-leukaemic events. Additionally, study of rAML cells or tissues outside of the body is limited because it is not yet possible to produce stable long term cell cultures of BM cells for growth in the laboratory. Mouse models of rAML, therefore, are invaluable in providing a better understanding of rAML development and the factors that affect this.

We have several specialized mouse models of rAML which have contributed to the understanding of rAML development and these are in demand from other researchers. As part of this project, we will be breeding these strains to provide frozen embryos for shipment to other laboratories for use in radiation research.

Clonal haematopoiesis of indeterminate potential (CHIP) is a condition where mutations in leukaemia- associated genes occur in BM cells, leading to cells with these mutations appearing in the blood. CHIP has been identified in a significant number of healthy elderly adults and is associated with an increased leukaemia risk.

Inflammation and altered immune reactions are key events in the development of rAML, favouring the growth of leukaemic blasts. It has recently been shown that inflammation increases the number of mutated blood cells in a novel mouse model of CHIP. In this project, we will use this model to determine how IR and inflammation effects growth and frequency of these mutated cells and how this may lead to Leukaemia development.

The insights gained from this project will contribute to developing accurate estimates of risk for rAML development in IR exposed populations. Also, identification of CHIP as a risk factor for rAML would be of significant interest to the Radiation Protection field, as this may lead to interventions that can reduce the risk of IR exposure or identify sub-populations that may be at greater risk .

What outputs do you think you will see at the end of this project?

The scientific outputs of this project will be new and increased knowledge of the radiation-induced leukaemogenesis process, and a better understanding of how this is influenced by inflammation.

This output will take the form of scientific publications in peer reviewed journals, poster and oral presentations at scientific meetings.

Other scientists will also benefit from the sharing of specialist genetically modified rAML mouse models which are not commercially available.

Who or what will benefit from these outputs, and how?

Current risk estimates of the UK population suggest 9000 cancer cases per year are attributable to IR exposure from an average annual exposure of 2.7 mSv, however over- or under- estimation is possible due to the uncertainty on the true risks. Information obtained in the proposed project would eventually feed into the development of refined models for risk estimation in human populations, particularly following low dose or dose rate radiation exposures.

Additionally, improved basic knowledge of radiation-induced carcinogenesis will be obtained during this project and in the longer term this work will aid the identification of early markers of radiation exposure and associated disease. Also, identifying potential mitigating or modifying factors for rAML risk, such as presence of CHIP, may eventually lead to realistic interventions or screenings to reduce risk for e.g. people undergoing radiotherapy.

Animal studies have contributed to the evidence base for providing advice and guidance to international organisations, government agencies, and the nuclear industry.

How will you look to maximise the outputs of this work?

All studies that yield sound results will be submitted for publication in the open scientific literature and thus add to the knowledge base available to researchers worldwide. This evidence will be consolidated through dissemination of project results and their implications to radiation protection specialists at the stakeholder meeting in the final year of funded projects, in addition to other appropriate scientific conferences and meetings e.g. European Radiation Protection Week (ERPW).

We continually seek to collaborate with other research groups across the UK and Europe, which provides significant benefits, as we can provide access to our specialised mouse models and archived biological materials derived from them for use in experimental systems that we do not have available to us. As part of this project, we are sharing transgenic mouse models of rAML we have developed and characterised previously with EU collaborators for the PIANOFORTE consortium.

We have previously provided mouse AML material for Transcriptomic and Proteomic Array analysis to laboratories in Europe, and spleen and brain samples for Whole Genome Sequencing. We have also provided rAML incidence/latency data and other

endpoint data to researchers specialising in statistical analysis and mathematical modelling of risk. These types of collaborations also lead to joint publications.

Species and numbers of animals expected to be used

- Mice: 4410

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 breed and use specialised mouse models sensitive for rAML. These models are used because they allow study of aspects of the rAML process that is not possible in humans or in laboratory cell culture, such as early genetic events in the bone marrow.

In this study we are using a model of CHIP to determine the effects of radiation-induced inflammation on growth and frequency of mutated blood cells and the risk of rAML. The mice are young adult mice (10-12 weeks old) when irradiated at the start of the experiments and they are then monitored for 2 years afterwards. This is to allow enough time for any rAMLs to develop (between 6-22 months post- irradiation).

Typically, what will be done to an animal used in your project?

The mice we are using have been modified to carry a specific mutation which is created by removal of one copy of a gene called Tet2 in BM cells (as seen in CHIP) when the mice are around 6-8 weeks of age. This is done by administration of a drug called Tamoxifen, either orally or by injection which activates an enzyme to cut out the gene.

Mice will be irradiated with X-rays, which involves transferring mice with their cage mates into the X- irradiator in transport boxes for the duration of the irradiation (usually no more than 10 minutes).

Mice are then maintained for up to 2 years with daily health checks, or until they reach their scientific end point, when they are humanely killed, and biological samples are collected for use in our analysis.

Mice will undergo blood micro sampling each month, which involves collecting a small amount of blood (up to 50 micro litres) from a vein in the tail .

What are the expected impacts and/or adverse effects for the animals during your project?

Tamoxifen administration and blood sampling require restraint, which may cause some temporary handling stress for 1-2 minutes each time. Injections only cause mild temporary discomfort.

Tamoxifen administration can reduce appetite, leading to weight loss during the dosing period. This is usually transient with weight regained within 2-3 weeks following the last Tamoxifen dose.

During long term studies (up to two years old) mice would be expected to develop leukaemias and other cancers associated with radiation exposure. Daily checking by trained staff and use of a welfare sheet developed after extensive experience with these types of experiment mean that all these mice are humanely killed as early as possible when symptoms are first identified.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Around 8% of animals would experience moderate severity and 92% would experience mild

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not possible to achieve the aims of this project without using animals because currently no laboratory-based system exists that adequately recreates the complex multi-stage process of leukaemia development as it occurs within a living organism or tissue.

It is not possible to maintain the long-term growth of primitive BM cells outside of the body in laboratory cell culture conditions as these cells will begin to develop mature blood cell characteristics and stop dividing after a few weeks.

It is difficult to obtain primary human radiation-induced AML samples for study where the exact exposure received is known. Additionally these samples cannot be used to identify early pre-leukaemic events and so would not be appropriate for this project.

Although mice will be used for irradiations and blood sampling in this study, we will also be collecting BM cells at specific timepoints and BM cell culture assays will be used wherever possible. Results from animal and cell culture approaches are complementary and comparison between the two can lead to further reductions and refinement, e.g., successful BM cell culture methods may allow for reduction in the number of mice used in future irradiation studies.

Which non-animal alternatives did you consider for use in this project?

There are an increasing number of methods and techniques available, from specialised suppliers or described in the literature, for short- and longer-term BM cell culture that we will use as part of this project.

Additionally, there are numerous commercially available human and mouse AML cell lines for study, which can be grown indefinitely in the laboratory without losing their characteristics.

We have improved our BM cell cultures by using customised liquid cell culture media with additional nutrients to grow the cells, optimising the amount of cells initially placed in culture and by using low oxygen incubators for cell growth which mimic the environment inside the BM. This has allowed progress with characterising BM cell responses to X-ray exposure in the laboratory, replacing the need to irradiate individual mice.

All these studies generate important data and inform experimental design for related mouse studies.

Why were they not suitable?

Most of the methods described above still do not produce reliable, stable long-term BM cell culture beyond about 6 weeks and so cannot be used for long-term study.

However, we can use them to optimise our short-term culture techniques where appropriate, for instance, cell cultures derived from the mouse BM will be used to identify how radiation-associated genetic changes affect the growth rate of these cells.

Human and mouse cell lines are available, however AML is a very variable disease and each cell line originates from a single AML sub-type which may not be representative of rAML development. Cell lines have also undergone significant genetic alterations, known as transformation, to allow indefinite growth in culture, making them unsuitable for study of early pre-leukaemic events where these alterations would not be present.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Throughout the course of this project licence experimental animal numbers will be kept to the minimum required using statistical analysis techniques with advice on this

being obtained from qualified and experienced statisticians available in house and with colleagues in other laboratories.

These numbers will be based on previous experience and data generated in past studies, along with published studies in the literature with the same strains or techniques. This means that numbers of mice used will be the minimum required to give sufficient statistical power to each experiment.

The CHIP model is genetically complex and is generated from crosses of three separate parental strains. Due to this complexity and the length of the project, a high number of breeding animals is needed to maintain these strains for the duration of the study. Additionally, breeding colonies of three different leukaemia models will be needed to provide frozen embryos for shipment to other laboratories, which contributes to the breeding numbers on this project, although storage of strains as frozen embryos reduces the numbers needed to be bred in the longer term.

Breeding animal numbers have been carefully calculated using background stock breeding information, new born survival rates and the expected proportion of animals with the desired genetics. Opportunities to reduce these numbers will be identified by careful monitoring of breeding colonies throughout the study, e.g. higher than expected new born survival rates or litter sizes means that fewer breeding pairs will be needed overall. This way total numbers will be kept to a minimum by avoiding over breeding whilst achieving the experimental numbers statistically required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To determine the minimal numbers of animals needed to achieve the scientific output for this study, we use various resources, including;

- related experiments or techniques described in the published literature where there is a well- established experimental design available

- consultations with technical experts at specialist companies to optimise in vitro assay methods and therefore reduce the number of in vivo irradiations or number of donor mice needed

- guidance available on websites such as the NC3Rs and the NC3Rs Experimental Design Assistant, the ARRIVE guidelines and also the PREPARE guidelines on the Norecopa website.

Breeding of genetically modified mice is made as efficient as possible by experienced animal house staff using planning tools such as Excel or other specialised mouse breeding programmes to produce an accurate estimate of the total numbers of mice required for the study.

These programmes aid in complex long term experiment planning, which incorporates multiple variables and the breeding needed to support this, including bulk up of stock animals and maintenance of parental strains for the experiment duration.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We continually revise and improve protocols for harvesting and high quality preserving of tissues and extracted biological material (e.g. DNA, RNA and protein) from experimental animals, allowing increased harvest from individual animals and thus increasing the data available from each experiment.

Tissues or other biological materials will be placed in long-term storage for future use and for distribution to colleagues and collaborating laboratories for alternate analysis.

These improvements have also allowed the use of archived historical material for cost-effective large scale screening methods, such as DNA sequencing, unavailable when the samples were first collected, therefore reducing the need for passage or repeating of AML-induction experiments to obtain more material.

Both male and female mice can be used for these studies, thus reducing the number of mice needing to be bred.

Production of frozen embryos for specialist Genetically Modified strains removes the need for continual maintenance of breeding colonies, and also the need for transport of live animals to other Laboratories

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

These mice have been modified to accurately model CHIP in humans and to monitor the appearance of mutated cells in the blood. There are no side effects from these modifications and CHIP itself has no symptoms. As such, this model is a refinement over previous CHIP models which needed high radiation doses for BM transplantation which causes significant side effects.

This model is highly representative of CHIP in humans, increasing the relevance of the data produced in this study. This model has a single gene mutation in the BM cells, which is identical to most CHIP cases in humans.

We can also adjust the percentage of mutated BM cells that appear in the blood of these mice, so they match that seen in humans with CHIP by altering the amount of Tamoxifen, the drug which activates the mutation. This also means that we can use a relatively low dose of the drug, thereby reducing the risk of adverse effects.

These mice are an ideal model for investigating the effect of IR-associated inflammation on CHIP and they have previously been used to show other sources of inflammation can cause changes in frequency of mutated blood cells in the blood. So, there is already an established protocol for relevant endpoints, data collection and analysis to use in our study.

We have experience in blood sampling techniques where minimal handling is used to reduce stress. We have also refined the blood sample analysis so only micro-sampling of the blood (50 micro litres maximum) is needed.

Why can't you use animals that are less sentient?

We are unable to use a less sentient species, or mice at a more immature life stage as this study requires monthly blood sampling for up to two years of age for data collection and analysis. This is to give sufficient time for appearance of the mutated blood cells and for AML development, which may take up to 22 months post-irradiation. It is important for the study to choose a model that develops CHIP/leukaemia in the same way that humans do, which means we must use animals throughout their lifespan.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will minimise animal suffering by identifying potential adverse effects and ensuring that humane endpoints are developed and applied under these circumstances.

We have developed welfare sheets and will ensure that staff involved in the day-to-day care of the animals are trained in using them.

Under guidance from the Vet, we will use the appropriate local anaesthesia when carrying out tail vein blood sampling when needed and use trained and experienced staff to handle the animals undergoing such procedures so as to minimise stress.

Mice on protocols which lead to leukaemia development are checked every day to identify symptoms at the earliest opportunity.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We base our leukaemia diagnosis and symptom presentation as set out in guidelines described in the Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice (Scott C Kogan et al, Hematopathology subcommittee of the Mouse Models of Human Cancers Consortium Blood.

2002 Jul 1;100(1):238-45).

We also use the advice on the 3Rs website (www.nc3rs.org.uk/the-3rs) and also the ARRIVE guidelines (https://arriveguidelines.org/) to refine experimental techniques/protocols and experimental design

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly review the scientific literature and consult with technical experts at specialist companies for new methods or techniques relevant to our research area, particularly for improvements to BM cell culture techniques, which we may be able to apply or use in our project. These techniques can generate data and help inform animal experimental design, and so reduce the number of mice we need to use.

Regular attendance at scientific meetings and presentations also provides information about up-to-date methods and techniques in the field which can be used to maximise the data we obtain from our research and provide opportunities to organise collaborative projects where we can share material/tissues generated in our animal studies, increasing the scientific output from each sample set throughout the course of the project.

Expert members of the local ethics and welfare committee can provide regular updates on new techniques and methodology, as well as providing access to relevant literature, training courses and workshops.

74. Mechanisms of resilience in the brain - protection from neurodegeneration

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Dementia, Ageing, Therapy, Brain cell fitness, Neurodegeneration

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim is to increase our understanding of how brain cells keep fit and healthy when they are young, and why they lose this fitness causing them to malfunction and die with age and in diseases such as dementia. We will use this knowledge to find new treatments that will prevent or slow dementia by making old cells work like young ones, boosting memory and preventing them from dying.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Dementia is rapidly becoming a leading cause of death world-wide. The diseases that cause dementia, which include Alzheimer's, Parkinson's, frontotemporal dementia and related disorders ("neurodegenerative diseases"), are all essentially untreatable. There are no widely available medicines that can cure or slow down these diseases. The medical, social, societal, economic impact of dementia is enormous, and finding treatments is internationally recognised as an urgent unmet need. Today, more than 55 million people have dementia world-wide, with two thirds of these in low- middle income countries. Age is the major risk factor for these disorders, and the global population is ageing. Dementia already costs £1 trillion annually: as the world ages, cases are predicted to double every 20 years, reaching a predicted 129M in 2030, costing a predicted £2.3 trillion. Treatments aimed at the specific causes of Alzheimer's disease – such as reducing 'toxic' proteins - are unavailable to most people, hugely expensive and only effective in selected people. An increasingly powerful approach is to develop therapies to help brain cells to remain alive and functional and prevent them from dying: to boost 'resilience'. The advantage of this is that resilient brain cells will be protected from all of the disorders causing dementia – including but not confined to specific diseases such as Alzheimer's or Parkinson's - and from age-related loss in brain function. Another major advantage is that there are many ways to boost resilience, so we could have many new medicines to use alone or in combination. The aim is to find ways to 'functionally rejuvenate' the brain: to make old brains work like young ones, so that old age is not blighted by dementia and incapacity, with all the repercussions not only for sufferers but for their families, carers, and for society. There is extensive evidence from laboratories worldwide that boosting brain cell function through various key processes is highly effective in mouse models of dementia, with several drugs already in clinical trials. Further understanding of these processes and using this knowledge to make new treatments: to make old brains work like young ones, is the aim of this work.

What outputs do you think you will see at the end of this project?

The work covered by this license is expected to further advance our knowledge of why brain cells lose their fitness and ability to function during aging and disease that renders us vulnerable to diseases such as dementia. This will enable us to find new ways to help stop/delay the onset of memory loss and brain cell death in these disorders, that can be developed to bring new treatments for dementia.

This work will also help develop blood tests that can help detect disease even before it is noticed by patients and that can help monitor response to treatment ("biomarkers").

Findings will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings. The key results will be communicated to the public through press releases. Any mouse models developed will be made available to other scientists interested.

In the short term, the scientific community will benefit from this work as it will advance our knowledge of dementia and why it happens. In the medium term we

hope it will drive first clinical trials of new treatments in patients. In the longer term, it is hoped this work will contribute to changing the clinical course of dementia and diminish its devastating personal, societal and economic burden.

Who or what will benefit from these outputs, and how?

Publications and scientific communications will benefit researchers in the science community, contributing to the progress in the field. Ultimately, we hope the new scientific knowledge identified to lead to new treatments for dementia. As outlined above, the unmet need for drug to slow dementia is enormous – the human suffering, societal cost and global economic burden of dementia in an ageing population means that neurodegenerative diseases are amongst the most important conditions affecting humankind today. Even a modest slowing of these diseases through new understanding and therapies would have profound benefits at the level of individual suffering through to economic impact.

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 in 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 integrity and output.

Species and numbers of animals expected to be used

- Mice: 18,650

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Neurodegenerative diseases are fatal disorders for which there is no cure. To understand the fundamental molecular mechanisms and develop rational therapeutics, clinical, behavioural and pathological outcomes in experimental models are essential. The complexity of the nervous system and the need to use cells or tissues that accurately model neurodegenerative paradigms with clinical outcomes, means that ultimately there is no substitute for animal experimentation. Invertebrates do not share sufficient commonalities in their CNS (central nervous system) to make them appropriate models for human disease. Rats and mice share many similarities in brain structure with higher vertebrates.

Biochemically and physiologically many of the mechanisms, process and pathways are identical to those in humans. Furthermore, the availability of transgenic animals with particular protein knockouts or overexpression make them useful tools for testing the importance of particular proteins in neurodegeneration model systems both in vitro and in vivo. Thus, these species are the most appropriate for testing basic hypotheses, which can be relevant to human health before moving into higher vertebrate species. However, all experiments will be designed with a commitment to refinement, reduction and replacement, with particular attention to matching supply and demand of mice, to minimising suffering by emphasis on defined end points, and to using mice only after using in vitro systems to the full.

In addition, mice are the most used species in biomedical research due to many factors, including their small size, easy maintenance, highly productive breeding output and a genome and biochemistry that closely resembles that of humans. We will use techniques and well established protocols and fully characterised transgenic lines of mice to conduct our studies.

Life stages

Throughout this programme of work, we are comparing aged and ‘younger’ adult mice. In the latter category, we will typically use adult mice aged approximately 3-6 months for most surgical procedures, cooling and dosing studies. In this age range their bodies and systems are fully developed and they are expected to be in good physical condition and of sufficient size and weight to tolerate, and swiftly recover from, intense procedures such as stereotaxic surgery and cooling.

A number of mice will be aged beyond 15 months in this project in order for us to study the changes that occur in the ageing brain. Given that ageing is the major risk factor for the onset of neurodegeneration, we want to understand the mechanisms that lead to the loss of neuronal resilience to stress over time, and to investigate whether or not this can be rescued. Extra will be post- operatively and post-procedurally with old mice when being compared experimentally to younger animals.

For standard prion disease studies not involving aged animals, we will inoculate juvenile mice aged 3-6 weeks, as performed in our and other laboratories for over 30 years as their skulls are still relatively thin at this stage allowing for easy penetration with a needle, leaving minimal damage at the injection site, which heals quickly in these young animals.

Newborn pups (within two days of birth) and embryos will be used so that we can harvest their neurons for primary cultures.

Typically, what will be done to an animal used in your project?

The vast majority of mice used in this project will be generated via standard breeding before being moved to experimental protocols. Some other mice may be bought in from reputable suppliers (typically aged wildtype mice) specifically for use in experiments once they have acclimatised.

Prion studies

Prion infection itself is the most accurate model of human neurodegenerative disease as it completely recapitulates the human disorders biochemically, histopathologically, clinically and in infectivity. It also follows a very well-defined and predictable time course of disease progression, and thus serves as a standard bioassay for testing therapeutic interventions on any of the pathways of interest, with reliable, repeatable readouts in biochemistry, behaviour, histology and clinical signs and, critically, survival. We will use both a rapid mouse transgenic model of prion disease, tg37 hemizygous mice (confirmatory signs of disease appear within about 12 weeks of inoculation) as well as wild type and other trangenic mice on a wild type background (confirmatory signs of disease appear within about 22-24 weeks of inoculation) in this programme of work. In both, direct intracranial injection of prion inoculum is perform in 3-6 week old mice, except where aged mice are inoculated. The tg37 mice have served as an invaluable model for us for many years under four previous PPLs, and we have published many research papers over the last 20 years using this strain of mice. Where mechanistic analyses about disease progression and pathways and proteins are the focus, animals will be humanely killed before any clinical signs during the course of disease. Only where therapeutic interventions are being tested, are the animals allowed to progress to early clinical signs when they are humanely killed according to specific pattern of early and/or confirmatory signs.

Cooling studies

In previous publications we have shown that cooling diseased animals can lead to neuroprotection, associated with the induction of the cold-shock protein RBM3. We want to further investigate how cooling and rewarming animals can change the cellular landscape at the translational, transcriptional and epigenetic level, and assess how this differs in aged and diseased states. We will also conduct cooling experiments in wild type and a range of transgenic mice to assess the roles that particular genes/proteins play in the maintenance and restoration of synaptic plasticity and neuronal resilience. We are developing alternative methods that allow for the induction of the neuroprotective RBM3 protein without subjecting the mice to hypothermia. This can be done by dosing mice with small molecules that induces RBM3. We have also designed AAVs (adeno-associated viruses) and ASOs (anti-sense olignoculeotides) that can lead to increased RBM3 expression in the brain.

Surgical procedures

Typical surgical procedures will include stereotactic injection of substances directly into the brain, including AAVs, lentiviruses, nanobodies, plasmids, labelling agents, or small molecules and compounds that do not pass the blood brain barrier.

Other procedures

Tail vein injection for administration of viruses; intraperitoneal injection of drugs and other compounds, such as transgene inducers eg doxycycline, tamoxifen, etc; oral administration by gavage or in water/food for therapeutic compounds. Where long-term dosing of a compound is needed, we may opt to use osmotic minipumps which can be placed in either the intraperitoneal or subcutaenous cavity Osmotic minipumps can be filled with compounds that are slowly released into the mouse's

system over a designated period of time, avoiding the need of regularly handling and injecting the animals.

The insertion of microchips subcutaneously may be carried out in a number of mice.

Example scenario 1

A cohort of Tg37 hemizygous mice are bred using standard breeding methods and between the age of 3-6 weeks are inoculated with infectious prions. At 8 weeks post-inoculation (wpi), animals will undergo daily dosing with either trazodone or a vehicle for 2 weeks. At 9 weeks post-inoculation (wpi), the mice will be given labelling agent AHA (L-Azidohomoalanine) in their drinking water for 1 week, for the labelling of newly produced proteins in the brains of both treated and control mice at an advanced stage of disease. At 10wpi, mice are humanely killed and their brains taken for biochemical analysis of the proteins that are present in the brains of control and treated mice.

Example scenario 2

A cohort of young (3 month old) and aged (18 month old) wildtype mice are ordered from a reputable supplier to be used for a cooling experiment. Mice are left to acclimatise to their new environment for 1 week before they undergo any procedures, and the aged mice will be subjected to weekly health observations and weighing until the end of the experiment. Once acclimatised, they will all undergo stereotaxic surgery for the delivery of an AAV into the brain to increase levels of RBM3 (with some mice being injected with an empty viral vector as a control). 3 weeks later, all mice will undergo the cooling procedure. Some mice will be killed by perfusion fixation under terminal anaesthesia immediately following cooling, and others will be left to rewarm back in their home cage for 24 hours before also being killed via perfusion fixation under terminal anaesthesia. When young mice are cooled, with their body temperatures dropping to around 16-18c, the synapses in their brains disassemble, and then when they are rewarmed and their body temperatures return to normal, their synapses regenerate. Aged mice fail to regenerate their synapses following cooling. The brains taken from the mice in this study would undergo histological analysis of whether or not the raised levels of RBM3 in the brain from the AAV injection prior to cooling has rescued synapse regeneration following cold-induced disassembly in aged mice when compared to young animals.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected impacts/adverse effects that we anticipate mice encountering in this project are as follows;

- Ageing: Some mice will be left to age naturally beyond 15 months of age and as such may develop signs of ill health associated with ageing. The adverse effects seen in aged animals can include an intermittent hunch, piloerection, poor grooming, abnormal breathing, intermittent tremor, weight loss/gain, mild loss of co-ordination and/or growth of internal/external tumours. They will be monitored weekly for the onset of these signs, and daily once one sign appears. We will

breed transgenic mice that are models of neurodegenerative diseases and have the potential to display a harmful phenotype. Very few animals will be left to reach the point where they present with these clinical signs, but those that do can develop a mild loss of co-ordination, hind- limb clasping, hunched posture, poor grooming, rigid tail and/or an abnormal gait. They will be monitored weekly from a specific time point depending on the individual strain, and then daily once one of these signs appears.

- Cooling: During cooling, mice will become very subdued with decreased respiration and piloerection, which is normal and expected as they enter a state of hypothermia. Rarely, they may display bouts of shivering for a few minutes as they return to normal body temperature (normothermia is complete by 2-4 hours after the procedure).

- Many steps in this project require substances to be administered to mice via injections, typically in the intraperitoneal cavity. This will mostly only provide transient and mild distress to the animals, but long-term daily dosing has the potential to cause anxiety, stress and/or aggressive behaviour. Bruising or irritation may also develop at the injection site(s) with repeated dosing. We will try and avoid this by using osmotic minipumps for substance administration if required long- term.

- Animals that undergo surgery can potentially experience post-operative pain or discomfort, particularly those dosed with novel compounds or ASOs. Rarely, they may recover slowly from the surgery and show a subdued demeanour, hunched posture, mild loss of motor-coordination and/or piloerection post-operatively for minutes-hours. Some mice may also show weight loss over the first few days following surgery. Wet mashed diet will be added to their cages and a course of analgesia will be administered as advised by NVS (named veterinary surgeon).

- For most labelling agents and transgene inducing agents, the optimal method of administration is through drinking water. Although we will add sweeteners to try and make treated waters more palatable, a number of animals may still show signs of dehydration and/or weight loss.

- Prion inoculation can lead to the development of clinical signs over a consistent and predictable timescale specific to the model of mice being used. When they start to show early signs of prion disease (~7 weeks in tg37 homozygous, ~10/11 weeks in tg37 hemizygous and ~17/18 weeks in C57Bl/6) - a rigid tail, hunched posture, subdued demeanour, piloerection, mild loss of co- ordination, erect penis, intermittent tremors and/or hind limb clasping. Confirmatory signs of prion disease will follow shortly after, which can appear as the dragging of limbs, impairment of righting reflex, ataxia, sustained hunched posture or abnormal breathing.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-threshold - 48%
Mild - 10%
Moderate - 42%

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Neurodegenerative diseases are lethal conditions that currently lack a cure. Mice serve as an exceptional model for various aspects of human disease, and they are particularly suitable for prion disease studies as they show all the primary characteristics of the disease, including extended incubation periods, impairment of regular brain function, and cell death. They also recapitulate the loss of brain cells, motor skills and cognitive abilities seen in other neurodegenerative disorders like Alzheimer's disease. Through the use of mice with prion disease, we have discovered new causes for brain cell loss and new targets for dementia treatment. We now need to further understand the role of this in broader neurodegeneration and the effects of its modification for new dementia treatments.

Further, due to the brain's complexity and the necessity for systems that can accurately model neurodegenerative diseases, animal experimentation remains irreplaceable. Despite rapid advancements in the field, there's still a lack of sufficient information to create precise computer models that can predict the complex responses of brain tissues in disease. While many studies can and will be conducted in cultured cells, an intact brain with its full complement of brain cells is the only system where mechanisms can be fully tested and therapies accurately evaluated. Mice share many similarities in brain structure with higher mammals, including humans, and many of the mechanisms, processes, and pathways are identical to those in humans. Moreover, the availability of genetically modified animals with specific gene knockouts or overexpression makes them useful tools for testing the significance of particular processes in neurodegeneration. Therefore, this species are the most suitable for testing basic aims, which can be relevant to human health.

Whenever possible, we will use cell culture and primary cultures to test new drugs for treating neurodegenerative diseases. However, the clinical validity of these and their relevance to human disease ultimately necessitates validation in mouse models.

Which non-animal alternatives did you consider for use in this project?

There are certain experiments that we can perform in cell lines and primary neuronal cultures that can increase our knowledge about the roles of particular pathways, proteins and genes of interest.

However, these can only confirm or indicate pathways that require testing in intact animals for confirmation of relevance. Similarly, in silico models that use machine learning can take existing data about the health effects linked to the molecular structures of certain compounds to predict how similar substances would perform, allowing us to predict the efficacy of drugs before administering them to mice. In silico models can help us to rule out any drugs that may be unsuitable, however any promising candidate drugs will still ultimately need to be tested in animals, as these models can not currently predict how a brain suffering from neurodegeneration will be affected by new drugs.

We use and consider primary neurons and astrocytes generated from both wildtype and transgenic newborn pups within two days of their birth. Although pups do need to be sacrificed to generate cultures, they provide a model for the investigation of neuronal structure and function at a high resolution and in relatively high quantity.

We are also doing some experiments in humans. We measure RBM3 in cooled humans' blood and in blood of dementia patients and we measured protein synthesis rates in human brains of Alzheimer's patients and controls to assess UPR* (unfolded protein response) activation in living patients for increased translational relevance of our in vivo studies.

Thus, we will use in vitro/ ex vivo methods for experiments wherever these can meaningfully replace animal models. For example:

- we will use reporter cell lines for UPR* activation for drug screening for possible neurodegeneration treatments, avoiding testing in mice
- we are developing cell reporter assays to screen more than 1000 repurposed compounds for their effect on structural synaptic plasticity, avoiding testing in mice
- where possible, we will use human blood and tissue samples to confirm the significance of key targets in human disease and to find biomarkers corresponding with the loss of synaptic plasticity during neurodegeneration.

* The unfolded protein response (UPR) is a cellular process that happens when there are too many misfolded or unfolded proteins in a part of the cell called the endoplasmic reticulum (ER). Proteins need to be properly folded into specific shapes to do their jobs correctly. When proteins don't fold properly, they can cause problems, like a puzzle piece that doesn't fit. The UPR is like the cell's quality control system and it tries to fix these misfolded proteins or, if they can't be fixed, it signals the cell to destroy them. If the UPR can't handle the number of misfolded proteins and the situation gets out of control, it can lead to cell death. This process is important in many neurodegenerative diseases.

Why were they not suitable?

In vitro and in silico models are valuable tools in neurodegenerative research, but they cannot fully replace in vivo models, such as mice, for several reasons.

- The nervous system is highly complex, with intricate interactions between various cell types, neurotransmitters, and signalling pathways, many of which are still not fully understood. In silico and in vitro models can simulate or replicate certain aspects of these interactions, but they cannot fully capture the complexity of the whole system as it operates in a living organism.
- Mice have intact physiological systems, allowing us to study neurodegenerative diseases in the context of a whole, living organism, including the influence of factors like the immune system,metabolism, and ageing, which can't be fully replicated in vitro or in silico.
- Mouse models allow for behavioural studies, which are crucial in neurodegenerative research. Changes in behaviour are often the most noticeable symptoms of neurodegenerative diseases in humans, and so it is important that we are able to observe and recognise deviations from normal behaviour in mice so that we can assess the effects of our interventions. In vitro models can't replicate, or predict, behavioural aspects.
- In vivo models can be used to study the progression of neurodegenerative diseases over time, which can't be achieved in non-animal models. In vivo models are essential for preclinical testing of potential treatments. They allow for the assessment of a drug's effectiveness, its distribution in the body, metabolism, side effects, and toxicity.

While in vitro and, ultimately, in silico models are continually improving and can provide valuable insights, they are currently complementary to, rather than replacements for, in vivo models in neurodegenerative research.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For most of the quantitative experiments, we will use previous experience (ours, or from the literature) to select sample sizes. In terms of the numbers of animals required, in general, we will use:

- 10-15 group housed mice for statistically valid behavioural testing 4 – 6 mice for semi- quantitative biochemical analyses

- for histology, previous experience has shown that group sizes of 4-6 are sufficient to detect even subtle differences, which are qualitative.
- mice will be grouped into cohorts large enough to allow sufficient numbers for sampling at regular interval
- where in vivo validation will require the administration of a disease modifying inhibitor or activator of a target molecule, pilot studies will be performed to determine the appropriate dose of this, if these are not already known.
- we have developed neuron-astrocyte co-culture models to probe mechanisms of synaptic plasticity impairment and non-cell autonomous mechanisms of neurodegeneration in vitro. Typically, from an average litter (6 mice) we are able to investigate 4-6 different parameters in triplicate (i.e. 12-18 different samples). To do this in vivo we would need ~18 mice. It also allows
- us to share different cells type between users thereby maximising the data we can get from a single animal.
- we will use repurposed drug libraries meaning that, in most cases, safe dosing limits and adverse effects are known therefore reducing the number of animals needed for screening and testing.
- for all mice, multiple tissues will be taken for multiple analyses e.g. the brain was dissected in half, one half for histological analysis the other half separated into different brain regions for biochemical analysis. Other organs may also be taken such as pancreas, liver, kidney, spleen and blood on compound treated mice.
- suitable experimental controls will be used e.g. normal brain homogenate for prion infection, vehicle only controls for dosing studies, scrambled/control cDNA sequences for studies using virus vectors. These will be selected based on each study and their specific aims.
- where possible excess stock (i.e. wild type mice) or ex-breeders are taken for tissue/blood samples for protocol optimisations or standard tissue references. Occasionally, excess mice will be humanely killed and used for protocol training e.g. intracerebral inoculations/ stereotaxic injections

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use the minimum number of mice needed in all experiments for reproducible results and statistical validity in line with the ARRIVE guidelines www.nc3rs.org.uk/ARRIVE. We use published protocols to guide statistical validity in all our experiments. We also use the PREPARE guidelines (https://norecopa.no/prepare) and the NC3RS Experimental design assistant (https://www.nc3rs.org.uk/experimental-design-assistant-eda) to help plan and design our experiments. We are committed to keeping the numbers of mice to a minimum by maximising the use of each experimental animal (see below), e.g. taking tissues from different halves or parts of the brain for biochemistry and

histology after behavioural assessment and neurophysiology, therefore maximising readouts from any one animal. In fact, we have pioneered a tissue sharing initiative at our company, where we take any tissues required by other groups (oesophagus, liver, skin, heart, kidneys, gut, tail, pancreas, sciatic nerve, spinal cord) through coordinated dissections of mice. The result has been >1000 mouse lives saved (ie sharing tissues from mice has saved having to sacrifice 1024 wildtype mice in 2023 alone, and 455 so far in Jan-Feb 2024). For genetically altered animals, where suitable lines already exist, animals will be obtained from the relevant supplier. Otherwise, we will have the required lines made by reputable companies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For our prion model we will adopt a batch breeding regime so that large cohorts of animals are regularly produced (i.e. one batch of animals per month) to be inoculated on a set date. Using this method we can generate enough mice to provide for everyone in the group each month, and having a set date for inoculation allows users to plan their experiments accordingly.

We will plan experiments months in advance and not generate mice until we have the relevant protocols, equipment and compounds in place to commence the studies.

We occasionally breed transgenic mice and humanely kill pups aged P0-P2 (within two days of birth) so that we can harvest their neurons for cultures. These matings will be staggered to ensure that litters are produced in manageable amounts and frequencies, so that no animals are produced that can’t be used.

When we need to take tissues from animals as untreated controls, we will liaise with other research groups within our institute to see if we can share tissues from the same animals. For example, we work alongside other groups in the institute that harvest skin and oesophagi from young and aged WT mice, but they often don’t have a need for the brain that we would be interested in using.

Tissues collected from stock animals, or excess tissues that were collected from experimental animals, will be labelled, stored and organised in a way that is clear and makes them easy to locate. These will then be available to every researcher working under this project licence, and can prevent the need for additional animals to be generated or humanely killed. Our group also has an experienced technician employed specifically to oversee the colony management and procedural work that will be performed on this project.

Further, to minimise animal wastage, prevent the unnecessary production of animals showing adverse effect and to ensure that animal breeding is inextricably linked to research requirement, we will ensure high standards of animal care, welfare and utilise the most appropriate breeding methods.

- ensure that colony sizes are monitored and adjusted within a formal forecasting system to meet the requirements of the research programme

- ensure that breeding colonies are always kept to their minimum size so as not to over produce. Detailed breeding records will be kept, enabling the selection of the most appropriate breeding stock.

- where possible, use single lines or crosses over multiple transgenics to reduce numbers   verify that before setting up or creating new transgenic lines a full search is carried out to

- ascertain that there is no other worldwide availability.

- ensure that researchers and technicians working on this project are appropriately trained and suitably competent to enable a high success rate to be achieved and thus minimise the number of animals used.

Furthermore, as part of good laboratory practice, we will write a study plan for each experiment including: a statement of the objective(s); a description of the experiment, covering such matters as the experimental treatments, the size of the experiment (number of groups, number of animals/group), and the experimental material; and an outline of the method of analysis of the results.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Models

The availability of transgenic mice with particular protein knockouts or overexpression make them useful tools for testing the importance of particular proteins in neurodegeneration model systems both in vitro and in vivo. For example, tg37 mice infected with prions rapidly and reliably model prion neurodegeneration and allow us to test new treatments in a validated model. Selection criteria of mouse models are: 1) presence of misfolded disease specific protein associated with known neurodegenerative disease e.g. mutant tau (AD, FTD, PSP and other tauopathy-dementias); mutant APP/PS1 (Alzheimers models), AND 2) presence of neurodegeneration (rTg4510, some AD models), OR synaptic phenotype. These are the most refined models available at present that most closely mimic either early (synaptic phenotype) and/or later (neuronal loss phenotype) neurodegeneration. All these models have been extensively phenotyped (and numerous publications exist), so they provide a measurable standardised readout against which we can assess the role of the pathway and the effects of intervention. Prion infection itself is the most accurate model of human neurodegenerative disease.

Methods

We will use validated procedures from our previous work and publications under previous PPLs (stereotaxic surgery for lentiviral/AAV/ASO-mediated gene modification; administration of neuroprotective compounds; behavioural analyses; and cooling-induced synaptic plasticity*) as these have been so informative in our work so far. We will also use PET (Positron emission tomography) imaging as a new means to diagnose incipient neurodegeneration, a method validated by others in mice and known to be relevant to human disease. PET imaging can reveal the size, shape and structure of the brain, and could allow us to identify very early signs of neurodegeneration in mice to which we can tailor our treatments.

Many studies within this project will involve brain injections via stereotaxic surgery. These are required to administer substances such as viruses, disease modifying agents and labelling compounds directly to the brain, minimising off-target effects that could arise if administered using alternative methods. We will adhere to the general constraints outlined in this licence relating to surgery, and follow the latest published guidance by NC3R's/LASA on aseptic surgical techniques.

*- Cooling-induced synaptic plasticity refers to the changes in the structure and function of synapses in the brain in response to low temperatures. Our cooling procedure induces hypothermia and lowers mouse body temperature down to 16-18c. The response of synapses to hypothermia can differ between young/aged and healthy/disease animals.

Why can't you use animals that are less sentient?

Regarding life stages, neurodegenerative diseases that we plan to study in this project are often diagnosed and treated at late stages in life. As such, it is important that we factor this in when designing our studies to boost the translatability of potential treatments that we might develop. We are also interested in preserving/increasing neuronal resilience in aged brains, and so to do this we need to work with naturally aged animals.

Invertebrate models can be useful for studying structure of neurons and toxicity testing, but they can not provide an insight into how disease treatments might affect pathophysiological and behavioural responses in larger and more sentient species such as mice. There are also no species of mammals less sentient than mice that are available currently for reliable behavioural testing.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use validated procedures from our previous work and publications (stereotaxic surgery for gene modification; administration of neuroprotective compounds; behavioural analyses; and cooling) as these have been so informative in our work so far. We will also use PET imaging as a new means to diagnose incipient neurodegeneration, a method validated by others in mice and known to be relevant to human disease.

Animal suffering will be minimised by:

- performing all surgical procedures (stereotaxic injections; prion inoculation) under general anaesthesia. For stereotaxic surgeries we will provide analgesia to all animals along with 7-day post-op checks as standard. These checks will include the provision of palatable mashed diet to prevent or ameliorate any weight loss in the days immediately following the surgery. We will use a stereotaxic frame connected to a digital co-ordinate reader which can guide the user to particular brain regions with great accuracy. Brain injections will be performed using ultra-fine Hamilton Neuros syringes to minimise structural damage to the brain.

- minimising the number of surgical interventions: most animals will receive one set of bilateral injections and not more than two, on separate occasions, not less than 1 week apart. In the case of ASO, mice will receive the whole dose through just a single injection. Animals that undergo surgery will receive mashed diet and 7 day post-op checks as standard

- administration of disease modifying agents / labelling compounds / transgene regulators with due care and pain relief if appropriate, as well as staged dosing, in general not more than daily dosing. All administration protocols adhere to published guidelines, LASA Good Practice Guidelines, and as advised by the NC3Rs. For some compounds, daily intraperitoneal injection/oral gavage dosing may need to be performed for more than 30 days (until the onset of clinical signs or defined endpoints). In these cases, mice will be monitored for adverse reactions and body condition (as standard for all dosing studies). Pain relief may be given as recommended by a NVS/ NACWO if mild signs of discomforted noted. Dosing will be stopped and the NACWO/ NVS contacted if moderate signs develop.

- when injecting mice in the intraperitoneal cavity repeatedly, we will alternate the side of the mouse that we inject each day to avoid bruising and irritation. In dosing studies that require daily injections for longer than three weeks, we may opt to administer compounds via an osmotic minipump (a small pump implanted into the mouse that slowly releases compound over time). Although the mice will have to undergo a minor surgical procedure for this, it will avoid subjecting animals to the cumulative stress of daily handling and injections for a prolonged period.

- we will use the most refined compounds possible with minimal adverse effects. The majority of new compounds will be identified from repurposed drug libraries and therefore information regarding dosing routes, concentration, frequency may already be published and the minimal effective dose will be used. Other compounds will be identified from the literature.

- for prion studies, most mice will not be allowed to develop clinical signs of disease and will be humanely killed at early time points during the course of disease progression. However, a few animals in designated experiments need to be left to develop diagnostic signs of clinical prion disease to confirm incubation times and assess effect of treatment. Prion disease can usually be confidently diagnosed at relatively early stages, and suffering is limited. Very rarely it can lead to rapid onset of prostration (severe severity) and in these cases it will be reported to the HOI.

- when using novel compounds or viruses we will conduct pilot studies (typically involving no more than 3 mice per condition) to check for a) tolerance of the substance by the mice with no adverse effects and b) expected performance of said compound/virus after biochemical/histological analysis. We will pay particular close attention to animals in pilot studies immediately after administration of the compound and in the days that follow.

- cooling can be induced, without general anaesthetic, by giving synthetic 5-prime-adenosine- monophosphate (5'-AMP). This induces a reversible deep torpor-like state by inducing hypometabolism in non-hibernating mice, and mimics the molecular signalling for torpor.. This is the optimal method of cooling of core body temperature and inducing a hibernation-like state.

- mice that undergo the cooling procedure will be monitored very closely throughout. We will use a record sheet for each cage of mice to record the time of their 5'AMP injection, the time that they enter/leave the fridge and also their temperature at regular intervals throughout the procedure.

- By doing this we will ensure that the animals' body temperatures do not fall below our target range, and we can balance this by removing the animal from the fridge for short periods throughout. Having this manual control over the mouse's temperature is a major refinement in comparison to other techniques of inducing hypothermia in mice. We have noted that mice aged beyond 18 months can take longer to recover from the cooling procedure, and so they will be watched closely to ensure that they return to showing normal behaviours before being left unsupervised. Once all mice return to the holding rooms we will check them again 30-60 minutes later just to make sure that they have settled back into their home cage and are not fighting.

- housing mice in groups, with enriched environments as far as possible, as this improves quality of life

- any time that we administer compounds via drinking water, we will supplement the water with a sweetener such as maltose to make it more palatable and prevent dehydration.

In addition, after our experience under our previous licence we have introduced the following specific refinements:

- providing analgesia (e.g. Metacam) after intraperitoneal injection if discomfort is noted. Analgesia will be provided under the guidance of the NACWO/NVS

- checking pH of substances (compound and vehicle) to be administered by intraperitoneal injection to avoid unpredicted side effects of new/unknown substances

- close monitoring of all mice after intraperitoneal administration for signs of discomfort and its duration as occasionally mice will be dosed multiple times and some substances may become an irritant

- using oral dosing over intraperitoneal injection administration where possible, as this reduces the chances of injection site irritation and is more relevant to clinical applications
- All new researchers that are due to perform the more complex procedures under this licence (e.g. stereotaxic surgery, prion inoculations etc.) will undergo thorough training using only cadavers until they show that they can perform the procedure with great consistency, accuracy and competency before moving on to live animals under close supervision.

Severity endpoints have been chosen to minimise animal suffering whilst obtaining the most valid scientific output.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are many online resources that provide guidance on how to conduct procedural work on mice in a refined manner. We will follow this guidance to ensure that we minimise the harm, distress and suffering that any animal may experience in this project.

The NC3Rs website provides a comprehensive index of all things relating to legislation, ethical considerations, latest advancements in the 3R's and colony management. They have also written many guides summarising best practices for carrying out procedures on animals, ranging from how to handle a mouse and recognise ill health, right through to conducting complex behavioural work and surgical procedures. Similarly, LASA have produced many guidelines that are useful, particularly in regards to adopting aseptic surgery techniques.

There are many video tutorials provided by JoVE (Journal of Visualized Experiments) that include detailed, instructional guides on how to carry out specialised techniques and procedures. The RAT (Research Animal Testing) website also has a great collection of videos that demonstrate refined methods and techniques for carrying out procedures.

As mentioned previously, we will utilities the NC3R's EDA, and we will consider the PREPARE guidelines when designing our experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will check the NC3Rs website regularly to keep tabs on advances in the 3R's and will discuss the use of any new methods or techniques with our NACWO and/or NVS before implementing anything.

Our NIO will also regularly send out regular updates/newsletters/bulletins.

Representatives from our lab will be encouraged to routinely attend symposiums, webinars and conferences held by LASA, NC3Rs and other organisations that promote knowledge sharing across the industry. These gatherings often provide a

great insight into progress that is being made relating to animal welfare and 3Rs that may not yet be published or adopted as common practice.

Our company also has a dedicated in vivo hub and a mouse user group that meets regularly to share knowledge, discuss best practices and explore new ideas relating to the 3R's.

75. The role of the peripheral nervous system in gut and lung health and disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Gastrointestinal tract, Lung, Nervous system, Immune system, Inflammation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how the nervous system of the gut and the lungs contributes to health and disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Healthy function of the gut and the lungs is achieved through cooperation of several cell types including those of the immune system and the nervous system. Disrupting this balance, in response to an infection or antigens, can adversely affect the function of these organs and lead to inflammatory conditions with severe consequences for human health. It is now well understood how immune cells contribute to these diseases, but unfortunately treatment options are limited. Until now, the mechanisms leading to gastrointestinal and lung pathologies remain poorly understood, pointing to a crucial role of other cell types such as the understudied neural cells (neurons and neuronal support cells called "glia") of the local nervous

system. Recent reports in the brain, skin and gut uncovered roles for the nervous system in physiology, immunity, and wound repair, suggesting that it may take on similar jobs in the lung.

Uncovering the roles of innervation in gut and lung health and disease is the focus of this project. The ultimate aim of this research is to provide new targets for the pharmaceutical industry in order to eventually develop successful treatments for gut and lung inflammatory conditions.

What outputs do you think you will see at the end of this project?

We anticipate several distinct outputs to have occurred by the end of this project:

New knowledge - This work will yield cellular, molecular and mechanistic knowledge that will advance our understanding of complex chronic inflammatory disorders affecting the lungs and the gut, such as inflammatory bowel disease, old-age related constipation, viral and bacterial infections, asthma, chronic obstructive pulmonary disease and fibrosis. The cellular, molecular and mechanistic knowledge emerging from this work will likely lead to improved disease phenotyping through the discovery of glial- based biomarkers and pathways for glial-targeting therapies for lung inflammatory and infectious diseases.

Publications - we will publish our results in open-access journals to communicate them to the broader scientific community, and ensure that the advances in knowledge that we generate can be widely utilised.

Intellectual property - we anticipate that the identification of disease pathways and therapeutic targets will generate intellectual property, which will be owned by the establishment and used to ensure wide accessibility to potential candidate therapies.

Who or what will benefit from these outputs, and how?

There are likely to be both short and longer-term beneficiaries from these outputs:

The short-term impact of this study will be beneficial to the wider scientific community (both basic and clinical).

The long-term and principal beneficiaries are likely to be patients affected by gut and lung inflammatory diseases and their families, as we foresee these outcomes will ultimately pave the way for the development of new treatments, thereby expanding the availability of innovative therapeutic options.

We expect that the complete realisation of the potential therapeutic advantages stemming from this project will not occur until after the project's conclusion. It is highly likely that additional work extending beyond the project's timeframe will be necessary to achieve this.

How will you look to maximise the outputs of this work?

We will seek to maximise the outputs of this work at multiple stages within the research process.

Throughout our research endeavours, we will engage in active collaborations with other research groups. This collaboration aims to facilitate the exchange of our expertise and knowledge while also benefiting from their insights and expertise. Such collaborative efforts will serve to optimise our progress, enabling efficient operation, accelerating our work, and ensuring the generation of reliable, reproducible data. Ultimately, this collaborative approach will provide genuine insights into disease biology, benefiting both our team and others in the field.

To ensure other researchers will benefit from this work, we will share our data and findings by publishing in open access journals. We will use both platform and poster presentations at major and local conferences to disseminate the findings of this work as well as give invited seminars. In addition, to maximise the impact of the research outputs, we will make all raw data (when appropriate) open access and disseminate findings in a timely fashion using pre-print servers. This will allow other researchers to investigate our data for their own research purpose. We will also widely share our tools and write resource and methods papers to ensure reproducibility of results and applicability to other experimental systems.

We aspire not only to publish the outcomes of our successful experiments but also to share our unsuccessful approaches through communication with collaborators, publications, presentation at conferences. Our goal is to highlight these unsuccessful experiments to minimise future failures for other research groups.

Species and numbers of animals expected to be used

- Mice: 20500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are an excellent model of the mammalian nervous and immune system and the least sentient species possible to deliver results in complex disease models reflecting most aspects of human disease.

Broadly speaking, mice serve as an effective model due to their ability to closely mirror the anatomical organisation, physiological functions, and pathological processes observed in humans. This comparison holds especially true when compared with simpler and frequently utilised experimental animal models like worms (Caenorhabditis elegans) and fruit flies (Drosophila melanogaster).

Specifically, when examining the development, structure, and functionality of the peripheral nervous system, mice recapitulate most aspects of neuroglia biology with remarkable similarity to that of humans, making them an ideal choice for understanding the fundamental aspects of the these cells. Furthermore, the identification of numerous clinically relevant molecular pathways has been facilitated

through genetic analyses conducted in mice, establishing them as a valuable model for understanding human biology and diseases.

Previous research has collectively shown that the proper assembly and optimal functioning of the peripheral nervous system during adulthood relies significantly on biological processes occurring during earlier stages of life, and that re-activation of developmental programmes occurs during disease pathogenesis (either spontaneously such as during ageing or in response to inflammatory stimuli).

Therefore, we will use tissues from embryonic, fetal, postnatal, adult and aged life stages of mice to identify cellular and molecular pathways and ultimately leading to a comprehensive understanding of the underlying causes and consequences of disease processes.

Gut and lung inflammatory models are well characterised in mice and their immune system is better understood than that of other animals, making mice an appropriate model. In addition, the commonness of mouse-based experimentation means that there are many reagents and genetically modified strains readily available that are directly applicable to this project. These tools include neuroglial reporter mice and inducible knockout mice

Typically, what will be done to an animal used in your project?

Genetically altered mice will be bred using conventional methods of breeding. The mutations in these mice are not expected to cause any harm that is more than mild. Some of our mice will be of a germ- free status so that we will understand the effect of microbes on the organisation and function of the peripheral nervous system.

Tamoxifen will be administered to some mice for a variety of reasons. These are to activate silencing of genes or induce a phenotype by activating genes. It will also be used to achieve cell ablation, where we actively destroy or remove cells of interest. In some mice we will achieve cell ablation through administration of depleting antibodies or through the use of adeno-associated viral vectors.

Some animals will have inflammation of the gut induced by chemical (Dextran Sulphate Sodium), via intestinal parasites (helminth Heligmosomoides polygyrus) or via known pathogen (Citrobacter rodentium). These will cause self-limiting colitis which does resolve in short period of time. The typical duration of these experiments is two - four weeks.

Some mice will have inflammation of the lung induced by the administration of pathogens which are typically influenza, Haemophilus influenzae, respiratory syncytial virus, house dust mite (or a modified allergen cocktail containing House Dust Mite, Ragweed, Aspergillus fumigatus) or a helminth (Nippostrongylus brasiliensis). Typically, these experiments can be up to seven weeks in duration.

Some mice will undergo blood sampling at selected time points so we can assess the effect of the inflammation.

Some mice will be given other substances which will label cells for study postmortem.

Some mice will be given antibiotics and biological modifiers that could have a therapeutic effect.

In a small number of cases animals will be allowed to reach 24 months of age for studies into aged gastrointestinal inflammation diseases.

Some germ-free animals will be given a cocktail of commensal gut microbiomes.

The administration of substances throughout our project will be via commonly used routes which are not expected to cause any harms, nor will we administer volumes that are not in line with published best practice.

All animals will be humanly killed at the end of the experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice that undergo induction of gastrointestinal diseases may develop soft stool which is normally self- limiting and resolves in 14 days after the disease induction.

Mice that undergo induction of lung diseases may develop a compromised overall state of discomfort which is normally self-limiting and resolves completely within 14 days after the disease induction.

Mice that undergo targeted cell ablation in the gut or lung may develop inflammation in those organs.

All the effects are expected to be transient and in some cases mice can experience a weight loss of up to 15% (and in some cases even up to 20% within a 72 hours time window). If the animals exhibit signs of pain, distress or suffering, they will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Subthreshold = 45%
Mild = 45% Moderate=10%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is necessary to use animals to study the mechanisms underlying the physiological and pathophysiological function of the peripheral nervous system in the gut and the lung as the regulation of this cellular system and the complex interactions with other cell types such as the immune, epithelial and stromal system cannot be currently modelled in cell-culture or computer-based models. The peripheral nervous system spans several tissues and relies on a diverse microenvironment to perform accurately; thus it cannot be cultured without changing its intrinsic genetic programmes. In addition, the inflammatory diseases we are studying are complex immunological disorders that cannot be recapitulated in vitro.

Which non-animal alternatives did you consider for use in this project?

Whilst we rely on animal models to delineate mechanisms underpinning complex cellular interactions in tissues, we inform and complement our animal experiments with cell-culture or computer-based models. We will employ ex vivo approaches, such as precision-cut-tissue-slices, organoid or primary culture systems to model aspects of cell-cell interactions. In addition, we will harness available datasets from human tissue to inform and prioritise our in vivo mechanistic studies. In addition, we will perform literature searches and discussions with colleagues at conferences to continually develop and expand our in vitro non-animal research models to address elements of our research questions.

Why were they not suitable?

Mammalian cell culture models offer a valuable amount of information and aid in generating hypotheses. However, the interactions between the peripheral nervous system and other tissues within the gut and the lung are exceptionally complex and cannot be faithfully replicated by the existing cell culture models. However, these models are suitable for aspects of our work, such as defining viral targeting strategies to specific neural cell populations or confirming specific and direct ligand-receptor interactions. Therefore, collectively, they enable us to decrease the number of animals that would otherwise be necessary.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimation of animals needed to accomplish our objectives is derived from past experience on animal numbers used for breeding and maintenance of specific lines and experimental numbers. This calculation is based on historical data and the number of genetic lines we are currently managing. Our overarching goal is to consistently minimise the quantity of animals utilised per experiment, aligning with

the principle of the 3Rs (Replacement, Reduction, Refinement). This objective is accomplished through well-designed experiments, appropriate controls, and accurate analyses. We anticipate that, typically for our studies, a range of 6-8 animals per experimental and control group or genotype is necessary to obtain statistically significant results. Throughout the duration of this project, we will engage with statisticians for continuous consultation to ensure that the number of animals used is regularly evaluated and adjusted to the minimum required while still achieving our scientific objectives. This collective approach ensures a reduction in animal use while upholding the reliability and reproducibility of the results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We employ the Experimental Design Assistant in our study design, a free resource offered by the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs). This tool helps us ensure that each experiment employs an appropriate number of animals and provides randomisation strategies for the allocation of animals to groups, aligning with ethical considerations and the principles of the 3Rs (Replacement, Reduction, Refinement) in animal research. Together with the PREPARE guidelines and sample size calculations, these tools allow us to carefully design our animal experiments in detail, aiming to ensure robust statistical power capable of detecting biologically significant effects of the expected size. This design strategy is informed by prior knowledge and is executed rigorously to enhance the likelihood of capturing meaningful outcomes within our research.

The NC3Rs’ ARRIVE guidelines will be used to design optimal animal research experiments with the smallest number of animals necessary to achieve statistically meaningful results, collect data and report results.

In addition, we harness our extensive expertise and comprehensive knowledge of peripheral nervous - immune system interactions to help us decide which effects are true versus which occur randomly; while resources such as the NC3Rs Experimental Design Assistant is a useful tool to suggest analyses to account for variables and covariates within the experimental design.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

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. Furthermore, we take steps to cryopreserve all our genetically-modified mouse lines, preserving them either as frozen embryos or sperm. This practice minimises the number of active lines we maintain at any given time. Additionally, it positions us to readily share these lines with other research groups upon request, facilitating collaborative efforts and the broader scientific community's access to these resources.

In experiments in which we test new parameters or examine the effects of new genes, we will first perform small-scale pilot studies prior to embarking on properly powered experiments. We take measures to optimise the timing of our experiments to ensure reliable data collection. For instance, in studying the gut transit time of mice using coloured dye ingestion, we noticed significantly less variability in experimental data when conducting this experiment during nighttime. This timing coincides with the animals' heightened activity levels, resulting in more consistent results. Consequently, shifting the experiment to the nighttime period has not only enhanced data consistency but has also contributed to reducing the overall number of animals required to achieve statistical power.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use mice. The particular models and methods we plan to employ typically do not subject animals to more than moderate discomfort. Furthermore, these methods have previously undergone refinement to minimise any distress experienced by the animals involved in the research.

Specifically, we will use the following models:

● Gene silencing or overexpression models: we will prioritise the use of tissue and/or cell type- specific genetic manipulations whenever feasible. This approach is preferred as it is generally less harmful compared to germ-line gene deletions or ubiquitous overexpression. This strategy allows for more targeted and specific modifications, reducing the overall impact on the animals.

● To study the microbial impact of the microbiome on the phenotype and function of the peripheral nervous system, we will use germ free mice. Germ free mice are an established model to study the impact of the complex microbiota on animal health. These mice will be maintained in a sterile environment from birth, while maintenance of these animals has been refined over the years to minimise distress experienced by the animals. We are planning to reconstitute the microbiota

(conventionalisation) in some animals, which will re-set their microbial status and intestinal physiology returns to a normal state.

● Some mice will be aged (up to 24 months), however, they will be closely monitored to ensure no pain, suffering, distress or lasting harm will be experienced.

● Gut pathology and inflammation (induced through administration of DSS or pathogens, such as Heligmosomoides polygyrus and Citrobacter rodentium) and

lung pathology and inflammation (induced through administration of house dust mite (or a modified allergen cocktail containing House Dust Mite, Ragweed, Aspergillus fumigatus) or pathogens, such as influenza, Haemophilus influenzae, respiratory syncytial virus or Nippostrongylus brasiliensis): We have chosen established and commonly utilised models, as they aim to induce the least possible pathology, depending on dosage. They have been extensively characterised and refined over many years in laboratories globally to minimise any potential pain, suffering, or distress experienced by the animals involved in these procedures.

- Targeted cell ablation in the lung and gut (achieved through inducible genetic systems): This strategy of tissue-specific targeted cell ablation is carefully adjusted or titrated to minimise the number of cells and tissues affected. While animals might show lung or gut pathology and inflammation as a result of local tissue-specific cell ablation, the goal is to precisely control the ablation process in such a way that it reduces the impact on other cells and tissues to avoid adverse effects on animal health.

All animals exhibiting inflammation and pathology will undergo frequent monitoring. They will be humanely killed at timepoints that are either earlier than or coincide with the onset of adverse symptoms to ensure their welfare and prevent unnecessary distress or suffering.

All the proposed models may involve various steps aimed at comprehending the involved biological processes. These steps can include procedures such as administration of drugs, and general phenotyping, including the withdrawal of blood from a superficial vessel. It is important to note that these methods have undergone refinement to minimise any potential suffering experienced by the animals. For instance, blood withdrawal will be limited to not exceed 10% of the total blood volume at any given time to prevent adverse impacts on the animals' physiology.

Additionally, apart from these experimental procedures, our research will also encompass standard breeding and maintenance methods for genetically altered mice.

Why can't you use animals that are less sentient?

Invertebrate models (such as the fruit fly Drosophila melanogaster or Caenorhabditis elegans) are not suitable to provide relevant results to achieve our aims as the organisation, development and function of their nervous and immune system is fundamentally different to that of vertebrates organisms. In addition, equivalent inflammatory models employed for our research, that recapitulate many of the features of human disease, do not exist for less sentient vertebrates, such as zebrafish, but have already been established in mice and are extensively utilised in scientific research.

For developmental aspects of our studies, we will use embryos or very young animals, however for complex analysis of tissue homeostasis we require animals to have developed a mature immune and nervous system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are committed to ongoing reviews of our experimental protocols to identify opportunities for enhancing welfare and reducing adverse effects. This involves assessing our experimental design, techniques, and animal care practices to make improvements. We will evaluate our performance internally while also integrating external knowledge and adopting best practices that emerge.

Additionally, we will maintain comprehensive records detailing our experimental procedures, aiming to share this information to benefit other research groups. Our goal is to continuously enhance animal welfare, minimise adverse effects, and contribute to the wider scientific community by sharing detailed insights into our methodologies.

To minimise adverse effects linked to genetic alterations, we will prioritise inducible or conditional alleles whenever feasible. This approach allows for the selective deletion of gene activity from specific tissues, as opposed to affecting the entire animal. By employing inducible or conditional alleles, we aim to achieve a more targeted and localised impact, minimising potential adverse effects associated with genetic modifications.

To minimise stress levels during breeding and maintenance, we will adhere to best practice guidelines such as the implementation of refined husbandry protocols, which may include environmental enrichment strategies and ensuring an adequate supply of nesting material for mice. These practices aim to create a more conducive and comfortable environment, promoting the well-being of the animals and minimising stress during their breeding and maintenance.

To minimise suffering during experimental procedures, animals will be closely monitored to be able to detect any adverse outcomes.

To minimise suffering of animals that undergo a new procedure or that are generated as a new line, we will undertake a pilot study with increased monitoring to establish optimal experimental conditions and confirm the appropriate humane endpoints.

We will employ anaesthesia and analgesia whenever deemed appropriate, including pre-emptive administration, to mitigate discomfort or pain in animals involved in our studies.

When working with specific models, we will ensure that necessary adaptations are implemented to cater to their specific needs and ensure their well-being. For example, for animals undergoing inflammatory models, wet diet will be placed on the bottom of the mouse cage to improve hydration, if necessary. For animals undergoing ageing experiments, group sizes will be increased to accommodate for loss of animals and to avoid single housing due to animal losses due to old age. If any adverse effects are observed, animals will be treated accordingly, and animals that develop unexpected adverse effects will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Prior to initiating any experiment, we will conduct a comprehensive review of various resources to ensure the refinement of our experimental procedures. This process

involves examining guidance provided by the NC3Rs, particularly focusing on disease models or common procedures such as blood sampling, accessible via the NC3Rs website. Additionally, we will refer to publications from the RSPCA Science Group or https://journals.sagepub.com/doi/10.1258/0023677011911345, which offer insights and refinements specific to certain procedures and models.

We will also follow the LASA Principles for preparing for and undertaking aseptic techniques and follow the PREPARE and ARRIVE 2 guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will have regular meetings between the project licence holder (PPL) and all personal licence holders (PIL) involved in this project. These meetings aim to facilitate efficient communication and discourse regarding the principles of Replacement, Reduction, and Refinement (3Rs), as well as any modifications or legal updates to the project licence and its execution. The 3Rs will also be actively discussed during lab meetings, departmental gatherings, and conferences, fostering discussions among colleagues and collaborators. The team will participate in seminars and relevant events dedicated to 3Rs topics, such as NC3Rs events and workshops that are opportunities to stay informed of 3Rs advances and new approaches. Animal procedures and routine husbandry and handling will be constantly refined by keeping abreast of technological developments in the fields communicated by the NC3Rs newsletter that we have signed up to.

76. Assessment of novel entities for the treatment of metabolic disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- 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

Insulin resistance, Diabetes, Obesity, Organ dysfunction, Novel therapies

Animal types	Life stages
Mice	juvenile, adult, aged
Rats	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to provide highly specialised preclinical data to our clients to support either the development of novel, candidate compounds for the treatment of metabolic disorders (e.g. obesity, diabetes, NASH, renal dysfunction) or to assess compounds with a different primary therapeutic indication to produce metabolic effects which may be considered of benefit (secondary indication) or be a potential side-effect.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit -

these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Metabolic disorders are highly prevalent worldwide affecting hundreds of millions of people in both developed and developing countries. The term metabolic disorders encompass a range of related conditions including, obesity, Type 2 diabetes and dyslipidaemia as well as related complication such as kidney or liver disease (see graphic below).

The World Health Organisation (2022; https://www.who.int/news-room/fact-sheets/detail/obesity-and- overweight) estimates that 890 million adults are obese and 1.6 billion are overweight worldwide.

Importantly, the obesity epidemic is no longer restricted to Western cultures, but is becoming a global burden with countries such as Mexico, Brazil and China currently most affected. In the absence of suitable intervention, the global epidemic of obesity will become a leading cause of morbidity and mortality driven by an increase in

related life-threatening metabolic disorders including dyslipidaemia, hypertension (a risk factor for stroke), cancer and Type 2 diabetes.

The metabolic system is a highly coordinated and intricate network of biochemical reactions that enables the body to function, grow, repair, and maintain health. Various organs and tissues contribute to metabolism, including the liver, kidneys, heart, muscles, adipose tissue (fat) and the pancreas.

This licence is primarily for the evaluation of customer compounds that have been developed to treat not only obesity but key-related aspects such as liver fibrosis, inflammation, loss of glucose or insulin control. These conditions are interconnected and, for example, a drug that reduces weight loss may be expected to improve related factors (e.g. glucose control, liver inflammation). The nature of the licence reflects the interlinked metabolic end points.

Metabolic disorders are a huge public health concern and burden because of their widespread occurrence. Addressing them has a huge positive impact on the overall health and well-being of populations as they often lead to chronic health issues, disability, and reduced life expectancy. The work on this licence is important in the discovery of new medicines and approaches for the treatment of obesity and related metabolic disorders. Recently marketed drugs such as semaglutide and tirzepatide have been demonstrated as being effective in causing weight loss in man. However, they have side effects and compliance issues since they are increasingly linked with sarcopenia (skeletal muscle loss) and administered by injection rather than taken orally.

What outputs do you think you will see at the end of this project?

The outputs from this project relate to generating data that will inform of:

Mode of action/efficacy: physiological data e.g. primarily body weight data, levels of glucose, food intake data as well as other relevant biochemical and molecular readouts

Side effect data: physiological data and behavioural data

Pharmacokinetics (PK): the fate of a drug within the body can be determined by tissue (e.g., blood, plasma, brain) levels of that compound over time after administration.

The data generated in this project will be used to provide our clients (principally from the pharmaceutical industry) with data to support their development of candidate compounds with potential for the treatment of metabolic disorders. The data may be used to decide whether or not a compound can progress to clinical trials and may be included in the data submitted to Regulatory Authorities for licensing new treatments.

In summary, the outputs of this project are primed to advance our understanding of effects of potential therapies developed for metabolic disorders or related complications and may contribute to the scientific literature upon client permission, and potentially result in innovative products with the potential to benefit individuals affected by these conditions.

Who or what will benefit from these outputs, and how?

The beneficiaries of these outputs encompass various stakeholders and their impact may be evident in both the short and long term. Initially, our clients will benefit from the generated outputs as the data produced will enable informed decisions on potential clinical applications of test items.

Benefits to client:

Access to high-quality research services: Clients will benefit from our expertise and state-of-the-art facilities, enabling them to advance their research projects with confidence.

Accelerated drug development: By leveraging our in vivo services, clients can expedite their preclinical studies, ultimately speeding up the drug development process and reducing time-to-market for new therapies.

Short-term benefits:

Data Generation and Initial Findings: Customers, whose compounds we test, will benefit in terms of providing useful data to determine whether or not a compound can progress to clinical trials.

Immediate Access to Specialized Services: Clients can quickly initiate in vivo studies without the need to invest in their own infrastructure.

Medium-term benefits:

Publication and dissemination of results: As studies progress, clients may expect to generate robust data that can be published in scientific journals, contributing to their own and our scientific recognition and validation.

Intermediate milestones in drug development: Clients may achieve key preclinical milestones, facilitating regulatory submissions and advancing their projects towards clinical trials.

Long-term benefits:

Successful advancement to clinical trials: Comprehensive in vivo data will support the transition from preclinical to clinical stages, increasing the likelihood of successful outcomes.

Broad impact on therapeutic development: The insights gained from these studies will not only benefit individual projects but also contribute to the broader field of metabolic dysfunction research, potentially leading to new therapeutic approaches.

Benefit of providing this project as a service:

Offering this project as a service aligns with our objective to support the scientific community and industry partners by providing high-quality, reliable, and efficient research solutions.

How will you look to maximise the outputs of this work?

Our customers will use the knowledge and data produced under this authority to inform project progression. This may involve dissemination of information to investors, developmental partners and publication where appropriate. The outputs will also be used to guide further development within projects, both in-house and externally, that are focused in relevant disease areas.

Collaboration with research groups and institutions will be actively pursued. By sharing data, insights, and expertise, we aim to accelerate progress, validate findings, and generate innovative solutions that may not have been achievable through solitary efforts.

We are committed to sharing the new knowledge and discoveries generated from this work with the wider scientific community whenever possible. This will include presenting our findings at conferences, workshops, and seminars, as well as publishing research articles in peer-reviewed journals.

Disseminating our results ensures that the scientific community and stakeholders can benefit from the insights gained.

Recognizing the value of transparency in scientific research, we will also consider publishing and presenting information about unsuccessful approaches and negative results. This can prevent duplication of efforts by other researchers, provide valuable insights into the research process, and contribute to the overall advancement of scientific knowledge and reduce the use of animals in research.

Species and numbers of animals expected to be used

- Mice: 11,250
- Rats: 4,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We need to use animals to investigate the potential efficacy and side effects of customer compounds on metabolic disorders because they provide a living system that closely mimics human physiological processes. Indeed, the models used in this licence translate well to humans (e.g. the generation of an obese phenotype through access to diets high in fat) Animal models are essential for understanding complex biological interactions, testing the efficacy and safety of novel therapeutics, and obtaining data that cannot be replicated in vitro or through computational models alone.

Rats and mice are the chosen models because they are the lowest sentient animals

suitable for our research, aligning with ethical considerations. They offer several advantages:

- Genetic and Physiological Similarity to Humans: Rats and mice share many genetic, physiological, and metabolic characteristics with humans, making them ideal for studying metabolic dysfunction and related diseases.

- Established Models: There is a wealth of existing research and historical data on the validity of the animal models used in the project, providing a strong foundation for our studies and facilitating the reproducibility and validation of our results.

- Practical Considerations: Rats and mice have relatively short lifespans and rapid reproductive cycles, allowing for the observation of disease progression and treatment effects within a manageable timeframe.

The choice of life stages for the animals in this project is based on the following factors:

Disease Relevance: The selected life stage must align with the specific disease or condition being studied. For example, young animals are used to investigate the onset and early progression of metabolic disorders like diabetes, as they are more susceptible to these conditions. This helps in understanding the initial mechanisms of the disease.

Ethical Considerations: We aim to minimize animal suffering by choosing life stages that align with humane principles and the specific requirements of the research. This ensures ethical integrity while maximizing scientific validity.

Specific Research Questions: Different life stages are chosen based on the research objectives. For instance, to study age-related complications, older animals are required. The life stage must reflect the developmental and physiological characteristics relevant to the human condition being modelled.

Precedent and Practicality: The choice is also informed by existing scientific literature and previous studies, ensuring consistency and comparability. Practical aspects, such as the availability and ease of handling animals at specific life stages, are also considered.

In summary, the use of rats and mice, and the selection of specific life stages, are carefully justified by their scientific relevance, ethical considerations, research objectives, and practical feasibility. This approach ensures that our studies are robust, ethical, and capable of providing valuable insights into metabolic dysfunction.

Typically, what will be done to an animal used in your project?

The project is a service-based licence focused on testing the efficacy of customers compounds in animal models of human metabolic diseases. While understanding the mechanisms underlying these diseases is essential, our primary objective is to evaluate the therapeutic potential of various pharmacological agents in preclinical models that simulate human metabolic conditions.

These studies will involve animals undergoing surgical procedures or being exposed to non- lethal/potentially therapeutic doses of substances to induce changes in metabolism, ultimately leading to the development of pathology reflecting human diseases. The test substances, challenges, and stimulants will be administered through various routes and with various dose regimens, chosen based on published research articles, prior experience, and customer requirements and scientific need. Blood sampling may also be conducted to collect samples for pharmacokinetic analysis. Drug doses will generally be selected from PK studies or acute feeding studies. Doses may be adjusted as the study progresses.

Each experimental model will be monitored daily following intervention and animals will be assessed for any signs of distress with supportive measures provided. Procedures will be undertaken using the most appropriate anaesthetic where required and analgesia will be given to any animal undergoing a surgical intervention. The mode of substance administration will be chosen to cause the least harm and distress to the animals. Any new substances or route of administration will be tested in a small pilot study and the animals monitored daily for signs of distress. Humane endpoints will be strictly adhered to.

The following models will be established:

Obesity and its complications

Genetic and induced obesity rodent models are essential for understanding the underlying mechanisms of obesity, testing potential interventions, and studying the associated metabolic and health conditions. Models induced by high fat diet or strains of rodents genetically predisposed to obesity are used to gain insights into the complex interplay of genetics, environment, and lifestyle factors in obesity development and progression.

Typically, once the model is established, animals will be given either vehicle or test compound(s) via an appropriate route. Body weights and food (and usually water) intake will be monitored, typically, daily during a suitable baseline period (the time when animals are typically administered with vehicle) and during test compound(s) administration. In advance and during the procedure, animals may undergo repeated blood sampling (e.g., for PK and/or glucose tolerance tests and/or paracetamol absorption test) and determination of body composition (fat, muscle, bone) by x-ray imaging (a technique used in humans) in mice only. It is important to note that not all procedures will be applied to each animal, as the optional steps are relevant for a specific model only. For instance, animals undergoing a glucose tolerance test typically will not be subjected to a paracetamol absorption test, as the main aim or focus of the study will be evaluating glucose sensitivity, not gastric motility and upper small bowel absorption.

Diabetes and its complications

Rodent diabetic models are vital in diabetes research due to their physiological and genetic similarities to humans. These models offer insights into disease development and progression. Rodent diabetic models are also used to study complications associated with diabetes, such as nephropathy (kidney damage), retinopathy (eye retina damage), and neuropathy (nerve damage). By manipulating variables like diet,

genetics, and environmental factors, we can gain a deeper understanding of the molecular pathways involved. These models serve as invaluable tools for testing potential therapeutics and exploring novel treatment approaches, ultimately contributing to advancements in diabetes management and treatment strategies.

Typically, once the model is established, either by pharmacological induction e.g. STZ or using genetically modified animals, animals will be given either a vehicle or test compound(s) via an appropriate route. In advance or during the procedure, animals may undergo repeated blood sampling (e.g., for PK and/or creatinine clearance test). It is important to note that not all procedures will be applied to each animal, as the optional steps are relevant for a specific model only. For instance, animals undergoing uni-nephrectomy (surgical removal of one kidney) typically won't be subjected to body composition assessment by x-ray imaging using a DEXA scanner, as the main focus will be on evaluating kidney function.

Models of kidney disease

Models of kidney disease are typically induced through various methods, including chemical (e.g. chemotherapeutic, STZ), genetic, dietary (high fat diets), as well as surgical interventions (kidney removal). They are crucial for studying the mechanisms, progression, and potential treatments of renal conditions. These models contribute significantly to our understanding of kidney diseases, such as chronic kidney disease (CKD) and acute kidney injury (AKI). Kidney disease models can replicate certain conditions or pathologies closely related to human kidney diseases. Typically, kidney disease is induced by a toxin or immune active molecule or surgically e.g. unilateral obstruction of ureter. The model will be chosen based on the specific research questions and ethical considerations. Both types of models are important for advancing our knowledge of kidney diseases and developing effective treatments.

Typically, once the model is established, animals will be given either a vehicle or test compound(s) via an appropriate route. In advance or during the procedure, animals may undergo repeated blood sampling (e.g., for PK and/or creatinine clearance test) and determination of blood pressure. It is important to note that not all procedures will be applied to each animal; for example, animals that will receive chemotherapeutics as a way of inducing glomerular damage will not undergo surgical procedures such as unilateral ureter obstruction. The steps are optional and relevant for a specific model only.

Models of liver disease

Models of liver disease are invaluable tools in research for studying liver conditions without invasive procedures. These models principally involve dietary interventions. They offer a way to explore liver disease mechanisms, disease progression, and potential treatments. The selection of a model hinges on the specific research inquiries and ethical considerations inherent to each study. Such models play crucial roles in advancing our understanding of liver diseases and the formulation of effective treatments. They are typically established by feeding animals modified diet, rich in fat and sugar.

Typically, once the model is established, animals will be given either a vehicle or test compound(s) via an appropriate route. In advance or during the procedure, animals may undergo repeated blood sampling e.g. PK. It is important to note that not all procedures will be applied to each animal on every study.

What are the expected impacts and/or adverse effects for the animals during your project?

The models used in this project are expected to lead to the onset of metabolic disorders including obesity and/or diabetes, or mild liver injury (NASH) or kidney damage. In the case of obesity, diabetes (and complications) and NASH, these phenotypes (as in humans) may develop over several months (e.g. 6 or more). However, based on experience these models are not associated with pain or behavioural changes but may be associated with effects comparable to those seen in man; e.g. increased urination, increased weight gain etc. It is expected that novel test compounds will treat these symptoms.

The well-being of animals involved in experiments is a priority. Measures will be taken to minimize any discomfort or harm during substance administration, and if an animal experiences more than mild, transient suffering, it may be euthanized unless quick and effective remedies are possible. Enhanced care will be provided until full recovery, and if improvement is not swift, euthanasia will be considered.

Model induction and substance administration might lead to temporary behavioral changes, with potential signs like abnormal respiratory movements or reduced weight gain. Daily assessments will gauge overall animal well-being, considering factors such as activity, demeanour, and body weight. Animals in poor condition will be euthanized.

For anaesthesia, careful control is crucial to prevent pain or harm. Monitoring methods, guided by experts, will ensure animals remain at an appropriate level of consciousness. If anaesthesia cannot be maintained or controlled, euthanasia will be administered immediately.

This approach prioritizes animal welfare, emphasizing responsible and humane practices in research.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild – 40% mice and rats
Moderate – 60% mice and rats

What will happen to animals at the end of this project?

- Killed
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of whole animals in our research is crucial due to the complex and systemic nature of metabolic dysfunctions. While in vitro methods, such as organ-on-chip technologies, offer valuable insights and can mimic certain aspects of organ function, they lack the ability to replicate the intricate interactions between multiple organs and systems within a living organism.

Metabolic diseases often involve multifaceted processes that affect various tissues and organs simultaneously. Whole-animal models are essential for observing the integrated physiological responses, pharmacokinetics, and pharmacodynamics of therapeutic interventions. These models allow us to study the comprehensive effects of a drug, including potential side effects and long-term impacts, which cannot be fully captured by in vitro systems alone.

Furthermore, animal models provide critical data on the drug's efficacy and safety profile, guiding the subsequent stages of drug development and clinical trials. Therefore, despite advancements in alternative methods, the use of whole animals remains indispensable for advancing our understanding and treatment of metabolic dysfunctions.

While we recognize and actively pursue the principles of the 3Rs (Replacement, Refinement, and Reduction), replacing the use of whole animals in the final stages of drug testing for metabolic diseases is not currently feasible. This is because our work represents the crucial final phase of drug evaluation, where comprehensive, systemic responses must be observed to ensure safety and efficacy.

All preliminary tests, including in vitro methods and alternative models, have already been conducted by our clients in earlier stages of research. These preliminary tests are essential for initial screening and mechanistic understanding. However, the complexity of metabolic diseases and the need to observe interactions within a whole living organism necessitate the use of animal models at this stage. The use of animal models remains essential in this final stage to guarantee the reliability and translatability of our findings, ultimately safeguarding human health.

Which non-animal alternatives did you consider for use in this project?

Ethical considerations and the desire to reduce the use of animals in scientific studies have led to the exploration of various alternatives in metabolic research. Here are some replacement methods and technologies that are increasingly being used:

- In Vitro Studies with Human Cells
- Organoids and 3D Cell Cultures
- Microfluidic Devices
- Bioinformatics and Computational Models

- Patient-Derived Samples

All in vitro and ex vivo drug screening has been performed by our clients in earlier stages of research. Some of all of the above were used depending on the client project requirements. These preliminary tests ensure initial safety and efficacy. Our work now focuses on the final stage of drug testing, where whole-animal models are essential to comprehensively evaluate systemic responses and potential side effects. The specific animal model used will vary depending on the project and its unique requirements.

Why were they not suitable?

While these alternatives to in vivo research in diabetes are promising, it is important to note that none of them can fully replace the need for in vivo studies, especially in later stages of drug development and safety testing. However, by combining multiple approaches, researchers can reduce the reliance on animal models and make diabetes research more ethical and efficient.

In the context of this project, a meticulous exploration of non-animal alternatives was undertaken with the aim of minimizing the reliance on whole-animal models. A paramount consideration involved the assessment of cell lines and organoids, as well as other pertinent technologies, for their potential utility in replicating complex physiological systems, in particular in the disease state. These alternatives, while valuable in specific contexts, were ultimately deemed unsuitable as primary substitutes for in vivo models, owing to their inherent limitations.

Cell lines, a stalwart tool in the realm of in vitro experimentation, offer the advantage of controlled environments and relatively facile manipulation. Nevertheless, they fall short in replicating the intricate interplay of physiological networks that pervade living organisms. Cell lines often consist of singular, or at best, isolated groups of cells, thereby omitting the profound systemic interactions inherent in the physiology of an intact organism. Consequently, they were deemed insufficient in capturing the holistic effects of candidate substances under investigation in this project.

Organoids, while exhibiting commendable potential for mimicking specific organ functions, similarly present constraints. These three-dimensional cellular structures, though closer in resemblance to the corresponding organs than isolated cell lines, remain unable to encompass the full complexity of an organism’s intricate physiological networks. Their focus on individual organs or specific cell types limits their capacity to replicate the systemic effects and interactions that are imperative to a comprehensive understanding of the candidate substances.

In essence, while non-animal alternatives such as cell lines and organoids offer valuable insights into discrete aspects of drug evaluation and testing, their inherent limitations in replicating the multifaceted physiological networks found within a whole organism rendered them unsuitable as stand-alone replacements for in vivo models. As such, the utilization of whole-animal models remains imperative in this project, ensuring a comprehensive evaluation of candidate substances and their effects within the intricate tapestry of a living system. Importantly, prior to testing within this project, candidate substances will likely have been identified on the basis of techniques including those detailed above.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used in the project over its duration and the group sizes of animals used in the individual studies is based on extensive experience from running such projects and experiments. The numbers are based on the previous project but will be affected by customer demand for the services which may differ over the next 5 year period. Sample sizes for our experiments are estimated from past experiments with input from statisticians within the company.

Statistical modelling

A typical study consists of 60 rats or mice (e.g., control group, model induced group, 2 drug treatment groups and a positive comparator with 10 animals per group). Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of 10 to achieve the quality of results we need. An average of 40 rat studies and 60 mice studies per year are carried out, thus 11,250 rats and 4,500 mice during the 5-year lifetime of this project.

Surgery considerations

Sham controls provide a robust means of controlling for the ancillary effects of a procedure, optimizing the ability of the investigator to evaluate for a placebo or procedural effect in an unbiased fashion. In addition, control animals provide baseline biomarker measurements which are then used within the statistical analysis to show a statistically relevant increase in circulating biomarkers following dosing.

Sham control animals will not be used unless there is a strong scientific reason or during model development phase to establish model induction and severity of the model.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During the experimental design phase of this project, several steps were taken to reduce the number of animals used, in alignment with ethical considerations and regulatory requirements. Mainly we used the expertise of our statisticians who can perform power calculations to ensure that studies are suitably powered to detect changes. Similarly, the statisticians advise on experimental design to improve statistical power and reduce animal usage. Use of the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) Experimental Design Assistant will also be considered.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In addition to good experimental design, several measures will be implemented to optimize the number of animals used in this project while maintaining scientific rigor and ethical considerations. These measures include pilot studies to establish responses to challenge agents, continuous monitoring and adjustment, sharing tissues and samples to minimize the overall number of animals used. Tissues and samples collected during experiments will be shared across research teams where if appropriate. We also apply an ongoing assessment of aims to identify opportunities to maintain the statistical power of the studies while reducing the number of animals required. By fine-tuning experimental protocols based on real-time data, unnecessary duplication or excess animal usage can be avoided. Although rare in this project, animals may undergo re-use which will lead to a reduction in the total number of animals in used across the lifetime of the project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have selected specific models of metabolic diseases based on their well-established ability to mimic human disease pathophysiology closely. Each model provides valuable insights that are crucial for our research aims while adhering to the principles of minimizing pain, suffering, distress, and lasting harm.

It is recognised that each disease model has its limitations. However, to answer specific scientific questions accurately, it is essential to use models that closely mimic human pathology. Therefore,having a variety of models under our licence is crucial to ensure that we can select the most appropriate model for each research objective. This approach allows us to obtain the most relevant and reliable data, ultimately contributing to advancements in understanding and treating human diseases.

As mentioned earlier valid in vitro and ex vivo drug screening has been performed by our clients in earlier stages of research. The work outlines in this project focuses on the final stage of drug testing, where whole-animal models are essential to comprehensively evaluate systemic responses and potential side effects. The specific animal model used will vary depending on the project and its unique requirements.

Models of obesity:

The chosen models are globally validated and have been used in our lab for the past 20 years. The induction of obesity in animals typically involves altering the animals' diet to induce obesity gradually. This approach is non-invasive, avoiding surgical procedures, which can cause significant pain and distress. Obesity is induced through the animals' natural feeding behaviour typically by providing a

high-fat diet. This method simulates human dietary habits and results in a more physiologically relevant model of obesity without causing undue stress or discomfort. Moreover, using strains of rodents genetically predisposed to obesity allows us to study the disease's development and progression in a manner that closely mimics the human condition. These models help elucidate the complex interplay of genetics, environment, and lifestyle factors in obesity.

Once the obesity model is established, animals are administered either vehicle or test compounds via appropriate routes with minimal handling, reducing stress and anxiety levels. Routine procedures involve monitoring body weights and food and water intake, typically done daily during the baseline period and test compound administration.

Throughout the study, animals are regularly monitored for health and well-being. Any signs of distress or health issues are promptly addressed with supportive care, including nutritional adjustments and veterinary care as needed.

Depending on the study’s objectives, animals may undergo specific procedures such as repeated dosing, blood sampling for pharmacokinetics (PK), glucose tolerance tests, or paracetamol absorption tests. These procedures are carefully chosen to minimize distress and are not applied to every animal. For example, animals undergoing glucose tolerance tests are not subjected to paracetamol absorption tests to avoid unnecessary interventions. The length of the study depends on several factors such as disease characteristic (chronic diseases may require longer dosing), drug properties (PK/PD), selection of appropriate markers e.g. development of fibrosis is longer than changes in glucose tolerance, hence studies focusing on the glucose tolerance are typically very short.

We use advanced technology such as body composition, including fat, muscle, and bone mass, is determined using x-ray imaging, a non-invasive technique also used in humans. This method provides valuable data while causing minimal discomfort to the animals.

Models of diabetes:

The chosen rodent diabetic models are essential in diabetes research due to their physiological and genetic similarities to humans such as changes in glucose/insulin tolerance, development of neuropathy or nephropathy. Hence, these models offer insights into disease development and progression and are used to study complications associated with diabetes, such as nephropathy (kidney damage), retinopathy (eye retina damage), and neuropathy (nerve damage). The chosen end-point and biomarkers selection will depend on the specific project requirements.

Diabetes in rodents is typically induced either pharmacologically, using agents like Streptozotocin (STZ), or by utilizing genetically modified animals predisposed to the

condition. STZ selectively damages insulin-producing cells, effectively modelling diabetes with minimal intervention. Genetically modified rodents provide a natural progression of the disease, mimicking human diabetes closely.

Once the diabetes model is established, animals are administered either vehicle or test compounds via appropriate routes with minimal handling, reducing stress and anxiety levels. Routine procedures include monitoring blood glucose levels, body weights, and food and water intake, typically done daily during the baseline period and test compound administration.

Depending on the study's objectives, animals may undergo specific procedures such as repeated blood sampling for pharmacokinetics (PK) or creatinine clearance tests. These procedures are carefully chosen to minimize distress and are not applied to every animal. For instance, animals undergoing uni-nephrectomy (surgical removal of one kidney) typically won’t be subjected to body composition assessment by x-ray imaging, as the main focus will be on evaluating kidney function.

Animals are regularly monitored for health and well-being. Any signs of distress or health issues are promptly addressed with supportive care, including nutritional adjustments and veterinary care as needed.

While not applicable to all studies, some animals may undergo body composition analysis using non- invasive x-ray imaging techniques like DEXA scanning. This method provides valuable data on fat, muscle, and bone mass while causing minimal discomfort to the animals.

Models of kidney disease:

The proposed rodent models are critical for studying different aspects of nephropathy, these could be

e.g. inflammation, fibrosis or specific changes in podocin expression. These models allow for the investigation of disease mechanisms, progression, and potential therapeutic interventions. While some models, such as the adriamycin-induced nephropathy model, involve model induction by chemotherapeutic the use of these models is justified by their ability to provide robust and reliable data necessary for advancing our understanding and treatment of kidney diseases.

Despite the severity of some models, measures are taken to minimize pain, suffering, and distress. For instance, animals are closely monitored, and supportive care, such as hydration and nutritional support, is provided to alleviate adverse effects.

Animals may undergo procedures like repeated blood sampling for pharmacokinetics (PK) and kidney function tests (e.g., creatinine clearance). These procedures are necessary for assessing disease progression and treatment efficacy, however the type and frequency of tests is chosen carefully to minimize distress. Continuous health monitoring is conducted throughout the study, and any signs of distress are promptly addressed with appropriate care. Maintaining animals in an enriched environment with suitable bedding, nesting materials, and social housing where possible helps to reduce stress and improve their overall quality of life. Humane

endpoints are established to prevent unnecessary suffering, and animals are humanely euthanized if they exhibit severe distress or illness.

Models of NASH

Chosen rodent models of liver disease are vital for understanding the mechanisms, progression, and potential treatments of hepatic conditions e.g. steatosis, lipid deposition, hepatic ballooning. These models closely replicate human liver diseases, such as non-alcoholic fatty liver disease (NAFLD), hepatitis, cirrhosis, and liver fibrosis, providing critical insights for research and therapeutic development. Common methods for inducing liver disease in rodents include chemical agents (e.g., high fructose diets, chemotherapeutics), genetic modifications, and dietary interventions (e.g., high-fat diets).

Liver disease models, such as those induced by high fructose or high-fat diets, are selected for their ability to closely mimic the pathophysiological conditions observed in human liver diseases. Dietary models gradually induce liver disease through natural feeding behaviours, reducing the need for invasive procedures. Depending on the research objectives, animals may undergo specific procedures such as repeated blood sampling for pharmacokinetics (PK), liver function tests (e.g., alanine aminotransferase [ALT], aspartate aminotransferase [AST]), and imaging techniques to monitor liver structure and function. These procedures are carefully chosen to minimize distress and are not applied universally to all animals. For instance, animals undergoing chemical induction of liver disease will not be subjected to genetic modifications.

Throughout the study, animals are regularly monitored for health and well-being. Any signs of distress or health issues are promptly addressed with supportive care, including nutritional support and veterinary care as needed. Animals are maintained in an enriched environment with appropriate bedding, nesting materials, and social housing where possible. These measures help to reduce stress and improve the overall quality of life for the animals. Humane endpoints are established to prevent unnecessary suffering, and animals are humanely euthanized if they exhibit severe distress or illness.

All animal on every protocol will undergo handling and baseline phase: Handling and baseline dosing protocol for rats and mice was established over the years which ensures little or no weight-loss upon the start of dosing. The advantage of doing this are illustrated in a recent mouse dietary induced feeding study where:

Body weight change during vehicle baseline dosing (Day -6 to 1, all 40 animals): Mean = -2.02g, Standard deviation = 1.26g

Body weight change during week 1 of compound dosing (Day 1 to 8, 10 vehicle animals): Mean =

-0.19g, Standard deviation = 0.62g

In absence of vehicle dosing during baseline, we would expect the mean weight reduction at baseline with corresponding variation during week 1 of the study instead. The mean reduction could hide a compound that caused a decrease in body

weight. The higher variation would require 4 x as many animals to detect the same size treatment effect.

In some cases a pilot studies might be executed. In order to minimise adverse events during the conduct of the studies outlined in this project licence pilot studies are adopted. These are performed when there is no or limited in vivo data. However, even if there are considerable in vivo data we often employ a small pilot cohort of rats or mice (maximum of 3 rats per dose) that run (typically) 7 days in advance of the main study as we often see higher exposure in obese rodent models than in lean rodents. If adverse events are observed the doses in the main study are revised as appropriate. Pilot studies help to address the power calculation and possibly reduce the n number in the main studies.

Why can't you use animals that are less sentient?

The choice to use mice and rats in this project over less sentient or immature animals is based on a combination of scientific, ethical, and practical considerations. Mice and rats are selected as research subjects due to well-characterized and minimally severe metabolic models that have been developed for these species.

Mice and rats are mammals, and they share many biological similarities with humans, including genetic, physiological, metabolic, and immunological systems. These similarities make them valuable models for studying human diseases and potential treatments. Researchers can gain insights into disease mechanisms and test therapeutic interventions more effectively by using these species.

It's important to note that terminally anesthetized animals are not suitable for this research. This is because the evaluation of metabolic processes often requires observation over a long period of time. Additionally, the relatively short lifespan of mice and rats is advantageous for studies where age is a crucial factor, allowing researchers to investigate various aspects of the disease during aging processes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To enhance the well-being of animals in research and gain satisfactory data while minimizing severity, several strategies will be implemented.

Regular and frequent monitoring of animals' body weight will be conducted, allowing for early detection of changes that may indicate distress or adverse effects. This proactive approach enables timely intervention and ensures the welfare of the animals. Handling technique will be implemented to reduce the stress associated with experimental procedures.

Drug administration.

Vehicle and test items will be administered by the least severe route and administration will be performed by highly skilled staff, using appropriate dosing techniques, dose volumes and solutions (e.g., sterile solutions, appropriate pHs) to minimise any stress and discomfort to the animals.

Appropriately sized needles will be used, and a separate needle will be used for each animal for systemic injection to maximise welfare and reduce the chance of inter-animal infections. Oral administration to rats will normally be by gavage using flexible catheters to minimise oesophageal trauma. Short oral dosing needles are typically used for mice which are likely to bite the flexible tubing . Refinements to oral administration of drugs such as training the animals to consume material voluntarily from a syringe have been considered but would not be practical as we could not guarantee the full dose had been swallowed leading to the potential for greater variability in data and requirement for study repeats. Due to the novelty of the test items requiring dosing we could not be sure of their palatability and any potential for adverse influence on absorption.

Where prior knowledge exists regarding administration of the test item in vivo this will be used to optimise the choice of doses and route of administration wherever possible.

The dosing regimen for substances administered will be optimized to achieve the desired effects with minimal impact on animal welfare. This may involve refining dosage levels, administration routes, or frequency, considering guidelines and best practices outlined in relevant literature and established protocols.

Surgery

We constantly strive to improve the techniques used to reduce the likelihood of suffering in the animals and to improve our surgical and dosing/sampling techniques to minimise the experimental failure rate. Aseptic technique will be applied at all times to reduce the risk of infection, based on the latest Home Office guidance and in keeping with the Laboratory Animal Science Association's (LASA) latest guidelines.

To reduce stress in the animals prior to them undergoing surgery, they will be allowed to acclimatise to their environment for at least 7 days prior to surgery being undertaken. They will usually be weighed and handled for at least three days prior to surgery to familiarise them to handling and to provide information on their growth curve and general welfare.

The veterinary surgeon regularly observes our staff performing surgery and inspects the animal's post- surgery regularly.

Rigorous postoperative care protocols will be established. Continuous monitoring for signs of discomfort or complications will be conducted, and any deviations from the expected recovery trajectory will be promptly addressed. This includes attentive care during the night if necessary.

Analgesia

Careful consideration has been given to the need for pre/post-operative analgesia in conjunction with the Named Veterinary Surgeon (NVS). The surgical sites are made as small as possible to allow access for implantation of minipumps, catheters or access to organs for direct injection. For all experiments animals will be treated pre/post-operatively following consultation with the NVS (e.g., with a non-steroidal

anti-inflammatory drug such as carprofen). The time course of the analgesia will be considered to ensure that it is beneficial from the time the animal may first begin to experience pain. An animal will not be used in an experiment until it is in a suitable condition, e.g., not displaying signs of experiencing pain, stress or discomfort. Animals are monitored for behavioural signs of pain by our staff who have extensive experience of post-surgical animals, by the NVS and by the Named Animal Care and Welfare Officers (NACWOs). Observations are recorded on individual post-surgical records by our staff. The Grimace Scales to recognise pain and assess its severity in post-operative animals (https://nc3rs.org.uk/grimacescales) will be used.

Additionally, biomarkers specific to the disease models will be monitored, allowing for a more nuanced understanding of the animals' health status. This comprehensive approach ensures that subtle changes indicative of distress are captured early.

Blood sampling

The taking of blood samples (anticipated to be primarily from the tail vein) is likely to be a frequent procedure undertaken. Heated chambers may be used to aid blood sampling. Restraint in a suitable chamber or manually may be used to facilitate the procedure. The use of local topical anaesthetic to reduce any pain and discomfort during blood sampling will be considered. Limits on volume of blood samples will be taken from the guidelines listed below. The use of a topical local anaesthetic to reduce any pain and discomfort during blood sampling will be considered.

By implementing these strategies and consulting established guidelines, such as the NC3Rs guidelines for specific disease models, the research team aims to refine experimental procedures, reduce severity, and establish earlier endpoints. This approach aligns with ethical considerations and contributes to the overall improvement of animal welfare in experimental research.

Housing

Animals will be group housed unless there is a scientific or welfare reason to single house an animal.

Where animals have been surgically prepared it may be necessary to single house due to possible damage a cage mate may cause to the surgical site or the implant itself. Fighting in male mice is well recognised as an issue and where fighting is seen in group housed animals, cages will be split down to prevent injury or death from fighting. Mice specifically will be kept in their delivery groups to reduce the likelihood of fighting. Where food and water measurement is taken (such as in obesity studies) or where multiple test items are being investigated animals may be single housed to prevent cross contamination of biological samples or where delivery groups don’t breakdown into pre-determined test groups. Food and water will be available at all times and animals will be given increased environmental enrichment beyond the standard enrichment supplied (e.g., extra toys to play with). When animals are placed in metabolism cages enrichment will be included for duration of samples collection.

Animals housing depends on the model to be employed, strain of mouse and study design. Every effort will made to group house, however, where accurate monitoring

of food and water is needed animals may undergo single housing. In addition, where some strains of aggressive mice are used (e.g. BALBc that are normally used in ADR studies) mice will be singly housed to obviate aggression (especially where mice might be returned to a group housed environment after a urine collection where they are singly housed). Importantly, reasons for single housing are also a consequence of the fact that the project is primarily focused on the development and use of drugs for the treatment of metabolic disorders. Accordingly in some studies accurate determination of food and water intake may be required since it may be of importance to understand whether doses that improve kidney function are also those that affect food intake.

For all NASH studies mice will be singly housed as C57Bl6J mice are aggressive so group housing even when it is limited to litter mates and involves weight matching as often unsuccessful (with between 25 and 50% of mice in a study having to be singly housed due to aggressive behaviour). Also as the mice are exposed to a diet either lacking in a nutrient or high fat in nature if the mice are group housed we get a lot of variation in the level of fibrosis compared to when mice are singly housed and this impacts on the validity of the data and reproducibility.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidance outlined in the PREPARE and ARRIVE guidelines for experimental planning and publication of data produced during this project.

We will also follow guidance issued by the Home Office, LASA, NC3Rs and RSPCA on animal re-use (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/470 008/Use Keeping_Alive_and_Re-use_Advice_Note.pdf), blood sampling (https://www.nc3rs.org.uk/microsampling and https://www.nc3rs.org.uk/general-principles), substance administration and needle use (https://www.nc3rs.org.uk/news/re-use-needles-indicator-culture-care, https://www.rspca.org.uk/webContent/staticImages/Downloads/AdministrationOfSubstances.pdf and http://www.procedureswithcare.org.uk/ASMS2012.pdf), aseptic technique (http://www.lasa.co.uk/wpcontent/uploads/2017/04/Aseptic-surgery-final.pdf and https://researchanimaltraining.com/articlecategories/aseptic-technique/), and housing (https://www.nc3rs.org.uk/3rs-resources/housing-andhusbandry/rodents).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Staying informed about advances in the 3Rs (Replacement, Reduction, and Refinement) and effectively implementing these advances during the project is crucial for ensuring the highest standards of animal welfare and ethical research. Here are some strategies that will be employed, including using websites, meetings, and scientific conferences related to animal welfare:

- NC3Rs Website: Regularly visiting their website and subscribing to their newsletters can provide updates on the latest advancements, guidelines, and best practices in animal welfare and alternatives to animal testing.

- Scientific Conferences and Seminars: Attending scientific conferences and seminars focused on animal welfare and the 3Rs, organizations like the British Laboratory Animal Science Association (LASA) and the Universities Federation for Animal Welfare (UFAW) often host such events.

- Collaboration and Networking: Engaging in collaborations with other research institutions and laboratories involved in similar research can facilitate the exchange of information and best practices related to the 3Rs. Collaborators may bring new insights and methods for optimizing animal use.

- Internal Training and Education: Organize internal training sessions and workshops for the project team to keep everyone informed about the latest 3Rs principles and techniques. Encourage ongoing education and discussions among team members to ensure everyone is aware of and committed to the latest advances.

- Regular Review of Protocols: Periodically review and update research protocols in response to new 3Rs advances. This ensures that the most ethical and efficient methods are being employed throughout the project's duration.

- Data Sharing: Engage in data sharing and collaboration with other research institutions. Sharing data and experiences regarding 3Rs implementation can help advance the field and promote best practices.

- Continuous Improvement: Foster a culture of continuous improvement within the research team, encouraging members to proactively seek and implement new 3Rs methods and strategies as they become available.

77. Function of brain-wide circuits controlling behaviour

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain circuits, Sensory processing, Movement control, Behaviour, Zebrafish

Animal types	Life stage
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim is to discover how brain-wide neural circuits control and modulate complex sequential behaviour.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The most important and evolutionarily conserved function of the brain is to control behaviour. Although basic neuroscience research has made considerable progress in understanding many aspects of neurobiology, including the cell and molecular biology of individual nerve cells, the most poorly understood aspect of brain physiology is how the 100 billion individual nerve cells that comprise the human brain function together, as a network, to perform the computations that control our actions, emotions and thoughts. This lack of basic scientific knowledge represents a major obstacle to understanding how genetic abnormalities, trauma, degenerative loss of specific cell types, and pharmacological agents affect the functions of neural

networks. In this project we will uncover fundamental principles about how entire brain networks are functionally organized, and how they carry out the computations that control flexible behaviour. Such a “systems-level” understanding of how neural circuits function in the healthy brain is necessary for understanding circuit abnormalities during diseases, and for developing improved diagnostic tools and treatments.

What outputs do you think you will see at the end of this project?

This is discovery research that we anticipate will provide new information about the organisation and operation of brain-wide circuits that control behaviour and in so doing, delineate general principles about brain function that are applicable across vertebrate species from fish to Man. These findings will primarily be disseminated through peer reviewed publications and conference presentations. We will also share our anatomical and physiological datasets and analysis code with the broader scientific community to support the reproducibility of our findings and maximise the scientific insights that can be derived from our work.

Who or what will benefit from these outputs, and how?

In the short term, the research community will benefit from (a) the scientific knowledge we contribute about how brain circuits are organised and how they function and (b) the experimental paradigms, datasets, and analysis tools that we develop, which can be used to explore a broad range of questions about nervous system function.

In the longer term, our findings, experimental assays and tools may guide the development of drugs or other treatments for nervous system disorders, in particular in the context of pharmaceutical and biotech companies using larval zebrafish a model for drug discovery and testing.

How will you look to maximise the outputs of this work?

We will publish all our research findings, including “negative results”, in peer reviewed scientific journals and ensure all research papers are open-access. We will share datasets (anatomy, genetic and neurophysiology data), analysis code and custom instrument designs with the research community. We will also share the genetically modified fish lines that we develop, for example by depositing these lines in international stock centres (ZIRC, EZRC) that serve the global zebrafish research community. We will regularly present our findings at international scientific conferences. We already have a number of active scientific collaborations with both experimentalists and theorists in the UK and internationally and will seek to maintain and expand this network.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 56600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The aim of this project is to understand how circuits that span across the vertebrate brain process information to control behaviour. Larval zebrafish is an ideal model organism for these studies because (a) they possess an archetypal vertebrate brain plan and therefore our findings will be relevant to all vertebrates, including humans; (b) the brain is very small and optically transparent, which allows us to use minimally invasive optical methods to monitor and manipulate nerve cells throughout the entire brain during behaviour. This is not possible in any other vertebrate model system.

Typically, what will be done to an animal used in your project?

Adult fish are used almost exclusively for breeding to generate baby fish (larvae) that are used for experiments.

A typical larval fish will be genetically modified such that its brain cells either emit light when they are active and/or can be controlled using patterned light stimulation. This enables us to monitor brain activity at single-cell resolution using advanced fluorescence microscopy and test hypotheses about circuit control of behaviour by manipulating brain activity patterns.

These approaches require the animal to be immobilised under a microscope and therefore the larval fish is briefly anaesthetised to embed it in gel, but sections of the gel are cut away so the animal can freely move its eye and tail. Restrained animals are presented with sensory stimuli such as visual cues, which encourage specific behaviours such as hunting.

To test hypotheses about the involvement of specific neural pathways, some animals will be treated with drugs, which fish take up directly from the water, or small groups of brain cells will be ablated using a laser or genetic methods.

In some experiments, substances will be injected into the brain, under anaesthesia, to visualise specific nerve cells or pathways.

Typically, experiments take less than 24 hours.

What are the expected impacts and/or adverse effects for the animals during your project?

Imaging of the brain is non-invasive. However, the animals are restrained in gel and they find this mildly stressful for a short period immediately following restraint, as assessed by a brief period of more vigorous swimming. However, behaviour returns to baseline over the course of a few minutes (typically less than 15 min) and restrained fish engage in natural behaviours such as routine swimming, hunting and social interactions with other fish.

In experiments in which we manipulate the brain using drug treatments, or ablate

small numbers of nerve cells, or alter brain activity patterns using light, the animal can experience mild or moderate behavioural alterations. These might last for the duration of the experiment, which is typically less than 6 hours.

In experiments in which we inject substances into the brain, animals are likely to experience mild discomfort following the procedure, similar to that produced by a subcutaneous injection.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For larval animals: 90% mild, 10% moderate.

For breeding and maintenance of GA zebrafish: 90% sub-threshold, 10% mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project aims to uncover how complete neural circuits in the vertebrate brain process sensory and contextual information to generate and modulate complex behaviour. The structure and function of brain circuits and the principles of their operation are still very poorly understood and therefore discovery research of this nature necessitates experiments using intact, behaving animals.

Which non-animal alternatives did you consider for use in this project?

We considered (i) existing neurophysiology datasets from published larval zebrafish studies, (ii) in vitro datasets and experimental approaches, (iii) computational modelling.

Why were they not suitable?

Although a limited number of larval zebrafish neurophysiology datasets are available, these are currently not suitable for addressing our research questions. Principally, this is due to limitations in the experimental design and/or quality or breadth of the recorded data. We do however make extensive use of community brain atlases (e.g. ZBB and mapzebrain) to evaluate neuroanatomy and gene expression patterns, which means we do not need to duplicate such experiments in our own lab.

Although certain specific aspects of nervous system function can be investigated

using in vitro preparations and/or using computational models, these methods are substantially limited in both scope (network size and complexity) and fidelity (there are many properties of nervous system function that we still do not understand). We do however make extensive use of computational modelling to interpret neural activity/connectivity data and formulate and constrain hypotheses that we can then test experimentally. We also stay well-informed about work within in vitro systems that can guide certain aspects of our work.

Overall, the size and complexity of the brain-wide circuits that control behaviour fundamentally requires us to study circuit operation in intact, behaving animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The breeding of zebrafish has been well-established for more than 25 years. I have estimated the number of animals that will need to be bred to adulthood based on the number of distinct genetically modified lines that are needed for this project, the breeding cycle that is required to maintain healthy fish, and the experience of my lab over the last 10 years. Thus, to maintain 100 genetically distinct lines for 5 generations with a typical group size of 50 fish will necessitate 25,000 adults in total for breeding and maintaining all lines over the next 5 years.

Almost all experimental procedures will use zebrafish at larval/juvenile stages (5 to 21 days old). We examine behaviour in freely swimming animals in response to a variety of naturalistic stimuli to discover rules by which specific patterns of sensory information are converted by the brain to behaviour. We also use drugs and advanced optical and genetic means to manipulate brain circuits in intact animals to observe how behaviour changes. To obtain statistically robust data we need a group size of around 30 animals per experimental condition and this equates to 20,000 larvae in total over the course of 5 years.

In addition, we do a variety of experiments using tethered larvae where we can both monitor their behaviour as well as using microscopes to observe, and manipulate, brain activity at very high resolution. These experiments will reveal the structure and operation of the brain circuits that control behaviour and will require 10,000 animals in total over 5 years.

Finally, to test hypotheses about the roles of specific brain cells, we will need to ablate these cells using either a laser or genetic method and then observe how brain activity and behaviour change. These experiments will require 1000 animals in total over 5 years.

Thus, in total we will require 56,600 animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A key feature of our experimental design is that it we collect comprehensive, multimodal data about the structure and function of brain circuits from individual animals. This allows us both to do better science but also reduces animal numbers. For example, our “whole-brain” imaging capability enables us to collect data from thousands of nerve cells across the entire brain during a single procedure in one animal. This allows us to discover complex relationships between different cell populations as well as reducing the number of animals needed to survey activity across all brain regions. Furthermore, using appropriate combinations of transgenes, we can both monitor and manipulate brain activity in the same animals and inter-relate circuit physiology with neuroanatomy. By measuring these different aspects of brain structure and function using a small number of refined procedures, we can obtain statistically robust results using fewer animals.

For certain experiments, we make use of the NC3Rs Experimental Design Assistant (eda.nc3rs.org.uk) to ensure our experiments are well designed, confounding factors are minimised, and statistically robust results will be obtained. For instance, when we are assessing the effects of a pharmacological treatment, animals from the same clutches are randomly assigned to treatment groups and the experimenter is "blinded" to the treatment to reduce the influence of genetic background confounding variables and bias, respectively. Where relevant, we first perform pilot experiments and statistical tests known as power calculations to help ensure we use the correct number of animals to obtain statistically robust data, whilst minimizing animal numbers. We exploit the power of “paired statistics” wherever possible; for instance, individual animals (and even individual neurons) are measured both before and after specific experimental manipulations. This helps account for variability between animals to provide a better measure of the effects of treatments. For experiments in which we are testing the effects of multiple independent variables we would typically use a factorial design and analyse the data using an N-way ANOVA or linear mixed effects model with two-tailed significance thresholds.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For some types of experiment, for example where we are testing the effect of a drug for which limited dosing data exists in zebrafish, we will refer to data collected using in vitro systems or other vertebrate species and conduct pilot studies to allow us to optimise drug doses and animal numbers.

We make extensive use of computational modelling and advanced statistical approaches that can enable us to identify principles of circuit function using complex, high-dimensional data from fewer animals.

In terms of maintaining genetically altered fish lines, our facility has developed procedures that enable us to maintain colonies with excellent standards of welfare and the smallest possible numbers of breeding adults. We also cryopreserve lines where possible to reduce the number of live fish.

Clutches of eggs are usually much larger than required for experimental testing. To reduce the number of protected animals used, we will only grow to free-feeding larval stages the number required for experimentation or line maintainance. This is facilitated by screening for genetic alterations at embryonic stages.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Larval zebrafish is ideally suited for our research question, enabling us monitor and manipulate the activity of entire brain circuits, at cellular resolution, during behavior. This is currently impossible in any other vertebrate model organism.

Because larval zebrafish are very small and transparent, we are able to use minimally invasive optical methods to probe the function of brain circuits. This is associated with less pain or discomfort than invasive electrophysiological methods or equivalent optical approaches in rodent models (involving implanted windows/lenses/fibres).

In some experiments the larval zebrafish must be partially restrained to allow us to examine the brain under the microscope. To minimise stress, we have refined this procedure by briefly anaesthetising the animal and the gel mount itself causes no damage or lasting harm to the fish: they display naturalistic behaviours whilst tethered and will continue to do so for at least 72 h and larvae can be subsequently released from the gel and grown to maturity with no obvious physical or behavioural defects.

In experiments in which we use a laser to ablate cells, this is performed under general anaesthesia. Using a laser to ablate small numbers of precisely targeted cells represents a substantial refinement as compared to more invasive and less precise mechanical ablations. We typically observe very specific behavioural changes following such ablations and the vast majority of the larval behavioural repertoire is unaltered, suggesting the larvae suffer little if any discomfort or distress.

Why can’t you use animals that are less sentient?

Zebrafish likely represent a less sentient species than `higher` vertebrates such as rodents, cats or macaques, which are traditionally used for systems neuroscience. Moreover, the vast majority of our experiments will be performed at embryonic/larval stages. Zebrafish are also the least evolutionarily complex vertebrate model organism in common research use and yet offer numerous advantages for understanding vertebrate brain function. Because we want to understand the

principles of the vertebrate brain we cannot use simpler laboratory model organisms such worms because these are not vertebrates.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During experiments, animals are continuously tracked with a video camera on computers that we can monitor for adverse outcomes. Any behavioural indications of adverse responses are therefore readily detectable, allowing for immediate intervention or termination of the experiment.

For breeding of genetically modified animals, we will investigate less invasive procedures for obtaining a DNA sample for screening. This is currently taken by fin clip, which regenerates in fish, but there are other methods to obtain swabs that are under development and we will test and take on board such procedures if they prove reliable.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the 3Rs as elaborated by the NC3Rs and the Laboratory Animal Science Association (LASA) guidelines, make use of the NC3Rs Experimental Design Assistant (https://eda.nc3rs.org.uk/) and refer to published best practice guidelines including Alestrom et al, 2019 `Zebrafish: Housing and husbandry recommendations` (https://journals.sagepub.com/doi/10.1177/0023677219869037) and RSPCA Guidance on the housing and care of Zebrafish Danio rerio (tinyurl.com/4vv5vu4j).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Members of our zebrafish community attend conferences with a 3Rs focus and we consult resources including Norecopa (https://norecopa.no/), the ZFIN zebrafish protocols database (https://zfin.atlassian.net/wiki/spaces/prot/overview) and publications related to fish welfare (https://mdpi-res.com/bookfiles/book/1802/Welfare_of_Cultured_and_Experimental_Fishes.pdf).

Our Aquatics "Animal Welfare and Ethical Review Body" and zebrafish user group are excellent forums for sharing best practices as well as new 3Rs advances. Our fish facility staff also provide practical guidance on how best to implement refinements.

78. The impact of tumour heterogeneity on disease spread and response to therapy.

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cancer, heterogeneity, metastasis, therapy, vasculogenic mimicry

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overarching aim of this project is to understand how different types of tumour cells interact with the normal cells of the body. How these influence spread of the tumour from where it started (the primary site) to other organs within the body (metastatic sites). We are particularly interested in how these interactions can effect response to therapy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Over the last decade it has become increasingly clear that tumours are very complex and made up of lots of different kinds of cells, including cancer cells and normal cells of the body. These different types of cells communicate with each other to influence how the tumour grows and spreads from where it originally formed (primary site) to other organs within the body, a process called metastasis, which is responsible for around 90% of cancer-related deaths. Despite having developed many therapies that

slow the growth of primary tumours, as a field, we have a poor understanding of whether these therapies also stall the growth of metastases or whether in some cases they are ineffective or cause increased growth of metastases. Emerging evidence suggests that immunotherapies, which activate the body's own protective systems to attack the cancer cells, often fail because of growth of new metastases, suggesting that the location of the tumour cells may play a role in treatment response. It is therefore important to understand how tumour cells behave differently when growing or undergoing treatment, in different parts of the body, so we can help improve therapies for patients with metastatic growths.

What outputs do you think you will see at the end of this project?

The immediate outputs of the work will be a greater understanding of how differences within a tumour (tumour heterogeneity), ie. different cell types, impacts growth at different sites, and how they respond to therapy. This work will likely result in the publication of our results and providing new information to other research groups.

Who or what will benefit from these outputs, and how?

In the short-term, the scientific community will benefit from a greater understanding of how tumour heterogeneity influences therapy response and will potentially benefit from being able to further analyse the large-scale datasets that we will generate as part of this project. Ultimately, in the long- term (beyond the end of the project) we hope that our findings will influence clinical practice and positively benefit patients.

How will you look to maximise the outputs of this work?

We will maximise the outputs not only through publications, but also, when the opportunity is available, through conference presentations and discussions with potential collaborators. We actively engage with collaborators who want to use our approaches/technologies in a very hands-on fashion and will continue to do so for this project.

Species and numbers of animals expected to be used

- Mice: 3995

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the least sentient, and most understood (in terms of what we know already of how their tumours behave and how we can use the tools that we have developed) animals that represent mammalian biology. They allow us to recapitulate human disease in a way that other, less sentient, animals cannot do. We have many years of experience working on breast cancer in this animal model and have developed

tools and strategies that are most suited to mice. We will work primarily with adult mice. Adult mice are more tolerant of the methods we wish to use, and they are more similar in life stage to humans who develop cancer than juvenile animals.

Typically, what will be done to an animal used in your project?

Most animals will be implanted with tumour cells via a needle injection through the skin into the mammary tissue area (known as the fatpad) or in the same area via a small incision of the skin (a surgical procedure), this will be done under anaesthesia. For metastases experiments the 'primary' mammary tumour may be surgically removed while small (typically ~5mm), enabling the mouse to form established metastatic tumours - where cells leave the original site of injection and travel throughout other areas of the body (which will initiate slower than the growth of the primary tumour). The animal may receive therapeutic treatment once a palpable tumour is identified, typically via intraperitoneal (into the abdomen) injection 2-3 times per week, or in their drinking water, and this would continue for the duration of the experiment. The animal may undergo imaging under anaesthesia to track the tumour cells thoughout the body. The animal will be killed, anticipated prior to reaching the humane endpoint, at the end of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will experience brief disorientation following any procedure under anaesthetic lasting a few minutes on waking. Mice may experience a short-lived discomfort following any injection through the skin (e.g. tumour cells into the mammary fatpad) however this is not noticeable in our experience and typically does not require any analgesic. For surgical procedures the discomfort and pain may last 1-2 days however with analgesic this can be mitigated.

One adverse effect we have experienced is the development of skin defects at the site of tumour growth, for example ulcers. This is more likely to occur when injections are carried out directly through the skin (as opposed to via a surgical opening of the skin). However, in most cases these are mild and do not seem the cause the animal any pain or discomfort, this will typically be seen towards the later stages of the experiment.

Mice receiving therapy may experience some transient (<7 days) weight loss which can be often ameliorated through increased fluid uptake, ie. gel food. Mice with overt metastases, may show early clinical signs of weight loss and decreased overall body score, depending on severity this is likely to last no more than 24 hours as here the animals will have reached the humane end point of the experiment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild 20%
Moderate 80%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cancer is a complex disease that develops in the context of a whole organism. It is now widely appreciated that interactions between the tumour and the “normal” cells of the host, the so-called tumour microenvironment (TME) influence tumour evolution, metastases and patient outcomes. We are particularly interested in these tumour-TME interactions which are poorly modelled with cells in a culture dish. In particular, metastasis is a systemic phenomenon which cannot be truly studied in cell culture system.

Which non-animal alternatives did you consider for use in this project?

We have considered the use of cell co-culture assays in a dish, which we will indeed use to support our findings in animals, but given the complexity of multi-cellular interactions this reductionist approach can only be useful once a specific interaction has been identified in the setting of a whole animal. We will aim to carry out in vitro assays wherever scientifically feasible.

Why were they not suitable?

They are suitable in some instances but mostly in support of findings from animal models. Cell culture approaches allow us to assess specific interactions and manipulate the system but are irrelevant if the same phenomena don’t occur in an animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have based our estimates on the type of measurements we are making in a given experiment i.e. tumour growth or gene expression changes, and the inherent variability within those measures from previous experiments. We have then made calculations based on our previous experience as to numbers of replicate animals

necessary to achieve statistical significance for a specific effect size. For newer types of experiments where we don't have a good expectation for the necessary sample sizes, we will perform pilot experiments and then revise sample sizes from those measurements. To estimate numbers of GAA animals we have used the Jackson Lab breeding colony size planning tool (https://www.jax.org/-/media/jaxweb/files/jax-mice-and- services/colonysizewksht.pdf?la=en&hash=8C1CB11B342A9E8177B31DD2D7A7BFC6414628AC) which estimates we need 780 animals to achieve 240 experimental animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We design our experiments such that we get a wealth of information from each animal by using data rich methods such as single cell RNA-seq. We also propose to use multiple different kinds of analyses on tissue generated from one animal. In addition, we use the results from one experiment to help reduce numbers in the following experiments. We have followed the best available guidelines on experimental design, including these listed on the NC3R website. We screened the existing literature to inform ourselves on the best standards currently applied in terms of sample size determination.

Wherever possible, we compare multiple treatments to the same control, reducing the number of control animals required. Importantly we also use our prior experience for a given experiment to help guide the above criteria.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will make use of pilot studies for some of the newer areas of research such that we can identify levels of variability between animals for different measurements. These studies will help us optimize our animal usage. We will share rich datasets with the community and frequently share tissue to maximise usage. We will also follow the advice from our experienced animal facility to ensure efficient breeding strategies where required and will seek the advice of a statistician.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 primarily be using mouse models, in particular transplantable models of breast cancer, and in some cases lung cancer, in which tumour cells derived from a mouse with cancer are grown in culture and then implanted back into mice that are genetically identical to those in which the tumour first developed. These models are

ideally suited to studying aggressive tumours and their response to therapy in an animal with an intact immune system. In most instances we implant tumour cells by directly injecting them through the skin into the fat tissue surrounding the mammary gland (the mammary fatpad) (in the case of breast cancers) avoiding unnecessary surgery. Where surgery is required, we have optimised procedures to ensure the minimal area of open/cut skin, thus allowing a much more rapid healing process. We aim to use the least invasive methods when delivering drugs to the animals to avoid undue distress.

Why can’t you use animals that are less sentient?

Studies on metastasis in particular require a whole animal to study and adult mice most resemble the life stage of humans that develop the cancer types that we study, primarily breast and lung cancers. We endeavour to use terminally anaesthetised animals where possible and we use this approach in particular when introducing fluorescent tracers to the blood stream.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are constantly monitoring ways to refine our procedures. We have previously instituted some procedures under terminal anaesthesia to minimise harms. Likewise, we have previously optimised treatment regimens that retain the desired effects while minimising toxicity.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow published best practice guidelines including (1) Guidelines for the welfare and use of animals in cancer research. P Workman et al; British Journal of Cancer (2010) 102, 1555-1577. (2) Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. Laboratory Animals (2001) 35, 1-41 (3) the LASA guiding principles for preparing for and undertaking aseptic surgery 2017.

Additionally, our establishment has a series of guidelines regulating many of the procedures routinely done to animals, for instance how often an animal can receive an injection, where, and how much, or how often it can give a blood sample. We will follow all of these, as well as several other standard operating procedures that were developed by a team of specialists at our establishment for the explicit purpose of minimizing animal suffering and are periodically updated. While designing experiments we will follow a series of guidelines existing in the literature to design and report our experiments, and will consult a biostatistician to ensure that we’re using as few animals as possible for our studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our establishment routinely circulate advances in the 3Rs and we will always seek to identify ways these can be incorporated in our project, while ensuring they do not

affect the (statistical/ biologically relevant) consistency of our data collection.

79. Examining new ways to understand and treat dementia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Dementia, Alzheimer's disease, Memory, Inflammation, Renin-angiotensin system

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand what goes wrong in dementia and to test ways to improve the disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Dementia is a syndrome (a group of related symptoms) associated with an ongoing decline of the brain and its ability to function. The main symptoms associated with dementia are memory loss and problems with language and understanding, but also many other changes in behaviour are seen in people with this condition. One in three people over the age of 65 will develop dementia and currently there are almost 1 million individuals in the UK affected. The most common cause of dementia is

Alzheimer's disease. The annual costs incurred by UK society associated with dementia are over £25 billion. Currently there is no cure for this condition or drugs that can significantly slow progression.

Therefore, dementia represents a significant medical and social problem. If an appropriate treatment or a cure is not found, it is predicted that 1.4 million people in the UK will have the condition by 2050.

What outputs do you think you will see at the end of this project?

We will achieve a greater understanding of the biological events that underlie dementia. By doing this, we also hope to identify new treatments for dementia and show that they can reduce the symptoms of the disease.

These findings will be widely disseminated through publishing scientific papers and participation in conferences. We will make our data available to others to use in their studies.

Who or what will benefit from these outputs, and how?

The main benefits of this research will be the generation of new knowledge on what happens during dementia, and identifying new biological processes and biomarkers. We also hope to determine if we can alleviate the symptoms of this disease with new treatments. In the short term, our research will benefit other researchers, the pharmaceutical industry, and clinicians studying the development of dementia and Alzheimer's disease. In the long term, we hope that our research will help guide the development of future therapies and ultimately could provide the basis for new clinical trials for people with dementia and Alzheimer's disease.

How will you look to maximise the outputs of this work?

We will publish our findings from these studies in respected, open access journals, present our data at leading national and international conferences, and utilise pre-print servers to maximise the dissemination of our research. We collaborate with other establishments, to assist with identification and development/repurposing of effective treatments.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mouse models of dementia including those that are genetically altered and compare these to control mice (without the disease). These mice will be bred in-

house and used as adults. Dementia predominantly affects the elderly, so we will need to allow the mice to age so they start to develop symptoms. Memory loss is one of the predominant symptoms in people with dementia and we can effectively measure memory in mice. In people with dementia one of the first problems they have is with recalling recent experiences and the mouse models of this condition also have similar issues that we can measures using specific tests. As in some studies we are aiming to test if treatments can reverse the symptoms of the disease, we therefore need these symptoms to be present before we start to treat the mice.

Typically, what will be done to an animal used in your project?

Mice will be bred and allowed to age until approximately 9-12 months to allow for their symptoms (e.g. memory loss) to develop. During this time blood might be taken and their behaviour (e.g. testing memory) assessed using tests that do not cause any harm or distress. For example, one test involves using the animals' innate curiosity to explore novel things. In this test, the mice will be placed in an arena containing two identical objects and the time they spend exploring the objects will be measured. The mice are then removed for a short period of time and then placed back in the arena where one of the objects has been replaced with a new one. Mice with a good memory will spend more time exploring the new object and those with poorer memory will not. Mice will be culled at various time points and tissues (such as the brain) taken to identify what changes are happening. In some studies, mice will be given treatments to try to reduce their symptoms and, the usual way we will do this is by giving them drugs in their diet, especially if we are going to treat for a number of weeks. However, in some experiments we might give drugs by injection for a short period of time. At the end of all experiments, animals will be humanely killed, and blood and tissues taken to assess modulator/mediators and pathological changes in more detail.

What are the expected impacts and/or adverse effects for the animals during your project?

The mice that we will use develop the major symptom of dementia and Alzheimer's disease specifically memory loss. These memory changes only become apparent when measuring their performance in specific tests. Memory loss is one of the first symptoms to be detected in these mouse models (similar to humans). Otherwise, mice appear healthy and develop/grow the same as the control mice for time period we propose to keep them for. We do therefore not expect any major 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)?

Sub-threshold/Mild (70%)
Moderate (30%)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice will be used in this project. Studying the mechanisms involved in dementia and Alzheimer's disease is extremely complex and involves understanding the interactions between several physiological systems (e.g. nervous, immune, and vascular). It is very difficult to mimic such complex interactions ex vivo, and whole animal in vivo experimentation is therefore vital in order to obtain a greater understanding. In addition to the pathological changes in the brains of Alzheimer's disease patients, the disease is characterised by deficits in learning and memory, and as such this behaviour is very difficult to model in vitro or assess in lower species (e.g. zebrafish). In addition, in vitro experiments do not allow fully the study of interactions between different body systems (i.e. immune and vascular), which are critical for this project. Thus, the questions and hypotheses to be addressed cannot be fully studied in vitro alone and require in vivo studies.

Which non-animal alternatives did you consider for use in this project?

We have considered using non-animal alternatives (in vitro models) that we routinely use in our work including culture systems using human induced pluripotent stem cell (iPSC)-derived neurons and cerebral organoids. We will use data from these in vitro models to help us design our experiments using animals, and therefore help to reduce animal numbers.

Why were they not suitable?

While these non-animal alternatives will allow us to study some aspects of the disease (e.g. amyloid beta accumulation; an abnormal protein produced in the brains of people with dementia) they not allow us to assess memory loss and whether we can improve this with interventions and we will therefore need to use animals for this part of our work. However, if any relevant non-animal alternatives become available during the project, we will implement these in our studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used our previous experience of performing experiments of this type to help us decide the best way to answer the questions we are asking while using the

minimum number of animals. For example, estimates of animal use is based on i) previous work and experience using the relevant methodologies, the parameters to be studied, and specific mouse models; ii) the scope and objectives of the current project; and iii) careful consideration of experimental design.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Several factors lead to a reduction of animal numbers, including reducing variation (e.g. keeping the environment consistent), good experimental design (including the use of the NC3R's Experimental Design Assistant) and the use of appropriate statistics. In particular, statistical tests will be used to ensure that we use the minimum number of animals possible to reliably interpret our data, and also so we can refine our questions to then design the most informative experiments. Whenever we get new data, we will always re-do our calculations in order to make sure we are still using an appropriate animal number to achieve our aims.

We will also assess memory over time in the same group of animals thus eliminating the need for separate groups and reducing animal numbers.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will minimise the number of animals bred by using efficient breeding strategies and by using mice of both sexes. Most of the mouse models of dementia and Alzheimer's disease will be used as heterozygous, meaning they have only one copy of the altered gene (+/-). We will therefore breed heterozygous (+/-) mice with control (-/-) mice and all the offspring produced (50% +/- and 50% -/-) will be used. In the rare event that animals will not enter a protocol, tissue (e.g. brain) will be taken for use in other studies. In addition, we usually take several tissues from the animals at the end of the experiments for multiple analyses (and sharing of tissue), which often leads to additional scientific questions. Whenever we perform analyses that leads to a large quantity of data (such as RNA-seq to analyse all genes) we will make our data freely available to other groups so they can analyse it to answer their own research questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Models: There are no in vitro alternatives to study the pathological and behavioural changes that occur in Alzheimer's disease and dementia. Transgenic rodent models are currently the most relevant models available to study Alzheimer's disease and

dementia (e.g. APPswe/PS1ΔE9 mice and APP23) or models of accelerated aging (e.g. SAMP8) and no surgery or injections are required to induce the disease. These mice naturally present with memory deficits and pathological changes in their brains over time, but otherwise they are healthy.

Measurements: For longer term studies, animals will be monitored over time for well-being (e.g. body weight checked) and they will be housed with other animals with enrichment. Animals might also undergo a series of behavioural tests. These tests are well described in the literature, and we have experience that these do not cause any lasting harm or distress for the animals. For example, we use tests that rely on natural behaviours in rodents (e.g. exploration), and we will not use any adverse stimuli or food restriction. However, throughout the project we will review the literature and engage with colleagues/collaborators to learn of any new refinements to the protocols that could be implemented.

For administration of substances over time we will use the least stressful method where possible such as administering agents in the diet. Studies will stop as soon as we see a relevant effect (e.g. improvement or reduction in memory).

In some studies, we will require a cardiac blood sample followed by perfusion with fixative in order to best preserve the tissue for subsequent analysis, and this will be done under terminal anaesthesia.

Why can’t you use animals that are less sentient?

Our objectives cannot be fully achieved using less sentient animals (such as fish/insects) or with very young (neonate) mice mainly due to the time taken for the disease to develop differences in terms of the pathology and cognitive changes, and adult mice are therefore needed. We have considered lower species (e.g. zebrafish) and where appropriate these could be used (e.g. injection of amyloid beta into the brain). However, zebrafish do not always show such similarities to humans and methods to assess memory and cognition have to be performed in adult fish (so also needs Home Office Licence approval). These methods (e.g. to assess memory) in zebrafish are not as well established and/or reliable as in rodents, but we will continually review this during the course of this licence.

As most of our studies involve keeping animals for months and assessing cognition, terminal anaesthesia cannot be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Most of our studies will involve long-term maintenance of animals with intermittent behavioural assessments and sometimes the mice will be treated with agents that we hope will improve outcome. The mouse models of dementia we will usually use have already been well characterised and we know when memory deficits should be seen. We will therefore be able to plan our experiments appropriately and use animals as early as possible. If we see a desired effect of any substance at an earlier time point than anticipated, we will complete the study earlier than planned. For our behavioural studies we will not use any aversive stimuli and will use tests that assess the animals’ natural behaviour.

For all studies and at all times, mice will be monitored frequently, handled appropriately by trained researchers (e.g. using tube handling for movement in and out of cages) and suitable home cage enrichment will be used.

In a minority of studies a small device (osmotic mini-pump) might be implanted under the skin of the mouse in order to deliver drugs that we hope will reduce the symptoms of dementia. In all cases appropriate post-operative care and pain management will be used in the short-term as this is a relatively mild procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will stay up to date with the literature, publications and recommendations from the most appropriate bodies such as the NC3Rs and LASA, as well being informed from communication with the NVS and NACWO and developments within the scientific community in general. We will follow the PREPARE guidelines (https://norecopa.no/prepare) for all our experimental work and design experiments so we can use ARRIVE guidelines (https://arriveguidelines.org/) for publications. In addition, prior to any animal studies we will prepare and submit a full experimental study plan to our animal unit to ensure all studies are carried out in line with best practices.

For refinements involving injections we refer to https://researchanimaltraining.com/articles/an- introduction-to-the-administration-of-substances/, and/or Morton et al 2001 "Refining procedures for the administration of substances" in Laboratory Animals (2001) 35, 1-41 and/or Turner et al 2011 “Administration of substances to laboratory animals: Routes of administration and factors to consider”, J Am Assoc Lab Anim Sci 50(5):600-613.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will at all times aim to implement any advances in techniques that adhere to the 3Rs and improve the welfare of the animals.

We will stay up to date of any advances through, for example:

-NC3Rs literature/newsletters and recommendations

-Establishment newsletters and seminars

-Discussions with colleagues

-Scientific literature

-Discussions with the NACWO

80. The influence of altered coagulation on wound healing

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Wound healing, Coagulation, Cardiovascular disease, Clot structure

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Wound healing complications (including non-healing and chronic wound formation) affect millions of people worldwide and can result in death, limb amputation and disfigurement. To date there remains little in the way of effective treatment strategies to target chronic wounds and scarring. Consequently, there is an urgent unmet need to develop such treatment strategies and reduce the disease burden on society and healthcare systems. Our overall aim is to define how alterations in coagulation contribute to, or impair, healing of skin wounds in healthy and diseased states and how this is influencing repair outcome. Through this understanding we will develop novel therapies to help improve skin repair using small molecule approaches or other biological agents with clinical potential that target the identified alterations.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Impaired wound healing is a major and increasing clinical burden as a result of an aging population and increased prevalence of risk factors including diabetes and obesity. In the UK alone, more than a million patients (6.5 million in the USA) suffer from non-healing wounds each year, with treatment costing the NHS around £3.2 billion ($25 billion in USA). These wounds are debilitating (physically and psychologically), have a high risk of infection, are the commonest cause of limb amputation and have a 5-year mortality rate of 45% (equivalent to colon cancer). Despite the profound socio-economic impact of wounds, they have long been under-researched, making the development of a treatment challenging. As we do not understand the mechanisms behind chronic wound formation, intervention only tends to happen once the wound is well established (after 4 weeks of non-healing).

Clot formation occurs in the first phase of healing, but the role of the clot in the healing process has been shown to extend far beyond the cessation of bleeding. However, the influence of changes to coagulation on healing has not yet been explored. Changes to the initial clot in a wound may compromise tissue repair from the first phase. Understanding the role of the clot in healing may provide new therapeutic targets at a much earlier stage in the healing process.

What outputs do you think you will see at the end of this project?

Our research objectives, focussed on clot architecture in wound healing, will lead to an improved understanding of:

- The role of the blood clot in healthy wound healing
- The role of the blood clot in non-healing wounds
- How alterations in coagulation seen in disease contribute to development of non-healing wounds
- How modulating the blood clot in the wound can improve healing

Together, this new understanding will elucidate processes in coagulation that contribute to healthy healing and non-healing and highlight new areas to target for therapeutic intervention.

This information will be disseminated through a minimum of 3 research papers from the studies described in this project licence to high-impact, peer-reviewed scientific journals.

Who or what will benefit from these outputs, and how?

The most significant short-term benefits of this programme of research will be the new knowledge disseminated to the scientific community of how changes in the components, structure, and breakdown of blood clots in a wound contribute to the disruption of tissue repair. This will later (medium-term to long term) translate into the identification of new key contributors to impaired healing and the identification of new therapeutic targets for the earlier treatment of non-healing wounds aiding the clinical

community and patients. For example, we will provide an improved understanding of how alterations to the blood clot that is formed in a wound influences the recruitment of immune cells to the site of injury and how this has downstream influences on repair outcome. This will not only provide insight into the mechanisms underpinning normal healing of skin or tissue, but also uncover new mechanisms that contribute to the derailing of the healing process which often results in either chronic or non-healing wounds. This new information will help us determine how we might clinically modulate the clot or other aspects of the wound to enhance beneficial aspects and dampen the components that impair healing. In the long-term this will lead to the development of novel therapeutics to treat these conditions at earlier time points which could then have far reaching social and economic consequences. It is also possible that detailed characterisation of blood clot alterations that influence the healing process could lead to the identification of specific markers that are predictive of failure to heal, providing prognostic information or alternatively diagnostic information that clinicians can use to inform treatment approach.

How will you look to maximise the outputs of this work?

We will look to maximise the output of this work by collaborating with colleagues who are already investigating the impact of blood clots on wound healing and the initiation and resolution of inflammation in wounds. This will potentially lead to the development of new techniques and models which would help us all with our research goals.

In addition, we will also look to disseminate new knowledge through presentations at scientific conferences and publication of successful and unsuccessful approaches. We will also communicate outputs to the lay audience as appropriate through press releases.

Species and numbers of animals expected to be used

- Mice: We estimate we will use approximately 4000 mice in the lifetime of this license. We estimate using 1000 of this total for breeding and maintenance.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the most appropriate species to use to address our research goals for the following reasons:

Mice are often chosen for wound healing studies because their bodies are quite similar to ours in terms of how they function, their immune system, and their overall structure. The wound model we have chosen is widely recognised and studied in scientific articles and mimics how human skin repairs itself, making it a useful model for research. Mice have a well-explored immune system that resembles ours, with similar types of cells. Additionally, in this model repair can be impaired or prevented,

offering the ability to study chronic wound formation. This kind of research is challenging to conduct in simpler animals like zebrafish or flies because their immune systems and anatomy are quite different from ours.

We have many tools specifically designed for studying mice available through collaborators or commercially that help us investigate and achieve our research goals. These tools include things like specially modified mice, antibodies, and substances that can block certain processes. Unfortunately, similar tools aren't readily available for studying pigs.

For our study on wound healing, we're going to use mice that are 7-12 weeks old. We've chosen this age range because at this time, they are in a phase where their hair cycle is at rest (telogen). Wounds heal faster when mice are injured during the active hair growth phase (anagen), but we want to avoid the impact of hair follicles on the healing process.

To understand the role of blood clots in healing, we'll either change the clotting process by giving mice certain substances or use mice that have specific genetic changes in their clotting ability. We have access to these genetically altered mice through licenses at our institution. These include mice with changes to proteins or cells in the blood. In addition, we want to see how changes in blood clotting affect wound healing in more complicated situations that often lead to slower healing. Therefore, we'll use three models of type 2 diabetes: which naturally develop diabetes, a model for obesity-induced diabetes, and mice injected with a chemical which damages the pancreas, combined with a high-fat diet.

The mice which naturally develop diabetes reflect many aspects of humans with Type II diabetes including delayed healing of wounds. Many researchers use these mice because their characteristics closely resemble those of humans with Type II diabetes, making the studies highly relevant to real-life situations.

The murine model for obesity-induced diabetes simulates human obesity-induced Type 2 diabetes and metabolic syndrome. These mice become diabetic between 10-12 weeks on a high fat diet (10-11% fat), with greater than 85% of mice diabetic by 18 weeks. Unlike other mice models induced by obesity, these mice don't overeat or have high levels of stress hormones, making them a more realistic model for human Type II diabetes. They are also easier to breed. These mice develop only a moderate level of obesity but develop higher blood sugar levels at earlier time points than observed in other obesity induced models.

Lastly, we will use a third model of diabetes where mice are given a low dose (that does not induce diabetes in mice on a normal diet) of a substance called streptozotocin (STZ) along with a high-fat diet. This combination induces insulin resistance (a feature of Type 2 diabetes) and high blood sugar levels. This model will be used as an alternative to the obesity-induced diabetes mice as it has a number of previously described changes to its coagulation. This will help us understand how certain changes in blood clotting contribute to poor healing in conditions similar to Type 2 diabetes.

Typically, what will be done to an animal used in your project?

We will start by altering coagulation in mice. This may be through mice with

genetically altered coagulation (obtained from other licences authorised to breed these mice) or by altering coagulation through injection or minipump altering levels of specific proteins or increasing inflammation in the blood. Additionally, diabetes may be induced which is known to alter coagulation (db/db mice, GA mice with HFD or low dose streptozotocin injections into C57BL/6 mice combined with HFD). This will help us to discover new disease mechanisms that may help to develop new treatments for non-healing wounds.

These mice will then be transferred to the protocol used for creating the wounds, which involves creating two or four wounds on the back of the mice using a 4-6 mm punch biopsy tools. The procedure is carried out under general anaesthesia and perioperative pain relief is given as routine. Further analgesia may be provided if indicated by our animal welfare score sheet. This procedure normally lasts no longer than 30 minutes. Follow up wound evaluations, including both macroscopic and optical imaging would typically take place under general anaesthesia 1-3 times a week in sessions lasting less than 30 minutes. Wound size and stage of healing will be assessed and optionally, substances applied to promote or delay healing. Typical studies are expected to last 21 days following wounding, with a maximum follow up period of 6 weeks. At completion of the study, animals will be killed by a schedule 1 method and tissues collected for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Based on our own studies using similar protocols, and the advice of other researchers with over 20 years of experience in wounding models we believe the protocols to be followed in this project to be very well tolerated.

The adverse effects we expect to observe in animals following protocols in this project are: localised wound pain, diabetes and its complications and weight loss.

Post-surgical pain

Animals may experience transient discomfort after surgery which is usually mild and self-limiting. Animals will be wounded under general anaesthesia, and will be given appropriate levels of pain- relieving drugs at the time of surgery (and thereafter) in order to reduce post-surgical discomfort. Animals will be monitored regularly for the development of adverse effects and any animal found to be displaying signs of distress or discomfort, that does not respond to remedial actions (as advised by the NVS), will be killed by a schedule 1 method. A specific score sheet has been developed and included within the relevant steps to aid in the monitoring for pain post-wounding.

Diabetes

We will use three models of type 2 diabetes. Db/db mice which have a mutation in the leptin receptor leading to the development of a diabetic phenotype (high body weight, reduced glucose tolerance, hyperinsulinemia, and changes in serum lipids). Whilst the db/db mice used in this protocol have the potential to exhibit a progressive

harmful diabetic phenotype we will ensure that all db/db mice are used under 7 months of age to limit severity.

A genetic model (NONcNZO10/LtJ) of type 2 diabetes that develops when male mice are weaned on to a 10-11% fat diet and results in the development of hyperglycaemia by eight weeks of age. The breeding of these genetically modified mice should not incur any adverse effects unless a HFD is provided.

We will also use a second model of type 2 diabetes using low dose streptozotocin-injected C57BL/6 mice (commercially sourced) combined with HFD. HFD leads to insulin resistance and low dose streptozotocin is known to induce a mild impairment of insulin secretion which combined replicate important features of the later stage of type 2 diabetes. High doses of streptozotocin have been shown to cause acute kidney damage in animals due to non-specific cytotoxicity. However, we will only use multiple injections of low dose streptozotocin which greatly reduces the development of any adverse effects. At low doses streptozotocin use can result in weight loss.

Diabetogenic/obesogenic diets lead to an increase in body weight and oily coat condition which is not harmful. Animals which develop diabetes may exhibit increased drinking and urination, poor thermoregulation, and increased propensity towards infection. Murine models of diabetes are also associated with delayed wound healing and wound erosion.

Other murine strains

Mice with altered coagulation obtained from other licences (Factor XIII (FXIII-/-, FXIII-L34V) or fibrinogen (FGA-/-, FGG3X, FGA4X, FGN7X), Platelets (GPVI-/-, PF4-Cre), endothelial cells

(BACE1-/-, CD36-/-, AC6-/-, VE-Cad-Cre), adipocytes (Adiponectin-Cre)) are not expected to present with any specific adverse effects related to this project licence.

Weight loss

Repeated anaesthesia, initially for the purpose of wounding and subsequently for follow-up assessments and re-application of substances can result in some weight loss. While this is typically small and limited in wild-type (normal) animals, it is more common and more extensive in diabetic animals. Animals will be monitored regularly for weight loss, and where greater than 5% loss is observed in wild-type (normal) animals, or greater than 10% in observed in diabetic animals (relative to their starting weight) they will be provided with an enriched more palatable softened diet. The provision of such enriched diets normally results in weight stabilisation and often in gain. Any animals that display greater than 20% loss in body weight will be humanely killed.

Cumulative impact

There is the possibility of cumulative impact for mice on this licence when travelling through all three protocols on this licence. This will be closely monitored for these mice to ensure they are not suffering because of this and methods will be refined if this causes issues.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

MICE: 10% mild, 90% moderate

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are three key reasons why the aims of the project cannot be achieved without animal studies:

1. At present, there are no suitable laboratory alternatives that are able to replace animal studies, as the studies require viable intact tissues, blood vessels and natural blood circulation, which currently cannot be cultivated or obtained using tissue culture.

2. Mice are a good model for wound healing, because basic mechanisms regulating wound healing are similar in mice and humans.

3. Genetically modified mice allow for targeted and precise testing of the role of certain genes, cells and coagulation factors.

In all experiments, in which the experimental results can be obtained without involving animals, in-vitro approaches will be employed. These will include experiments exploring in-vitro cell migration and angiogenesis assays using human cells. Prior to starting animal experimentation, we will undertake a comprehensive literature search and scientific discussion to ensure that the factor that we plan to study is indeed very likely to be involved and to exclude the possibility that a similar study has already been published.

Which non-animal alternatives did you consider for use in this project?

Human skin explants, and in vitro tissue culture.

Why were they not suitable?

Human skin explants cannot be used as the blood vessels and lymph vessels are disconnected and we need to be able to see how immune cells in vessels arrive and leave wounds. Although in-vitro cell culture experiments cannot recreate complex

multicellular interactions nor the impact of disease states, we will continue to use these approaches where possible to add value to the animal studies performed and to minimise the need for in-vivo experimentation as much as possible.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of mice needed for the studies have been estimated based on 1) prior data and the variation in this data using similar mouse studies, 2) prior data and the variation in this data using laboratory methods directly underpinning the studies, and 3) statistical methods to estimate the minimal number of mice needed to test the hypotheses (objectives) of each part of the studies, based on the size of the effect that we (expect to) observe and the variation in the data, with a particular degree of certainty (normally >95%).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We took the following steps in experimental design to reduce the number of mice to be studies in this project to the absolute minimum: 1) laboratory experimentation wherever possible to replace or underpin any animal study, 2) used the NC3R's Experimental Design Assistant during experiment design to ensure that we used the most appropriate group size and included appropriate control groups so that experimental data will be scientifically interpretable, 3) statistical methods to estimate the minimal number of animals required to test the primary hypotheses with >95% certainty, 4) avoiding any possible repeats of previously published literature, 5) creating more than one wound per animal (whilst remaining within the moderate severity limits), allowing for within animal controls (when possible) to reduce the total number of animals required and 6) obtaining as many tissue samples as possible from all animals for laboratory testing.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use a number of measures to optimise and reduce the number of animals we plan to include in our studies. This includes but is not limited to: 1) efficient breeding strategies to minimise the number of breeding pairs needed to achieve the required numbers, while being mindful of avoiding possible inbreeding and its effects, 2) laboratory studies using human cells and proteins instead of animal studies where possible, 3) carefully storing samples from experimental animals such that additional future experiments can be undertaken on archived material wherever possible. This material will be made available to other research groups on request so that

additional experiments involving animals will not be required. And finally, 4) the models we use will be refined to reduce variability and therefore enable us to use the minimum number of animals possible. We will continue to explore the potential of small animal imaging to allow collection of data at multiple time points to further reduce numbers, and to maximize and refine the collection of multiple pieces of data from individual mice. Pilot studies will be used when new substances that haven't been used in wound healing studies before are to be administered. For this, we will first test the desired effect(s), dosing regiments and timing in-vitro and on ex-vivo whole mouse blood. Once established, we will then test appropriate dosing and timing in- vivo using an initial set of 3 animals. Following a successful pilot with three animals this will be expanded to a maximum of 10. Based on the data and their variability obtained, we will then perform power calculations to determine the number of animals needed for each experimental aim.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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're going to investigate how changes in blood clotting affect the healing of wounds in mice. To do this, we'll make wounds on the backs of mice using a common method (punch biopsies), and this will be done while the mice are asleep from general anesthesia. After that, we will give the mice pain killers and let them recover, and we will follow how their wounds heal for up to 6 weeks.

Most wounds in mice usually heal within 21 days, but since we're specifically looking at how changes in blood clotting affect healing, we expect that some of the things we do to the clotting process might slow down the healing. These changes could include increasing the levels of certain proteins in the blood , adding drugs that prevent blood clots or introducing substances that cause inflammation (e.g. bacterial products) to the blood or wounds. We might also use mice with genetic changes that alter or prevent formation of certain proteins or cells involved in blood clotting.

Additionally, we know that diabetes can affect blood clotting and wound healing. So, we'll use three different models in mice that mimic diabetes in our study: Mice which naturally develop diabetes due to genetic alteration, mice with genetic and diet-induced type 2 diabetes, and mice with type 2 diabetes induced by a combination of a chemical that damages the pancreas and a high-fat diet. Since diabetes is linked to delayed healing, we'll observe the mice for up to 6 weeks to make sure we capture all stages of the healing process.

The model involves surgery using careful aseptic technique and the smallest size and number of wounds necessary to achieve our scientific objective. We will also

provide pain relief prior to wound formation and as needed post wound formation as well as combining treatments into one general anaesthesia session when possible. All of these steps will minimise suffering of the animals. Any animal threatening to exceed moderate severity will be humanely culled.

Why can't you use animals that are less sentient?

The skin in which wound healing is explored will need to be fully developed and therefore immature animals cannot be used. Alternative models of healing, such as the Zebrafish model cannot be used due to differences in their healing mechanisms, and the wound being constantly submerged in water. Experiments that involve recovery are required to study wound healing as it occurs over time and is similar to wound healing in humans and will allow us to study the role of clot architecture in tissue repair in health and disease. Using the mouse thus offers the best opportunity to translate our findings into the clinic to benefit patients.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our overriding rule, is to use the model and methods with the least likelihood of causing pain, suffering, distress or lasting harm that is necessary to address the scientific question being asked.

For a given animal, we create the smallest and fewest wounds or implants under general anaesthesia, provide appropriate levels of pain relief, and undertake the fewest and shortest follow-up assessments and substance administrations by the most refined route, possible.

Wherever possible, environmental enrichments (e.g., forage food, nesting materials and wooden chew- sticks) will be provided to animals following our protocols.

Our protocols include the use of strong analgesia perioperatively followed by use on an as needed basis according to our welfare and wound score sheet. Frequency of wound monitoring will be increased at an early stage based on wound appearance and clinical signs of the animal. This allows for early intervention if remedial actions (eg. analgesia, antibiotics or enhanced supportive care) are required and timely application of humane endpoints should those remedial actions prove ineffective.

Mice undergoing the induction of diabetes will be visually inspected and weighed on a weekly basis prior to wounding. After wounding, animals will be visually inspected on a daily basis for the first two weeks after injury, and weekly thereafter. Close attention will be paid to the provision of water and frequency of cage cleaning in these animals. The adverse consequences of diabetes induction will be minimised by the provision of additional bedding/nesting materials, strict adherence to aseptic surgical technique and the provision of supplemental heat sources during anaesthesia and recovery. Animals will be monitored regularly for weight loss, and where greater than 5% loss is observed in wild-type (normal) animals, or greater than 10% in observed in diabetic animals (relative to their starting weight) they will be provided with an enriched more palatable softened diet. The provision of such enriched diets normally results in weight stabilisation and often in gain. Any animals that display greater than 20% loss in body weight will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow best practice guidance published on the LASA, LAVA and NC3Rs websites for research and testing using animals including LASA guiding principles for preparing and undertaking aseptic surgery, NC3Rs mouse grimace scale, EDA and guidance on blood sampling and administration of substances. To aid with the development of our studies and help promote the use of the three Rs we will use PREPARE guidelines. We will review these guidelines on a regular basis and also be guided by the facility's NVS for advice. With regards to field-specific developments in wound healing animal models, we will consult up-to-date publications on wound healing models for improvements. Implication of any developments will first be discussed with the facility NACWOs and NVS, and subject to approval of protocol amendments by the Home Office. To help improve the reporting of our research and maximise the quality and reliability of our published work we will look to the ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in the 3Rs through regular (annual) refresher courses, through communications from the establishment named persons and updates through the NC3Rs website and monthly newsletter. For the implementation of any advances in 3Rs, I will regularly review the protocols in use with the personal licence holders on the project and NACWOs to determine if any refinements can be implemented. Any such modification will first be checked with the facility NVS and amendments approved by the Home Office.

81. Investigating mechanisms of nociception and chronic pain

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

pain, neuroscience, neuronal malaadaptation

Animal types	Life stages
Mice	adult, juvenile, neonate
Rats	adult, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to better understand how neurons, which control our pain, become activated and cause long lasting (chronic) pain. This project will determine how neurons respond to disease or injury through adapting to alterations in the surrounding neuronal environment to cause long lasting pain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A large proportion (~50% of people in the UK) of people suffer from chronic pain (long lasting heightened pain). Current painkillers don’t work very well or cause severe side effects such as addiction. Despite this clinical need and knowing many patients suffer from chronic pain, there is still poor understanding of how chronic pain

develops. We know sensory neurons are damaged and become active to cause chronic pain but little progress is being made to make new painkillers.

It is known that neurons are not the only cell type that controls our pain but lots of differing cell types contribute to this. Blood vessels, immune cells and resident supportive cell types also become damaged during chronic pain. These subsequently communicate with the neuron, causing alterations in our pain perception. This project explores how these differing systems interact to control our pain. By better understanding how our pain is controlled by this diverse cell population we can improve painkiller treatment and improve patients' quality of life.

What outputs do you think you will see at the end of this project?

The outputs of this project will include; Publications in Open Access Journals.

Dissemination of information at Local, National and International Conferences
Providing accessibility to datasets via data repositories

Identify a treatment approach that could progress to a clinical trial.

Who or what will benefit from these outputs, and how?

The immediate benefit from this project will be for basic science by producing world-leading and internationally recognised work that will be published in high impact journals.

Output from this project may be utilised by others including preclinical academics, industrial partners and clinicians. We will disseminate our results via both National and International conferences and workshops.

While Basic science will benefit in the short-term (1-3 years), clinical impact may take a longer period to be achieved. This may have the potential to be a therapeutic or diagnostic tool.

The time taken to fully evaluate a given therapeutic or diagnostic tool is likely to go beyond the five year duration of the programme of work due to the complexities involved in the research.

How will you look to maximise the outputs of this work?

Research findings will be communicated where possible via preprint repositories and via open access publishing. Personal communications at local and global research events will be pursued to access the key stakeholders of this work.

Species and numbers of animals expected to be used

- Mice: 1900
- Rats: 900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our current state of understanding is based upon research involving rodents – rat and mouse. The underlying physiological systems that control our pain perception are similar between rodents and humans. The outlined experimental approaches can be performed in these rodents as demonstrated by our own expertise and published literature and can be compared to comparable approaches in human. This project incorporates adult rodents as well as neonatal rodents to enable us to explore pain perception in differing aged mammals. This is important as the pain system develops and grows during adolescence. In addition, as the pain systems becomes older as age increases this system becomes damaged. Rodent models of disease or injury are similar to human situations for example alterations in diet causes diabetes and pain. This allows direct comparisons to be made between these experimental studies and a patient. We continually refine our methods using animals, adopting new experiments to minimise animal use, whilst providing the required experimental information. Despite using refined methods to reduce animal use, it is still necessary to use animal models. Furthermore, advances in technology allow these rodent models to greater expand our understanding using genetic models (viral, transgenics) to explore new molecular targets.

Typically, what will be done to an animal used in your project?

In this project, mice will be bred to have specific genetic modifications which are not expected to be harmful and will be bred using natural mating and maintained for use in this or other protocols.

Typically actions in this project include:

Behavioural studies Administration of substances Tissue extraction

Animals will be humanely killed at the end of the experiment

The animals in this project may be injected multiple times with the candidate items that will be tested by one or more of the following routes - subcutaneous, intravenous, intraperitoneal, intrathecal or diet to assess mechanisms that control pain.

In some studies rodents will be anaesthetised and recordings will made from neurons and other tissues. At the end of these experiments animals will be humanely killed.

The rodent models to investigate pain will be set up and the candidate therapies tested. In some studies, blood samples will be taken from a superficial vessel followed by a sample taken. The typical duration of a study will vary from between 2-12 weeks and beyond dependent on the study design.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will experience short periods of discomfort during projects e.g. through administration of substances or application of sensory stimuli. Experience to the application of sensory stimulus is short and repeated stimulation to the same paw is controlled whereby stimuli are alternated between paw. Nociceptive behavioural assays are generally carried out in perspex enclosures to which the animals are habituated to prior to onset of behavioural testing. Tests involving application of stimulus are applied to the rodent hind paws. In the case of polyneuropathy (following intraperitoneal or intrathecal administration) alternate paws will be assessed in order to reduce the discomfort of exposure on one area. All tasks are escapable (apart from instances of experimental agent administration as well as chemical induced pain assessment). All evoked pain behaviours will be applied with an inter stimulus interval. In addition, some tests have a cut off (e.g. time) to minimise the risk of sensitisation to stimulus and tissue damage.

Expected severity categories and the 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 rodents will experience and exposed to short mild discomfort from administration of agents and/or application of sensory stimuli due induction of pain processes and the protocols adopted are classed as moderate severity. Mice and rats will be utilised under protocols 2-4. The protocol steps to performed are the same for both rodent species and all strains. As outlined the majority of studies performed rodents will experience an expected severity limit of moderate under these protocols. Under previous Home Office PPL authority ~60% mice and 100% rats under similar protocols experienced moderate. This severity will be applied to most instances to allow comparison of behavioural performance during different experiments. Protocol 1 involves breeding of genetically altered mice.

These mice will experience upto mild severity due to procedures requiring tissue biopsies for genotyping. Under previous Home Office PPL authority 40% mice did not exceed mild severity on a similar protocol.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Perception of pain is a whole mammal sensory experience. In all mammals this involves differing tissues and cells within our body, working together to control

how we control and react to pain.

Neurons are at the centre of this. However, the nervous system is made up of lots of different cell types (e.g. endothelial, astrocytes, neurons, immune cells); which all contribute to differing aspects of the perception of pain. Initial proof of concept studies can be carried out in cell based studies though no study can replace the extensive interactions these differing cell types have within an animal. Therefore, animal studies are a key aspect of pain research whereby pain behaviour can only be measured in the living animal; in addition, these measurements can also be made in response to differing neuronal modulating agents.

Which non-animal alternatives did you consider for use in this project?

Cell based experiments are performed in the laboratory to allow us to investigate molecular and cell function in response to a drug or by altering the environment in which the cells are grown i.e. less oxygen. This provides us with understanding about what controls the cells responsible for pain. Cell based experiments in the laboratory inform us into how best to perform in vivo experiments. These cells are derived from animals, as these cells are not available from humans. The use of in vitro models may provide information that could allow us to review our in vivo approaches, which could lead to a reduction in the numbers of animals undergoing experiments and/or provide us with an opportunity to refine our current experimental techniques. In addition, viral induced approaches to initiate GAA reduces the animal number by reducing breeding.

Why were they not suitable?

This project requires many body systems to work together to understand how pain is controlled and develops. These studies can only be done in animals to allow us to appreciate the intricate networks in play where differing cells and tissues communicate to control the perception of pain.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our experimental group sizes are determined from past experience, available research resources such as publications and communication with experienced researchers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will ensure that we use the minimum number of animals required to answer the scientific question by performing power calculation studies. We may also apply the NC3Rs experimental design assistant tool for appropriate experimental planning. We will regularly consult qualified statisticians about experimental design and statistical analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Both sexes of rodents will be used as well as pilot studies will be performed on smaller cohort sizes to optimise experimental protocol. Where appropriate breeding will be monitored to prevent excess breeding, though enabling sufficient animal numbers for experimental protocols. Breeders may be used for tissue collection to further analysis. Where appropriate pilot studies will be performed to monitor new experimental approaches e.g. administration of substances and induction of transgenes and will typically utilise 3 rodents/sexes/experimental intervention to optimise experimental design. Following on from this sample size calculations can be performed to determine the required number of animals needed per study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically altered mice bred and maintained are of a non-harmful phenotype, with no observed alteration in normal rodent behavioural repertoire

Substances are prepared and administered using sterile technique. The route of administration is via the least invasive method appropriate to the model. The volume of substances to be used will be in accordance with the Laboratory Animal Science Association (LASA) good practice guidelines.

Nociceptive behavioural testing involves a short-lasting application of sensory stimulus to the hind paw

e.g. typically no more than 5 seconds for mechanical von Frey application. The transient nature of this experience is short lived and the rodent is freely able to move away from this stimulus if they choose to.

Rodent models of diabetes can be induced either chemically (e.g. by administration of streptozotocin, STZ) or through diet. These approaches provide a controlled induction of diabetes that can be applied to differing strains of animal, with instances providing a model comparable to the human clinical setting to enable improved understanding of diabetic neuropathic pain.

Rodent models of chemotherapy induced neuropathic pain present clinically translatable research outcomes, with presentation of alterations in the sensory nerve structure and development of exaggerated pain responses. Chemotherapy agents are administered typically via intraperitoneal injection, with clinically relevant outcomes e.g. pain, loss of intraepidermal nerve fibres, present in rat and mouse models of chemotherapy induced neuropathic pain. Doses of chemotherapy are utilised that are the lowest concentrations known to cause sensory neuronal cytotoxicity or induce the onset of neuropathic pain behaviours.

Surgical procedures will be performed under anaesthesia. These include stereotaxic injections, recording of neurophysiological measures that allows for recording for example neuronal activity or vessel perfusion, which will be performed under terminal anaesthesia. These include recording of neurophysiological measures that allows for recording for example neuronal activity or vessel perfusion, which will be performed under terminal anaesthesia. Additionally, recovery from anaesthesia for surgical procedures or for injection ie intrathecal maybe required for administration of substances

i.e. injection into CNS. These procedures require animals to undergo anaesthesia and in experiments involving administration of substances, rodents maybe recovered.

All animals will be humanely killed by a Schedule 1 method.

Why can’t you use animals that are less sentient?

Rat and mice are the least sentient animals that can be used to meet the objectives stated in the licence. Experiments we have performed previously under our PPL have involved rats and mice allowing us to fully explore the mechanistic avenues using an array of differing methodologies that include exploration of behaviours and using terminal procedures to acquire understanding about neurophysiological systems. The use of a mammalian model will allow us the opportunity to explore translationally relevant experimental findings.

Although rats and mice can be used to meet the objectives in the licence, mice allow for the utilisation of genetically altered model systems. Experimental techniques are established in rats to allow the assessment of physiological systems that may require surgical interventions to introduce electrophysiological measures as they are a larger model and require less technical manipulation.

The adopted rodent models are robust methodologies that are well documented, internationally recognised by leading academic and medical associations to enable clear comparisons between rodents and humans due to similarities in the pain anatomy and experience between these mammals.

The use of alternative model systems such as Zebrafish do not allow studies to be performed to meet the objectives of this license. Zebrafish only provide an opportunity to explore mechanistic avenues and more specifically relating to developmental biology. These organisms have little similarity with mammals, demonstrated by the lack of key physiological features ie organs, sex and genetic traits, inherent differences with the nervous system – anatomy and pharmacological

efficacy due to reduced blood brain barrier, a less heterogenic and complex immune system than that in mammals and would not provide results that meet our objectives of this PPL. All these components demonstrate that rodents are the least sentient species as the perception of pain requires a multifaceted processing of an experience relying upon differing hierarchical orders of the nervous system, communication between multiple differing physiological systems including blood vessels, immune system and neurons, which coordinate nociception and pain perception. Fundamental understanding of nociception in zebrafish is unclear and not enabling progression in achieving the objectives of this project license.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The research laboratory has extensive experience in animal handling and designing appropriate in vivo experiments to minimise animal distress. Animals will be monitored regularly in order to identify the onset of any adverse clinical signs that may cause a deterioration to the animals health. Additional animal bedding and environmental enrichment will be provided. Methods of administering substances will be assessed and to ensure these are delivered in an appropriate manner, with LASA guidelines referred to. Surgical procedures will be conducted with aseptic precautions according to LASA principles and advice from the veterinary surgeon. The veterinary surgeon will also advise on appropriate analgesia where appropriate. If signs of adverse events are identified, steps will be taken to alleviate those signs or the animal will be humanely killed immediately. Under our previous Home Office authority, we developed alternative strategies to induce genetically altered animals by utilising injection of viruses to induce genetic alterations in mice as this reduces the total number of animals bred.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Experiments involving rodents are designed with referral to appropriate guidelines that may include such as the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments), PREPARE or LASA Good Practice Guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will maintain close interactions with the relevant welfare, training and information officers as they oversee and perform in vivo studies. The PPL holder will stay informed of advances in the 3Rs by regularly checking the NC3Rs webpages (https://nc3rs.org.uk/the-3rs) and the newsletters which are circulated monthly. Moreover, the PPL holder will attend appropriate seminars, symposiums and conferences deemed suitable.

82. Brain Regions In Learning, Memory, and Motivation

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Hippocampus, Navigation, Space, Memory, Networks

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, aged
Rats	neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to identify how hippocampal cells interact with cells in other parts of the brain to enable an animal to navigate, to remember places, to identify objects which might be found there including rewards or punishments, and to value these places positively or negatively. In addition, we plan to characterise how these properties deteriorate in mouse models of Alzheimer's disease, Downs syndrome, and other neurodegenerative and psychiatric diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Currently, the functional anatomy of the hippocampal formation and its connected areas is very poorly understood, and little is known about the functional roles that various brain regions connected to the hippocampal spatial cells play in spatial

memory and navigation.
This project aims to continue to uncover the properties of individual cells related to spatial memory and navigation. The work to be carried out under this project licence will take us to the next step in which we will characterize the functional relationships between the different cell types and provide important data for the generation of computational models of spatial behaviour and navigation.

This project is important for advancing our general knowledge of hippocampal function and its role in the broader operations of the brain. In addition, we expect to develop specific behavioural tests to uncover the functions of these broader networks that could be translated to human clinical work for early diagnosis of hippocampus-related neurological impairments, such as Alzheimer's disease.

What outputs do you think you will see at the end of this project?

Outputs from this project will include data and information generated on functional anatomy of the hippocampal formation and its connected areas, as well as the functional roles that these various brain regions play in spatial memory and navigation. As well as this, this project will generate new information on the broader spatial system of the brain and the functions of its components. This information will be used to investigate how these properties deteriorate in mouse models of Alzheimer’s disease, Downs syndrome, and other neurodegenerative and psychiatric diseases.

The detailed descriptions of behavioural tests developed during this research, as well as programmes written for data analysis, will be made available to a wider scientific community. Findings will be made available through publication in peer-reviewed journals and presentations at scientific conferences and meetings.

Who or what will benefit from these outputs, and how?

This project will benefit the neuroscience research community, as the knowledge of anatomy gained will facilitate our understanding of the brain, and the creation of appropriate computational models.

This research will also benefit the wider research community, as the results may lead to early diagnosis of human neurological impairments, such as Alzheimer's disease.

Our results will be used to inform drug development research, to aid earlier diagnosis and provide treatments aimed at slowing down or stopping disease progression.

Tracing the functional connectivity between hippocampal spatial cells and those in anatomically connected regions

Short-term benefits
We will map out the areas connected to hippocampal spatial cells and study their functional significance. We expect that some of these will be involved in providing inputs to hippocampal spatial cells and creating the spatial properties while others will take the outputs from the hippocampal spatial system and use it to assign positive or negative values to specific environmental locations or to enable the

animal to navigate to one or more goals.

Medium-term benefits
As part of this programme, we expect to uncover the broader spatial system of the brain and the functions of its components, which enable us to predict the effect of damage to these regions which in turn could be translated to medical research for early diagnosis of hippocampus-related dementia, such as Alzheimer's disease.

Long-term benefits
This research is curiosity driven, i.e. basic research, and the primary long-term benefit is the generation of fundamental knowledge about the properties of spatial cells in the brain.

Importance
Currently, little is known about the functional roles that various brain regions connected to the hippocampal spatial cells play in spatial memory and navigation.

Our current programme of research will address this deficiency in our knowledge.

This is important for advancing our general knowledge of hippocampal function and its role in the broader operations of the brain. In addition, we expect to develop specific behavioural tests to uncover the functions of these broader networks that could be translated to human clinical work for early diagnosis of hippocampus-related neurological impairments, such as AD.

<u>Describing hippocampal functional anatomy</u> Short-term benefits
We will characterize the functional connections between spatial cells, namely place, grid, head direction and boundary vector cells in the hippocampal formation.
Medium-term benefits.

More generally, we will investigate and characterise the functional properties of the broader network of cells in areas anatomically related to the hippocampal formation;

Long-term benefits
This research is curiosity-driven, i.e. basic research, and the primary long-term benefit is the generation of fundamental knowledge about the properties of spatial cells in the brain. In addition, knowledge of anatomy will facilitate our physiological and behavioural understanding of hippocampal function and the creation of appropriate computational models. Together, these will facilitate translation of the results to human clinical work for early diagnosis of hippocampal-related neurological impairments, such as AD (see below).

Importance
Currently, the functional anatomy of the hippocampal formation and its connected areas is very poorly understood. My laboratory has been uncovering the properties of individual cells related to spatial memory and navigation. The work to be carried out under this project licence will take us to the next step in which we will characterize the functional relationships between the different cell types and provide important data for the generation of computational models of spatial behaviour and navigation.

Characterising changes in neuronal firing patterns in the hippocampal formation and connected regions in AD mouse models

Short- to medium-term benefits
We will characterise the behavioural deficits in a mouse model of AD over time and relate these to the spread and accumulation of pathological proteins, changes in glia-neuron interactions, and synaptic and neuronal loss. We will investigate how neuronal loss leads to cognitive impairments associated with the disease (e.g. impaired performance on spatial and non-spatial tasks).

Importance
Currently, it is not possible to study the progression of AD in humans from the onset to fully developed dementia. The disease is believed to begin in the hippocampal formation and then spread to other parts which makes this the ideal place to study it.

Our results will be used to inform drug development research, for improvement of early diagnosis methods and for facilitating the design of effective intervention methods to slow down or stop disease progression.

How will you look to maximise the outputs of this work?

Research findings will be made available through publication in peer-reviewed journals and presentations at scientific conferences and meetings, as well as through local channels such as retrospective review meetings and project presentations.

Species and numbers of animals expected to be used.

- Mice: 9000
- Rats: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use both mice and rats over the course of the project. Most of what we know about the functions of the hippocampal formation comes from work on rats, and the information gained from this project will be directly comparable to previous work and will build on current knowledge.

Mice offer the advantage of allowing genetic manipulations to identify cells and modify their activity, which can allow for targeted approaches in testing and manipulation when focusing on various areas of the brain.

Typically, what will be done to an animal used in your project?

The majority of animals used in this project will be used under breeding and maintenance protocols, including ageing.
Some of animals in this project will undergo behavioural tasks, and of these the vast majority will be rewarded with food for correct performance. Some animals will undergo surgical procedures to have tiny electrodes implanted into their brains or brackets for holding measuring devices affixed to their skulls.

Recordings of brain activity are taken from animals as they perform in spatial and non-spatial learning tasks, and the brain has no sensory receptors and thus the animals are unaware of the brain recordings. Stimulation of brain areas is not intended to cause pain or discomfort but to elicit natural behaviours or to modify such behaviours.

In a minority of cases, experiments will require mice to be head-fixed and running on an air-suspended ball while performing a task in virtual reality. Food reward is then used to encourage navigation reaching desirable performance after 3 to 5 days of experience. Once familiar with head-fixation, mice willingly explore virtual environments, similar to what they do on running wheels in their home cages.

In some cases, drugs will be injected into their brains or damage made to small areas of the brain under anesthesia in order to assess the role of these brain regions in spatial and other types of learning and memory.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals are not expected to experience any pain during or following surgical procedures, as operations are carried out under deep anaesthesia and animals are given painkillers both pre- and post-operatively to minimise pain and discomfort during recovery.

Rats and mice usually recover within a day, as assessed by normal consumption of food, running on wheels and building a nest in their home cages. It can take up to 5 days for animals to recover from surgery when a brain region is damaged, during which time they mostly rest and sleep. In all cases animals will be given sufficient time to recover.

Some animals show transient dislike of head fixation via a brief increase in urination and/or defecation which disappears several minutes after the first exposure. Food reward is then used to encourage navigation reaching desirable performance after 3 to 5 days of experience. A small percentage of animals may not acclimatize to restraint within 5 days and these will be removed from the task.

Expected severity categories and the 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: The majority of animals (60%) are expected to experience Sub-Threshold

severity due to being used in breeding or ageing protocols and experiencing no adverse effects as a result.

Animals used solely for tissue collection (10%) will experience a Non-Recovery severity.

A proportion of animals (20%) will experience a Mild severity as they will only undergo behavioural tasks with no prior surgery.

Animals undergoing surgical procedures (10%) will experience a Moderate severity.

Rats: 10% of animals are expected to experience Sub-Threshold severity due to being used in breeding or ageing protocols and experiencing no adverse effects as a result.

Animals used solely for tissue collection (5%) will experience a Non-Recovery severity.

A proportion of animals (30%) will experience a Mild severity as they will only undergo behavioural tasks with no prior surgery.

Animals undergoing surgical procedures (55%) will experience a Moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We use rodents because it is not possible to study the role of the hippocampus and related brain structures in real world navigation without using behaving animals.

It is possible to genetically modify mice to replicate some of the pathological processes underlying Alzheimer’s dementia and to use these animals to study the biological mechanisms of Alzheimer’s. By studying the pathological anatomical and physiological changes in the brains of these animals and the effect of these on the animal’s ability to use its spatial mapping system and remember places we hope to understand some of the mechanisms of Alzheimer’s.

Which non-animal alternatives did you consider for use in this project?

We will use cadavers when living tissue is not required and unconscious anesthetized animals to study brain conductivity and other aspects of hippocampal function which do not require behaviour. Where possible, we use computational

modelling to direct our experiments and to minimize animal numbers.
Why were they not suitable?

For studying the brain and behaviour, and to address the aims and objectives of this project, there are currently no suitable methods to fully replace the use of animal models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based on previous experience and retrospective review of previous project licences and annual returns.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the latest behavioural and physiological techniques that allow us to increase the amount of data collected per animal, including the use of neuropixel probes.

Studies will be conducted in collaboration with local computational scientists at the establishment, in order to efficiently analyse data obtained from this project, to maximise the overall outputs as well as outputs from individual studies and data sets.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible, we will use techniques that allow for targeted genetic manipulations, which minimises the number of animals necessary for research through reducing the need to breed transgenic animals that may be expressing or lacking target genes.

We will employ efficient breeding practices through collaboration with the local animal facility staff, including the nominated GAA Breeding Lead, and lab colony managers. The breeding strategy for genetically altered animals will be dependent on breeding performance of individual lines, litter sizes and demand. Where possible, lines will be maintained in a homozygous state, thereby obviating the generation of excess offspring with inappropriate genotypes. When heterozygous breeding is required, wild-type littermates will be used for control experiments. We will use computer models to predict and understand the results of experiments and this will enable us to use the minimum number of animals. Where possible tissues will be shared.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rats and mice are used in these studies since they are very good at navigating in familiar environments and remembering what has happened to them there. We know a lot about the anatomy and physiology of their brains and in particular of the parts of the brain to be studied in this project.

They are the most widely used experimental animals for combined behavioural and electrophysiological studies; furthermore, most of the detailed anatomy of the hippocampus has been elucidated in these species.

While rats are docile and display high levels of spatial memory and navigational abilities, using mice offers unmatched access to genetic tools, allowing us to induce specific gene mutations relevant to diseases such as Alzheimer's disease, as well as using genetic techniques to record from and manipulate functionally/genetically/anatomically defined ensembles of cells. It is possible to genetically modify mice to replicate some of the pathological processes underlying Alzheimer's dementia and to use these animals to study the biological mechanisms of Alzheimer's.

In general the methods used are not intended to cause pain or suffering and any unintended pain or suffering will be minimized using painkillers or other appropriate methods.

Why can't you use animals that are less sentient?

Less sentient, non-mammalian, species have a brain structure is vastly different from the human brain, and may not display the complex behavioural repertoires used to study memory, navigation and learning, so do not allow for accurate translation into human biology and disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use unconsciousness anesthetized animals to study brain conductivity and other aspects of hippocampal function which do not require behaviour. We will use cadavers instead of live animals wherever possible. Optimal results in behavioural experiments require that the animals are healthy, in good spirits, and motivated to perform well. For this reason, the majority of our behavioural tests involve food and liquids as rewards. We also use minimum levels of food restriction to motivate the animals to work for these rewards. Professional operative techniques and post-operative care ensure a minimum of adverse effects and the minimum level of

suffering from any surgical or other procedure.

Animals under active testing usually live in enriched environments with ample space and several toys which are chosen to be compatible with the experimental procedures. Because both rats and mice are highly sociable animals, the animals are housed in groups in large home cages where possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Refinements to rodent head fixation and fluid/food control for neuroscience. Barkus C et al., J Neurosci Methods. 2022. Nov 1; 381:109795
Guiding Principles for Preparing for and Undertaking Aseptic Surgery. Jennings, M. and Berdoy, M., 2010. A Report by the LASA Education, Training and Ethics Section.
PREPARE: guidelines for planning animal research and testing. Adrian J Smith, R Eddie Clutton, Elliot Lilley, Kristine E Aa Hansen, Trond Brattelid 2018 Lab Animals Apr;52(2):135-141.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep up to date with 3R developments through the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs), including recommendations for best practices in neuroscience rodent experiments.

We will further be informed by the local 3Rs group at the establishment as well as animal facility staff and Named Persons, who we will work closely with to continuously implement 3Rs advances.

Members of the lab will attend regular 3Rs meetings and events, and relay key information back to the research group.

83. Development of Novel Deubiquitinylating (DUB) Enzyme Inhibitors for Fibrotic, Cardiac, Musculoskeletal and Neurodegenerative Diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Neurodegeneration, Fibrosis, Mitochondrial disease, Cardiac disease, Protein degradation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To develop new treatments for serious diseases by discovering drugs that inhibit a specific class of enzymes, termed DUBs (short for DeUBiquitinylating enzymes).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
We have developed molecules that can inhibit many of these enzymes and we want

to develop them to treat diseases of significant unmet medical need: Musculoskeletal disorders such as muscular dystrophy are a group of degenerative disorders that have a high unmet medical need. Some of the more severe forms of disease are clear from birth and are fatal within first 15 years of life. These diseases affect sufferers through the whole course of their lives, having a large impact on their quality of life.

Currently drugs approved in Europe to treat these disorders focus on relieving the symptoms rather treating the underlying conditions. There are good reasons to believe that drugs that inhibit DUB enzymes will improve disease progression and add significant quality of life to patients.

Cardiac diseases including cardiomyopathy and heart failure are highly impactful upon quality of life and have high levels of mortality. Blocking DUB enzymes is expected to improve the energy availability to the heart and prevent the progression of these disorders.

Fibrosis is a condition that leads to scarring and a build-up of connective tissue in various different organs after injury such as the kidney in acute kidney failure, lung in pulmonary fibrosis, heart following a heart attack and liver following liver disease. This fibrosis leads to much reduced function or even organ failure. DUB enzymes are thought to play a critical role in the inflammatory process that contributes to fibrosis and therefore have the potential to prevent the development of fibrosis, or even reverse existing fibrosis once it has developed.

Neurodegenerative disorders, such Parkinson's Disease are well known for their catastrophic impact on patients' quality of life and overall life span. They are also well known for being extremely difficult to treat. The most obvious symptoms of Parkinson's disease are shaking, slowness of movement and difficulty of walking, however dementia can also be common on later stages of the disease. Current standard of care focusses on improving symptoms, rather than reversing or curing disease. Through their unique mechanism of action DUB inhibitors have the potential to delay the development of such diseases, or even reverse the diseases once they are diagnosed. Development of such agents are of great interest across both the pharmaceutical and medical industries.

This programme of work will be carried out to provide 4-5 clinical candidate drugs within 5 years. Each will a) be well tolerated b) demonstrate predictable circulating concentrations within the blood and c) show therapeutic potential in established models of disease. These drug characteristics can only be effectively assessed with co-ordinated use of cell-based techniques and animal models.

What outputs do you think you will see at the end of this project?

This project will result in 4-5 candidate compounds to be developed as clinical drugs in 5 years. Each will a) be well tolerated b) demonstrate predictable circulating concentrations within the blood and c) show therapeutic potential in established models of disease.

Compounds will arise from the companies research efforts and developed internally.

Candidate compounds will be patent protected and developed outside the scope of this license to proceed to clinical trials either funded by the company or supported by partner organisations. Clinical trials will begin approximately 12 months after completion of studies under this license.

In addition, through building the understanding of how inhibition of DUB targets impact the disease progression, new information will be generated. It is anticipated that packages of work using studies with cells combined with experiments performed under this license will form the basis for further high impact publications.

Who or what will benefit from these outputs, and how?

In the short term, information generated and published at conferences or in journals will advance the field of DUB research and focus the field on the key DUBs involved in disease biology.

In the long term, this research will lead to new efficacious and better tolerated treatments for neurodegenerative, metabolic and fibrotic diseases thus benefiting patients with potential economic benefit for companies marketing the novel drugs.

How will you look to maximise the outputs of this work?

We will collaborate with key researchers in the field (both academic and industrial) as well as charities supporting disease research. It is expected that this will lead to publications and conference presentations on both successful and unsuccessful approaches. Through continual relationships with Key Opinion Leaders, active in Clinical Studies or Research we will have access to patient groups.

Where there are relevant charities or patient groups for a particular disease we will approach them to share information and progress.

Species and numbers of animals expected to be used.

- Mice: 2160
- Rats: 230

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The majority of animals used in the project will be adult mice. Mice are the most appropriate species due to being widely used as an experimental model. The disease models that will be supported by these studies are established in mouse and have been used to develop some effective and safe treatments for neurodegenerative, cardiac, fibrotic and mitochondrial disease in humans. Adult

animals will be used as the majority of diseases being treated will be in adult humans with the exception of muscular dystrophy which is more comment in juveniles and as such juvenile mice may be used. Rats will be used in the rare situation that mice are not a suitable species, for example if the drug target is not expressed in mouse, the compound pharmacokinetics are predicted to be significantly better in rat or the planned disease model is in rat.

Typically, what will be done to an animal used in your project?

Typically, test compounds will be given to the animals, most likely through the mouth with a tube. Studies will either look at short term (under 24 hour) changes within the animal after a single dose of the compound or longer term (one to four weeks) impact with daily dosing of the compound. Animals will be maintained in groups in their normal cages throughout studies.

In generation of genetically modified mouse strains, short surgical procedures to implant embryos or vasectomy may be performed in a few animals.

What are the expected impacts and/or adverse effects for the animals during your project?

It is possible that the compounds administered have some side effects, such as causing transient nausea so animals will be observed to ensure these are mild.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity is expected to be mild in the majority of cases (98% of mice and rats). Moderate severity is possible in up to 2% of mice and rats.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

A successful compound needs to have the characteristics to leave the stomach, enter the circulatory system and reach the site of disease in order to have an effect. In order the determine this, live animals are required as several body systems are working together to deliver the compound to the target site.

Whereas it is possible to grow cells in culture, it is not possible to create the complex interaction between many different cell types that are involved in disease biology in mammals.

In vitro (cells grown in the laboratory) assays will allow us to address important early-stage criteria such as enzyme potency, selectivity, off-target effects and to make predictions about the compound's metabolic stability before it is dosed in vivo (to animals). While the predictive capacity of these in vitro approaches is continually improving they cannot currently replace animal studies. This is because new drugs need to be tested in complex, biological systems with integrated functional pathways and this requires the use of animals. Sole reliance on in vitro biochemical and cell-based assays are not suitable to determine and identify key properties required of therapeutic agent in a complex biological system. Pharmacokinetics and biodistribution are examples of crucial endpoints that require integrated responses of the body to a drug that include components of absorption, distribution, metabolism and excretion. In vitro systems are getting better at predicting absorption with the use of flux across cell layers and and metabolism through profiling how compounds are metabolised by human liver cells, however they are not perfect. Moreover, distribution and excretion of a drug or its metabolites is impossible to accurately predict with in vitro methodologies. Indeed, it is recognised that co-ordinated use of both in vitro and in vivo experiments allows robust translatable predictions to human systems that would not be achievable by in vitro or in vivo studies alone.

In our specific case additional complications arise from the mechanism of action of our compounds as they are designed to act as reversible tight binding (covalent) inhibitors of DUB enzymes. This means that the compounds have the potential to bind to the target enzyme for longer than might be expected. The potential time disconnect between test compound concentration and initial effect cannot be replicated to sufficient detail in cellular or sub-cellular experiments without testing this in experiments involving the integrated system of a living animal. Moreover, potential covalent interactions with unidentified off-target proteins could result in physiological impact that would not be predicted from in vitro assessments.

Which non-animal alternatives did you consider for use in this project?

Our drug discovery programme uses many non-animal techniques such as cell-free biochemical assays, cell assays and computer modelling to address important early-stage criteria such as drug potency, selectivity and potential side-effects; metabolic activity is often initially assessed using liver or other tissue preparations (artificially, outside the animal). While these reduce the numbers of animals used they do have limitations and cannot (currently) fully replace animal models.

Before being tested in animals, the compounds will be profiled through experiments using human cells that exhibit relevant biology to the disease. This will form a large part of the project as a whole, with many compounds being tested and their progression continued or stopped based on experiments using non-animal alternatives.

Why were they not suitable?

These models will contribute to the research but they cannot recreate the complexity of human physiology and re-create the digestive, circulatory and tissue environments working together.

Additional biological complexity is created in terms of our therapeutic mechanism of actions. The molecules that we produce bind to their target enzymes in a manner that is unlike the majority of drugs. Therefore, complex biological models are essential to adequately assess how they will behave, particularly with respect to how long they stay within a tissue relative to concentrations within the blood stream. Since the human DUB system shares much in common with that of other mammals, in terms of enzyme structure and distribution within the body, mice and rats can be effectively used as surrogates to show the impact of active drug levels in a biological system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Mouse usage is split between support protocols and efficacy. Approximately **500** mice are estimated to be used in support protocols to allow breeding of GEMs (Genetically Engineered Mice). These will not transfer onto other protocols. Approximately **1000** GEMS will transfer onto efficacy protocols and as such have only been counted once in the total animal number.

Approximately 1450 mice and 230 rats will be used of efficacy protocols. This is split between two protocols -
Dose Finding and Tolerability Number of Mice - 250 Number of Rats - 50

Animal numbers are based on 5 mouse studies per year using 10 animals and 1 rat study per year using 10 animals.
Pharmacokinetics and Pharmacodynamics Number of mice - 1200
Number of rats - 180
Animal numbers are based on
PK studies: 5 mouse and 1 rat studies per year, each using 9 animals.
PD studies: 6 mouse studies and 1 rat study per year, each using 30 animals (5 groups of 6 animals per study).

Group sizes are based on previous experience with protocols and results from similar studies.
What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The exact number of animals required varies between experiments but studies are

designed to get the maximum amount of data from each animal, for example by testing more than one compound in a study with a single control group. Online tools such as the NC3R's Experimental Design Assistant have been used to further refine studies and provide information about experimental design, sample size and statistical analysis methods. Studies are designed to ensure that appropriate statistical power is achieved for each study through access to statistical expertise during planning.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

New experimental protocols will be set up by running small pilot studies with smaller group sizes guided by literature and our own data. This will then be used to determine the minimum number of animals required for each study. Study design will ensure that the maximum information is generated from the minimum number of animals, for example by testing more than one compound in a study with a single control group.

Measures to keep animal numbers to a minimum:

- Strict screening criteria with clear stop/go decisions to ensure only our best compounds progress to in vivo studies.
- Adapting the study type - studies employing serial micro-sampling, reduced time-course, or cassette dosing (dosing more than one compound at the same time) will reduce animal numbers; consequently, full time-course studies will be performed downstream where fewer compounds are tested, reducing animal numbers further.
- Avoiding duplication - if data is available elsewhere, some experiments may not be necessary; however, some may be replicated for validation purposes.
- Whenever possible, genetically modified animals will be obtained from researchers/suppliers to avoid re-creating these lines and to utilise their knowledge.
- Minimal numbers approach in tolerability studies with stepped dosing.
- Experimental design principles - pilot studies and statistics:
- Each of the protocols described herein are established and standardised throughout the drug discovery field with well accepted animal numbers required for robust translational predictions. They have been run under previous or current licenses within the organisation.
- Pilot studies will be employed when working with compounds that have not undergone in vivo testing before. These will be in minimal numbers of animals using dose-escalation procedures to ensure animals are never unintentionally administered non-tolerated doses of a given drug.
- Sources of variation and bias will be reduced whenever possible through after-action reviews of studies to ensure future learnings. Moreover, advice will be sought from Establishment Licence Named Individuals, e.g. Named Veterinary Surgeon (NVS) and Named Animal Care and Welfare Officer (NACWO), so we are able to follow current best practice.
- Factorial experimental design and blocking will be applied whenever possible and animals randomised to treatment groups.
- Appropriate statistical methods will include independent t-tests and ANOVA on

sequential and end-point data sets and regression analyses for time-courses. Frequently used models will be monitored for robustness and integrity (e.g. using trend plots). Application of these methods will give confidence that any apparent difference between datasets is not down to chance and that experiments perform consistently and as such will help data interpretation of a meaningful compound effect across studies.

- Routine statistical power analysis of conducted experiments will be undertaken to ensure that we are able to identify meaningful effects at 5% significance level with a power of 80%, and to adapt experimental design if our studies are over, or under powered. Separate power calculations may be necessary when considering experiments with male and female animals or both sexes together, and this will be taken into account.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animal models run during this project involve the administration of test compound(s) to mice and rats and sampling of blood or CSF either from a superficial vein (blood only) or under terminal anaesthesia or after the animal is dead. Other organs and tissues will be samples once the animal is dead. In some studies, animals will be observed for clinical signs.

These models/methods cause the least pain, suffering, distress or lasting harm to the animals because they are controlled through running studies for the minimum length of time in which to see a drug effect and by administering compounds at doses that are tolerated by the animal. Volumes of blood sampled are as low as possible.

Why can't you use animals that are less sentient?

Immature animals are not appropriate as they are not representative of the adult human. Similarly, these protocols cannot be done under terminal anaesthesia as this would only be suitable for very short term studies.

Zebra fish have been previously used to complement our research efforts but mouse better reflects the presence of our targets and their interactors. Mice and rats are the lowest species in which the (patho-) physiological function of DUBs in mammals can be studied and translated to the human physiology.

The physiology and architecture of mammals is required to accurately model the complex process of ubiquitinated protein processing.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

When anaesthesia/analgesia are not required (e.g. dosing/sampling) we will use the least invasive route/method (e.g. in-dwelling cannulae or micro-sampling from superficial vessels); we will ensure that the volumes dosed/sampled will not be detrimental to the health of the animal. Where blood sampling is required, methods are optimised to reduce the volume of blood needed to a few microlitres to reduce the burden upon the animal.

Housing, husbandry and care: animals will be housed and cared for according to best practice. Licensees will be trained in recognising pain and suffering and abnormal changes in animal behaviour. Experiments will be supported by a well-trained and experienced centralised husbandry team. Surgery is performed aseptically under the standards outlined in the "Guiding Principles for preparing for and undertaking aseptic surgery" (LASA 2010).

Humane end-points: Animals will be checked closely for the onset of clinical signs and the frequency of these checks adjusted accordingly. Studies will be terminated at the earliest possible point to minimise adverse effects without affecting the science. Behavioural and facial patterns can be used to identify sick animals early.

We will use our knowledge and expertise (or that of other researchers) to predict and manage adverse effects; we will ensure that appropriate humane endpoints are developed, applied and refined as more information on the model becomes available.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the ARRIVE guidelines as recommended by the NC3Rs Laboratory Animal Science Association (LASA) best practice guidelines will be adhered to for blood sampling.

The Experimental Design Assistant will be consulted when establishing a new experimental protocol and is available through the NC3Rs website.

There is valuable guidance for best practice training and protocols available through the NC3Rs website, for example, handling and restraint, euthanasia, humane endpoints, welfare assessment, microsampling, anaesthesia, analgesia. Personal License (PIL) holders who are required to undertake any of these activities will be signposted to this website. Local establishment guidelines are in line by those endorsed by the NC3Rs and will be adhered to as well as incorporating refinements into our working practices. For any new procedures, the NC3Rs guidelines will be followed.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Attendance of NC3R-related seminars and monitoring the NC3Rs website. Staff

working on the license will be encouraged to bring the 3Rs into their work and it will be part of their performance objectives to do so.

The establishment is a shared facility and the implementation of the 3Rs is encouraged throughout with periodic meetings sharing refinements to welfare and additionally interactions with the NVS, NACWO and Named Information Officer (NIO).

Attendance at conferences and formation of key collaborations to keep abreast of advances in non- animal alternatives, such co-cultures with several cell types, organoids or tissue in more physiologically-relevant environments.

84. Development of veterinary medicines for farm animal species and human medicines using farm animal species

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.

Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes.
Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Veterinary medicine, Farm animal, Efficacy, Safety, Human medicine

Animal types	Life stages
Cattle	neonate, juvenile, adult, pregnant, aged, embryo
Sheep	neonate, juvenile, adult, pregnant, aged, embryo

Animal types	Life stages
Goats	neonate, juvenile, adult, pregnant, aged, embryo
Pigs	neonate, juvenile, adult, pregnant, aged, embryo Domestic fowl (Gallus gallus domesticus)
Turkeys	neonate, embryo, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?
The overall aim of the programme of work is to develop safe and effective means of

preventing and controlling disease in farm animal species, however it also includes work on human medicines.

The aim of this project is to generate data from in vivo efficacy and safety studies in the livestock animal species to be used:

To develop and market products for the control of disease in farm animal species.

In dossiers submitted for the registration of products for the prevention and control of disease in farm animal species and on a limited scale human medicines.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The overall aim of the programme of work is to develop safe and effective means of controlling disease and promoting the health and welfare of livestock species worldwide. Additionally, to provide data to allow the development of human medicines using animal models.

What outputs do you think you will see at the end of this project?

The primary benefit of the work conducted under this licence is the production of reports which are fit for the purpose of submission to the relevant regulatory authority e.g., for a marketing authorisation to enable new substances to be available for prevention and control of disease in livestock species.

Who or what will benefit from these outputs, and how?

The programme of work would contribute to the development of a number of safe and effective products for the prevention and control of disease in farm animal species and developing human medicines. As there is a mandatory requirement to conduct studies to demonstrate the safety and efficacy of such products before they can be marketed in the UK, EU and worldwide, the consequences of this work not being carried out would be that safer, more effective products would not be available to treat disease. Whilst it is accepted that other establishments may be able to provide this service, the combination of skilled staff and the large-scale, purpose-built animal facilities at this facility makes for an ideal site to conduct this type of work.

How will you look to maximise the outputs of this work?

Data and reports from studies are included in dossiers for submission to regulatory authorities to allow for the use of safe and efficacious products.

Species and numbers of animals expected to be used.

- Cattle: 1800

- Sheep: 1200
- Goats: 300
- Pigs: 2500
- Domestic fowl (Gallus gallus domesticus): 9100
- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The European Parliament and Council Directives 2001/82/EC as amended by 2004/28/EC lay out the Community Codes relating to veterinary medicinal products.

Article 1 of the directive defines a veterinary medicinal product as any substance presented for treating or preventing disease. Further, any substance or combination of substances which may be administered with a view to making a medical diagnosis or to restoring, correcting, or modifying physiological functions is also considered a medicinal product. The scope of this licence is inclusive of chemical pharmaceuticals, pharmaceutical products derived from biotechnology including gene therapies and monoclonal antibodies, vaccines, serums, allergens, excipients of medicines and foods or feed additives intended for curative purpose or to maintain health.

A new veterinary medicinal product can be placed on the market within the UK or EU only when the applicant has obtained a national or European marketing authorisation. The Community Codes relating to medicinal products (2001/82/EC, Article 12 as amended by 2004/28/EC) list the particulars and documents that must be provided in support of an application for marketing authorisation. These include the results of physico-chemical, biological or microbiological tests, toxicological and pharmacological studies, and clinical trials.

Other countries and supra-national organisations have also developed their own guidance documents to ensure high standards of assessment of new veterinary medicinal products before they can be distributed to the public. These independently developed and disparate national guidelines have been progressively standardised over a period of decades by experts of many countries working through organisations such as the Organisation for Economic Co-operation and Development (OECD) and the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH). VICH guidelines present both detailed specifications of study designs and strategies for combinations of studies that will characterise the effects of a new medicinal product in the most efficient and effective manner.

Guidance on the conduct of efficacy studies for vaccines and immunosera is available in a range of European Pharmacopoeia monographs and varies

considerably between products. The dose used is that quantity of the product to be recommended for use and containing the minimum titre or potency expected at the end of the period of validity. For live vaccines, the most attenuated passage level that will be present in a batch of vaccine should be used. The efficacy evidence must support all the claims being made, for example onset and duration of immunity. For vaccines, the influence of passively acquired and maternally derived antibodies on the efficacy of a vaccine should be evaluated. The efficacy of each of the components of multivalent and combined vaccines should be demonstrated using the combined vaccine. Demonstration of efficacy is usually undertaken under well controlled laboratory conditions by challenge of the target animal. The challenge should usually mimic the natural conditions of infection, with regard to the amount of challenge organism and route of administration for example.

Data from target animal safety studies are required for registration of veterinary medicinal products in the regions participating in VICH. International harmonisation of standards for essential target animal safety studies facilitates adequacy of data and minimises the need to perform separate studies for regulatory authorities of different countries.

Bioequivalence studies are often part of applications for generic veterinary medicinal products to allow bridging of safety and efficacy data associated with a reference veterinary medicinal product. The EMA guideline (EMA/CVMP/016/00) provides guidance on the design, conduct and evaluation of bioequivalence studies for pharmaceutical forms with systemic action.

Non-Steroidal Anti-Inflammatories (NSAIDs) have become an important class of veterinary medicines for most mammalian animal species. The extent of usage and the range of indications for NSAIDs have increased in recent years. The EMA guideline on the conduct of efficacy studies for NSAIDs (EMA/CVMP/EWP/1061/2001) focuses on recognised pharmacological actions with potential therapeutic benefits of anti-pyretic, analgesic, anti-inflammatory or other anti-thrombotic effects.

Dose determination and dose confirmation studies are required for demonstration of efficacy of NSAIDs and antimicrobials. Dose determination studies should be conducted in the target species using a range of doses selected on the basis of preliminary studies, parameters that are relevant for the anticipated effect and a dose range that is considered appropriate for further use.

Pharmacokinetic studies are essential to employ veterinary medicinal products under the best conditions of efficacy and safety, to establish effective plasma concentrations and correct therapeutic schedules or to proceed to dosage adjustments in particular cases. This particularly applies to products with a narrow therapeutic range and to those for which a close relationship between plasma concentrations and therapeutic or toxic effects may be demonstrated or expected. Just like with veterinary medicines all human medicines must also undergo regulatory approval before they can be used in the main stream. Prior to clinical studies in humans there are regulatory requirements to undergo initial efficacy and safety work in animal species that provide scientifically robust relevant and comparative data. Whilst a lot of work is initially carried out in rodents and small

animals, there is some work that is more applicable to farm animal species. For example evaluating products for birth control (change in the cervical mucus to be impenetrable to sperm) or vaginosis in sheep as they have similar vaginal mucus profile to the female human, or products to treat arthritis as certain joints of animals (i.e the stifle in sheep) are also similar to humans (knee) and have been used to support the development of products for arthritis and other joint related conditions (https://www/oarsijournal.com/article/S1063-4584(10)00235-9/pdf). There products must however satisfy all the requirements for approval, such as safety and efficacy data. The data generated from these studies would form part of a dossier of evidence for product registration for use in humans and furthermore, improve/enhance product development prior to evaluation in humans. Therefore, where these occasions occur and procedures match those of this project then studies investigating human medicine may also be carried out.

As a corporate research organisation there is a requirement to provide our clients with the ability to carry out the above tests and associated studies/investigations in several species (cattle, sheep, pigs, goats, poultry) at all possible life stages to ensure required products can be registered. Animals must be used as registration requires evaluation of the test product in the target species.

Typically, what will be done to an animal used in your project?

Procedures vary greatly depending on the study, as the majority of the work covered by this licence is commercially driven. A client provides a study outline which is assessed by a senior manager and project licence holder before a decision is made as to whether to quote for the work. This initial assessment includes an ethical aspect to determine whether the work is within the scope of the ethical policy and within the capability of the resources available. Study designs (treatment replication and animals per replicate) will be discussed with a statistician based on expected differences between treatments. All study outlines are reviewed and approved by the AWERB prior to the study starting.

The project licence holder checks the study procedures against the project and personal licence authorities and is responsible for ensuring that the study outline is presented to the AWERB.

Animals will usually be screened as healthy and sero-negative for the antigens / antibodies of interest well in advance of the study starting. This can be achieved in a number of ways i.e. screening on their source farms, screening on arrival, sourcing SPF eggs for incubation and hatching on site or ensuring that animals do not consume colostrum. Animals may be maintained on the source farm for the study to be conducted in situ or animals may be transported to site. Immediately prior to the start of the study, animals selected for participation may be screened again to prove that they are sero-negative for the antigens / antibodies of interest at the start of the study. An inspection by a veterinary surgeon will also usually be carried out to ensure that the animals are healthy before the study commences.

The test product will usually be administered to a number of the animals by the recommended route and method of administration. The administration may need to be repeated a number of times. A number of animals will usually remain untreated

(or receive a placebo) and / or a number of animals may be administered a comparator product as a positive control. The positive control will usually be a product licensed for that indication in the same species. Unless otherwise justifiable, positive and negative controls will be housed alongside those in receipt of the test product and staff conducting assessments will be blinded to treatment. For studies with live vaccines, vaccinated and unvaccinated animals may need to be housed separately with appropriate biosecurity to avoid sero-conversion of the controls.

Following administration, animals will usually be sampled on a number of occasions at various time intervals in order to determine response to the vaccine / immunosera.

Animals may be challenged with the disease the vaccine / immunosera is claimed to protect against. Challenge can either be by direct challenge to the animal or by contact with animals which have the disease (either naturally or by direct challenge themselves). The challenge material will usually be a recent field isolate and will usually have been tested to ensure that it produces symptoms of a sufficient degree to allow efficacy to be determined. Following challenge, animals will be sampled on a number of occasions and various assessments (clinical observations, rectal temperatures, etc.) will be carried out in order to determine if the test product has been efficacious. Animals will be monitored closely in order to try to ensure that they do not exceed the protocol severity limit. This may necessitate monitoring outside of the normal working day.

At the end of the study animals will either be euthanased, sent directly to slaughter or rehomed following inspection by a competent person, in compliance with FSA and VMD regulations to protect the human food chain.

The number and type of animal being used on study will vary dependant on the required study design. The following steps/procedures will be used throughout this project:

Animals may be administered a suitable course of antibiotic on farm of origin or following transport at DAH as a prophylactic measure.

Suitable animals may be transported in excess of the maximum distance / duration indicated by the Welfare of Animals (Transport) Order 2006.

Suitable animals may be transported immediately after birth.

Suitable animals may be deprived of colostrum.

Animals may be restrained by physical means or confined within a restricted area for sample collection, test item administration or tattooing of injection site.

Poultry may be kept on slatted, mesh or perforated floors.

Animals may be housed according to the Defra code of recommendations for the welfare of livestock.

Animals may be penned individually within sight and sound of their compatriots and on the majority of occasions with physical contact with compatriots as well, in commercial space allowance in accordance with Defra codes of recommendation, for a maximum of 12 consecutive weeks.

Administration of test article or control on up to 12 occasions (if more applications are required for a specific protocol, these should be agreed by the AWERB) by intramuscular, subcutaneous, intradermal, intraperitoneal, intravenous, intranasal instillation, spray, wing web, intra-mammary or topically to the skin / conjunctiva. Administration of test article or control by the oral route (including on feed or bolus /capsule) up to a maximum of twice daily for 40 consecutive days.

Administration of test article in feed or water, which will be offered ad libitum to appetite or on a restricted daily scale.

The area near the inoculation site may be tattooed in order to allow easy location subsequently.

Administration of challenge material or control by intramuscular, subcutaneous, intradermal, intraperitoneal, intravenous, intratracheal, into an air sac (poultry), intranasal instillation, intramammary, spray, wing web, orally (including by gavage), via the cloaca, by natural exposure (including exposure to animals which have been challenged) or topically to the skin / conjunctiva according to the following. Veterinary or husbandry treatments may be withheld.

Blood sampling with / without the topical application of a vasodilator, including following non- surgical cannulation of blood vessels. A maximum of 10% of the estimated total volume on one occasion and up to 15% in a 28-day period.

Collection of samples from the nasal passage, naso-pharyngeal passage, cloaca or vagina using a swab.

Collection of faeces with a swab, faecal loop or finger.

Measurement of body temperature per rectum.

Withholding of feed or water to allow for collection of samples e.g. bile, for clearer assessments of gut lesions, or collection of gut samples or to encourage uptake of treated feed or water when offered.

Collection of urine from sheep for urinalysis by inducing anoxia. Sampling may be attempted up to 3 times in one day.
Synchronisation of oestrous and ovulation according to normal farm practice i.e., insertion and extraction of intravaginal sponges or CIDRs, and intramuscular injection of Pregnant Mare Serum Gonadotrophin (PMSG) or equivalent.

Insemination of semen to the vagina / opening of the cervix using a syringe and/or rubber tube and a vaginal speculum to open the vaginal walls.

Administer cloprostenol (or equivalent) and steroids if needed to terminate

pregnancies at or before 40 days after insemination.

Use of speculum to examine the vaginal track and cervix pre and post test item application or collection of test item, mucus, or secretion samples, on up to 8 occasions in one day.

Animals may be killed by a schedule 1 procedure. Alternatively, birds may be killed by a method approved under EC 1099/2009 i.e. exsanguination / decapitation following captive bolt or head only electrical stunning; birds less than 3 kg may be killed by manual dislocation of the neck; birds less than 5 kg may be killed by mechanical dislocation of the neck.

What are the expected impacts and/or adverse effects for the animals during your project?

This will be very varied based on the type and model of study. Depending on the species and disease model clinical symptoms may include weight loss, reduction in weight gain, inappetence, dullness, lethargy, behavioural changes, diarrhoea, nasal discharge, ocular discharge, vulval discharge, raised temperatures, injection site reactions etc. This list is not exhaustive and all symptoms are not applicable to all studies.

All studies will be kept to the minimum severity limit possible, with the majority being mild and some moderate. Animals are inspected a minimum of twice daily with extra obs in place when needed. Any animal with adverse effects evident will be monitored closely and treated or withdrawn from study as applicable under veterinary advice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on previous PPL the estimated severity would be mild and moderate with 90% of animals at mild and 10% at moderate

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

When testing efficacy and safety of veterinary medicines the European guidance documents require that the target species of animal is used.

Which non-animal alternatives did you consider for use in this project?

None are applicable for registration studies.

Why were they not suitable?

Target animals are required for registration studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers included have been estimated based on historical data from previous projects of work to develop livestock veterinary medicine products. However, actual numbers will be customer driven and will be monitored and may need to be adjusted depending on the work profile over the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where there is a European guidance document detailing the requirements, we will comply with these. Where there is no guidance document, we will take the advice of a statistician.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Following EU guidance documents, sharing control groups for multiple treatment comparisons, collecting supplemental samples in addition to the primary objectives.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain

why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal species, methods and models we propose to use are as dictated by European guidance documents and agreed with clients and regulatory authorities.

Why can't you use animals that are less sentient?

The animal species we propose to use are as dictated by European guidance documents.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

After each study is complete they are reviewed by AWERB and any potential refinements proposed and discussed. This are then actioned by the PPL holder alongside all personnel with Home Office responsibilities to instigate throughout the establishment.

The PPL holder is the key Study Director and NTCO and is directly involved with all studies on site, constantly monitoring the progress of studies, the welfare of the animals and potential outcomes.

Technicians are in daily communication with the Study Directors and PPL Holder about welfare and health status of the animals and adjustments are made immediately as required to ensure optimum welfare and reduced severity.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Home Office, DEFRA and RSPCA guidance documents along with breed guides are followed to ensure optimum environments are provided for all animals. Study designs follow EU guidance documents where applicable. Studies are run to comply with GLP and GCPv regulations.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through seminars, conferences, literature, sharing information with other institutes.

85. Functional genomics in African trypanosomes

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes.

Key words

African trypanosomes, Functional genomics, Infectious disease, DRiF-Seq

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To apply contemporary genetic technology to study African trypanosome parasite gene function at genome scale.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

African trypanosomes and related species affect >20 million people per annum, as well as having a huge detrimental impact on meat and dairy production, killing over 3 million cattle each year, and creating losses of ~$4 billion from developing

economies in Africa, Asia and South America. There is no vaccine available, diagnostic tools are limited, and drug resistance has emerged.

The function of trypanosome genes and their role in disease are of great scientific interest, including for understanding parasite biology, identifying virulence factors and tackling drug resistance. African trypanosomes contain >10,000 genes, most of which are hypotheticals (genes of unknown function). We have developed and applied a new highly-parallelised, high-throughput technology to create genome-scale libraries of mutants in multiple species and strains of trypanosomes. This enables the first genome-scale forward genetic screen in this important parasite of humans and livestock.

Application of this in animal models provides understanding of how parasite genes affect disease establishment, outcome and transmission. This is critical, for example, in identification of virulence factors and mechanisms of drug resistance, which affect how we treat infected individuals.

What outputs do you think you will see at the end of this project?

Successful generation of novel methodologies and biological knowledge about mechanism of disease that effectively and efficiently meet the demand of research colleagues, vaccine development pipelines and drug discovery centres. The resulting methods and knowledge being incorporated into research programmes that will expand scientific understanding and/or have a direct translational impact on the development of new therapeutic and diagnostic approaches for African trypanosomiasis affecting both humans and livestock.

Who or what will benefit from these outputs, and how?

As a result of their significance for human and animal disease, there is a large international academic community of scientists studying African trypanosomes specifically, and closely related organisms more widely. The demonstration of generally applicable and transferrable, high power genomic methods will directly benefit this large scientific community, with potential impacts across the wider parasitology community.

The immediate beneficiaries will be researchers working in the models developed and used during this project license (which include models of both human and animal disease). For these researchers, the cell lines and plasmids can be used 'off the peg'. In addition, the experimental and analytical approaches which will be used here can be adapted to other types of functional genetics work in unrelated disease organisms such as Leishmania, Toxoplasma and Plasmodium spp.

In terms of translational medicine/veterinary, functional genomics is an essential prerequisite for priming vaccinology pipelines or development of new diagnostics and antibody-based drug delivery. Existing high-throughput vaccinology pipelines will enable immediate opportunity for testing the genes identified here for potential in protection against African trypanosomiasis. Equally, gene function identified that appeared to be potentially druggable will be quickly communicated to the established pipeline for the development of new anti-parasite interventions.

Finally, product-development partnerships, the UN Food and Agriculture Organisation (FAO), and national programmes for African trypanosomiasis in South America and Africa are also key stakeholders that will benefit from the outputs of the this license.

How will you look to maximise the outputs of this work?

Maximising the outputs of this work will primarily be through dissemination of results (including open access Publications and Conferences in advance of publications), sharing of data and methods (especially Resource Sharing and integration to Online Resources), and engagement with end users (Collaborations and Conferences).

In addition, we will keep our current funders up-to-date with research outcomes to allow dissemination via their website, and utilize the institution's animal welfare and ethical review body and the NC3Rs regional Programme Managers for local dissemination.

Importantly, much broader impact is anticipated by incorporation of data and analysis generated during this license into field-specific curated informatics resources, which provides common infrastructure and analysis methods for integrated genomic/post-genomic data from trypanosomes and Leishmania. It is the community's major resource for access to diverse functional genetic data.

Species and numbers of animals expected to be used.

- Mice: 200
- Rats: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Type:
African trypanosomes only infect mammals. Thus, the smallest available animal models are rodents.

Research over many decades has shown that rodents can support the growth of trypanosomes without significant discomfort to the animals when the disease is monitored closely, and animals are humanely killed at the appropriate time (as the clinical signs of distress are highly predictable).

For infections requiring low inoculum, parasites will be inoculated into a mouse and maintain symptoms of disease associated with mild to moderate levels of pain and distress, with the resultant infection reflecting normal progression of disease.

For infections requiring millions of parasite inoculum, this amount inoculated into a mouse would create symptoms of disease linked to moderate to severe levels of pain, and non-physiological host reaction. Severity can be reduced by using a larger rodent model – rats. One million cells injected into a rat maintains symptoms of disease associated with mild levels of pain and distress, and the resultant infection reflects normal progression of disease.

Life stage:
This licence will use adult purpose-bred animals of assured health and genetic status, typically obtained within the UK or the EU from commercial suppliers.

Typically, what will be done to an animal used in your project?

Typically, animals will experience mild, transient pain and no lasting harm from inoculation of trypanosomes by injection using standard routes.

Before/during trypanosome infection, animals may be supplied orally with doxycycline, and/or may be administered with cyclophosphamide (animals will experience mild and transient discomfort from inoculation of cyclophosphamide).

Animals will experience clinical signs associated with trypanosome infection: initially poor coat condition, dull/sunken eyes, temporary shivering, progressing to hunched back, staring coat, piloerection, intermittent shivering with reduced mobility at upper limit.

Animals will be routinely monitored for clinical condition and parasite numbers through blood count to assess and control the impact of parasite infection. To that end, animals will experience mild and transient discomfort from blood sampling.

Animals will experience non-recovery anaesthesia, in which the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain.

Infections will typically last 15-21 days. The scientific endpoint for the studies proposed here will be no longer than 28 days for all animals.

What are the expected impacts and/or adverse effects for the animals during your project?

Impact from parasite infection:
Trypanosome infections are consistent in terms of disease development and ensure a predictable progression through clinical signs that minimise potential harm to rodents. Research over the past 70 years has produced good and solid knowledge of the likely progression of parasitaemia for various parasite and animal strains and inocula, route of infection, parasite growth kinetics, animal symptoms, clinical changes, experiment size and endpoints, safe immunosuppressant treatment, effective administration of doxycycline in drinking water, adverse effects, and several other parameters of animal infection.

This enables accurate projection of the course of infection through animal body

condition (following the FELASA guidelines on pain and distress) and parasite number in blood. This is routinely used in research labs, and classes the proposed work as moderate severity by the HO.

Specifically, rodents infected with trypanosomes develop symptoms of disease associated with mild levels of pain and distress (clinical signs of poor coat condition, dull/sunken eyes, temporary shivering), progressing through to moderate levels of pain (hunched back, staring coat, piloerection, intermittent shivering/tremors with reduced mobility). Through routine monitoring the animal wellbeing status for its clinical condition, parasite numbers through blood count, and experience of the kinetics of the infection profile, it is possible to assess and control the impact of parasite infection.

For instance, clinical conditions observed following inspection schedules: Mild signs=> poor coat condition, dull eyes-score, transient shivering/tremors
Moderate signs=> hunched, staring coat, piloerection, intermittent shivering/tremors with reduced mobility and/or responsiveness.

Any animal that presents the moderate clinical signs will be humanely killed by terminal anaesthesia and exsanguination, or by a Schedule 1 method.

Other impacts:
During infection, when animals are blood sampled in a conscious state, refined techniques in line with LASA and NC3Rs guidelines will ensure that any pain is mild and transient in nature. Blood collection limits will accord with HO policy. On rare occasions (less than 5% of times), a bruise or swelling at the site of blood sampling may form which should subside within 3 to 5 days.

Doxycycline is a broad-spectrum antibiotic of the tetracycline class. When required, it will be used to switch on the transgenic parasite gene silencing machinery in vivo.

Cyclophosphamide is a chemotherapy treatment for several different types of cancer.

When required (to enable establishment of infection for certain parasite-animal strain combination), it will be used to short-term suppress the animal immune system.

At end of infection experiments, animals undergoing non-recovery step for the collection of blood and/or tissue will typically be exposed to an anaesthetic gas or the experience of an injection of an anaesthetic drug. The anaesthetic agents will be administered in such a manner as to minimise the risk of panic and the animals will gradually lose consciousness and enter a stage of deep surgical anaesthesia. At this point, blood and/or tissues will be collected. The loss of blood during the procedure will ensure that the animals will not regain consciousness. However, humane killing will be completed and confirmed according to Schedule 1.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected maximum severity experienced by 100% of the animals undergoing trypanosome infections will be moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of experimental animals is essential to address questions related to an infectious agent, particularly those questions related to the identification of targets to inform therapeutic interventions.

Which non-animal alternatives did you consider for use in this project?

African trypanosomes can be grown axenically in vitro in culture flasks at human-body temperature in nutrient-rich media.

Why were they not suitable?

After being transmitted by the bite of a tsetse fly, African trypanosomes create cyclical waves of parasitaemia. Initial passage of infectious forms creates a first wave that is controlled by parasite quorum-sensing and host immune response to the surface coat. However, within this peak are cells that have switched coat and are able to go on to create a subsequent parasitaemic wave. Successive waves enable the parasite to establish a chronic infection. Parasite virulence is hence a combination of factors affecting establishment/growth (ascending wave), peak height and duration (1st peak/descending wave) and chronicity (subsequent waves) (Figure 3). This infection dynamics cannot be replicated in vitro.

In the host blood, trypanosomes exists as two different lifecycle stages: a replicative stage (which evades the host immune attack) and a quiescent stage (which is pre-adapted to survive in the insect vector). This parasite cell differentiation cannot be recapitulated in vitro.

Asymptomatic individuals with human trypanosomiasis (long-term seropositives) have undetected parasitaemia in their blood. This is because trypanosomes migrate to other tissues and organs in the body, where they remain undetected but can re-emerge at a later stage. Parasite tissue tropism cannot be replicated in vitro.

Ultimately, trypanosome infections result in parasites crossing the blood-brain barrier and causing the fatal neurological disorders characteristic of its disease (African trypanosomiasis is also known as "*Sleeping Sickness*"). Despite recent advances in 3D organoid cultures in our collaborator's laboratory, parasite interactions with the

host brain cannot currently be replicated in vitro.
All the above aspects of human and animal disease cannot be replicated in vitro using the culturing systems currently available. As such, it does not represent a non-animal alternative to the proposed work.

References for the statements above:
Trindade et al. (2016) Cell Host Microbe 19(6):837. Capewell et al. (2016) Elife 5:e17716. Silva Pereira et al. (2019) Open Biology 9(5):190036.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

African trypanosomes only infect mammals; thus, the smallest available animal models are rodents. For **Objective 1** of this license, ~50,000 cell inoculum is required. This amount can be inoculated into a mouse. For **Objective 2** of the license, ~1 million cell inoculum is required. This amount inoculated into a mouse would create symptoms of disease linked to moderate to severe levels of pain, and non- physiological host reaction. Severity can be reduced by using a larger rodent model – rats. One million cells injected into a rat maintains symptoms of disease associated with mild-to-moderate levels of pain and distress, and the resultant infection reflects normal progression to parasitaemic peak.

Establishment of infection with animal-adapted parasites is extremely efficient, with nearly 100% of cells infectious (see Figure 5) – i.e. for the parasite and mouse strains proposed here, trypanosomes will always establish infection and grow in vivo.

Experimental design:
African trypanosomes create successive waves of parasitaemia due to an interplay of parasite plus host factors. Parasite virulence is a combination of factors affecting establishment/growth (ascending wave of infection), peak numbers and longevity (first peak/descending wave) and ability to survive long term (subsequent waves). By sampling mutants at both ascending and descending wave, this work will be of significance to the maximum number of possible end users: those who assess fitness during initial infection establishment (the most assessed point due to its amenability to testing), but also those wishing to measure fitness after first peak (a stage where traditional approaches have low statistical power without very large group sizes; see Figure 3).

Sample size:
For quantitative power, it is vital that sampling of mutants in the inoculum is reasonably high. In **Objective 1**, we will assess fitness of gene-set library of mutants in vivo at multiple points during infection, to provide quantitative data on both survival

and initial establishment of infection (first wave) and also longer-term persistence/survival of the host immune response (subsequent waves). Infection of mice with ~50,000 cells allows individual gene effect sizes as low as 25% to be detected (based on both simulated data and experimental tests from our preliminary studies). Sampling from 4 mice detects changes in individual clones of 2-fold or more, in addition to providing an estimate of gene variance between infections. Thus, 4 mice will be used for each library timepoint during a wave of parasitaemia.

For quantitative power in **Objective 2**, whole genome fragment libraries will be transfected into human and animal African trypanosomes to produce 15 distinct populations of 10,000 independent mutants each. Each of these population of genetically-distinct parasites will be inoculated into two rats [15 libraries x 2 rats = 30 rats per parasite species] and also sampled at different points during infection (as per Objective 1) during ascending or descending wave. This group size – 100 cells per mutant – allows detection of changes in parasitaemia upon gene silencing with analogous power as Objective 1. A typical gene will be represented by 7 RNAi mutants stochastically distributed between the 15 libraries, each used to inoculate an individual rat. This approach is preferable to dividing a single library between the rats as it systematically captures variation between clones and also animals (with >99% of genes expected to be represented by more than >1 mutant).

Quantitative fitness measurements seen in competition (library approach) will be validated as individual gene experiments (gene-by-gene approach), with mouse allocation to groups by pre-experiment randomisation across animals in all cages.

For **Validation**, we will introduce an individual RNAi fragment into trypanosomes to silence a specific gene upon induction in vivo (plus control, i.e. RNAi that has not effect). A group size of 5 mice offers sensitivity to >2-fold change in parasitaemia (as has been used for numerous experiments in the literature).

Based on the above, 200 mice + 200 rats will be used during the lifespan of this license.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To meet the Objectives of this licence, it is important to have a high coverage of mutants within a library. To be able to identify the true positives (those that will then be exploited in a clinical setting in the future) within the testable set (either a gene-set library in **Objective 1**, or a genome-wide library in **Objective 2**), it is appropriate to set a reasonably high power (see more in *Protocols* section). In very simple words, when proposing to take lead candidate genes forward into clinical R&D pipelines, one should only be prepared to be wrong 5% of the time, at the same time as only missing a true positive hit 10% of the time. Performing the proposed work with any less power would represent unnecessary (and wasteful) animal use.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The overall aim is to phenotypically profile the entire parasite genome during host

infection. African trypanosomes encode >10,000 genes. The traditional and now largely superseded way of carrying out such test would be to generate mutants for each of those genes individually and test them in vivo in a piecemeal gene-by-gene approach. This would represent a huge cost in terms of animals.

Instead, in this license we will exploit recent technical advances in genomic science to demonstrate the effectiveness of a highly-parallel parasite mutant analysis method in vivo. The approach (which uses a library format to combine 1000's of functional gene tests in a single animal) is based on in vitro and pilot in vivo data, suggesting a very high chance of success, and could substantially reduce experimental animal usage (by 70-99%, depending on the individual study design), while obtaining better scientific translatability, and with further potential for replacement of large animals for large-scale gene analysis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal model used during this project will be rodents (mice and rats). The method will be infections of models with trypanosomes.

- for infections requiring millions of parasite inoculum, this amount inoculated into a mouse would create symptoms of disease linked to moderate to severe levels of pain, and non-physiological host reaction. Severity can be reduced by using a larger rodent model – rats. One million cells injected into a rat maintains symptoms of disease associated with mild to moderate levels of pain and distress, and the resultant infection reflects normal progression of disease.
- for infections requiring low inoculum, parasites will be inoculated into a mouse and maintain symptoms of disease associated with mild to moderate levels of pain and distress, with the resultant infection reflecting normal progression of disease.

Why can't you use animals that are less sentient?

African trypanosomes only infect mammals; thus rodents are the smallest, least sentient animal models of disease.

Immature life stage has an immature immune system and, as such, is unable to support parasite infections without developing severe symptoms of disease.

Adult animals are required for infected blood/tissue collections as their larger body size correlates to a larger blood/tissue volume. This represents a Reduction in the

number of animals required to obtain solid scientific translatability.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Housing:
The welfare costs to animals will be minimised by the provision of group housing with environmental enrichment, bedding and or nesting material as standard.

Accurate monitoring of disease progression:
Trypanosome infections in rodents are consistent in terms of disease development, and ensure a predictable progression through clinical signs of infection and assessment of humane end-points that maximise the generation of scientific knowledge at the same time as minimising potential harm to animals. Several years of research in the trypanosome field has generated good and solid knowledge of the progression of parasitaemia for different strains of parasites and of rodents, and of the associated clinical signs (as parasitaemia levels in peripheral blood correlates with expected clinical signs). Thus, regular sampling during an infection allows good monitoring of individual animals and identification of those which might be reaching the upper limit of the moderate category (e.g. Figures 2B & 3A). This enables the accurate projection of the course of infection that follows the FELASA guidelines on pain and distress and their table of clinical signs representing the upper limit of severity in each category. Through regular monitoring of body condition, along with the FELASA guidelines and regular sampling, the impact of parasite infection is controlled through measurements based on strain, clinical condition and experience with the infection profile in animals, which will enable ***a constant and overall assessment of animal welfare***.

Handling:
Animals will be handled and restrained with the minimal effective restraint. Full records of procedures undertaken, daily monitoring and veterinary requests will be maintained using both hard copy and a proven electronic facility management software. All licensees and animal care staff will be trained, supervised and signed off as competent for the procedures they will undertake.

The use of doxycycline:
Tetracycline/doxycycline is required to switch ON the parasite gene silencing machinery by RNA interference (RNAi) in vitro and in vivo, which enables the effective study of gene function at scale and in parallel: i.e. testing hundreds of gene knockdowns at once. This representing a significant reduction in the number of animals required (see Reduction section). Animal infections with RNAi mutants typically add doxycycline to drinking water. Colleagues at other institutions and us through collaborative work have shown this regime to be successful both in terms of triggering RNAi and also in terms of animal welfare. Several other research labs have also successfully replicated such an administration method, and their results are available through publications in peer-reviewed journals.
In the unlikely event in which signs of dehydration (which are readily recognisable in rodents) are observed as a result of the use of doxycycline in drinking water, this licence will consider supplementing the water with 5% sucrose (as validated by peer-reviewed publications) or the use of doxycycline in diets as a method of refinement.

Routinely-used suppliers provide doxycycline- containing diets for tetracycline-regulated systems. Between the two oral routes, sweetened water delivery is the preferred option, as chemical compound delivery through diet makes it difficult to ensure that the animal has eaten sufficiently to activate a tetracycline-regulated system.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice guidelines from LASA and NC3Rs will be followed with regards to refining blood sampling and injection technique.

Home Office Code of Practice will be used to ensure animal care and housing is appropriate. This guidance will be used in conjunction with the advice available in the NC3Rs Resource Library for both housing and handling of animals.

The NC3Rs Procedures with Care web resource will inform personal licensees of refinements in the conduct of the minor procedures undertaken in this licence.

The NC3Rs grimace scales will be used to monitor animals for potential signs of distress.

Home Office and FELASA severity information will be used to ensure the severity experienced by the animals can be recorded and limits within this licence adhered to.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Myself and research group members who are licence holders and academic users of this project licence will stay up-to-date with the NC3Rs' advances through the information on their webpages and cascaded via their newsletters, and regularly attend NC3Rs’ webinars, training sessions and symposia. Hardcopy journals relating to laboratory animal science (via the institutional membership of LASA and the IAT and links to FRAME) are also accessible to us.

86. Initiation and resolution of inflammation in skin wounds

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Wound, Skin, Inflammation, Therapy, Repair

Animal types	Life stages
Mice	juvenile, adult, aged, pregnant, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the wound inflammatory response to inform the development of new therapies to improve wound healing and reduce scarring in humans.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Wound healing complications (including scarring and chronic wound formation) affect millions of people worldwide and can result in death, limb amputation and disfigurement. To date there remains little in the way of effective treatment strategies

to target chronic wounds and scarring. Consequently, there is an urgent need to develop such treatment strategies and lessen the disease burden on society and healthcare systems.

What outputs do you think you will see at the end of this project?

1). A greater understanding of the cell biology of skin wound inflammation during repair, in chronic wounds and scarring which will inform the development of more effective treatments for animals and human patients with these conditions.

The effects on chronic wound treatment are potentially far researching. Effective therapies for non- healing wounds might avoid the need for amputation and the resulting risk of death or disfigurement altogether. This would lesson the burden of these wounds both on the afflicted patient and healthcare systems.

Identification of new pathways that can dampen and resolve wound inflammation could impact the treatment of a wide range of inflammatory disorders, including, but not limited to wound healing, for example arthritis, heart disease and cancer.

The research will be published in peer-reviewed journals and summarised in press releases.

New therapies may be developed.

The provision of a preclinical chronic wound model to the scientific community.

Who or what will benefit from these outputs, and how?

Beneficiaries of this program of work include academics and clinicians working in dermatology, aging/gerontology, immunology, multiphoton imaging, human patients with chronic wounds, acute wounds (including trauma and surgical wounds), or scarring, animal patients with chronic wounds, acute wounds or scarring may also benefit (dogs such as whippets and greyhounds commonly suffer from chronic wounds in later life).

The benefits of this research are expected to extend beyond skin repair and will likely be applicable to a wide range of tissues in which damage and/or chronic inflammation lead to scarring and associated pathologies (eg. chronic inflammation and fibrosis of the liver and lung). The work will therefore be of benefit to pharmaceutical companies and biotechs interesting in developing therapeutics that modulate the inflammatory response, wound repair or macrophage (Mphi) biology.

The work proposed on the role of immune-modulators particularly lends itself to development of novel therapeutics, for example through development and use of synthetic receptor specific agonists or utilising endogenous agonists. Our research will address fundamental principles in Mphi biology. Since immune cells are known to perform a diverse array of functions in health and disease, this work will have wide ranging impact, likely well beyond the skin repair field. One possibility we will explore here is immune cell adoptive transfer to determine whether acute wound immune cells can be delivered to chronic wounds to rescue the healing process as a potential

therapeutic strategy.

How will you look to maximise the outputs of this work?

Intended outputs of this programme of work will include publications and presentations (short-term), and may include filing of patents, most likely regarding novel therapeutics to treat chronic wounds or fibrosis (medium-term). It is likely that if candidate compounds are identified and progressed to phase 1 clinical trials that this would be beyond the duration of the current PPL.

Species and numbers of animals expected to be used.

- Mice: 7500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used due to the ready availability of genetically modified animals for study of disease pathways and processes. We have selected models of skin repair and chronic wounds that equate to human disease and we have extensive experience with these models. Young adult animals will be used to equate to young adult humans and 12-24 month old mice used to equate to middle age to elderly humans to understand the role of aging in the healing response.

Typically, what will be done to an animal used in your project?

The majority of animals will be anaesthetised, given a pain killer once asleep (by injection under the skin), have their back fur trimmed very short and small circular wounds made to the skin. Some animals will have substances added to the wound to to understand their effects on skin repair. Others may have a transparent dressing put on the wounds, similar to those used on people. Images of the wounds may be taken whilst the animal is asleep, this is usually when a dressing is being changed or a treatment being given. Experiment duration will be between 1 and 14 days in the majority of animals.

What are the expected impacts and/or adverse effects for the animals during your project?

We plan to use different models of skin wounds in young adult and elderly mice. We expect so see minimal weight loss (< 10%), no overt pain or abnormal behaviour.

The experiments will typically last up to 14 days, with pain killers provided when the wounds are made to ensure minimal discomfort. The animals will be culled humanely at the end of the experiment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The breeding and maintenance of mouse colonies, including elderly mice is of mild severity (100%) For the skin wound protocols we expect to see moderate severity in all animals (100%)

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use mice to study skin wound repair because we cannot study complex multicellular interactions nor the impact of disease states on immune cells or wound repair in cell cultures. Human skin explants cannot be used as the blood vessels and lymphatic vessels are disconnected and we need to be able to see how immune cells in vessels arrive and leave wounds.

Which non-animal alternatives did you consider for use in this project?

Larval zebrafish, fruit flies, cell culture, tissue culture of human skin explants.

Why were they not suitable?

Important differences between fish, fly and mammals mean we cannot replace mice with invertebrates, although we will continue to explore their potential use. Zebrafish, for example, are unsuitable as the skin structure is very different to mammalian skin due to the presence of scales and water environment. A vital component of this program of work is the mouse model of chronic skin wounds. It is not currently possible to model chronic wounds in non-mammalian systems. The mouse model therefore improves our ability to translate findings into the clinic to benefit patients. We will continue to use in vitro and ex vivo approaches with human tissues and skin cells where possible to add value to the animal studies performed and to minimise the need for in vivo experimentation as much as possible.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate we will use approximately 7500 mice in the lifetime of this license. We estimate includes using 3500 for breeding and maintenance.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When experiments are planned we receive input on experimental design and power calculations from in-house statisticians and using NC3Rs experimental design assistant. We also attend training courses on experiment design to ensure that we use the most appropriate group size and include appropriate control groups so that experimental data is scientifically interpretable.

We carefully store samples from experimental animals such that additional future experiments can be undertaken on archived material wherever possible. This material will be made available to other research groups on request so that additional experiments involving animals will not be required. The models we use have been refined to reduce variability and therefore enable us to use the minimum number of animals possible.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continue to explore the potential of small animal imaging to allow collection of data at multiple time points to reduce numbers and will maximize and refine the collection of multiple pieces of data from individual mice.

We carefully store samples from experimental animals such that additional future experiments can be undertaken on archived material wherever possible. This material will be made available to other research groups on request so that additional experiments involving animals will not be required.

Pilot studies will be performed where appropriate, for example to inform dose selection of a potential therapeutic. Animal breeding will be carefully controlled to minimise surplus.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the most appropriate species to use to address our research goals for the following reasons:

Mice have comparable mammalian physiology, immune system and anatomy to humans.

The excisional wound model is extensively published in the literature as a prototypic model of skin repair and provides analogous histological changes to human wounds.

The immune system in the mouse is well characterized, with similar leukocyte subpopulations as in human.

Repair can be impaired or prevented, offering the ability to study chronic wound formation and persistence.

The plethora of murine tools (transgenic animals, antibodies, inhibitors) available either through collaborators or commercially, enable us to investigate the proposed systems to achieve our research objectives and are not available in porcine systems.

Using the mouse to study wound repair therefore offers an excellent chance of obtaining clear interpretable and reproducible data that could be translated to human systems for patient and societal benefit.

Why can't you use animals that are less sentient?

We cannot use more primitive model organisms such as zebrafish and fly as it is not possible to study age-impaired healing or create chronic wounds in these systems, in addition, the immune system is much less comparable to human and the skin anatomy is almost entirely different to mammalian systems.

A more immature life stage cannot be used as the systemic effects of advanced age are required for the models to generate useful data that could be translated to human systems.

The protocols are not brief enough for them to be carried out under terminal anaesthesia.

Ex vivo skin and wound samples from human amputated limbs will be used when available and relevant to the question being addressed. They are not a substitute for mouse experiments as they lack a circulatory system to study immune cell recruitment and emigration and tissue perfusion.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Previous studies by us show that acute and chronic mouse wounds are very well

tolerated, providing basic needs such as maintaining body temperature during (using a heat mat) and following anaesthesia (using a warm box) are met. Mice are kept under anaesthesia for a short period after wounds are made to ensure that the i) immediate pain signals from wounding have died down (such that the animal is not in moderate pain as recovering from anaesthesia) ii) topical gel application has had sufficient time to set (where applicable) and iii) to allow wounds to be photographed at time zero. Chronic wounds are also well tolerated throughout the course of the model and are of reliably moderate severity, however appropriate analgesics are provided (as in many human patients) including at time of wounding and following any wound debridement.

Blood collection will be done under general anaesthetic for animals with open or chronic wounds to avoid trauma to the wounds during restraint. Occasionally animals attempt to remove their wound dressing (eg. transparent dressing such as Tegaderm) . This is minimised by ensuring wounds are made closer to the cranial end of the dorsal skin and making sure that dressings are not excessively large eg covering skin near the hind legs which could irritate the animals during movement. Further refinements include trimming the animals nails, single housing animals and providing additional environmental enrichment to serve as a distraction. Animals will be single housed for the shortest time possible to achieve the scientific objectives and will be provided with additional environmental enrichment.

Analgesia will be used as standard at time of wounding and for protocol 3 at time of wound debridement. No severe models are required in this project. Where more refined approaches become available during the course of this licence either through personal communication, publications or veterinary advice, we will investigate their use following discussion with the NVS and Home Office Inspector. Animals displaying >15% weight loss, rough fur, inappetence, inability to groom, reduced mobility or meeting the humane endpoint criteria described in protocol scoring sheets will be removed from the experiment and killed by a schedule 1 method.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow NC3Rs guidance, ARRIVE and PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institute is very proactive in distributing information relevant to the 3Rs, including seminars and workshops held either locally or by third parties on a range of topics such as husbandry and handling. This information will be digested and incorporated into the project where possible.

87. Investigating disease mechanisms and therapy for Friedreich's ataxia (FRDA)

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Friedreich's Ataxia, Therapy, Antioxidants, Iron chelators, Metabolomics-based drugs

Animal types	Life stages
Mice	adult, juvenile, pregnant, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to investigate the molecular mechanisms of FRDA with the view to provide novel therapeutic strategies for FRDA diagnosis and treatment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Our studies will provide new insights into the treatment of FRDA and will provide further stimulus in the scientific community opening new lines of investigation for the development of new therapies. There is currently no therapy for FRDA. Therefore, the outcome of this project by providing new compounds, therapeutic approaches and/or novel targets will provide relief from suffering and better quality of life for FRDA patients worldwide.

What outputs do you think you will see at the end of this project?

To date, there are no effective treatments to halt Friedreich's ataxia (FRDA) progression and there is an urgent need for the development of valid therapies to slow down the progression of FRDA thereby providing a better quality of life to patients. This lack of therapies is due at least in part to a substantial lack of understanding of the early pathophysiological mechanisms. The aims of the proposed work strongly fit within the scope set out by published research priority setting exercises; in particular by preventing the disease progression and finding an effective treatment for this disorder. In addition, it will improve our current knowledge on the disease. All data from these studies will provide valuable information for the potential translation to the clinical trials. We expect that the findings of this proposal will provide a unique opportunity to devise novel therapeutic strategies for FRDA patient diagnosis and treatment so that children with FRDA may, one day, experience long-term survival opportunities. The findings of this research proposal are likely to be of interest to scientists across various disciplines, both in basic and applied research fields, which may not only be limited to FRDA therapeutics, and could be also useful for pharmaceutical companies which are active in developing drugs for different neurological disorders. The outcome of these projects will be published in peer reviewed journal articles.

Who or what will benefit from these outputs, and how?

Friedreich's ataxia (FRDA) is the most common form of the hereditary ataxias accounting for at least 50% of cases of hereditary ataxia. The estimated disease incidence, based on carrier frequency of 1 in 85, is 1:29,000. Generally, in Europe prevalence is quoted as between 1 in 20,000 to 1 in 50,000, with some geographical variability (the highest levels observed in northern Spain, south of France and Ireland, and lowest levels in Scandinavia and Russia). The prevalence of FRDA in the UK is ~1 in 54,000 or an estimated 1100 patients in the UK. In the US about 1 in 50,000 people are affected with an estimated carrier prevalence of 1:100. Individuals with FRDA usually present in early childhood with difficulty in walking due to poor coordination or "ataxia". The condition progressively deteriorates, leading to the development of debilitating musculoskeletal deformities and immobility, while the majority die in early adulthood due to heart failure or associated complications. FRDA is caused by a defect (mutation) in the FRDA gene which codes for "Frataxin," a protein which plays an essential role in mitochondrial health within the cell. A deficiency of this protein leads to disordered iron metabolism within mitochondria, leading to increased production of toxic molecules, oxidative cell stress and, ultimately, cell death. There is currently no effective therapy for FRDA. As outlined in Ataxia UK 2010 Vision Research Strategy and in line with Friedreich's Ataxia Research Alliance (FARA)'s strategic priorities, it is an absolute priority to find effective treatments that can stop disease progression or cure it.

This project has three main aims; firstly, we aim to investigate the disease-specific changes in our FRDA mouse models and to identify potential and novel targets for the development of therapeutic strategies in this childhood disease. This study will improve our current knowledge on the disease. In addition, it will open up new opportunities for the identification of novel biomarkers for FRDA or other neurodegenerative diseases. Secondly, we aim to investigate the use of compounds with antioxidant properties as a form of treatment for FRDA. The outcome of this research proposal may lead to an encouraging treatment option to attenuate associated symptoms and prevent FRDA disease progression. The safety and pharmacological effects of the proposed drugs have already been tested in human with various disease conditions including diabetes, cancer or early chronic kidney disease. Therefore, following successful *in vivo* safety and efficacy studies of these drugs in our FRDA mouse models, they could progress rapidly to the next stage of clinical trials. Finally, we aim to investigate the role of key regulators of sphingolipid metabolism in FRDA and to identify potential and novel targets for the development of therapeutic strategies in this childhood disease.

Short term benefits: The proposed research projects would enhance our understanding of disease pathogenesis and have a potential to deliver new candidates for the development of effective therapies for FRDA. The results of these studies will be disseminated at national/international conferences and will be published in peer-reviewed journals.

Medium- and long-term benefits: These studies would be of interest to many researchers and pharmaceutical companies who are working on neurodegenerative disorders including FRDA. The findings of this research could yield novel disease targets for pharmaceutical therapy so that FRDA patients may, one day, experience long-term survival opportunities. This not only would have a huge impact on UK health system but would also benefit patients suffering from neurodegenerative disorders worldwide.

How will you look to maximise the outputs of this work?

I am collaborating with several collaborators nationally and internationally. We meet regularly to discuss the progress of the projects and the plan for future grant applications. In addition, with help from my collaborators, I was able to hold preliminary discussions with clinicians. It became apparent that a clear and detailed appreciation of the translational pathway from mechanistic discovery to potential treatment development and clinical introduction would be highly worthwhile from the outset. Moreover, it was suggested by my peers, that I keep the research objective clearly focused on delivering results that can be rapidly translated, as well as adopting the good practices learned from previous trials in drug development. In addition, I am the Director of a recently established multi- disciplinary research network on Rare Diseases, which aims to enhance progress in rare disorder diagnosis and treatment. This involves research into the origin of the disease, diagnosis, developing therapies, preventing strategies and interventions, disease mechanisms and models and health and social care. The outcome of this project will be presented at meetings/seminars organised by this network as well as other national and international conferences. These events will provide a forum for the discussion of new discoveries and development of new collaborations and exchange

of ideas and technical expertise. Moreover, I have close ties with patient communities, and I am an active member of leading national and international charities for people affected by ataxia. These charities are responsible for improving care and providing support for ataxia patients. As such, we would be able to liaise with patient advocates and their families to ensure the patient voice is integrated into the research project, acknowledging the importance of patient engagement in all stages of the research and development process. The results of these studies will be disseminated at national/international conferences and will be published in peer-reviewed journals.

Species and numbers of animals expected to be used.

- Mice: Over the 5-year project we expect to use maximum of 3,000 mice.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

There is currently no effective therapy for FRDA. In order to better understand the disease and to assess treatment strategies, a living mammalian animal model with complex systems, organs and tissues similar to humans is considered necessary. Use of animals is now required to extend the initial information that has already been achieved from studies of lower organisms or cells grown in the laboratory in order to provide a more complete understanding of this human disorder and to undertake testing of novel drugs before progressing to human clinical trials. This PPL aims at investigating the therapeutic efficacy and safety of compounds previously validated in vitro as a single agent or in synergy with other therapies in appropriate disease models in particular mouse models. This step is fundamental before proceeding to clinical trials in patients.

Typically, what will be done to an animal used in your project?

We will assess the safety and efficacy of treatments with different compounds to ameliorate the FRDA- like disease phenotype of genetically altered mice. We will use age- and sex-matched groups of 5 young adult YG8-derived FRDA mice (2-3 months old). We will first proceed with initial short-term efficacy and toxicity pilot studies of the compounds. Mice will be subjected to different escalating dose treatment regimens using intraperitoneal injection (the most common and efficient delivery route for these compounds at this stage). This will then be followed by long-term efficacy studies of the FRDA mice (2-3 months old) with the most efficacious treatment regime, based upon the results from the short-term studies, with an end point of 5 months of age followed by analysis of the mice to detect any beneficial effect of the drug on the disease phenotype. After treatment, all mice will be killed by a schedule 1 method or Terminal anaesthesia and tissues will be collected.

What are the expected impacts and/or adverse effects for the animals during your project?

The work outlined in this project aims to characterise mouse models for FRDA and evaluate the effects of different treatment strategies (including antioxidant/iron chelating agents and targeting sphingolipid- metabolising enzymes) on reducing disease effects. The procedures undertaken in this study will include breeding and maintenance of genetically altered animals, injecting candidate drugs to ascertain how they are distributed in the body and trialling of potential therapeutic in models of FRDA disease.

The efficacy of candidate therapeutics would be tested by using benign behaviour test to ascertain improvement of mobility/agility. All protocols used in this project are classified as mild or moderate. All the behavioural tests for the assessment of disease associated neurological deficits are non-invasive and are unlikely to cause any pain, distress, or harm to the mice. At the end of the studies, all animals will be killed under terminal anaesthesia or by a schedule 1 method. Any other procedures including drug administration have been widely used and are not associated with any adverse effects. All animals will be inspected regularly, and any abnormal signs or symptoms will be discussed with the veterinarian. Any mice that show signs of pain or distress will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for the breeding and maintenance and behavioural testing is mild and for drug treatment procedures is moderate for all tested animals.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is currently no cure for FRDA, and studies with Omav have been more focused on neurological effects of the disease rather than cardiomyopathy, the main cause of death. Therefore, efforts are underway to understand the pathophysiological basis of the disease and to develop a therapy that will halt or reverse the progress of disease. Some information relevant to understanding FRDA and its treatment have been obtained from studies of bacteria, yeast, worms, fruit flies and human cells grown in a laboratory. We are currently performing *in vitro* cell

culture studies using human and mouse model FRDA fibroblasts to determine potential novel FRDA treatments. We also aim to establish further FRDA mouse model cell lines as part of this project using the previously published protocols. In addition, several groups around the world are now attempting to develop induced pluripotent stem cells (iPSC) from FRDA patient skin fibroblasts, followed by differentiation into neurons and cardiomyocytes, which may prove useful for future investigations of FRDA aetiology and therapy. We have recently generated sensory neurons from iPSC-derived FRDA patients that are used to further validate our findings *in vitro* before moving to preclinical studies. In addition, we have recently established *Galleria mellonella* larvae as a model for studying oxidative stress and efficacy and toxicity studies of compounds with antioxidant properties. However, in order to better understand the disease and to assess treatment strategies, a living mammalian animal model with complex systems, organs, and tissues similar to humans is considered necessary. Use of animals is now required to extend the initial information that has already been achieved from studies of lower organisms or cells grown in the laboratory in order to provide a more complete understanding of this human disorder and to undertake testing of novel drugs before progressing to human clinical trials. Mice are the lowest vertebrate animals in which a representational model of FRDA has been developed. At the same time, mice are also considered to be similar enough mammalian organism to humans to provide invaluable information from preclinical testing of potential therapeutic approaches before progressing to human clinical trials. The aim of proposed project is to utilise the available knowledge on FRDA disease progression to identify novel therapeutic strategies that can be then tested for safety and efficacy against molecular and behavioural deficits in FRDA. This can only be accomplished using animals.

Which non-animal alternatives did you consider for use in this project?

We use human fibroblast cell lines in our laboratory to determine potential novel FRDA treatments. We also aim to establish further FRDA mouse model cell lines as part of this project. In addition, we have recently developed sensory neurons from FRDA patient skin cells which are the most phenotypically relevant cell types affected by FRDA. Moreover, we have recently established *Galleria mellonella* larvae as a model for studying oxidative stress and efficacy and toxicity studies of compounds with antioxidant properties.

Why were they not suitable?

In order to better understand the disease and to assess treatment strategies, a living mammalian animal model with complex systems, organs and tissues similar to humans is considered necessary. Use of animals is now required to extend the initial information that has already been achieved from studies of lower organisms or cells grown in the laboratory in order to provide a more complete understanding of this human disorder and to undertake testing of novel drugs before progressing to human clinical trials.

Reduction

Explain how the numbers of animals for this project were determined. Describe

steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Calculations are carried out to determine the necessary number of animals for each experiment, ensuring significance of our results, but at the same time minimising the number of animals used. Breeding will be kept to the minimum amount necessary to obtain the experimental groups of mice for disease-like characterisation and therapeutic testing. For all of the experiments, each group will contain between 4 and 16 age- and sex-matched mice, which is the number needed to obtain the essential results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In this project we will minimise the number of animals used while ensuring the achievement of sufficient data to meet project's aims and objectives. The efficiency of the proposed therapeutic will be evaluated via a combination of molecular, biochemical, pathological, and behavioural analysis to maximise the amount of data that can be obtained from a single animal without compromising animal welfare. We have performed *in vitro* cell culture testing of potential FRDA therapeutic compounds to determine the best compounds to take forward to *in vivo* testing in mice. Different cell types will be used to validate the data prior to *in vivo* analysis to ensure that the observed effects are not specific to particular cell types, this includes human and mouse fibroblast cell lines and human sensory neurons. The efficacy and toxicity of the compounds will be further tested *in vivo* in our recently established *Galleria* models before moving to preclinical studies in mice. In addition, the number of mice used for pilot studies will be reduced by the use of already available data on DMF dosing rather than repeating these studies again. The animal group size for our experiments is based on our previous experience and statistical power calculations, so that the number of animals is sufficient to achieve statistically significant results. However, pilot studies will always be performed for new treatments (e.g. to assess feasibility and minor potential side effects) using the minimum number of animals (usually 5 per experimental treatment group). We interact with the technicians overseeing the husbandry of our mice to keep the numbers at an optimum level. We will consult with publications on sample size determination, the online Power/Sample Size Calculator, reporting standards for preclinical research design and using data from previous *in vivo* drug studies to assist our power calculations. Thus, typically each experimental group will contain between 4 and 16 age- and sex-matched mice, which will allow a significance level of 0.05 with 80% power to be achieved if the size of the effect is equal to a standardised difference of the mean of between 1.2. For all behavioural and molecular phenotype analysis, differences between groups of mice will be assessed by either the student's *t* test or ANOVA statistical analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The main step to reduce the number of animals required for this project is based on the screening analysis of the proposed compounds *in vitro* using our human and mouse fibroblasts and sensory neurons and *in vivo* in our recently established non-regulated *Galleria* models. We also adopted robust statistical analyses to calculate the minimum number of mice required to obtain reliable results. In addition, we perform pilot studies for the new treatment to assess the efficacy and toxicity of the compounds using a small number of animals. To minimise animal wastage, prevent the unnecessary production of animals showing adverse effect and to ensure that animal breeding is inextricably linked to research requirement, the project licence holder will:

Ensure high standards of animal care, welfare and utilise the most appropriate breeding methods.

Ensure that breeding colonies are always kept to their minimum size so as not to over produce. Detailed breeding records will be kept enabling the selection of the most appropriate breeding stock.

Ensure that Personal Licensees working on this project are appropriately trained and suitably competent to enable a high success rate to be achieved and thus minimise the number of animals used.

Ensure that Personal Licensees will work alongside highly qualified and skilled technical staff who will assist with best breeding practices and experimental procedures.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For any studies aiming at detecting any beneficial effects of the drug on preventing or reducing the disease-like phenotype of the FRDA mouse models, mice will be used from 2 to 3 months of age with an end point of 5 months of age. We have refined our experiments to have an end point (usually before 5 months of age) before mice become ataxic, cardiomyopathic and diabetic to minimise the potential suffering from potential earlier onset of disease.

The proposed antioxidants and iron chelators have already been approved to use in human and mice with no expected side effects. In cases where the drug has limited information regarding its testing *in vivo*, the proposed drug concentrations have been obtained from testing their analogues. The excipients used in both drug and placebo

groups will be at concentrations that has previously shown to be safe and would not be expected to have any adverse effects. Suffering of mice will be minimised throughout the project with the administration of potential therapeutic agents by the mildest available route of administration. However, the mice will be closely monitored post treatment, and should any mice show signs of mild pain or distress, suitable supportive care measures will be taken, such as supplying food to the bottom of the cage. Any mice that show signs of pain or distress will be killed immediately by a Schedule 1 method.

We have consulted with publications and data from previous preclinical studies to determine the best/mildest and most efficient route of administration for our candidate compounds at this stage. Oral admin might have a slower onset of action and reduced efficacy for some agents compared with ip delivery. Nevertheless, we will further refine our routes of administration when possible.

We have previously developed different behavioural tests to assess the early stage of FRDA-like neurological deficit which have progressively been refined over the past few years. This includes the use of rotarod apparatus and beam walk test that allows for accurate assessment of disease phenotype in our FRDA mouse models following treatment with the candidate compounds. The current apparatus stands no higher than 15cm above a padded surface and mice falling from the apparatus should be completely unharmed. These tests are all non-invasive and are unlikely to cause any pain, distress, or harm to the mice. In addition, mice will undergo each assessment on different days to ensure that suffering is kept to a minimum. The behavioural recordings will be made under direct observation at all time, ensuring that we do not stress the animals. However, if any of the mice do show signs of such disease during the behavioural tests, they will be killed by Schedule 1.

Why can’t you use animals that are less sentient?

In order to better understand the disease and to assess treatment strategies in Friedreich's ataxia, a living mammalian animal model with complex systems, organs and tissues similar to humans is considered necessary. Use of an adult, fully developed organism is required to extend the initial information that has already been achieved from studies of lower organisms or cells grown in the laboratory in order to provide a more complete understanding of this human disorder and to undertake testing of novel drugs before progressing to human clinical trials.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Suffering will be minimised by the administration of safe doses of potential therapeutic agents by the mildest available route of administration. Mice will be closely monitored after treatment and should any mice show signs of mild pain or distress, suitable supportive care measures will be taken, such as supplying food to the bottom of the cage. If mice show more than mild signs of abnormality of gait or coordination, lack of balance during walking, or movement they would be killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

High quality studies have been published in respected scientific journals which require high ethical standards in animal research. We will use these key scientific publications and also those guidelines published by other external organisations such as Laboratory Animal Science Association (LASA), Institute of Animal Technology (IAT), Royal Society for the Prevention of Cruelty to Animals (RSPCA) as guidance for our experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We can stay informed about advances in the 3Rs by consulting the NC3R website, reading the latest scientific literature, and attending conferences and research festivals. In addition, the Animal Welfare and Ethical Review Bodies meetings have a session dedicated to the latest developments in 3Rs.

88. Neuronal network adaptations underlying behaviour

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Psychiatric disorders, Neurological disease, Brain plasticity, Flexible behaviour, Cognition

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how adaptations occur to the neuronal networks that underlie flexible behaviour and how these processes are perturbed in neurological and psychiatric conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cognition is an emergent property of activity within complex neuronal networks. To understand the processes of cognition we need to understand how these networks operate and, crucially, how they adapt to changes in the environment to enable

flexible and appropriate behaviour. Disruptions to these cognitive processes are core features of mental illness and neurological disease that affect an estimated 25% of the population and constitute an enormous burden on society. There are currently very few effective treatments for the cognitive symptoms of these conditions. Whilst the development of better treatments would benefit many people, achieving this will require a better understanding of the fundamental biological processes that underlie cognition and how they are perturbed in neurological and psychiatric conditions.

What outputs do you think you will see at the end of this project?

The outlined studies are expected to generate data and publications that advance understanding of the neuronal processes that underlie cognition and how these are perturbed in neurological and psychiatric conditions with a focus on schizophrenia. The studies are also expected to test proof-of-concept approaches to enhance cognition with the results published in scientific journals and shared with online databases.

Who or what will benefit from these outputs, and how?

The knowledge gained from these studies will advance understanding of fundamental brain processes that underlie adaptive learning which will benefit scientists working in a number of fields within neuroscience including memory, mental health and computational neuroscience.

Longer term benefits may arise from advancing our understanding of cognition and the rational development of new therapies to enhance cognition in the treatment of neurological and psychiatric disorders. Realising the benefits of enhancing cognition will be achieved by engaging and collaborating with pharmaceutical companies.

How will you look to maximise the outputs of this work?

Dissemination of the outputs will be primarily by publication of research findings in academic journals but also including preprint publications sharing of raw data to enable rapid dissemination and open access to all data. Presentation of findings at academic conferences and seminars will also disseminate findings to targeted audiences.

Collaboration with pharmaceutical companies will achieve wider dissemination to enable the principles to be incorporated into the development of new therapeutic strategies for psychiatric and neurological disorders.

Species and numbers of animals expected to be used.

- Mice: 6000
- Rats: 350

Predicted harms

Typical procedures done to animals, for example injections or surgical

procedures, including duration of the experiment and number of procedures. Explain why you are using these types of animals and your choice of life stages.

The use of animals is essential to this project as currently there are no suitable alternative for studying the mechanism of adaptive learning in the mammalian central nervous system. Rodents have been chosen as they have been shown to be suitable for studying adaptive learning and importantly, their well-defined neural pathways share direct homology to that of humans. Furthermore, much is known about the properties of neural plasticity and adaptive learning in rodents. Mice are particularly useful for these studies as they are amenable to the genetic manipulations required to identify specific cell types and modify neuronal pathways. The outlined studies will investigate the cognitive impact of psychiatric disorders with a focus on adaptive learning. Cognitive impairment is evident in early development as well as emerging later in life, therefore, whilst the majority of studies will use adult mice, some will use neonatal, juvenile or aged animals. A small number of studies will be conducted using rats, as this species is able to learn the more complex behavioural tasks required to assess specific questions about learning.

Typically, what will be done to an animal used in your project?

The vast majority of animals used in the outlined studies will be mice (~95%), the remainder being rats. Around half of these will be genetically altered to enable specific cell types to be identified. These mutations are not expected to have any adverse effects on the wellbeing of the animals. Around 60% of all animals will be used in studies conducted on their brain tissue after they have been humanely killed. Of these, around 30% will have undergone a surgical procedure, two to six weeks prior to killing, to inject a non-harmful agent into the brain to either mark or modulate specific cell types. Surgery will be performed under general anaesthesia using full aseptic precautions. All mice will receive suitable pain relief appropriate for the species and will be monitored for any signs of post operative pain.

Following surgery, mice are expected to make an uneventful recovery and to resume normal behaviour within a few hours.

Around 10% of animals will be used in behavioural studies. Prior to these, animals may undergo up to three surgical procedures conducted under general anaesthesia and using full aseptic precaution.

Typically, the first of these will involve the injection of agents into the brain, as outlined above. One to three weeks later, a second surgery may be undertaken to implant head bar for head restraint, a cranial window to enable brain imaging or a recording or stimulation probe. In a small number of cases a third surgery may be performed where it is necessary to implant both a cranial window and a recording or stimulation probe where the geometry precludes combining approaches in one surgery. All mice will receive suitable pain relief appropriate for the species and will be monitored for any signs of post operative pain. In all cases, the animals are expected to make an uneventful recovery from surgery and to resume normal behaviour within a few hours. Following each surgery, animals will be allowed to recover for at least 1 week before any further procedures are undertaken.

The behavioural tests using both freely moving and head-fixed configurations rely on the animal's innate curiosity to explore their environment and novel objects within it and are not expected to have any adverse effect indeed, most animals engage readily in the tasks. Nevertheless, to minimise any associated stress, all animals will be habituated to the experimenter and experimental setup prior to testing. To motivate animals to perform in tasks involving a food or liquid reward, their access to food or water may be restricted for a period prior to testing. All animals on food or water restriction will be weighed at least once weekly and more frequently at critical time points e.g. during the first few weeks of restriction. Any animal that loses more than 15% bodyweight will be given additional food or water until suitable weight has been restored. During behavioural testing, the animal may be attached by a flexible light weight cable to enable brain recordings or stimulation to be undertaken whilst the animal is moving freely around the test arena. In some instances, agents that modulate brain activity may be given by injection prior to testing. Typically, animals will undergo behavioural training or testing for 1 hour per day for 2 months but occasionally this period may be extended to up to 4 months, after which they will be killed using a humane method. For some experiments (2%), the complexity of the task will necessitate the use of rats. In a small number of cases, animals will be anaesthetised and used in a non-recovery terminal procedure during which recordings will be taken from the brain before the animal is killed.

To enable high-resolution recordings or images to be made of brain activity and precise changes to the sensory environment whilst mice are performing tasks, some mice (20%) will be fitted with a head fixation device so that the head can be maintained perfectly still relative to the recording apparatus.

These mice will be trained to sit or run on a treadmill or similar moveable platform whilst their head is maintained in a fixed position, with the relative arrangement of treadmill or platform and head fixation positioned to minimise bodily discomfort.

Training/recording session typically lasts for 1 hour per day and mice are only head fixed during this period. Data from previous studies shows that provided the mice are appropriately habituated to head restraint they can learn to perform complex cognitive tasks. Task motivation is achieved as described above with reward delivery typically via a liquid spout placed close to the mouth. Typically, mice will undergo behavioural testing with head fixation for 1 hour per day for up to 12 weeks, after which they will be killed using a humane method to enable brain tissue to be collected for further analysis. A small number of mice (<1%) will be used in acute electrophysiological studies, which typically last 3-5 hours but may last for up to 9 hours to maximise data acquisition from each mouse. During these, the mouse will be given a 15 minute break at regular intervals of at least every 3 hours. At the end of these sessions, the mouse will be killed, using a humane method, and its brain tissue collected for analysis.

To investigate how neurological diseases, such as schizophrenia, disrupt learning, a number of studies (30%) will be undertaken using mouse or rat models that mimic aspects of the disease using the techniques described above. Whilst these animals will have impaired learning, they otherwise appear and behave normally. In some instances, the disease model is progressive in nature (for example models of neurodegenerative diseases), in these cases the animals will be killed at an age

before any suffering is known to occur.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of mice used in the outlined studies will be genetically modified however, these are not expected to have any adverse effects on the wellbeing of the animals.

All surgical procedures will be carried out aseptically under general anaesthesia and animals will be given pain killers as appropriate. Following surgery all animals are expected to make an uneventful recovery and to resume normal behaviour within a few hours.

Animals on food or water restriction are expected to experience mild hunger or thirst. These animals will be closely monitored and any that lose more than 15% of body weight will be given additional access to food or water until the target weight is achieved. Restriction will be maintained for the duration of behavioural testing which may last for up to four months. Data shows this is not unduly stressful for animals but does increase their motivation to perform cognitive tasks involving a food or water reward. Similarly, after appropriate habituation, head restraint or tethering has been shown not to increase stress levels (PMID: 32699235).

The disease models used for these studies only cause mild cognitive impairments and do not impact on the general wellbeing of the animal.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice 75% mild, 25% moderate.
Rats 50% mild, 50% moderate. (Rats are expected to account for ~5% of the animals used).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not possible to investigate the mechanisms by which adaptations within neuronal networks bring about changes in flexible behaviour, or to assess how these

processes are altered in neurological and psychiatric conditions, without the use of animal as these processes only occur in the intact living brain of highly developed animals like mammals.

Which non-animal alternatives did you consider for use in this project?

Human volunteers for non-invasive imaging or electrophysiology (EEG). Human patients with implanted electrodes.

Computational modelling.

Non-regulated species such as simple invertebrates. Non-regulated model systems

Why were they not suitable?

Human volunteers for non-invasive imaging or electrophysiology (EEG). These techniques offer very low spatial and temporal resolution and therefore do not allow analysis of the cellular mechanisms underpinning cognition.

Human patients with implanted electrodes. Cellular resolution recordings may be made from these subjects but the access to patients with these implants is intermittent at best and any data is inevitably confounded by the lack of health in the subject population. Furthermore, it is not possible to interrogate the mechanisms involved using human subjects.

Computational modelling. We aim to use in silico computational modelling as a complementary approach to guide and enhance the power of our experiments.

However, a computational approach will always be an approximation of the experimental system and is therefore not capable of fully replacing animal experiments.

Non-regulated species such as simple invertebrates. Whilst these systems can be useful for studies of cellular and synaptic mechanisms, they cannot offer insight into the circuit level bases of mammalian cognition.

Non-regulated model systems such as organoids or assembloids derived from human stem cells do not have the complex circuit architecture or sensory inputs to address the scientific objectives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of animals required for the breeding programme was calculated in collaboration with the assistance of technical managers working in my institutes genetically altered mouse breeding unit. Animal numbers required for experimental groups were calculated from expected effect sizes based wherever possible on previous studies in our laboratories or elsewhere and taking into account the required number of replicates per experiment, the expected attrition rate for failed experiments, the required age and sex (for most experiments we will use animals of both sex) and the genotype of the animal.

As an example, to test the effect of a specific loss-of-function resulting from a genetic mutation known to increase the risk of schizophrenia on the 'long term potentiation' (LTP) of neuronal synaptic strength would, based on previous findings, require n=11 replicates from each of wild type and heterozygous gene deletion mice. The estimated number of successful LTP experiments from ex vivo slices is 2 per mouse therefore, this experiment will require 6 wild type and 6 heterozygous gene deletion mice. To generate these mice will require the breeding of a wild type male with two heterozygous gene deletion females. This will result in the progeny (normal litter size 6 to 8 pups) of two litters split roughly 50/50 between wild type and heterozygous deletion mice. Therefore, to undertake this study will require a total of 15 mice (12 experimental animals (6 wild type and 6 heterozygous) and 3 breeding animals, (one male and two females).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Group sizes were calculated from expected effect sizes based wherever possible on previous studies in our laboratories or elsewhere and in collaboration with our partners in both academia and industry. This ensured the most effective study design that reduces animal use to the minimum necessary for the maximum statistical power. In addition, a statistician has been consulted for advice on power calculations and analyses for all experiments. To avoid pseudoreplication, we will use Bayesian linear mixed model analysis to identify and incorporate the sources of variability in our analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Efficient breeding procedures: For disease models we will use heterozygote-wild type breeding pairs that will provide littermate wild-type controls for our experiments. This is statistically the most effective study design and reduces the need to breed wild-type control animals. Transgenic lines to identify neuron subtypes will be bred to homozygosity where possible to reduce the number of non- experimental animals generated by the breeding programme.

Enhance the power of results and the validity of conclusions drawn: Data acquisition will focus on genetically defined subtypes of neurons. This will be achieved by the introduction of genetic material into selected neuronal populations by targeted injections coupled with transgenic mouse lines. This approach greatly reduces population variability thus increasing the statistical power of our results for any given effect size and thereby minimising the number of animals needed to obtain the

required data.

Ex vivo brain slice studies: Great care will be taken in the preparation of slices to maximise the number of successful experiments that can be achieved per animal to minimise the total number of animals needed. In addition, my group actively participates in my institute's tissue sharing networks to ensure the maximum scientific value is derived from each animal used.

Recordings from implanted animals during behaviour: Typically, these studies will be run in series and preliminary data analyses performed concurrently. This enables the effect size to be monitored experiment-by-experiment and power calculations revised accordingly, this approach ensures that only the minimum number of animals are used to obtain the required data.

High-resolution imaging and electrophysiology: Physical vibration caused by movement makes in vivo acquisition of high-resolution data from anatomically identified neurons extremely difficult. To overcome this, some animals will be head-fixed whilst undertaking cognitive tasks to enhance experimental productivity and thereby minimise the number of animals needed to obtain the required data.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rats and mice are the species of choice for these studies as their brain anatomy is comparable to that of humans and many of the cognitive functions attributed to specific regions of the human brain translate directly to the rodent brain. In addition, most human genes have analogous rodent genes, enabling manipulations to be undertaken to evaluate the genetic basis of human disease. Furthermore, the techniques required to investigate the genetic basis of human disease, e.g. genetic manipulation and high-resolution imaging and electrophysiology, are well established in rodents.

Live animal studies will focus on discerning the dynamic changes that occur in neuronal networks during learning using data obtained from chronic implanted recording and stimulation devices, such as microelectrodes and miniature light guides. Electrophysiology offers the most direct measure of neuronal activity at a cellular level, which is essential to understanding the mechanisms of information processing by neuronal activity. High resolution imaging allows measurement of subcellular activity from a large population of neurons. These techniques can be combined with behavioural, pharmacological and genetic manipulations to

understand the causal relationships between neuronal activity and behaviour. Minimisation of pain, suffering, distress or lasting harm will be achieved as follows: Breeding: The majority of animals used in the outlined work will have genetic alterations. Most of these, including transgenic mice for neuronal identification, are not expected to have any adverse effect on the wellbeing of the animals. Some animals with heterozygous genetic deletions that closely replicate human disease will be used. These deletions are associated with impaired cognitive function but do not adversely impact the wellbeing of the animals. In some disease models, such as the transgenic Alzheimer's disease mice, disease progression means that mice would experience suffering if allowed to age beyond a certain point. For all such strains, the animals will be used and killed before an age at which suffering is likely to occur.

Surgery: All surgical procedures will be performed under general anaesthesia and using full aseptic precautions and appropriate pain killers in accordance with LASA guidelines. Throughout the procedure, care will be taken to maintain body temperature and fluid balance. The weight of any cranial implants will be kept below 10% of body weight.

Why can't you use animals that are less sentient?

Understanding the neurophysiological bases of cognition relevant to humans requires the use of an awake behaving mammalian species. Rodents have the least sentience of mammalian species suitable for these studies. It is not possible to conduct these studies in less sentient species as they do not have neural systems comparable to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Genetic alterations: Wherever possible, selective, rather than systemic genetic manipulations will be used to minimise the risk of inducing a harmful phenotype. For example, mouse lines expressing cre recombinase only in cholinergic neurons. Disease mouse models: Models with a progressive harmful phenotypes will be terminated at the earliest possible time-point and before suffering is expected to occur, (eg. Alzheimer's disease model mice).

Surgery: All surgical procedures will be performed under general anaesthesia and using full aseptic precautions and appropriate pain killers in accordance with LASA guidelines. Throughout the procedure, care will be taken to maintain body temperature and fluid balance. The weight of any cranial implants will be kept below 10% of body weight.

Behavioural testing: Well characterised non-aversive cognitive tasks will be used. Prior to testing, the animals will be habituated to the handler and the experimental set up using a staged approach with head fixation duration increased each day. Training and habituation will be adapted to suit each animal. For example, some animals are easily habituated to head-restraint whereas others require longer. Some animals will be trained to perform virtual reality tasks whilst head fixed. This task exploits the animals innate drive to explore their environment. To minimise the

associated stress of head restraint, the duration of the test sessions will generally be limited to 1 hour per day and at all times the weight of the animals will be fully supported. In all cases, the frequency and duration of testing will be limited such that it does not result in tiredness or distress. During testing, the animals will be monitored frequently for signs indicative of distress and, if evident, testing will be stopped immediately.
Stimulation intensity by electrical or optical methods will be kept to the minimum required to elicit a measurable neurophysiological response. Stimulation intensity will be immediately reduced if any aversive behaviour is observed.

Food and water restriction: To motivate animals to engage in tasks involving food or water rewards, animals may be maintained on a regulated feeding or drinking schedules. However, animals will always receive food and water daily. Animals on food or water restriction will be weighed regularly, particularly during training and testing, and any that lose more than 15% of body weight, compared to age matched controls, will be given free access to food and water until normal body weight has been regained. A staged approach will be taken to determine the minimum food or water restriction required for animals to perform optimally. Indeed, data indicates that restricted calorie intakes coupled with physical exercise during task performance may be beneficial for the animal health. In addition, the behavioural tasks also support animal health by providing higher levels of stimulation.

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 published NC3Rs guidelines and follow the principles of the PREPARE guidelines. All surgical procedures will be undertaken in full compliance with LASA guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will stay informed about 3Rs developments through the information circulated by my institutes NIO and by attending 3Rs relevant events, such as the 3Rs annal prize giving events hosted by our ELH.

I set up and chaired the University's Head-Fixed Mouse Procedures working group which receives and analyses the most recent data on head-fixed mouse behaviour.

This includes information from the national NC3Rs working group on rodent behaviour. The work of the group has identified refinements in animal handling, habituation and water restriction to optimise the animal experience and performance during head fixed tasks.

89. Role of AMPA receptors in synaptic plasticity

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain, Synapse, Glutamate Receptors, Memory, Neurodegeneration

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand how information is stored in the brain. Brain functions like memory and learning rely on information being retained in the molecules that reside at the interphase between neurons, namely the specialised molecular machinery at synapses.

AMPA glutamate receptors at synapses, are central to this information storage and are the main focus of this project, as is the development of methods to visualise the native molecular architecture of neuronal synapses, ultimately this is crucial for an accurate understanding of the processes underlying information storage in the brain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Glutamate receptor channels are transmembrane protein complexes that respond to

and are activated by the neurotransmitter glutamate. Glutamate, a small molecule, is released from 'input neuron A' and activates 'receiving neuron B'. This activation is mediated by glutamate receptors, which bind to the neurotransmitter and consequently open a transmembrane pore enabling the flux of ions into neuron B, causing its activation (or excitation). This process, termed signalling, takes place at specialised cell junctions between neurons A and B, and these junctions are termed 'synapses'.

Glutamate receptors are central to brain development, and are also the main players in excitatory synaptic transmission and synaptic plasticity, a process underlying information storage such as learning and memory. On the other hand, malfunction of glutamate receptors contributes to various diseases rendering them an important drug target to treat diseases including epilepsy, motor neuron disease, depression, schizophrenia and dementias. Hence, understanding the glutamate receptor structure-function relationship and their operation at synapses is a core question in neurobiology. Our work focusses on the AMPA-type glutamate receptor (AMPAR), which initiates neuro-transmission at synapses by triggering (electrical) depolarisation of the postsynaptic membrane (of neuron B) in response to binding glutamate (which is released from neuron A). The number of AMPARs is also selectively upregulated in synaptic plasticity, such as in long-term potentiation (LTP), a leading cellular model for information storage in the brain. We are studying AMPAR regulation and modulation by AMPAR accessory (auxiliary) proteins and therapeutic ligands in vitro, and their action in synaptic plasticity in mouse brain slices.

Our ultimate goal is to i) understand the regulation of these central receptors, ii) unravel how AMPA receptor dynamics at synapses enable information storage, i.e. how memories are stored at synapses,
iii) explore aspects of AMPA receptor dysfunction that lead to memory loss and diseases such as dementia and epilepsy and iv) define the architecture of AMPA and other synaptic protein complexes in the content of native neuronal synapses. As demonstrated in our publications, we have a clear lead to understand a critical step in information storage (learning) - the mechanism underlying AMPA receptor recruitment into potentiated synapses.

What outputs do you think you will see at the end of this project?

Like in other basic research projects the main output will be an increase of knowledge. An in-depth understanding of AMPAR gating and their regulation at synapses in synaptic plasticity, will lead to a better understanding of how information is stored as memories. Our work also involves the development of AMPAR modulators, which (if successful) would be developed into therapeutics at a later stage. All our data are published in peer-reviewed journals.

Who or what will benefit from these outputs, and how?

The process of memory-formation remains one of the most fundamental, open questions in modern neuroscience and goes awry in dementias, such as Alzheimer’s disease. The decline of memory function with age and an ever-increasing ageing population have important implications for life-long health. Hence, an understanding of learning and memory mechanisms, and the precise role of AMPARs in this

process, will be essential for devising new strategies to promote life-long health and well-being. Beneficiaries would be the pharmaceutical industry and the general public, both in the longer term.

How will you look to maximise the outputs of this work?

We do collaborate on certain aspects of our projects. These include collaborations investigating the role of AMPAR modulatory lipids, the structure of AMPAR gating intermediates (captured with modulatory ligands), the development of modulatory ligands with medicinal chemists, and a collaboration with brain slice electrophysiologist to study physiologically relevant LTP mechanisms.

Further, we regularly publish our work in high-profile journals and in review format; we also present our data on both national and international scientific meetings.

Species and numbers of animals expected to be used.

- Mice: 20000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice as a model organism for our studies on AMPA receptor-mediated synaptic plasticity. These are the species of choice because it is possible to acquire genetically modified strains of various synaptic proteins including AMPA receptor subunits (Gria1-4). Moreover, there exists a battery of behavioural memory tests further supporting the use of mice for studying the molecular mechanisms of learning and memory. Papers on behavioural testing of AMPAR mouse mutants help interpreting our molecular findings. The age of our experimental animals varies, ranging between the ages of birth and day 6 for generating neuronal cultures, and adulthood mice (4-8 weeks) for tissue harvesting of the brain for in vitro studies (imaging synapses and brain slice electrophysiology).

Typically, what will be done to an animal used in your project?

The majority of animals on this licence will be bred to generate and maintain genetically altered lines. Many of the mice we use are not subject to regulated procedures. They are culled and brain tissue is collected for in vitro studies.

To introduce genes of interest into the brain of juveniles and adults, injection of virus-like particles into the skull of neonatal (P0) mice will be performed. This procedure takes place under anaesthesia, which aims to be done in under 7 minutes, and causes minimal distress to the pups (which develop into adults normally), as outlined in our publication: Ho et al., 2020 J. Neurosci Methods.

Very occasionally, we may also introduce transgenes into the brain of adult mice (4-6 weeks of age). This procedure also takes place under anaesthesia an is similarly performed in under 7 minutes.

Lastly, we will use in utero electroporation to introduce transgenes into mouse brains. In this method, plasmid DNA is electroporated into the brains of mouse embryos (E14, E15).

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will be housed in a social environment and will have ad lib access to food and water.

Surgery will involve administration of substances or altered genes into the brain; mice will be anesthetised prior to surgery and adult mice will be given pre-emptive analgesia. Surgical mice may experience some discomfort after surgery, and this is expected to last no more than 24 hours. Only a small proportion of animals used will undergo surgery, as described above ('injection of virus-like particles into neonates and adults'). The majority will be used for breeding purposes.

Very rarely after surgery the severity of clinical signs maybe such that the humane endpoints are reached. Unless otherwise specified, the administration of substances and withdrawal of body fluids will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than lasting harm.

Some mice will be killed under terminal anaesthesia for collection of tissue or perfusion purposes.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

None of our procedures are classified as severe. The majority will be mild (<20%), and subthreshold (70%), with a small percentage (< 10 percent) experiencing moderate (surgical-related procedures).

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our ultimate question, the role of AMPARs in synaptic plasticity and learning, can only be studied at neuronal synapses; in neuronal circuits, such has the tri-synaptic circuit of the hippocampus, which can be manipulated under realistic conditions.

These include acute excision of an AMPAR subunit (GRIA gene) by virally expressed Cre recombinase followed by viral replacement with a modified AMPA receptor in vivo, or through genome editing approaches in neurons obtained from mice. Hence, the need to use mouse models (such as floxed GRIA lines).

Alternative in vitro models, such as brain organoids do not possess the same neuronal organisation as the hippocampus does. Wiring between pre and postsynaptic neurons is partly arbitrary and thus not suitable for our synapse-selective studies on synaptic information storage.

Which non-animal alternatives did you consider for use in this project?

We have been investigating AMPAR regulation in in vitro preparations over the last 15 years and are continuing to do so. These include structural work of AMPARs (cryoEM, x-ray crystallography), testing AMPAR mutants through biophysics and cell biology measurements of receptor gating and trafficking in the HEK293 cell line, as well as in silico simulations of AMPAR conformations. In sum, these approaches allow a detailed study of the receptor complex.

Why were they not suitable?

These approaches answer complementary questions - they provide insight into receptor structure, organisation and gating kinetics, which is essential information that our neuronal experiments built on. They cannot, however, cannot address receptor behaviour in a synapse.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will use an absolute minimum number of mice, sufficient to give statistically significant results. Breeding will be strictly monitored to limit surplus mice throughout this project. Further, cryopreservation will be used to preserve essential mouse lines, allowing us to only maintain lines needed for specified experiments. We have optimised a number of experimental procedures, such as surgical injection of neonatal animals, improving targeting of brain regions, so that fewer animals are required for each experimental result to be obtained. We currently maintain seven

colonies of genetically altered (GA) mice, which we aim to maintain throughout the duration of this PPL.

For the experiments involving neonates we obtain about 10 brain slices per pup. An experimental cohort would include 2 pups; including two repetitions this would amount to 6 pups doe a gicen measurement.

For electrophysilogical recordings of brain slices from adult mice we require approximately 4 mice per condition.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

With regard to the husbandry requirements, we leveraged the data accumulated in our previous license that consisted of the same mouse lines. With regard to the experimental work, thanks to the work in our previous licence we have a solid understanding of our expected data distributions and we would be able to generate accurate power calculation models wherever possible

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All of our experiments are ex vivo recordings (or tissue harvests), an experimental setting that inherently uses a low number of animals - we obtain about 10-12 brain slices per mouse and these last a full working day. This will usually result in solid data sets that will have to repeated one or two times with an age-matched animal.

Cryopreservation will be used to preserve our lines and therefore lower the stock for extended periods. Cryopreservation of embryos and sperm will be used for long-term storage of genetically altered mouse lines. Rederivation will be undertaken should the health status of the animals be compromised in a way that would significantly affect the welfare of the animals or where the experimental results might be altered unduly.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The brain structure of mammals is overall similar and mice are best suited for this project as multiple mouse models have been developed to study memory mechanisms. For our purpose lines will be used from which glutamate receptors can

be genetically excised and replaced with altered versions.

Genotyping of our lines will be done in the most non-invasive way, using ear punches. Animal technicians are well informed about our lines which have no avert phenotypes and are overall healthy and overall normal even after excision of glutamate receptors. The ‘stargazer’ line, which harbours a mutation in the CACNG2 gene that causes absence epilepsy, is characterised by a smaller body size and slightly ataxic gait. This phenotype should not affect the animals’ normal behaviour such as the ability to eat and drink and in general mice of this strain may live without any problems for up to 2 years. Homozygous Stargazer mice are not used for breeding (but are only used for tissue samples) and usually only kept until the maximal experimental age, which is around 10 weeks.

Surgeries will be predominantly carried out on neonatal pups (transcranial injections) and on pregnant dams for in utero electroporation of embryos. Surgery will be carried out following a strict aseptic technique and by highly trained and competent individuals. All animal experimentation carried out under this project licence will comply with the document ‘Animal Usage Guidelines’

The duration of surgical procedures will be kept to a minimum to keep the occurrence of any possible adverse effects. We have refined the procedure for injection of neonatal animals through design of a custom 3D-printed pup immobilisation mould with incorporated anaesthetic mask, suitable to minimise dead space for more reliable control of anaesthetic delivered to the neonates. This improves both the specific targeting of brain regions that we are interested in, and improves pup recovery after procedures.

Why can’t you use animals that are less sentient?

The mouse is one of the simplest mammals in which to study memory mechanism. Moreover, at least a third of our experiments uses mouse pups at the age of 5-7 days old. Similarly, we routinely generate dissociated neuronal cultures (for electrophysiology and imaging studies) and these are derived from 1 day old mouse pups.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Every step of our work has been carefully assessed so to minimise welfare costs and it is constantly re-assessed as improved methods become available.

Moreover, we have recently patented a method for anaesthesia of neonatal (P0-2) mice, together with a custom 3D-printed apparatus for maintenance of anaesthesia during surgical procedures. This approach represents a refinement for anaesthesia of neonate rodents, which has been implemented by many laboratories worldwide.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our animal experiments are very standard, predominantly involving tissue harvest for

in vitro experiments, and administration of transgene either by in utero electroporation into embryos or by intra cerebral injection into neonates; these have been worked out well to minimise animal suffering. Other than that, we follow the Home Office Guidance on the Operation of the Animals and NC3Rs guidelines, specifically the ARRIVE guidelines (Kilkenny et al., 2020).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our Biological Services Group and NIO keeps us updated about the latest advances in the 3Rs, we follow the NC3Rs newsletter and their seminars and demonstrations.

90. Understanding metabolism in immunity and cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Cancer, Immune cells, Metabolism, Therapy

Animal types	Life stages
Mice	neonate, embryo, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to better understand the role of metabolic processes in normal immune responses and in cancers arising from cells of the immune system, so that we can develop new therapies for patients with lymphoma.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 aim of this research is to understand the changes that occur in the metabolism

of cells in the immune system when they are activated to fight infection. We also aim to understand whether the same changes in metabolism occur when immune cells are behaving abnormally causing autoimmune disease or lymphoma (a cancer of immune cells). Our recent work has discovered a particular part of metabolism required by normal and cancerous immune cells to grow, and that blocking this part of metabolism can be a treatment for lymphoma. This research will focus on closely related areas of metabolism that allow cells to make substances that they need to grow and make copies of themselves, such as DNA. We have reason to believe that investigating this will uncover new potential medicines for a variety of human diseases including lymphoma. We know that existing medicines, such as methotrexate, widely used for the treatment of these diseases, work by blocking the metabolic pathways that are the focus of our research. Therefore, the importance of this project is that by investigating the way immune cells use metabolism in health and disease, we can better understand how existing drugs work, and develop new ones to improve treatments for patients with lymphoma.

What outputs do you think you will see at the end of this project?

This research will generate significant data regarding the metabolism of immune cells. It will also generate data regarding the therapeutic value of targeting specific metabolic enzymes in cancers such as lymphoma. As a consequence, the results of this research will be of significant interest to the wider research community.

Therefore, we will disseminate our findings by presentation at local, national, and international meetings and conferences. The data will be assembled into scientific manuscripts with the aim of publishing the results in high-impact general journals to reach the widest possible audiences.

A further aim of this research will be to identify which metabolic enzymes make the best therapeutic targets for the treatment of lymphoma. Therefore, a longer-term output would the development of candidate compounds to be progressed into clinical trials. This could involve either the re-purposing of existing medications in rational combinations with other metabolic inhibitors, or the generation of novel molecules.

We will continue to involve patient and public advocate groups in all aspects of this project: for example to review the media used to disseminate this research and its outcomes, and to review applications for ethical approval, trial protocols and patient information leaflets for any future clinical trials arising from this work.

Who or what will benefit from these outputs, and how?

We expect that our research will benefit several groups including basic scientists, clinician scientists and clinicians, the pharmaceutical industry, patients and wider society.

Basic scientists: An enhanced understanding of the role of metabolism in immunology will have an impact on several ongoing projects at our University and further afield. This proposal also aims to improve our understanding the metabolic processes driving pathology in lymphoma which is an area of intense research interest worldwide. Additionally, this project will provide training and educational

opportunities to the next generation of young scientists. More specifically, postgraduates and postdocs will have train in our laboratories during the course of the fellowship with gain skills and experience that will be invaluable as they seek to continue their careers in other labs, industry or the NHS.

Clinicians and clinician scientists will benefit from the advancements in our understanding of the role of metabolism in immunology in health and disease. Given the existing widespread clinical use of drugs such as methotrexate, pemetrexed, leflunomide, and fluorouracil, there is a strong rationale for the clinical investigation of drugs that target closely related metabolic pathways that may show better efficacy and/or tolerability or may synergise with existing therapies. This research will directly inform the design of future clinical trials testing these novel approaches, particularly in lymphoma, but also in autoimmune diseases such as rheumatoid arthritis.

The pharmaceutical industry will also benefit from this, as this research will identify novel therapeutic targets and expand our understanding of existing therapies. We would anticipate partnering with industry to conduct clinical trials to test the concepts that arise from this work.

Patients: In the longer-term we hope for health benefits to patients suffering with lymphoma and autoimmune diseases such as rheumatoid arthritis. While these latter goals are likely to take of the order of ten years or more, we do anticipate the enhanced understanding of pathways and targets linked to metabolism to enable us to begin the development of these agents during and shortly after the proposed project.

Wider society: The proposed research is likely to be of benefit to wider society through the benefits to patients and industry described above. We also have a strong history of interaction with research charities as well as industry partners. Our work has and will be featured in their publicity and outreach activities, and we envisage the translational science proposed here leading to new concepts relevant to their goals, and opportunities for further research and fund-raising.

How will you look to maximise the outputs of this work?

As above, we will look to publish the results of this research in high impact scientific journals. We will always aim to publish in open-access journals so that other researchers can access our work without a paywall. Successful publication will be accompanied by press-releases and promotion on the relevant Institutes' websites and use of personal and institutional social media (e.g. twitter/X). In addition, the lead applicant will promote this work through presentations and through his links with cancer metabolism and immunology networks.

We have an existing network of local, national and international collaborations and are always looking for new opportunities. In particular, we will make any resources (e.g. the newly generated conditional knockout mice) available to other researchers.

We will also publish reports of any unsuccessful approaches, to avoid other researchers attempting the same experiments, particularly when animal use is implicated.

Species and numbers of animals expected to be used.

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mouse models are the most appropriate species to be used for the planned work. They recapitulate much of human immunology, metabolism and cancer biology, and observations in mouse models are frequently translatable into the clinic. In addition, we already have the tools to manipulate individual genes in these animals including the relevant mice to generate the conditional knockouts of particular molecules of interest. Furthermore, there are well characterised mouse models of human disease including human lymphoma. These points, along with our extensive experience with these animals, make mice the most appropriate species for this project.

We plan to use young adult mice for the majority of the experiments ranging from a minimum of 8 weeks (reflecting a typical age for the animals to develop a mature immune system) to a maximum of 15 months. We will be generating and maintaining genetically modified mice so these animals will include juvenile mice in addition young adults. Immunisation experiments will typically be performed in young adults aged from 8 – 12 weeks. We will also use young adult mice (8 – 12 weeks old) for the lymphoma cancer models. The mice used who spontaneously develop lymphoma will need to be older as previous research has demonstrated that lymphomas reach an incidence of 50% at 15-20 weeks in the specific lymphoma model that we plan to use. Therefore, we will plan to monitoring them for up to 15 months for the development of lymphoma.

Typically, what will be done to an animal used in your project?

We will generate and maintain genetically modified mice which will be used for subsequent experiments. Some of the proposed research will involve injecting the animals (typically either intravenously, under the skin or in the abdomen) to induce an immune response so we can study this. This injection will typically be done once but some animals will require drug treatment (typically given orally by gavage) for up 2 weeks. Most of the animals should remain well during this period but some may suffer side effects from the drug treatment such as weight loss, diarrhoea or infection. The same types of drugs will also be used in experiments to determine whether they are also effective treatments for lymphoma, a cancer that arises from cells of the immune system. Some animals will develop lymphoma spontaneously at around 4 – 5 months of age. We will monitor the animals closely during this period and kill them humanely if they have a large amount of lymphoma (this typically shows up and enlarged lymph glands in the neck, armpits and groins or a distended abdomen) or exhibit any major distress. We will then inject these cells into other

mice either intravenously to induce lymphoma in the lymph glands or spleen, or into the heart or brain to induce lymphoma in the brain. The mice will generally recover quickly from the initial injection but will be expected to develop lymphoma over the next 4 weeks. We will plan to humanely kill the animals before the develop significant amounts of lymphoma to minimise distress. Some of the experiments will involve genetic modification of the cells prior to injection, others will use new drugs/combinations of drugs to reduce the progression of the lymphoma. We will also inject some mice with human cancer cells under the skin and monitor the develop of that with various drug treatments. These mice will need to be immunodeficient (have weakened immune systems) so they do not reject the human cells, so they will need to be carefully monitored and looked after to avoid infection.

In the lymphoma experiments mice will typically be injected once with the lymphoma cells, treated with drugs for 2-3 weeks and then humanely killed.
There are no other specific surgical procedures planned.

What are the expected impacts and/or adverse effects for the animals during your project?

We are not expecting any adverse effects from the generation and maintenance of the genetically modified animals with the exception of the lymphoma mice.

Approximately half of these animals will spontaneously develop lymphoma between 4-5 months of age with other animals developing disease after this. This manifests as enlargement of the lymph glands in the neck, armpit and groins and swelling of the spleen. While this does affect the appearance of the mice they do not initially display any weight loss or abnormal behaviour consistent with pain or discomfort. As the tumours grow over a periods of weeks the mice can start to lose weight and demonstrate signs of distress. We will design our experiments so the will be humanely killed before they reach this point.

We are not expecting any adverse events from immunising mice other than mild and transient effects from the immunisation injection itself. While treatment with some of the proposed antimetabolic drugs are well tolerated, treatment with drugs such as methotrexate can cause adverse events in the form of bone marrow failure and gastrointestinal lesions. This would manifest in the mice as weight loss, diarrhoea and increased rates of infection.

We will inject other mice with the lymphoma cells from the mice who are genetically modified to develop this spontaneously. This will be done either intravenously, under the skin (subcutaneous), or directly into the brain. The intravenous injection and subcutaneous injections will only result in mild and transient discomfort. Some the mice will then be treated with antimetabolic drugs as above with the same anticipated side effects. The mice injected intravenously with lymphoma cells will have the comparable impacts to the animals who develop lymphoma spontaneously i.e. swelling of lymph glands and spleen. However, the time-course of the development of this is more predictable so we would anticipate being able to “time” the experiments to gain the scientific benefit while minimising distress to the animals. The mice injected subcutaneously with lymphoma cells may also have some transient discomfort at the injection site. We anticipate that they will develop a

tumour that grows over several weeks at this site. We will plan to start drug treatment when the tumour reaches a pre-specified size and continue this for up to 5 weeks.

We are anticipating that the tumours will respond and shrink but some may continue to increase in size (e.g. in control animals). Therefore, we will plan to humanely kill these animals when the tumour reaches a prespecified size before the animals exhibit significant distress. We are not anticipating the injection of the lymphoma cells into the brain to cause any impact from the lymphoma until several weeks after injection so we will plan to humanely kill the mice after 3 - 8 weeks (dependent on the cells used) to assess this. The lymphoma cells either will be genetically modified to remove the metabolic enzyme of interest before injection or the mice will be treated with antimetabolic drugs as above. It is possible that some mice may die as a result of the injection into the brain itself. We aim to minimise this by working with other scientists with significant relevant experience.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The breeding of the genetically modified animals is expected to have a mild severity whereas the experiments are expected to be of moderate severity. Therefore as the animals that have the appropriate genetics will be then used for the experiments we expect that 100% of the experimental mice will have a moderate severity.

What will happen to animals at the end of this project?

- Killed
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals for this project for several reasons. Firstly, a key discovery in the field of metabolism over the last few years has been the importance of the metabolic “microenvironment”: the specific concentration of nutrients such as sugars, amino acids, minerals and vitamins that are found in the vicinity of the immune cells or cancer. The concentrations of these nutrients in standard cell culture media are often very different from those found in body compartments in living animals leading to significant differences in metabolism. This has been shown to lead to potentially misleading experimental results which has hampered advances in immunology and cancer research. In addition, cells moving between body compartments will encounter different concentrations of metabolites which are maintained at stable concentrations by metabolic processes in living animals, factors which are both

difficult to recapitulate in the test tube. Secondly, a major feature of this project involves testing the therapeutic potential of targeting more than one metabolic enzyme/pathway. While methotrexate is a widely used and effective treatment for patients with autoimmune diseases and lymphoma, it can have significant toxicity. Given the complexity of biological systems, combinations of metabolic inhibitors do need to be tested in living animals to gain the best understanding of efficacy and potential toxicity, prior to clinical testing in human patients.

Which non-animal alternatives did you consider for use in this project?

We have considered using both in vitro cell culture models and data from human-based clinical studies/clinical trials in this project. We will be performing several experiments on human cell lines in the test tube to establish whether particular drugs work and whether they can act synergistically with other medicines, and to identify the mechanisms behind such interactions. We also have extensive information from the clinical use of existing anti-metabolic drugs in human patients such as methotrexate in lymphoma and autoimmune disease to guide our proposed work.

Why were they not suitable?

In vitro cell culture models do allow for allows for controlled experiments and provides insights into cellular responses. However, as outlined above they have many limitations for our work, due to the differences in the concentration of nutrients between the cell culture media and the body, and fluctuations between body compartments. This can lead to erroneous observations and misleading conclusions, meaning that it is crucial validate our observation in living animals. The ultimate aim is to develop novel drugs and drug combinations that have not been used in humans with these specific diseases before. Therefore, it will also be critical to gain knowledge of the potential efficacy and adverse events in murine models prior to testing in humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the numbers of animals based on our previous experience of blocking particular metabolic enzymes and observing the effect on immune responses and lymphoma development.

Experimental groups will be chosen in accordance with standard experimental design. For example, for treatment with pharmaceutical inhibitors, experimental animals will be treated with the drug prepared in an appropriate vehicle/diluent with control animals being treated in the same way with the vehicle/diluent but without the

drug under investigation. For the conditional knockout models, the control animals will typically be "Cre-only controls": animals that have one of the genetic alterations but without the other one meaning that the they still have the metabolic enzymes under investigation. We will maximise the data output by making detailed records of what happens while the mice are alive, including the impact of any treatments on factors such as weight, behaviour and side effects such as diarrhoea or infection. In addition, we will make full use of cells and tissue after sacrificing these animals by analysing lymph nodes, spleen, bone marrow and peripheral blood. Furthermore, we will also assess other organs if clinically relevant such as the gastrointestinal tract or liver if the animals exhibited any adverse effects due to drugs such as methotrexate. Where mice have the same genotype in experimental and control arms, we will plan to randomise them to the different interventions. The person performing the treatment will work with the laboratory team and staff from our animal tech service, who will be blinded to the treatment that the animals are receiving when they assess them. Our previous research has given us an indication of the size of the effect that we can expect to see in the proposed experiments and the potential vales of specific parameters (e.g. mean, standard deviation) to enable us to perform power calculations. However, as this is novel research, it may be that the effect sizes may differ from that predicted. However, we will anticipate this and rapidly refine the experiments if this occurs – for example, by reducing the number of animals treated if the effect size is larger than expected. Furthermore, we will use data from our in vitro experiments to inform the design of the animal work. For example, if a particularly strong effect is seen with a specific approach in human cell lines, we may perform a pilot experiment with a small number of animals (e.g. 3-4 animals in each group) to ascertain whether we can reduce the number of animals used for subsequent experiments. We have estimated the total number of animals required on all protocols (i.e. both breeding and experimental) based upon our comparable previous research.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We took and will continue to take several steps to reduce the number of animals being used in this project. We made use of the existing literature and our own experience and data to design the experiments to use the least number of animals possible. All in vivo experiments will be preceded by extensive in vitro modelling using lymphoma cell lines to identify the best therapeutic strategies and drug combination for testing in the mice and provide valuable insights into cellular processes. We have designed the experiments to assess the impact of genetic knockout or pharmacological inhibition on tumour bulk at a pre-specified timepoint rather than assessing impact on survival – this will mean that we can use less than a third of mice required for the latter. All of this was planned incorporating the use of the NC3R's experimental design assistant. All mice will be randomised to treatment/control where appropriate, along with blinding and close clinical monitoring.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have previous experience of breeding the genetically modified animals so will be

able to apply this experience to make the breeding as efficient as possible. In addition, we will harvest as many tissues as possible post-mortem. This will include lymph nodes, spleen, blood, bone marrow and brain tissue to gain the maximum amount of information from these animals. If the tissues are not used immediately, we will freeze them for later use or make them available to other researchers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using the most appropriate animal models for this project. We will use wild type and genetically modified animals to study the impact of blocking metabolic enzymes on the immune response. We will have to induce immune responses to study them, which will mean that the animals will need an injection of the substance typically either under skin, into the peritoneal cavity or intravenously. We plan to use 3 different approaches to model human lymphoma in these animals. We will plan to inject immunodeficient mice with human cancer cells under the skin and study how the cancer responds to different treatments. This approach will minimise distress to the animals as we anticipate that the cancer will just be restricted to one part of the animal where it can be easily measured. It also reflects the method used in the scientific literature, which led to the development of a novel drug treatment for this cancer, something we hope to be able to improve upon. Will also inject mouse lymphoma cells into other animals to induce lymphoma. We already have experience with this approach, meaning that we can anticipate when the animals will develop measurable disease, and when then may develop signs of distress. Therefore we plan to sacrifice the animals at a pre- determined timepoint (typically 14-28 days - dependent on cell type and model) which we have selected as the animals will detectable disease (allowing us to determine the impact of drug treatment), but will not have such extensive disease that it causes them significant distress. The last approach will involve intracranial injection to induce development of lymphoma in the brain and central nervous system. We will refine our approaches as much as possible, but it is not possible to use any other approach as the science is dependent on lymphoma development in this particular site. We are not anticipating any of the protocols being classed as severe.

Why can’t you use animals that are less sentient?

Non-mammalian animals are not appropriate for this work as they lack the specific type of immune cells that we are interested in and therefore the experimental results will not be relevant. We cannot use younger animals as mice typically do not have mature immune system until about 8 weeks of age.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animals will be closely monitored for the development and progression of lymphoma in the relevant protocols. If it becomes apparent that particular treatments or combination approaches are leading to improvements in outcome we will plan to refine our procedures to assess the animals at earlier timepoints during the course of the experiments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will plan to follow best practice as described in the PREPARE guidelines, following all of the recommendations in the PREPARE checklist.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have recently completed the project licence training and repeated the personal licence training so have recently received up-to-date training on this. In addition, we will regularly review advances on the NC3Rs website, we’ve signed up to the NC3Rs newsletter and will attend the NC3Rs events.

91. Understanding variation in cognitive indicators of animal affect and welfare: individual differences and computational approaches

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:

Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

cognitive bias, judgement bias, individual differences, computational modelling, rat

Animal types	Life stages
Rats	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To improve methods for assessing animal welfare by investigating how individual ('personality') differences affect welfare measures, and by using new data analysis approaches to better understand and enhance the reliability of welfare measures.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Accurate assessment of animal welfare is essential if we are to understand how animals managed by man, including laboratory, farm, companion and zoo animals,

are affected by the way that we house and look after them. It is also needed in order to assess the impact of changes to housing and husbandry systems, including those specifically designed as refinements to improve animal welfare. A key determinant of animal welfare is whether they are in a positive or negative emotional (affective) state. New 'cognitive bias' markers of animal welfare have been developed which use how animals make decisions as an indicator of their emotional states. They are now used in animal welfare science, neuroscience and psychopharmacology across many species (>200 published studies). **As a new method, it's important that cognitive bias tests of animal affective state and welfare are as accurate and informative as possible and there are still issues that need to be ironed out**.

Recent multi-study analyses show that they have validity but also that there is variation in findings. Important reasons for this include that there are a number of underlying mechanisms linking emotional states to cognitive biases which may exert contrasting effects. These can be disentangled using computational modelling of decision-making data. Additionally, longer-term individual ('personality') differences in cognitive bias may also generate variation in findings. Understanding and quantifying these effects will allow us to further refine the cognitive bias approach and increase its accuracy, validity and utility as a marker of animal affect and welfare. The work will focus on laboratory animals (rats) but, as for past developments in cognitive bias testing, it will be generalisable to other species.

What outputs do you think you will see at the end of this project?

The project will provide us with new information about the variability and reliability of cognitive bias measures of animal welfare and thus allow us to improve these measures by taking into account the effects of individual ('personality') differences, and by using new methods of data analysis. **This novel information should thus help us to enhance the accuracy, validity and utility of cognitive bias tests.** To this end, we will also produce code for computational modelling methods that can be used to analyse cognitive bias data. And we will provide new information on how individual differences affect findings from cognitive bias tests, and whether individual differences in cognitive biases can themselves predict how well animals cope with their environments, as they do in humans where 'optimistic' biases predispose better coping. Overall, this information should also allow us to refine and improve the design and analysis of cognitive bias measures of animal welfare. Findings will be published in peer-reviewed scientific journals and/or lodged on appropriate preprint servers.

Who or what will benefit from these outputs, and how?

Knowledge accruing from this project should help other scientists to design and interpret cognitive bias tests more effectively, including through the use of computational modelling code for data analysis. At a more fundamental level, increased understanding of the underlying mechanisms mediating links between cognitive and affective processes will be of theoretical value to other researchers, and generalisable beyond the cognitive bias test and species studied here. If individual differences in cognitive bias predict coping and resilience as they do in humans, this may be of wider public interest (e.g. popular science articles). It may also have practical consequences, including facilitating selection of individuals for

specific scenarios (e.g. tasks, housing) according to their likely future resilience, and manipulating early experiences to influence individual cognitive biases and thereby enhance animal welfare.

How will you look to maximise the outputs of this work?

Scientific publication will be a key method for disseminating findings, alongside popular science publications if results relating to individual differences parallel findings in humans. To further maximise dissemination of positive findings relating to individual differences in resilience, and discussion of how these may be applied to enhance animal welfare, we will hold a workshop on this topic for scientists and other relevant stakeholders (e.g. NACWO, NVS and animal care staff). We have a number of international collaborators on this project and they will also be able to disseminate findings through their local networks.

Species and numbers of animals expected to be used.

- Rats: 184

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 work with juvenile and adult rats because they have been studied extensively in cognitive bias research, and well-developed behavioural protocols exist for quantifying their cognitive biases and generating data for computational modelling analyses. This means that they have become a key model (and the first) in the development of these new indicators of animal welfare; results from research on rat cognitive bias have generated methods and principles that have helped to improve the measurement of welfare in many other species. **Whilst the 'cognitive bias' measure was initially developed in rats, it is highly translatable and has now been employed in studies of mammals (including humans and agricultural species), birds, fish, insects and, just recently, cephalopods**. Rats were also the third most used species in scientific procedures in 2022 in Great Britain and, therefore, better monitoring and improvement of their welfare remains an important goal.

Typically, what will be done to an animal used in your project?

Upon arrival in the unit, rats will be allowed to acclimatise for 1-2 weeks during which time they will be habituated to handling and food rewards. They will be housed socially (e.g. pairs or larger groups) in conditions that would at no point fall below the standards outlined in the Home Office Code of Practice. Following acclimatisation and habituation, rats will be trained on a cognitive bias task. This will be a *judgement bias task* in which rats learn to make one response (e.g. approach; nose poke; press

a lever) to a cue (e.g. a tone of a particular frequency) predicting a positive outcome (e.g. food) in order to acquire that outcome, and a different response (e.g. don't approach; stop nose poke; refrain from lever pressing; press a different lever) to a different cue (e.g. a tone of a different frequency) predicting a relatively more negative outcome (e.g. no food; airpuff) to avoid that outcome. Once trained, they will be occasionally presented with ambiguous cues intermediate between the training cues. Their responses to these will indicate whether they are anticipating a positive or negative outcome (termed 'optimistic' or 'pessimistic' responding) and will be used as markers of their cognitive (judgement) bias. Responses to ambiguous cues will then be measured weekly for between 1-6wks to get a measure of baseline individual differences in 'optimism' and 'pessimism'.

During the training and baseline testing period, a subset of rats may be exposed in groups to *play-pen enrichment* sessions. These will take place up to three times a week, with sessions being up to 1h long. Studies indicate that such sessions have beneficial effects including inducing positive affective states. The aim will be to see whether this enrichment enhances baseline 'optimism' relative to control rats who will not be exposed to play-pens.

Following baseline testing, rats may be exposed to treatments designed to manipulate their affective state and welfare. These may include:
opportunity increase and decrease: up to 3wks addition or removal of cage enrichment objects followed by removal or addition of cage enrichment objects to return to initial conditions *threat increase and decrease*: up to 3wks of unpredictable housing (e.g. exposure to short periods of damp bedding, altered light cycle) followed by return to initial (stable) conditions *short-term manipulation*: *Negative* - e.g. restraint by the tail / restraining device; exposure to an unfamiliar cage and/or odour from unfamiliar conspecifics; up to 30 short (<5sec) avoidable airpuffs. *Positive* - e.g. food rewards; 'tickling'; play opportunities. Treatments will last a maximum of 15min. Subjects will receive no more than three *negative manipulations* per day and ten in total during each manipulation period which will span a maximum of 2wks.

Each rat may be exposed to up to two of treatments (i) and (ii) (i.e. one experience of each treatment or two experiences of one treatment and none of the other), and up to five periods of treatment (iii), each separated by at least 1wk.

During baseline testing, the weeks when treatments are imposed, and weeks between treatments, rats may be exposed to judgement bias tests and *independent tests of rat affective state, personality and welfare* (e.g. open field test; elevated plus maze test; sucrose preference test). Faecal samples may be collected during these tests for subsequent assay of faecal glucocorticoid metabolites as non-invasive markers of physiological stress. *Home cage behavioural observations* may also be carried out. Rats will be exposed to a maximum of six behavioural tests in any one week (this could range from six different tests to six of the same test). Rats may also be weighed weekly throughout the training and testing period.

What are the expected impacts and/or adverse effects for the animals during your project?

The behavioural tests of affective state and welfare are either positively motivated

(e.g. sucrose preference test), involve both positive (food) and mildly negative (no food; airpuff) outcomes with the latter being avoidable (judgement bias tests), or may induce a mild and short-lived (e.g. 5-10min) negative state (e.g. open field, elevated plus maze). They are therefore not expected to cause suffering or, at most, that this will be mild and transient and should be alleviated once rats return to their home cages.

Play-pen enrichment sessions are expected to generate positive affective states. Aggression in these sessions is unlikely but they will be monitored so that any rat performing or receiving damaging aggressive behaviour can be removed and returned to its home cage.

Affect manipulations (i) and (ii) may cause mild suffering. This could result in the development of abnormal behaviour and/or temporary weight loss. It is anticipated that, should they occur, these effects will be reversed when initial housing conditions are reinstated.

Affect manipulation (iii) involves short-term treatments that may result in transient mild stress which should be alleviated once each treatment ends.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity level for all animals, tests and manipulations is mild.

What will happen to animals at the end of this project?

- Rehomed
- Killed
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project investigates cognitive indicators of animal affective state and welfare. Cognitive markers are empirically inferred from the behaviour (e.g. decision-making) of live animals and it is therefore necessary to use them in this work.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives are not applicable in this study

Why were they not suitable?

It is not possible to empirically evaluate how (manipulations of) affective states influence behaviour and decision-making without using live animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on previous work, we estimate that we will need 32 animals to detect individual differences in statistical parameters of cognitive bias responses for each sex (i.e. 64 rats in total using both males and females). These differences will then be related to independent measures of affective state and welfare in response to affect manipulations.

For studies investigating the effects of play-pen experience or other affect manipulation treatments, we estimate, on the basis of previous work, that we will require 20 animals per treatment group for between-subjects experimental designs (e.g. manipulation of play-pen experience), and a total of 20 animals for within-subject experimental designs (e.g. serial exposure to affect manipulations). We will carry out one between-subjects study (40 rats) and two within-subjects studies (40 rats), and may then add a further between-subjects study or two within-subjects studies (40 rats) giving a current estimate of 120 rats in total for these experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used power analyses based on data from previous related studies to calculate our sample sizes and thereby ensure that our experiments are adequately powered whilst minimising 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?

Computational modelling of decision-making data in the judgement bias task will allow us to extract several measures of the processes that underlie the links between affective states and cognitive bias (e.g. optimism bias, reward sensitivity, punishment sensitivity) and which would otherwise require separate experiments and/or test procedures. These processes can then be related to how individuals respond to affect manipulations.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 behavioural measures / tests of affective state and welfare. These are short-lived, involve mild challenges to the animals (and in some cases are positively motivated) and we anticipate that they will have no or mild effects on the animals which will end after the tests finish.

We will use non-invasive physiological markers of affective state and welfare (e.g. faecal samples for glucocorticoid metabolite assay) that should not affect the animals.

Play-pen enrichment sessions are likely to be positive experiences and are being encouraged for laboratory rats (e.g. https://nc3rs.org.uk/3rs-resources/rat-playpens). Therefore, we anticipate that they will not cause any harms.

Affect manipulations will involve batches of short-term or longer-term changes to environmental conditions. They have been designed to have only mild effects: short-term manipulations will be transient (up to 15min) whilst longer-term manipulations (up to 3wks) are designed to involve combinations of relatively small changes to the environment.

Why can’t you use animals that are less sentient?

Cognitive bias testing is designed to be able to assess the affective states and welfare of many different species including highly sentient ones (e.g. it has been used to study primate and cetacean welfare). To develop and refine the approach therefore requires the use of awake sentient species such as the laboratory rat which is likely to be of similar sentience to other domestic species managed by man whose welfare we need to assess.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will monitor animals during behavioural testing and terminate tests should subjects appear to be strongly affected (e.g. intense escape attempts).
We will monitor the effects of short- and longer-term affect manipulation treatments. Any animal that shows sustained (>2wks) and untreatable aberrant behaviour (e.g. over-grooming; frequent bouts of stereotypic behaviour) or weight loss of >10% will be removed from the treatment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow ethical guidelines as published in the journal Animal Behaviour (https://www.sciencedirect.com/science/article/pii/S0003347222002469), one of the first journals to develop such guidance. We will follow ARRIVE and PREPARE guidance for experimental design and related considerations.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am a specialist in animal welfare research and so regularly read papers and attend meetings relating to animal welfare and the 3Rs. I hold and have held senior positions in scientific societies and organisations relating to animal welfare research. This includes running meetings, workshops, online gatherings etc. My institute hosts events that actively promote the 3Rs and I participate in these. I'm therefore aware of many of the latest developments in the field, including before they are formally published. I will use this knowledge and other contacts (e.g. members of my lab are on ECR 3Rs committees at my University) to keep abreast of developments and implement them as appropriate.

92. The neural basis of complex cognition

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cognition, Behavioural neuroscience, Frontal cortex, Basal ganglia, Psychiatric

Animal types	Life stages
Rats	juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim is to identify how chemicals in different areas of the brain work together to generate the thoughts and behaviours that are commonly referred to as ‘executive functions’, for example, planning, goal-directed thinking, attention, expectation and anticipation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In humans, executive functions are compromised to varying degrees in many neurological and psychiatric disorders, such as schizophrenia, Parkinson's disease and Alzheimer's disease, as well as in the course of normal and pathological aging. How different diseases and disorders affect these executive functions is not yet fully

clear, so it is important that we develop and use the right tools to assess and understand specific symptoms, that we might also more effectively develop treatments to alleviate them. Rats also have executive functions, albeit not as well developed as in humans, but there are many similarities. By looking at rat behaviour under varying conditions, we can learn about the similarities and differences in the brains of different animals, and this will improve our understanding of the impact of human diseases and aging on these functions.

What outputs do you think you will see at the end of this project?

Our goal is to generate new knowledge about our understanding of how brain processes work together to underpin complex cognition, with a particular aim to elucidate how attentional flexibility, in both normal and dysfunctional brains can impact learning. We also aim to adapt (via automation) our well- established method for assessing attentional flexibility, such that it might be made more accessible to other researchers and their projects. Our outputs will take the form of scientific presentations of data, at conference or to industrial collaborators, and in open-access research publications in peer-reviewed international journals; datasets and means of analysis (e.g. behavioural data) deposited in public, open-access databases; novel behavioural task protocols reported in open access publications, online and/or in methods papers.

Who or what will benefit from these outputs, and how?

An expectation is that short-term benefits will arise from improvement(s) of pre-clinical models, with validation by cognitive assessment – allowing reduction of the numbers of rodents used, and possible replacement with lower order animals such as fish or even insects. We work closely with scientists in drug companies with the expectation that we can improve research techniques for pre-clinical testing of new drugs. We hope that this in turn might enable the medium-term benefit of progression of a new drug to clinical trials for the treatment of psychiatric illness, or at least in the capacity to increase the speeds at which novel compounds' efficacy can be established. The development of a more accessible and higher-throughput automated version of our attentional flexibility task might facilitate research where otherwise it prove too costly. A resulting long-term benefit might then be the establishment of a novel treatment for one or more human psychiatric illnesses.

How will you look to maximise the outputs of this work?

We will share our findings at scientific group meetings and with our international collaborators in the pharmaceutical industry, through scientific presentations at conferences, both local and international, and via open-access publication in leading peer-reviewed international journals. We are looking to collaborate further with industrial partners to develop, with them, a novel automated attentional flexibility task, and will share our results, both successful and unsuccessful with them - further discussing and sharing those results with other experts in the field, and where suitable in open-access publications.

Species and numbers of animals expected to be used

- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use adolescent and adult rats as they show similar executive functions, albeit not as well developed, as humans. Rats share a mammalian brain structure with humans, which whilst not identical, have sufficient similarity that we can identify homologous regions that mediate the cognitive functions we seek to understand, making them an ideal species to work with. We try to use behavioural tasks that have established translational validity - i.e., that rats and human (and other vertebrates) can undertake using the same basic cognitive processes. We can then learn about the similarities and differences in the brains of different animals, and this will improve our understanding of the impact of human diseases and aging on these functions.

Typically, what will be done to an animal used in your project?

A typical experiment involves measuring the behaviour of rats as they perform a particular task, which might be spontaneous behaviour (such as foraging for food in a maze or arena) or trained behaviour (such as pressing a lever for food). We measure changes in the animals' behaviour as a result of interventions, such as permanently, or temporarily, reducing activity of different brain circuits (for example, causing targeted and localised brain damage), which mimic the effects of clinical pathology, or using drugs to temporarily increase activity in some brain areas to mimic clinical treatment. Some of these interventions (e.g., those involving surgery) are up to a ‘moderate’ level of severity, causing transient pain or distress. As with human surgery, anaesthetics (general and local) are administered pre- and during surgery, and painkillers are administered the day before, immediately prior to, and the day after surgery (and for longer under NVS advice if necessary) to reduce post-operative pain. During behavioural testing, the effects of procedures are minimal, intended only to cause subtle changes to the behaviour we’re examining (for example, rats may receive an injection of a drug to subtly improve or impair their cognition prior to behavioural testing). We control access to food prior to testing so that the rat is sufficiently motivated to perform a task to get food treats, but they are maintained at a healthy weight and always fed a normal quantity of food daily regardless of whether they perform a task for food. Depending on the behavioural task, rats may need to be trained and tested daily, or only once a week. Behavioural testing can then continue over several months. At the conclusion of testing, the animals are humanely killed and their brain tissue may be taken for analysis post-mortem.

What are the expected impacts and/or adverse effects for the animals during your project?

Rats that have received surgery would be expected to experience moderate levels of pain, that we seek to alleviate with painkillers. There may be temporary weight loss after surgery, if the rats lose their appetite, but the rats should resume normal eating within a few days of surgery.

Surgeries where electrodes are implanted, or where an avirulent virus is administered, should return to normal within 48 hours of the surgery. Those rats that show weight loss of >15% would be humanely killed.

Surgeries where a lesion is induced may (depending on the toxin and the location of the lesion) might show recovery in stages - with some rats showing hyperactivity or stereotyped movements for 24-48 hours post surgery as the lesion develops, and a corresponding loss of appetite. Appetite may recover shortly after that 48 hour period, or may take longer to fully recover (1-2 weeks). Rats will be fed higher calorie, palatable foods to mitigate the harms that the loss of appetite might cause. Rats that show weight loss of >20% would be humanely killed.

Rats that receive an injection of a drug, or have blood withdrawn from the tail vein, would be expected to experience brief mild pain during the injection, and thereafter there should be little in the way of adverse effect. There may be a short-term, desired subtle change in their cognitive abilities after injection of drugs. In some cases, injections are repeated over several days, or a subcutaneous/intraperitoneal pump may be implanted, to induce longer term change to cognitive function, and there may be effects on appetite depending on the drug. Where we would expect weight loss, it would typically be about 5%, and we would feed the rats more palatable food to mitigate this, but rats that show weight loss of >15% would be humanely killed.

We may use older rats to investigate changes to cognition as the rats age. We would expect mild age- related decline, and will monitor rats more closely once they reach 12 months. We will monitor weight, and assess appearance (skin/fur condition), body condition score, respiration, dentition, movement and behaviour using a traffic light system: green is normal; amber requires increased monitoring or support; three or more clinical signs at amber, or any at red, and the rat will be humanely killed.
We do not expect any adverse effects resulting from our behavioural testing. If any are observed during development of novel tasks, we will stop testing, and discuss with the facility's technical staff and NVS if necessary, as well as seeking further advice from the establishment's Animal Welfare and Ethics Review Board (AWERB).
Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on the previous project, we would expect about:

- 15% of rats used on the project will experience subthreshold harms
- 25% of rats used on the project will experience mild harms
- 60% of rats used on the project will experience moderate harms

What will happen to animals at the end of this project?

- Killed
- Rehomed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are interested in the brain processes underlying behaviour. It is not possible to study behaviour in anything other than an awake behaving animal, which includes humans.

Which non-animal alternatives did you consider for use in this project?

We constantly monitor the literature for potential non-animals alternatives. Searches of PubMed and Web of Science for 'replacement' or 'alternative' with 'cognitive flexibility' and 'rodent' or 'animal', or 'artificial intelligence' with 'cognitive flexibility' suggests there are currently no viable non-animal alternatives that we can use to explore complex cognition. We do conduct limited research with human participants, principally to determine that when we adapt behavioural tasks in rats, the results we see remain comparable between the two species - as it is important that we can apply what we learn from the rats to the human condition. We have also begun to explore the use of artificial intelligence (AI), to investigate how AIs solve the tasks we set humans and rodents.

Why were they not suitable?

We cannot investigate the brain processes underlying behaviour in humans because it is not possible to systematically manipulate brain function in the same controlled manner that is possible in animals such as rats. With AI, whilst there have been great advances recently, there is currently insufficient understanding of how AI solves the behavioural tasks we use, and, indeed, insufficient understanding of the specific cognitive processes impaired in diseases like schizophrenia, to allow us to attempt to manipulate AI to replicate what we observe in the behaviour of humans and rats. Until we have a clearer picture of what specific cognitive processes are impaired, and how AIs solve complex behavioural tasks, and how they can be impaired to mimic human disorders, we cannot use AI to replace living organisms.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to

minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse. How have you estimated the numbers of animals you will use?

The estimated number of animals is based on prior experience on my current PPL, and PPLs held by the previous principle investigator prior to my taking over as group lead at the establishment. We also take regular advice from local statistics experts to ensure the numbers of animals we use are the minimum necessary to see the effects on behaviour that we expect. Our behavioural studies typically involved rats sampling and discriminating a number of different odours and digging media, in a series of stages. We counterbalance the order that they encounter these stimuli, and we have found that with the effect sizes and variability we typically observe, a group of 12 rats allows both perfect counterbalancing of those stimuli, and results in robust and replicable statistical significance. To ensure the minimum number of animals we try to obtain as much behavioural data from one animal as possible, for example, by testing them multiple times in the same task to improve confidence in the accuracy of measurements, and under multiple conditions (for example, before manipulations ('baseline') and after (or vice versa in a counterbalanced group when the effects of manipulations are acute), to measure change in behaviour as a result of a manipulation. This way, we can collect a substantial amount of data over several months from the same animal, without needing a large number of groups of different animals. The behaviours we use are initiated by the animal and ‘self-paced’, so we can use ‘rate of work’ to indicate effort and willing. This provides an important ‘check’ on welfare: the animal stops when it wants to.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We collaborate with a statistics advisor who offers support and advice in design and analysis and supports continual professional development, particularly in statistics. With his support and guidance, we have recently developed a novel approach to our data analysis using Bayesian inference. This enables us to gain more information from the data, so potentially increasing statistical power and enabling a reduction in numbers.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When developing new behavioural tasks, we use animals that experience subthreshold harms (controlled access to food sufficient to motivate food reward seeking), which allows us adapt the tasks as needed (based on observed results) without needing to use more animals than the initial cohort. We preferentially use behavioural tasks where repeated testing does not affect our results, which means a single cohort of animals can be used to collect a substantial amount of behavioural data with only subthreshold harms.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rats are the most suitable animals for this project because they are mammals, like humans – which allows us to more closely associate their behaviour and brain function with that of humans. In terms of the behaviour we are interested in, rats are a well established model, and there are a lot of data from rats that we can use for comparison in term of both brain function and behaviours of interest. The established behavioural tasks we use are ones that rats readily engage with – they are inquisitive and learn readily; and rats are a good size in terms of maintaining appetite to engage with food reward- motivated behavioural tasks for long enough that we can collect all the data we need from a single behavioural test without need for a break that might impact performance.

Our objectives - which rely on being able to measure behaviour - can only be achieved by minimising animal suffering, as the rat will not perform the behavioural testing if it is overly anxious or in distress. To that end, it is not always our intention to 'model' the entirety of a psychiatric syndrome, and it is unlikely that this would be possible anyway. By addressing individual symptoms or symptom clusters, rather than modelling all aspects of the psychiatric syndrome, we try to minimise the severity experienced by an individual rat. We therefore select methods (e.g., targeting brain areas with avirulent viruses, or systemic administration of a psychoactive drug) to manipulate cognitive function, and the subsequent behavioural tests that reflect those functions, to ensure the animals are affected only to the degree necessary to observe those subtle changes in cognition. To that end, when we can, we use viruses instead of lesions, because recovery from the surgeries where we inject viruses is quicker, and rats experience fewer harmful symptoms than they would during a surgery to induce a lesion. Rat cognition can then be assessed in behavioural tests when they have recovered from any surgery (during which time they are monitored to ensure normal recovery), or after injection of a drug (or where possible oral administration in a palatable jelly tablet) to observe an acute change in cognition.

Why can't you use animals that are less sentient?

To explore complex cognition, we observe behavioural changes after manipulations to brain function. It is necessary, therefore, that we use awake adolescent and adult animals - with juvenile rats often having not developed the capacity for the complex cognitions we are interested in. We use mammals because our goal is to be able to extrapolate our observations of rat behaviour to that observed in humans. Because there are brain circuits that have been conserved during evolution, the differences, as well as the similarities, between animals (e.g., humans and rats) provides

important information about how behaviour is organised in the brains of different species and how this gives rise to different, species-typical, behaviour.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Rats will be socially housed at all times to prevent stress from isolation, with objects they will find interesting to engage with (e.g., tunnels, and chew sticks) added to the their cages for enrichment. Rats are handled regularly, to ensure they are acclimated to engagement with researchers, minimizing.

We will conduct our surgeries under aseptic conditions, and to ensure the best possible welfare outcomes arising from any surgeries, we will work with our facility's technical staff and the NVS such that we use the most appropriate peri- and post-operative care methods and pain management for the given technique. Rats are kept warm after surgery, whilst they recover from anaesthesia, and are monitored for any welfare concerns. Once recovered from anaesthesia, rats are returned to their cages, and the social enrichment from their cage mates.

Where possible, we give the animals drugs in palatable jelly tablets, meaning we don't always need to inject to administer the drugs.

The behavioural tasks we use are intended to cause no harms to the animals, but as we seek to develop novel tasks to assess complex cognition, we will monitor performance and adjust as necessary to ensure that is the case.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will employ the PREPARE guidelines and the Guiding Principles for Behavioural Laboratory Animal Science (LASA, BAP, BNA, and ESSWAP) when planning and conducting our studies. We will follow LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017). In addition, we will use the 3Rs resource library for husbandry and in vivo techniques (e.g. Grimace Scales to assess pain).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will engage in regular communication with the highly skilled technical team at our facility, to ensure we stay up to date on techniques to ensure the best possible welfare outcomes arising from our research methods. I sit on the establishment's Animal Welfare and Ethical Review Body and chair a local ethics committee, both of which provide valuable insights into the principles of the 3Rs as applied to both regulated and non-regulated work. We will actively seek ideas for improvements using information sourced from organisations such as the Laboratory Animal Science Association and the National Centre for the Replacement, Refinement, and Reduction of Animals in Research, and the newsletters, resources and workshops we are informed of by our Home Office Liaison Contact.

93. Breeding and maintenance of genetically altered animals

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - 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

Genetically, altered, rodent, breeding, maintenance

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To produce, maintain, and provide genetically altered rodents for use under other project licences.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-

term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This licence is intended to provide an efficient service for the breeding of genetically altered rodents and to supply them for future use on other scientific licences. Using a range of sophisticated technologies, the DNA of rodents can be safely manipulated to allow for experiments that elucidate the role that candidate genes play in a range of biological processes. In turn, these insights can lead to treatments that can prevent or treat disease and optimise the health of both animals and humans.

What outputs do you think you will see at the end of this project?

The genetically altered animals produced under this licence will be used in a variety of models and studies. The high-quality genetically altered model outputs will be used in studies required by regulators, and other studies supporting this aim. This will help progress drugs into clinical trials, or prove the chemicals produced will be safe to be used by the public. The results from these models will be used as source of information in peer reviewed publications within the scientific community. Animals produced within this licence will contribute to further scientific benefits across other licences.

Who or what will benefit from these outputs, and how?

This project will benefit people and animals by revealing the genetic contribution to a range of biological processes that are involved in health and disease. Where genes play a role in disease processes, this may provide better diagnosis and/or earlier and better treatment of a disease. The ability to easily manipulate the DNA of rodents, enables further understanding of the role and function of particular genes in diseases. In the longer term this understanding may lead to improved treatments for diseases affecting humans.

How will you look to maximise the outputs of this work?

The importance of this project proposal is to introduce as little variation between animals as possible, by breeding in carefully managed colonies, so that fewer animals are needed to generate reliable results and the results are more reproducible.

The ability to share the same genetic lines with multiple researchers will reduce variability and improve consistency of experimental results.

Species and numbers of animals expected to be used

- Mice: 462080
- Rats: 25000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodents are biologically very similar to humans and can be genetically manipulated to mimic many pathological conditions, which is not the case for non-protected alternatives such as fruit flies. As this is a breeding licence, all life stages < 15 months may be used (i.e. all stages except for aged animals).

Typically, what will be done to an animal used in your project?

Rodents will be initially bred according to the strategy finalised during discussions between technicians with considerable experience in the breeding of genetically altered animals and the end users in advance of animals arriving. Most animals will not experience any other procedures whilst on this licence; animals will be transferred to other licences or humanely killed. Most animals bred will be identified by taking an ear notch and the tissue used for genotyping or the implantation of a microchip for identification.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals that are bred and produced under this licence will not show any phenotype that would cause a change from normal. A small number may have a genetic alteration that mimics a human condition e.g. Alzheimer's as they age. These strains will be bred as young adults to reduce the chance of the disease phenotype occurring. Other strains may have an impaired immune system, increasing their risk of developing infections, but these will be maintained in isolators or IVC caging to prevent infections occurring.

Expected severity categories and the 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 in this licence will be considered as sub threshold for severity.

25% mild

20% moderate

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although in-vitro assays are becoming more prominent in biomedical research they cannot always adequately model the complete array of behavioural, cellular, molecular and physiological interactions required to fully understand how genetic alterations result in normal or abnormal processes. Prior to acceptance of any program of work the necessary scientific justification will be received from sponsors and will include details of their experimental use and why there are no alternatives to the use of live animals available. Establishments will have Project Licence authority for the use of live animal models and this will include the relevant justification for use in their program of work.

Which non-animal alternatives did you consider for use in this project?

Requests for rodents to be bred under this licence must describe the non-animals steps that have preceded the in vivo stage. AWERB will determine that animal use is justified by confirming that, as far as is practical, all non-animal steps have been completed. For instance, and where relevant, studying the impact of genetic alterations in vitro (in cells) before progressing to genetic alteration in animals.

Why were they not suitable?

Where animals move to a scientific licence the sponsor is required to justify the requirements for the use of animals and have considered all relevant non-animals models. Reasons why non-animal alternatives are not suitable will be assessed on a case-by-case basis during AWERB review of ongoing projects.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers we have estimated are based on previous production figures and demand over the past 5 years. The breeding of any model will not commence without a proven justified requirement and a suitable colony management plan in line with forecasted demand. Maintaining a continuous overview of production and demand throughout the lifetime of any given colony allows us to monitor breeding programs in a detailed and proactive way.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have been working with most of the production models which are included under this PPL for many years and have also gathered production data from the 5 previous years in order to refine and reduce the number of animals being used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We manage our breeding program is the best possible way to avoid surplus production and refine our techniques. We also work very closely with our partners to clearly define the needs and adjust the breeding strategy accordingly.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the most widely used species in the field of genetic alteration. Standard protocols, methods and reagents have been optimised for this species and there are acknowledged benefits from their use. The rat is less frequently used. However, their larger size may make them the more appropriate model when surgical interventions are required or existing historical/background data is predominantly within this species.

Prior to receipt of any genetically altered (GA) line we will perform an extensive welfare assessment to ensure that housing, husbandry and specialist care can be provided according to the GA and any expected phenotypical traits.

Animals will be maintained in specific sized social groups in accordance with their gender and phenotype, allowing for natural hierarchical behaviour but avoiding overt dominance related aggression as well as reducing stress associated with single housing.

When genetic testing is required the least invasive method, the ear punch system, will be utilised for collection of ear tissue. A robust and thorough sampling process will reduce the need for re-sampling e.g. thorough cleaning of the ear punch between animals will significantly reduce the risk of sample contamination.

Why can’t you use animals that are less sentient?

Rodents are biologically very similar to humans and can be genetically manipulated to mimic many pathological conditions, which is not the case for non-protected alternatives such as fruit flies.

Nevertheless, as this is a service licence, the breeding request form will ask that requesters explain why their particular area of research cannot utilise non-protected alternatives or less sentient protected animals such as zebrafish. Rodents will only be bred if appropriate justification is provided.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will follow best practice guidelines and group house colonies as standard, utilise non-aversive handling, and ensure environmental enrichment is present in all cages. Attendance at training events and networks ensures that staff are made aware of any suitable refinements for the breeding and maintenance of genetically altered rodents.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

- 'Efficient Breeding of Genetically Altered Animals Assessment Framework' (Home Office)
- Materials produced by the NC3Rs' 'Expert Working Group for mouse breeding and colony management' and disseminated via the Breeding and colony management hub (NC3Rs)
- Welfare assessment recommendations from 'Assessing the welfare of genetically altered mice' (Wells et al. 2006, Lab Animals)

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, Named Training and Competency Officer and other colleagues in Animal Technology, and by attending appropriate training courses and conferences. Use of NC3Rs website and attendance at meetings will help share knowledge. We also hold regular 3Rs focus groups.

94. Measurement of avian heart rate, acceleration and flight performance.

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

heart rate, birds, flight performance, energetics, kinematics

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The long-term aims of the work are to measure the behaviour, energetics and kinematics of both captive and wild living birds during various modes of flight and investigate the relationship of these parameters to the fundamental biophysical principals governing the animal's morphological design and physiological performance. This could include studying the role of factors such as body mass, body shape, wing design and physiological functions such as heart size, cardiovascular performance and body temperature.

Project objectives:

1) To quantify how body and wing mass, along with body, wing and tail shape and feather enhancements, influence flight speed, kinematics and proxies of energetic costs (such as via changes in heart rate and 3D body dynamic acceleration).

2) To quantify how tag mass, shape, location and attachment methods influence flight speed, kinematics and proxies of energetic costs.

3) To compare and contrast measures of heart rate and acceleration and determine

the applicability and accuracy of the sinewave model of dynamic body acceleration for estimating flight performance of birds.

4) To measure and constrast the flight performance and energetics of birds flying in different energy scapes i.e. when flying alone or in flocks, or when flying over different ground elevations, or when experienceing different wind strengths and directions.

5) To quantify the repeatability and stability of values for heart rate, body and wing kinematics.

6) To measure the body temperature of birds in different environmental conditions and when under going different intensities of locomotion (either occuring naturally or following body mass manipulation).

The overall approach is to conduct a continuous series of short and long flight experiments on unrestrained birds instrumented with miniature data loggers capable of recording variables such as global positioning system geolocation (GPS), 3-axis accelerometry and angular velocity, heart rate, pulse oximetry and subcutaneous body temperature. Analysis of the results will include the testing and refinement of predictive aerodynamic and biomechanical models of flight and consideration of both intra- and inter- indivdual performance. Project plan

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

The aerodynamics, biomechanics, physiology and energetics of locomotion, particularly of flapping flight, are complex and are still quite poorly understood. Improved quantitative understanding of these subjects will be of direct benefit to the academic community but will also indirectly benefit those involved in conservation and concerned about the development and implementation of best practise in the tagging and handling of birds. Scientific application of miniature data loggers allows for technical refinement in that individual animals can be studied in more natural states and performing more natural behaviours. Modern technology also allows for higher resolution measurements and, thus, more accurate data interpretation and better physiological and biomechanical modelling. Wild animals can lead challenging lives and furthering the understanding of their evolutionary adaptations, ecological roles and energetic requirements will lead to better models for understanding their locomotor capacities, better predictions of their habitat requirements and a superior basis for which to make future management and conservation decisions. Improved understanding of the techniques utilised in these studies may also arise from analysis of measurement repeatability, precision and accuracy.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

The primary work will focus on the flight performance of homing pigeons (Columba livia). Around 300 birds may be used over 5 years.

Other species of birds e.g. parrots, birds of prey, waterfowl, shearwater, pied kingfishers may be studied in falconry centres or in the wild. This may involve up to a further 200 birds, subject to opportunity and funding.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Few adverse effects are anticipated. The only invasive procedure is the application of subcutaneous gold-plated electrode pins. These are very well tolerated by birds and infections are expected to be very rare. If detected, then pins will be removed and wounds cleaned and treated with anticeptic. Birds will be removed from experiments until fully recovered.

Addition of weights will initially done progressively. If birds demonstrate behavioural intolerance to the additional loads then experiments will not proceed to higher levels. Weights will either be maintained or reduced, depending on level of response e.g. slower return to the loft or nesting sites.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

The study of animal locomotion behaviour and ecology frequently requires the study of their natural behaviour in the wild. Studies of captive animals both inform the interpretation of wild animal studies and also provide valuable insights in itself and with the opportunity for more detailed and accurate data collection. The study of animal locomotion can be usefully informed by mathematical modelling but is a complex subject that requires real animal experimentation to feedback into the modelling process. Separation of the effects of drag against weight, or the details of the design and shape of a moving wing require direct testing.

Reduction

Explain how you will assure the use of minimum numbers of animals.

These studies are designed as a continuous series of experiments in which knowledge is gathered and refined from one experiment to the next. Thus, the work is designed around a single colony of homing pigeons that varies in size from around 40 to 100 individuals and constitutes a relatively small social group. Birds will fly as individuals but also enjoy flying in a group.

Previous experience has shown that homing pigeons fly with heart rates of around 640 beats per minute, with a SD between days of around plus/minus 10 beats min^{-1}. This work also shows that adding 5% body mass raised heart rate by a mean of only 10 bpm (or around 1.5%), which is only equal to the natural SD of the population heart rate. Thus, it is necessary to be able to have a chance of detecting a change in heart rate of around 1% (or 7 beats min^{-1}). In a paired test, using a total N of 26 birds would yield a statistical power of around 95%. Thus, I consider a total N of around 20 to 40 birds per experiment, to be a reasonable compromise between obtaining a satisfactory experimental resolution of changes in energetic costs and the desirability to reduce the numbers of animals used in experiments. Typical tests will include t-test (usually paired), ANOVA (usually repeated measures), regression and multiple regressions. If smaller numbers of birds are used then experimental measurements may have to be repeated a number of times for each individual in order to obtain sufficient statistical power. However, this approach will be necessary when studying other non-pigeon species where the number of individuals available may be limited.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

The group at Bangor is developing one of the world's smallest mulit-sensor data loggers, with current measurements of 46mm x 16mm x 8mm and a mass of 5 to 12g (or 1.25 to 3% body mass including battery and packaging). These miniature data loggers are well tolerated by the birds and have been shown to be excellent for recording high resolution variables. There will be some scope in the future to slightly reduce these tags in size and mass by simplifying their specification, which will further improve the refinement of the technological approach.

We have experimented with an improved elasticated harness design and light weight plastic backplates with velcro, which speed up handling times and seem to be tolerated very well. Previous reviews of our files found no evidence of additional mortality as a result of wearing data loggers compared with controls. We practice daily maintenance by experienced animal technicians and have many years of experience in pigeon husbandry. We are visited by the veterinarian and have had little disease or other medical issues.

The major procedures are the use of subcutaneous electrodes in order to record the electrocardiogram of the heart (ECG), and the addition of added mass up to 12% of body mass on the body of the bird (usually across the chest area as this eliminates additional increase in drag) or addition of added mass along the forearm of the wing

(up to 0.75% each wing). These are considered to be mild procedures and fully reversible.

1) (a) In the case of the subcutaneous electrodes, previous experience has shown that gold safety pins can be left in the skin of the birds for many weeks without causing any behavioural effects or evidence of infection. Insertion into the skin of the upper and lower back takes a few seconds and appears to cause very little concern to the birds and no evidence of any subsequent effects or suffering. (b) We will also be trialing Pulse Oximetry technology in the hope that, in the future, this non-invasive approach may be able to substitute for the ECG measurements in some experimental circumstances. (c) We will investigate the possibility of using external electrodes glued to the skin for ECG recording, but previous experience suggests that the signal quality can be very poor and that Pulse Oximetry (option (b)) may be a more likely route to refinement.

2) In the case of added mass, we typically use lead weights but are considering sourcing tungsten weights due to their higher density and, therefore, lower bulk (thus reducing drag even further).

Although the pigeons will be used in a continuous series of experiments, they will be handled and checked before every flight. As all the procedures are fully reversible within a short period of time, and the birds are fully trained and habituated to the experiments. Thus, it is considered that there is very little likelihood of any significant “carry over” effect from one experiment to the next. Natural breaks between experimental flights should be sufficient to allow for any recovery that might be necessary, either physical or psychological.

95. Sensory processing in birds

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Avian retina, Adeno-associated virus, photoreceptors, biosensor, 2-photon live imaging

Animal types	Life stages
Quail (Coturnix coturnix)	juvenile, neonate
Domestic fowl (Gallus gallus domesticus)	juvenile, neonate
Zebra finch (Taeniopygia guttata)	juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The Aim of the Project is to record light-evoked activity from nerve cells in the retina of birds, to better understand bird vision and how the avian retina functions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Birds have excellent eyesight, and in many cases can resolve details in visual scenes that remain invisible even to humans. This includes spatial resolution (how 'sharp' their image is), temporal resolution (how 'fast' their image updates), and what colours birds can discriminate. However, to date, lack of experimental access has

precluded direct investigation of the function of bird eyes. Accordingly, our understanding of how birds achieve their superior insight remains very limited.

b

The expressed biosensors (genetically engineered fluorescent proteins that change brightness in the presence e.g. of calcium, a proxy for neuronal activity) will be monitored in the ex-vivo (freshly extracted) tissue using 2-photon imaging (a specific form of fluorescence microscopy that uses "invisible" infrared light to excite biosensors). These measurements will help understand the function of retinal neurons. Moreover, since the retinas under investigation would be from three avian species, the measurements would be novel and help better understand the relationship between a bird's natural environment and lifestyle, and how their eyes function. Since the avian species under study are completely ground-dwelling (domestic fowl), partially ground-dwelling (quail) and arboreal (zebra finch), the activity patterns are expected to differ. This novel information is expected to manifest in publications exploring the various facets of bird retinal connectivity and functions.

Who or what will benefit from these outputs, and how?

The measured biosensor activity in the successfully targeted bird retinal neurons will be compared to similar activity measurements from other animals. This will assist in understanding the organizational and functional differences of bird visual processing. It may also reveal how retinal neurons exhibit functional and structural differences based on the animal's environment.

More generally, the project will contribute to an improved understanding and technical ability to affect gene expression in birds.

How will you look to maximise the outputs of this work?

Unpublished work suggests that different virus variants tend to target different neurons within the retina. This could help in targeting bird neurons both in the outer retina and the inner retina, thus expanding options in specifically targeting different and specific retinal circuits. This would be achieved through collaborations with labs which have expertise in creating and producing different virus variants.

Moreover, the viral injections into the eye have been partially standardised for small songbirds. This could assist in minimising the overall retinal damage, thus, reducing the animal number, and increasing the output.

The project results will also open new possibilities for viral transduction strategies (i.e. targeting) in birds in general.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 450
- Quail (Coturnix coturnix): 90
- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Avian vision is poorly understood. We seek to improve our understanding in how birds see, and what can we learn from them about the evolutionary history as well and the function and dysfunction of our own eyes.

We will use three avian model systems: domestic fowl, quail, and zebra finch. Domestic fowl and quail share a ground-dwelling environment in the wild, but notably differ in that quail can fly for extended periods of time, while domectis fowl is essentially flightless. Zebra finches are small passerines and inhabit a more arboreal habitat and routinely fly rather than walk or hop. Consequently, these three species form an excellent arc from flightless (chicken), to sporadic (quail) to routine use of flight (zebra finch), and this makes for a powerful comparison to understand how eyes and their function might link to flight. For this, we will investigate whether these environmental and behavioural differences impact how visual stimuli are transduced and processed in their retina pathways and whether retinal circuitry, function, and cellular dynamics change.

Juveniles and neonates from domestic fowl and quail species would be better suited for the experiments due to their smaller size and not having attained their adult size yet. On the contrary, both juveniles and adults can be used for the zebra finch species since their size does not vary.

Typically, what will be done to an animal used in your project?

Birds will be separated from group housing and food-deprived up to 3 hours before the ocular injection begins, and monitored during this period. This will be followed by intramuscular administration of an appropriate analgesic (pain relief). The bird will then be anaesthetised (put to sleep) using isoflurane gas anaesthesia directed through a custom-made and established beak holder and injection apparatus. In addition, the bird will be gently wrapped with a bandage to restrict wing motion.
For injection, the eyelid will be temporarily and gently pulled back to enable an unobstructed view of the eye. From experience, this step does not lead to injury. A fine needle will be used to puncture the eye at the sclera junction (front of the eye, where the transparent cornea meets the opaque sclera). This puncture will be used as the entry point for a highly precise syringe attached to a very fine blunt needle carrying the AAV serotype suspension (i.e. the to-be-introduced viral agent).
An ocular insertion of the needle will then be carefully made into the fundus (back of the eye) to prevent damaging the eye lens and the vitreous body (the transparent body between the lens and the back of the eye). The needle will be inserted inside the fundus, and the viral suspension will slowly be released into it. From experience , this process causes none or, rarely, minimal damage to the fundus. Post-injection, the needle will be carefully retracted, and the eyelid repositioned. The bird will then

be transferred onto a warming plate, with regulated warmth to assist the recovery from the anesthesia faster. Upon regaining consciousness, the bird will then be returned to the cage with sufficient water and food. Further analgesics and/or topical (surface applied) anesthetic may be administered to mitigate post-injection pain after discussion with the NVS. The full procedure will take up to 45 minutes depending on the effect of the anesthesia on the bird. The injected bird will be monitored over 3-8 weeks, after which the bird will be killed by a schedule 1 method for subsequent histology and/or 2- photon imaging (microscopy).

Immediately following injections, animals will be temporarily single-housed for typically up to 48 hours in clean enclosures to allow them to fully recover . Throughout this time they will be able to interact with conspecifics that will be housed in adjacent cages. Thereafter, injected animals will be returned to the colony, and tagged with a leg-band for identification. Constant monitoring of the injected bird would be undertaken daily by the animal facility staff, with the assistance of video recording.

What are the expected impacts and/or adverse effects for the animals during your project?

No significant post-experiment impact is to be expected once recovered from the procedure.

The bird used for ocular injection would be administered with an appropriate analgesic at surgery to mitigate pain. The recovery period will be carefully monitored and treated with analgesics if and when needed. No other significant impact is expected for the animal model during the project investigation.

Post-surgery, the injected birds would be kept in separate cages for up to 48 hours but still close to their cage mates.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities for every bird type in use, i.e., Domestic fowl, Quail and Zebra finch, would be mild. This would be due to the controlled injection environment, which will be carried out under gas anaesthesia, i.e., isoflurane preceded by an intramuscular injection of a non-steriodal anti-inflammatory drug such as meloxicam.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 avian models is essential for the project since we aim to understand the inner retinal workings and dynamics of the avian retina. For this, we need the birds to obtain the eyes. We also want to understand how visual signals are processed in different retinal pathways (i.e. types of retinal nerve cells). This would be achieved by targeting various different retinal neurons using different viral agents and then studying their neuronal activity patterns. The project has been planned *in-vivo,* where the successfully targeted retinal neurons would express fluorescent sensors for neuronal activity, something which would be not comparable when done in *in-vitro* cell culture.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives since the measurement of the neuronal activity using 2-photon imaging is best performed in the targeted retinal neurons from freshly taken retinas of injected model species.

Why were they not suitable?

Non-animal alternatives would not be suitable for this project since the measurement of the neuronal activity using fluorescence microscopy best performed in fresh retinas extracted within 5 hours from injected model species. Neuronal activity measurements performed in ex-vivo (freshly extracted) tissues are the closest to in-vivo (in the live animal) results, something which non-animal alternatives would not be able to provide. For the work we need intact tissue and connections between cells to study intercellular interactions. This cannot be recreated in non-animal alternatives, cultures or in silico (computer simulations). For best results, retinal injections will be carried out in live bird model species to express the fluorescent sensors in and near various retinal neurons.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Over the course of the 5-year project duration (60 months), we anticipate injecting around 15 animals per month on average. Of these, 50% will be domestic fowl, 40% zebra finches, and 10% quail.

60 months * 15 animals = 900 animals (450 domestic fowl (males), 360 zebra finches

(either sex), 90 quail (either sex))

The relative proportions between species relate to considerations in animal access as well as the species' differential relevance to scientific questions.

To date, most existing understanding on the structure and function of bird eyes comes from chicken. Accordingly, by primarily focusing our work on chicken we will be able to integrate with the largest possible body of previous knowledge, thus maximising overall outputs. However, chicken are largely flightless and heavily domesticated, and thus potentially not centrally representative for birds in general. To therefore begin to understand how the function of eyes might differ between birds, we will put a second main emphasis on zebra finches, a small flighted species that is readily kept and studied in the lab. Finally, quail can fly, however they spend most of their time on the ground, emulating a lifestyle that closely resembles that of chicken. Accordingly, the relatively smaller number of quail will be used to link insights gained from the work on chicken and zebra finch.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Half of all animals used will be male domestic fowl chicks sourced from the agricultural sector. These fowls would be used to establish a baseline of the experiment which would then determine how many more birds would be utilised. A better baseline would be the utmost priority in order to reduce the overall number of birds.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Undertaking correlated investigations together in the same animal model and carrying out smaller pilot studies for novel investigations to generate preliminary data before expanding the number of animals. Lastly, measures such as sharing tissue from the experimented avian model for more than one investigation will be done to reduce the total animal number.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Three bird model species will be used for this project: domestic fowl, quail and zebra finch. These three bird species would cover both, the ground-dwelling and the tree-

dwelling lifestyle of birds. All three bird species can be easily bred in captivity.
Eye injections can be undertaken using three prominent routes of administration in the bird eye; these are subretinal (beneath the retina), suprachoroidal (even deeper, below the choroid which sits behind the retina, but above the sclera which sits below the choroid), and intravitreal ('above' the retina). Out of these three routes, the intravitreal route of administration is known to be the least invasive procedure. It would cause the least amount of pain and discomfort to the injected bird since it does not cause breakage in the retinal tissue.

Analgesics (pain relief) will be given. The injected animal is carefully monitored after the injection for 24-48 hours in separate cages for any signs of post-surgery discomfort, which, in case it happens, is treated appropriately and according to NVS advice. Lastly, on completion of the designated experiment, the experimental animals would be killed using the appropriate schedule 1 method.

Why can’t you use animals that are less sentient?

The use of the three avian models, i.e., domestic fowl, quail, and zebra finch, is essential becasue the project is focussed around avian vision. The two prior species would be used in their juvenile or neonate stage for the ocular injections. In contrast, the latter is a passerine species that would be used in its adult stage. These investigations are essential for understanding the inner workings and dynamics of avian vision transduction and processing and identifying the neuronal network involved in these processing mechanisms. This investigation aims to be centred around avian retina vision processing and, therefore, cannot be done with less sentient beings.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The experimental procedures would be done in a precise and organised manner. The methods would be carried out carefully to minimise unnecessary harm to the avian model species under investigation. Moreover, every injected bird model would need increased monitoring and careful post-operative care.

Close monitoring will be carried out for the injected birds, which would be assisted by the use of remote video recordings. In addition, topical anesthetic analgesia and/or antibiotic eye drops will be administered as necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow best practice guidelines such as 'Animal Research: Reporting of In Vivo Experiments' (ARRIVE) and 'Planning Research and Experimental Procedures on Animals: Recommendations for Excellence' (PREPARE) insofar as applicable to our approach and research questions.

Moreover, we will stay informed by using the National Centre for Replacement, Refinement and Reduction (NC3Rs) as source of help, especially regular e-mail bulletins (NC3Rs News) that are forwarded to us by our NIO.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Appropriate webinars and symposia about the 3Rs would be attended to be up to date with the advances in the topic. Other sources such as the National Centre for Replacement, Refinement and Reduction (NC3Rs) new bulletins and newsletters would also be considered for extracting information about the 3Rs. Implementation of advances, if applicable, will be carried out as appropriate.

96. Evolutionary divergence among zebrafish relatives (Danionins)

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

speciation, hybrid incompatibility, development, comparative genomics

Animal types	Life stages
Zebra fish (Danio rerio)	adult
Danio nigrofasciatus	adult, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To investigate genomic and developmental divergence between close taxonomic relatives of the zebrafish (Danio rerio).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 existence of identifiably different species is one of the most fundamental properties of the natural world. For example, what is it that makes a horse a horse and a donkey a donkey? Why don’t we have a whole raft of variation between the two, from dorses to honkeys?

One of the most widely accepted definitions of species, the ‘biological species

concept', depends on the idea that species are reproductively isolated from (cannot breed with) each other, because they choose not to mate with each other or cannot produce offspring that are viable or fertile. It is common knowledge that 'good' species cannot usually produce viable, fertile hybrid offspring. Ultimately, this inability of good species to make hybrid offspring is due to 'hybrid incompatibilities': bits of the genome from different species that simply won't work together. In the case of horses and donkeys, they will sometimes choose to mate with each other, but the product is a mule (or a 'hinny', depending on whether the mother was a horse or a donkey). Both are viable animals, but they are almost always infertile, and this hybrid incompatibility prevents horses and donkeys from collapsing into a whole raft of variation. For mules, the infertility arises because horses and donkeys have different numbers of chromosomes, but many more closely related species have the same number of chromosomes yet still exhibit hybrid incompatibility, because particular combinations of genes do not work together.

The wider benefit of this work is that it will help us to understand one of the most fundamental of all problems in biology: what makes a species a species and how do they split to form new species. It is hard to overstate how novel and important is the study that we propose, which is highly interdisciplinary. In particular, it will shed new light on the genetic interactions that mean that hybrids do not 'work'. Understanding which genes are involved helps us to understand what determines whether genomes can work together coherently. The results of the work will therefore be of great interest to evolutionary biologists, developmental biologists, geneticists and genomicists.

Surprisingly, almost everything we know about hybrid incompatibilities in metazoan animals comes from fruit flies (Drosophila) and only a single vertebrate incompatibility has been well characterised, in mice. Human interest in biology is generally biased towards vertebrates, because of their greater relevance to ourselves, and a greater understanding of hybrid incompatibilities in vertebrates could tell us a great deal about the way that the different parts of the genomes of higher vertebrates are evolved to work with each other.

The zebrafish, Danio rerio, is one of the most well-characterised and researched vertebrate organisms, from the perspective of developmental and functional genomics. What is less widely realised in the scientific community is that the zebrafish has many close relatives (approximately 30 in the genus Danio and more in the genera Danionella and Devario), about which we know very little from a scientific perspective, although they are widely and easily kept as aquarist hobby species. It is known that viable crosses can be made between at least some of these species, and this knowledge has been used to map the genomic basis of colour pattern variation in Danio. No published work exists to quantify genome divergence between species in this group, nor to map the genes that lead to the accumulation of hybrid incompatibility. Counter-intuitively, because of a process known as "reinforcement", in which natural selection acts against the hybrid offspring of closely related species that live together, and may actually favour hybrid incompatibility, it is possible that some less closely related species actually form viable hybrids more easily than congeners. For this reason, we also include the possibility of working on species from Danionella and Devario, sister genera to Danio, although it is not our intention to do so in the first instance. However, in the first instance, we have chosen

a small set of Danio species that are widely available in the aquarium trade, are easy to keep, and which can be crossed with D. rerio, with some evidence of hybrid incompatibility.

What outputs do you think you will see at the end of this project?

We need to know about hybrid incompatibilities in order better to understand biodiversity and the natural world around us: why do things we call 'species' exist as recognisable entities? The study of hybrid incompatibilities has been central to our understanding of evolutionary speciation for at least a century and is currently being reinvigorated by the availability of whole genome resequencing data.

Primarily, this project will result in the production of inter-specific (and possibly inter-generic) crosses between Danio species and their close relatives. These hybrids will be used for the scientific investigation of the genetic and developmental incompatibilities between species.

Publications and scientific presentations about (i) the scientific husbandry and breeding behaviour of close relatives of the zebrafish; (ii) the potential to make crosses between different, close relatives of the zebrafish and the viability of hybrid embryos and fertility of hybrid adults; (iii) genome assemblies and comparative genomics of close relatives of the zebrafish; (iv) details of developmental abnormalities in hybrid embryos; (v) identification and genetic mapping of hybrid incompatibilities.

Who or what will benefit from these outputs, and how?

This project will quickly accrue scientifically useful information about the husbandry and breeding of close relatives of the zebrafish, which we expect to be immediately valuable to the research group and, following presentations and publication, to a much wider scientific community that studies zebrafish and speciation. As the project progresses we will also generate genomic and developmental information about zebrafish relatives and their hybrids that will be of very substantial and long-term interest to the wider scientific community studying zebrafish, development, comparative and functional genomics and speciation. In particular, we expect this work to be of very significant and long-lasting interest to evolutionary biologists, developmental biologists, geneticists and genomicists.

How will you look to maximise the outputs of this work?

Our results will be presented at national and international scientific meetings and published in open- access journals. Genomic data will be published on appropriate open-access servers. We will share data, animals and tissues with other research groups, on request and at appropriate times.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 480
- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The zebrafish, Danio rerio, is one of the best characterised of all species from a developmental and functional genomic perspective. Coupled with the fact that it has many closely related species, at least some of which it is known to hybridise with, makes this group of fishes, the Danionins, the only group of vertebrate animals in which it is realistic to study the nature, developmental consequences and genomic basis of hybrid incompatibility. The use of vertebrates is important because of the genetic and developmental homologies with other species of scientific interest, including mice and humans. In addition to the zebrafish itself, we intend to investigate a small number of other closely related species with which it is known or possible that D. rerio can hybridise. These include closer and more distant taxonomic relatives in the genus Danio (D. nigrofasciatus, D. albolineatus, D. kyathit) that are already known to hybridise with D. rerio. Species from closely related genera (Devario and Danionella) may also be of interest as we progress. Much of the work in this project will focus on the fertilisation of eggs and development of embryos, the earliest stage at which hybrid incompatibilities can be manifested. Developmentally normal embryos will be grown to adulthood, in order to be able to assay fertility. Hybrid incompatibilities are often evident as infertility, especially in the heterogametic sex.

Typically, what will be done to an animal used in your project?

Fish will be raised to adulthood in normal husbandry conditions and, once reproductively mature, used to make within- and between-species crosses. Whenever possible, these crosses will be made by natural mating but, for species that will not made naturally, we will use artificial mating techniques including the stripping of eggs from female fish under anaesthesia and the extraction of sperm from males under anaesthesia (https://zfin.org/zf_info/zfbook/chapt2/2.8.html). Fertilised embryos will be raised to hatching, when any abnormal fry will be killed. Normal embryos will be raised to adulthood to assay fertility.

What are the expected impacts and/or adverse effects for the animals during your project?

Embryos may develop abnormally. For example they may fail to develop beyond certain stages or may experience slow growth or stunted morphology. All such abnormally developing embryos will be euthanised before reaching the free feeding stage. Adult hybrid fish may experience partial or complete loss of fertility.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Breeding and maintenance of fishes 75% sub-threshold, 25% mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animal models to understand how genomic incompatibilities arise between diverging species. This is a very complex and poorly understood process for which no in vitro system exists.

Which non-animal alternatives did you consider for use in this project?

The most obvious alternative approach would be to use non-protected species such as fruit flies. The complex, whole-organism nature of hybrid incompatibility precludes other approaches.

Why were they not suitable?

The study of hybrid incompatibility in fruit flies serves as the basis for this project and has provided important information about the nature and genetic basis of hybrid incompatibilities (e.g. Presgraves & Meiklejohn 2021 Frontiers in Genetics, 12: 669045). However, firm scientific conclusions rely on their being generalisable, and we know almost nothing about the developmental and genetic basis of hybrid incompatibilities in any group apart from Drosophila. In vertebrates in particular, which have very different genomic and developmental organisation, but also better genome annotation and shared homology with humans, we know almost nothing. Further work on Drosophila cannot help us to understand the origins of genomic incompatibilities in vertebrates.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Danio species form shoals and are kept in family groups of 40 individuals per tank. We will keep three tanks per species to allow sufficient rest periods between matings and we will maintain four generations of each species over the course of the project. We want to make reciprocal crosses between four species. Pure species crosses (e.g. D. rerio female x D. rerio male) will provide controls. These are all wild, outbred species and individual variation in outcomes is therefore both unavoidable, but also inherent to the research e.g. because it is unlikely that all alleles contributing to hybrid incompatibilities will segregate in all crosses or individuals. In addition, while levels of hybrid incompatibility are known for some of these crosses (e.g. D. rerio x D. nigrofasciatus, Endoh et al. 2020 PLoS One, 15: e0233885), others do not previously appear to have been attempted (e.g. D. nigrofasciatus x D. kyathit). In the latter case we will conduct pilot studies with small numbers of fish, to determine whether they will mate naturally or will require in vitro fertilisation, and what the fertilisation and hatching rates of embryos is likely to be. Data output will be maximised by making as many fertilised embryos as possible from each individual cross. This will increase the number of embryos that we can assess for development and the number of subsequent adults that we can assess for fertility. Males and females for all crosses will be selected randomly whenever possible and embryos and adult fertility will be screened blind to cross type. Cross types will be compared for fertilisation and hatching rates, rates of developmental abnormality in embryos and rates of fertility in normally developing adults. We will also collect normal and abnormal embryos from some crosses to compare their genomes by re-sequencing them, in order to map the genomic basis of incompatibility.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To make experimental crosses we will only use fully grown, healthy adults that are clearly in breeding condition.

We are an established research laboratory, with abundant experience in experimental design and statistical analysis. We are also open to receiving advice from others. We always choose to use methods that reduce the number of animals used, while still obtaining scientifically valid results.

These websites may be utilised to provide additional information on statistics and experimental design: The NC3Rs experimental design assistant https://eda.nc3rs.org.uk/; The 3Rs – Reduction.co.uk site at http://www.3rs-reduction.co.uk/

In order to ensure that high quality, reliable and valid data is produced from the minimum number of experiments, the ARRIVE guidelines (Kilkenny et al., 2010) will be followed when reporting the results obtained from this project. http://www.nc3rs.org.uk/page.asp?id=1357

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use efficient breeding techniques including, for example, storage of sperm from males when relevant. Small pilot studies will be used when necessary to check

experimental protocols and to get an indication of likely results, especially in situations where species combinations are novel.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Fish are the vertebrates with the lowest neurophysiological sensitivity. The development and genomic basis of phenotypic traits in vertebrates are qualitatively different from invertebrates because vertebrates possess more sophisticated physiological systems and homologies are less common. Humans are also more interested in and attach greater significance to an understanding of vertebrates, partly because of their closer relatedness to us, and partly because we often have a greater interest in the management of their populations.

The zebrafish Danio rerio and its close relatives are the most appropriate species for our work because: (i) They are easy to obtain and straightforward to keep and rear in the lab. (ii) Knowledge of their development is unsurpassed in any other higher animal. (iii) They have a fully sequenced and annotated genome and outstanding genetic resources. (iv) The zebrafish is one of the most widely used of all model organisms, and our results will therefore be especially relevant to the scientific community. (v) All species in the genus Danio mature quickly and breed easily in captivity, and require very similar water conditions to D. rerio.

Why can’t you use animals that are less sentient?

The majority of our results will be obtained from embryos, but we need to make crosses in order to obtain these embryos.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our expectation is that the majority of experimental work will be sub-threshold. Nevertheless, we will monitor carefully the health of all husbanded fish, their water quality and diet.

During normal husbandry, fish will be provided with appropriate environmental enrichment including simulated gravel, artificial plants to promote shoaling and/or perspex tunnels to facilitate escape behaviour, where this does not compromise the need to maintain water quality.

Aquarium rooms have artificial lighting with appropriate photoperiod. The temperature of inside aquaria will be maintained between 24 ^{o}C and 29 ^{o}C which substantial experience in other labs has shown to be comfortable for zebrafish and their relatives.

Water quality is the most important factor in maintaining the well-being of fish and good filtration is the most important tool in maintaining water quality in aquaria. All fish will be maintained in tanks in Tecniplast flow-through systems using reverse osmosis water with automated salt dosing to maintain conductance between 150-1700µS and pH between 6.5 and 7.5 and dissolved oxygen between 5- 9ppm. Ammonia, nitrite and nitrate levels will be appropriately monitored and maintained below 2ppm, 2ppm and 25ppm respectively as per established guidelines (Alestrom et al., 2020 Lab. Animals, 54: 213-224).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In recent years there has been a surge of new guidance for scientific experiments involving fishes. We will follow general NORECOPA, American Fisheries Society and other guidance (for example the PREPARE guidelines, Smith et al. 2018, guidelines for severity reporting, Hawkins et al 2011, 'Guidelines for the Use of Fishes in Research', Jenkins et al 2014, and ethical considerations, Sloman et al. 2018). We will also continue to follow recommendations in technical publications for best practice in particular procedures. We already implement housing and husbandry recommendations for zebrafish (Alestrom et al., 2020) in Nottingham and will apply these for other Danio species which have highly similar physiology.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By reading relevant papers as they are published, consulting the 3Rs website and attending relevant training courses and workshops (e.g. run by the RSPCA). We will keep in touch with developments in alternatives to in vivo experiments (e.g. Schaeck et al 2013) and with societal considerations about the use of fishes in research (e.g. Message & Greenhough 2019).

97. Regulation of hippocampal synaptic function in health and brain disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Alzheimer's disease, synapses, leptin, oestrogens, therapy

Animal types	Life stages
Mice	juvenile, adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Using methods of study that are conducted in a standardised, systematic way we will assess how the hormones leptin and oestrogens influence:

1. The way that nerve cells communicate with each other as the brain ages.
2. the ability of neural networks in the brain to change through growth and re organisation (neuroplasticity).
3. the ability of the brain to learn and process information.

The studies will also let us investigate the possible use of compounds similar to leptin and oestrogens in treating disruption in communication in a specific area of the brain known as the hippocampus and in preventing damage/ cell death, and so changes in brain function, in models of disease such as Alzheimer's Disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In western societies life expectancy is increasing and as a consequence neurodegenerative brain disorders like Alzheimer's disease (AD) are becoming more prevalent. Although our understanding of AD has increased dramatically in recent years, there is currently no cure for AD, and very few therapeutic agents are useful in alleviating symptoms.

Nerve cells have small gaps between them (synapses) that allow nerve cells to communicate effectively. We will specifically look at the processes involved in communication at the level of the synapse, as this process is damaged in neurodegenerative disorders. Studying this will give us information on the aims above and consequently studies such as this are vitally important if we are to fully understand the unwanted brain changes that occur in AD, and if new drugs are to be developed to treat devastating brain diseases like AD.

What outputs do you think you will see at the end of this project?

In the short term this study will uncover novel information about the cellular processes that underlie the actions of leptin and oestrogens in the brain. It is already known that leptin is a hormone that is produced by fat cells and it plays an important role in controlling food intake, by sending signals to the brain after a meal to indicate a sense of fullness. We have found that leptin also influences the hippocampus, which is a brain region that controls how we learn and remember information. Here we aim to examine in more detail the cellular events involved in these effects of leptin in the hippocampus, and specifically how it influences communication at synapses. We will also examine the effects of oestrogen, which is more commonly known for its involvement as a reproductive hormone in females. Oestrogen, like leptin is also able to influence the functioning of the hippocampus, and in turn has an impact on learning and memory. Here we will also examine further the cellular events involved in the effects of oestrogens in the hippocampus and specifically the impact on communication at synapses.

In the medium term, this study will generate more detailed cellular information on the benefits of leptin- and oestrogen-based molecules in models of Alzheimer's disease. In the longer term, this study could lead to identification of novel compounds for therapeutic use in Alzheimer's.

Novel findings generated at each stage of this project will be published in scientific papers.

Who or what will benefit from these outputs, and how?

In the short and medium term, this work will build on and extend our previous findings to provide additional novel information on the cellular processes involved in

the effects of leptin and oestrogens on the functioning of the hippocampus in the mammalian brain. It will improve understanding of how alterations and/or abnormal changes in the leptin and/or oestrogen hormonal systems, during development and/or in adult, influence the cellular events that are required for the brain to learn and remember information. This in turn will provide valuable insight into how alterations in these hormonal systems leads to changes in cognitive function. These findings are likely to benefit scientists (neuroscientists, cellular/molecular biologists, and drug discovery scientists).

In the longer term, this programme of work will build on our recent studies that have found that small molecules derived from the large leptin peptide, have the potential to be developed into new drugs for use in Alzheimer's disease (AD). It will also build on our pilot findings that suggest a recently identified oestrogen receptor, known as GPER1 may have therapeutic potential in models of AD. Ultimately this work could identify novel molecules as lead compounds for an anti-AD therapeutic, or it could identify novel avenues for therapeutic intervention in a clinically important but currently untreatable brain disorder. In the longer term, these findings are likely to benefit patients, and people involved in the management of patients, as well as the pharmaceutical/biotechnology industry.

How will you look to maximise the outputs of this work?

To increase impact beyond immediate academic communities, our key findings will be highlighted on establishment websites. All major discoveries will be publicised locally and nationally via press releases that will be produced in conjunction with the establishment Press Office.

As our findings will be of interest to other stakeholders in pharmaceutical and biotechnology industries, we will fully engage with sector representatives, to ensure all potential collaborative ventures and routes for future development are explored.

This study is likely to be of significant interest to patients, families, and carers. Consequently, our findings will be disseminated widely to various end users and other stakeholders via our active participation in public engagement activities.

Species and numbers of animals expected to be used

- Rats: 50
- Mice: 150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Immature female and male rats or mice (7-25 day old) are required to obtain healthy

tissue for the preparation of brain slices. Immature rats or mice are used as the brain and the networks that we study are fully formed at this stage of postnatal development, and this is the optimal stage to study synaptic plasticity, which is the cellular process that drives learning and memory. The use of brain slices and neuronal cultures allows us to investigate the effects of hormones on communication between brain cells. Established cell cultures do not form tightly organised networks of connections found in the human brain, and thus animals are required as sources of primary tissue for these experiments.

To examine the impact of specific oestrogen receptors on neuronal function, genetically altered mice (immature and adult) that lack specific oestrogen receptors are required to obtain healthy tissue for preparation of brain slices. There is no alternative to using animals for tissue.

To examine the impact of altered leptin function, mice models of obesity (immature and adult) that fail to produce leptin, or are insensitive to leptin are required to obtain healthy tissue for preparation of brain slices. As above there is no alterative to using animals for tissue.

In order to examine the potential benefits of leptin or oestrogens on impaired neuronal function in Alzheimer's disease (AD), it is necessary to maintain wild type and various widely used mouse models of AD. Immature and adult AD mice are required to obtain healthy tissue for preparation of brain slices. As above there is no alterative to using animals for tissue.

Typically, what will be done to an animal used in your project?

We will maintain genetically normal ('wild type') animals and genetically altered (GA) animals and the animals will then be humanely killed for analyses of tissue from the brain.

What are the expected impacts and/or adverse effects for the animals during your project?

Typically the GA animals will have no phenotype, (are completely well and don't show any clinical signs related to the genetic changes) and will experience no pain or distress.

Some of the GA animals will have an obese phenotype and may suffer from diabetes. Animals will gain weight from 4 weeks of age, and develop associated clinical signs such as increased appetite (polyphagia), increased thirst (polydipsia) and increased urination (polyuria), that may peak around 15 weeks of age. The GA animals will not be allowed to develop serious health problems as we will not use them beyond 15 weeks of age.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Maintenance of GA animals: Those that are phenotypic will be used before development of clinical signs and will likely be mild (90%), with 10% moderate.

Humane killing of animals: 100% non recovery

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of animals is necessary as the animals will provide brain tissue for *in vitro* analyses (eg brain slices, neuronal cultures) of the complex events that occur at neuronal synapses.

There are at present no appropriate cell lines and/or model systems available that mirror the complex network of synaptic connections that occur in the brain.

Which non-animal alternatives did you consider for use in this project?

We did consider the use of neuronal cell lines (e.g. the SH-SY5y neuroblastoma cell line) or human stem cells and non-human embryonic primary tissues rich in progenitors, for some of our studies.

Why were they not suitable?

Neuronal cell lines are not suitable for use in our studies as these cell lines are not able to fully recapitulate the complex network of synapses that occur in the brain, or to mirror the aberrant brain changes that occur in brain diseases like Alzheimer's disease.

Although human stem cells and non-human embryonic primary tissues rich in progenitors may be ideal tools to investigate neurodevelopmental disorders, they are not suitable for use in our studies as they are not representative of adult phenotypes. Further, human stem cell based models are not good at modelling the synaptic and brain changes that occur in central nervous system degeneration.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may

include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used will be the minimum to satisfy experimental criteria. Statistical advice was sought to help with calculations using typical variations from our past experiments, and to enable calculation of the minimum number of animals, whilst ensuring statistical significance.

In general, brain slices from 5-6 animals will be sufficient to establish biological effects of the magnitude we expect for electrophysiological studies to monitor changes in the magnitude of synaptic transmission. For studies using neuronal cultures, a minimum number of 3 different cultures is sufficient to achieve the biological effects, and at a magnitude that we expect. Each neuronal culture preparation requires brain tissue from 3-4 animals.

We have used our annual return or procedures to estimate the number of animals we will need for our studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Statistical advice was sought to ensure that the sample size calculations are sufficient to control for the typical levels of variability we see from previous studies using these approaches.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In order to reduce the number of animals used, we will aim to share brain tissue where possible. For our studies using brain slices, we can produce up to 12 slices from the brain of one animal. My laboratory is set up for three people each to carry out experiments on 4 slices. Each of these twelve experiments can therefore reach the required group size of about five within one week, with the use of a total of five animals.

In our studies we will aim to harvest as many tissues as possible at post mortem. If the tissues are not used immediately, we will freeze them, and make available to other researchers working on similar questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain

why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The use of rats is appropriate as hippocampal synaptic transmission and synaptic plasticity are very well characterised in this mammalian species.
The use of mice is appropriate as there are at present no other species for which transgenic gene technology has been developed. Transgenic mouse models have been generated that replicate the gene mutations and aberrant brain changes that occur in human cases of Alzheimer's disease.

The use of mice is necessary as well established mouse models of obesity, and leptin insensitivity/deficiency exist that will be used under the authority of this project licence.

The animals used may be obese but they are not clinically unwell. We don't expect, indeed it would be detrimental to our studies if any animal deviated from essentially normal welfare.

In some instances (25%), we have to ensure that brain tissue that is anatomically undamaged and physiologically normal is retrieved from post-natal animals for further analyses. We therefore cannot use purely physical or purely chemical method of killing as listed in Schedule 1. Instead we wish to render animals unconscious by the delivery of a suitable anaesthetic (but not the overdose that would be required to kill them where tissue would be anoxic and thus unsuitable for our studies), and then to kill them by decapitation. We regard this method as being equally humane to those listed in Schedule

A premortem blood sample may be taken under the anaesthetic.

Why can’t you use animals that are less sentient?

Non-mammalian animals are limited in their use because they do not mirror the complex network of synaptic connections that occur in the brain.
We can't use embryos or very young animals as their brain networks are too immature.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All adult animals will be regularly weighed and their body condition will be regularly scored, to monitor signs of weight loss or loss of body condition. Any animal that is losing weight will be flagged to the Named Veterinary Surgeon (NVS) and monitored closely to ensure it stabilises or regains the weight, and any animal displaying >10% (not expected) weight loss would be withdrawn from the study immediately and killed.

Animals showing frank signs of diabetes (not expected), such as polyuria, polyphagia or polydipsia will be promptly killed. Any animal that develops a welfare problem other than those described, or in which the known adverse effect might be approaching the severity limit will be referred to the NVS, and/or withdrawn from the

study and killed humanely.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The PREPARE guidelines for planning animal research (Smith et al, 2018), and NC3Rs guidance and publications will be followed.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check information and updates on the NC3Rs website, and via the NC3Rs newsletter. We will attend 3Rs symposia, and webinars, as appropriate.