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Animals (Scientific Procedures)

Act 1986

Non-technical summaries for project licences granted January – March 2023

CONTENTS

DEVELOPMENTAL AND REPRODUCTION SAFETY TESTING OF MEDICINAL PRODUCTS USING SMALL ANIMAL SPECIES

Project duration

5 years 0 months

Project purpose

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

Key words

Regulatory, Safety Assessment, Developmental, Reproduction

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This project licence authorises the use conduct of safety studies in laboratory rats, mice and rabbits to evaluate the safety of medicinal products in terms of the risks to reproductive capability, fertility and thedevelopment of unborn, newly born and developing animals. In order to make sound regulatory decisions regarding safe human exposure levels to these materials, information is required covering exposure of adult animals and the impact on all ages of development from conception to sexual maturity.

These study types are required by global regulators to ensure potential pharmaceuticals are safe to betaken by humans in the clinic.

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

accrue after the project has finished.

Why is it important to undertake this work?

Governments require (and the public expects) that substances we are exposed to are safe or that theirpotential hazards are well understood and documented.

The data generated from the studies performed under this project will be used to inform decision-making processes on pharmaceuticals under development and, where appropriate, to satisfy governmental regulatory requirements necessary to gain marketing authorisation or product registration.

This safety assessment is of immense importance along with other non-rodent and non-animal studiesin demonstrating to governments and the public the safety of these substances or highlighting their known hazards and safe handling.

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

This project licence authorises the conduct of in vivo safety studies in laboratory small animal speciesto evaluate candidate pharmaceuticals and novel and currently-registered substances in terms of systemic toxicity, toxicokinetics and the impact of pharmaceuticals on all stages of development from conception to sexual maturity.

The overall benefit of this project is that it generates high quality data that is acceptable to regulatory authorities and enables internal decision making within our clients' organisations. This project will alsoensure that pharmaceuticals that the general population may take in the clinic are safe.

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

Our customers will benefit, as the data we generate will allow them to progress their pharmaceuticalsunder development and, where appropriate, to satisfy governmental regulatory requirements necessary to gain marketing authorisation.

It is a regulatory requirement that new medicines are tested for safety and tolerability prior to first-in human trials and to support longer term dosing in humans. Data from two species (a rodent and a nonrodent) are usually recommended within regulatory guidance's, including ICH M3(R2), ICH S6 (R1)and ICH S9 [1], with species selected based on similarity of pharmacokinetic and metabolic profiles to humans and relevant pharmacology (i.e. the target in the test species has a similar role to that in humans.

With specific focus on potential reproductive and developmental effects, the premise of this licence is the acquisition of data that will enable national and international regulators to decide if a new medicinalproduct can safely proceed through human clinical trials, potentially including women of child bearing potential (WOCBP) and/or infants, and then if it should be marketed, based on toxicological safety andmargins of exposure. It is a requirement under UK law that new medicinal products are subject to suchinvestigations in animals and undergo independent regulatory reviews by the MHRA before they reachthe consumer. With the exception of validation, exploratory drug candidate selection and exploratory range finding studies, all regulatory work carried out by Labcorp under this licence will be compliant with (and required under) international regulatory guidelines specified by ICH, OECD or EC. Data acquisition from exploratory studies will either support selection of suitable candidates for further evaluation studies or help to remove

unsuitable candidates from development at an early stage, thus saving animals and protecting human health.
European Council Directives 2001/82/EC and 2001/83/EC (Article 1) define a medicinal product as anysubstance presented for treating or preventing disease, or 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.

The expansion of scientific and medical knowledge has led to the development of drugs which can treat or alleviate the symptoms of many illnesses but there is still a need to develop medicinal productsto diagnose and treat many human conditions such as Heart Disease, Stroke, Obesity, COPD, Respiratory Infections, Cancer, Autoimmune diseases, Diabetes, Alzheimer's, and Schizophrenia, amongst others. When these needs are combined with the growing threat from antibiotic resistant bacteria the advancement of science and the development of new products are as important as ever.

This project licence authorises the conduct of studies in laboratory rodents and rabbits to evaluate thesafety, quality and effectiveness of medicinal products for the avoidance, prevention, diagnosis or treatment of the of disease, ill-health or other abnormality or their effects in human beings, in terms ofgeneral and reproductive toxicity and whole body system exposure.

Where possible, in vitro methods and assays are used to support the assessment of the hazard profileof a test substance, but in vivo safety assessment in full body systems remains a legal, regulatory requirement. Regulatory guidelines commonly require the use of both rodents and non-rodents in a programme of toxicity studies, which will generally follow both an established sequence and design.

Regulatory authorities will not permit exposure of humans until the relevant programme is completed. In development of a human medicinal product, volunteers and patients are used in clinical trials only after certain studies have been conducted and assessed; and formal marketing of that product occurs only once further studies are completed, the package of studies submitted to the relevant authority andthe outcome of the risk assessment is deemed acceptable by the regulator. In the case of, for example,a novel excipient, it is used in its target indication only on similar successful completion of an acceptable risk assessment by the relevant regulator.

The regulatory authorities, for whom toxicology safety assessment studies are performed, provide guidelines for the work, but not explicit experimental designs. The guidance given in the various guidelines of the International Council for Harmonisation of Technical Requirements for Registration ofPharmaceuticals for Human Use (ICH) and The Organisation for Economic Co-operation and Development (OECD) are generally followed in the design of safety evaluation programmes and in thedesign of studies. The ICH brings together the regulatory authorities of Europe, Japan and the United States to make recommendations on ways to achieve greater harmonisation in the interpretation and application of guidelines and requirements for product registration in order to reduce or obviate the need to duplicate testing. Development plans and study designs are tailored to the requirements of theclinical plan and so will vary depending on proposed clinical indication, target population and route of administration to humans/animals. No single experiment is capable of providing all of the information required to fulfil regulatory requirements. However, most regulators are pragmatic in their views and allow programmes of work to develop and progress in the necessary direction in order to allow the overall needs of the experiments & scientific objectives to be met.

Examples of the Regulatory Authorities to whom studies under this licence may be submitted include;the MHRA (Medicines and Healthcare products Regulatory Agency of the UK Department of Health),EMA (European Medicines Agency) and United States FDA (Food and Drug Administration).

The primary benefit of work carried out under this licence will be to allow regulatory authorities (who are totally independent from the commercial interests behind every marketing application) to come toinformed decisions, based upon safety data generated in these studies, regarding the risks to which humans are exposed when compounds are produced, transported or used.

For all categories of material covered in this Licence, studies conducted will help to remove unsuitablecandidates from development at an early stage, thus saving animals and resources. Successful studies will also allow identification of target organs and systems and effects on reproduction and development of offspring through one or two generations. In addition, they may provide biomarkers to allow monitoring and management of human exposure.

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

Where confidentiality permits, data, study design and best practice will be openly shared at conferences, workshops, webinars, blogs and publications.

As 3R's benefits are also realised under this project licence, these will be shared more widely withother establishments.

Species and numbers of animals expected to be used

- Mice: 22050
- Rats: 38750
- Rabbits: 5500

Predicted harms

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

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

Rodents (rats and mice) will be predominantly used in this project along with rabbits. All life stages ofanimals will be used (embryo, neonate, juvenile and adult, either direct dosing, usually to adults, or investigating any developmental effects of test substances).

Species choice and use of specific animal models is determined by the need to generate regulatoryacceptable data. Where a choice of species is possible, care is taken to select the most biologicallyappropriate species, and the species which most closely relates to humans. Studies to assess the types of material covered by this licence are usually performed on small animal species.

Generally, the rat is the rodent species of choice in Developmental and Reproduction Safety Testing. There is wide knowledge of the response of rats to various substances and a wealth of background literature. Rats are large enough to provide repeated blood

samples, and big enough litters of pups, thus requiring significantly fewer rats than mice to achieve the same objective. Mice may be used when considered a more appropriate species, for example, if they more readily absorb the test substance, are more relevant biologically or improved tolerance depending upon objective of the study.

Rabbits may be used when considered a more appropriate species, for example non-pregnant rangefinding studies prior to conducting reproductive studies in pregnant rabbits. The rabbit has achieved acceptance as a suitable secondary non-rodent species of choice and offers several marked advantages.

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

The animals used under this licence will be given the test substances in a similar way to that in which they are expected to be given to humans. As most medicines at taken orally the majority of animals willreceive the test substance directly by insertion of a flexible rubber catheter into the stomach via the mouth. Most animals are treated in this way once per day daily, although studies may occasionally require two or three doses within 24 hours. For some test substances, the oral route of administration may not be appropriate; for example, it may be broken down, not tolerated or not absorbed during the digestive process. In these situations, alternative administration routes such as subcutaneous or intravenous injection may be more appropriate. Animals may be manually restrained for “bolus” administrations lasting a few seconds or minutes.

For test substances which will be administered to humans intravenously over extended period of time due to their inherent toxicity or fast clearance from the body it may be more appropriate to surgically implant a permanent catheter (with appropriate anaesthesia and analgesia) to allow the animal free movement whilst being dosed in their home enclosure. Test substances intended to treat a localised condition may be administered to that body part or area for example to the skin. Blood and urine samples may be taken to measure the level of the test substance or its metabolites within an animal’s circulatory system. These may also be analysed to detect any changes in blood or urine chemistry, allowing in-vivo monitoring of body systems and organs for example liver or kidney function. Study animals are closely observed several times a day by highly trained technologists who monitor for any signs of discomfort. Other measures such as food consumption and body weight are used to closely monitor for treatment related effects. Veterinary surgeons are employed on a full time basis and are available 24/7 to provide clinical treatment, guidance on animal welfare and the conduct of proceduresincluding appropriate surgical technique, anaesthesia and analgesia.

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

The majority of animals are expected to have mild adverse effects of treatment such as reduced weightgain or changes in appearance or behaviour. A small number of animals (usually limited to the highest doses evaluated in early studies) may show more moderate adverse effects. The nature and type of effect varies dependant on the biological systems affected, however, these usually result in findings such as reduced food consumption, weight loss and changes in behaviour such as reduced activity.

Humane endpoints will be applied or dose levels reduced if animals show excessive effects.

Many of the endpoints measured on reproduction studies do not adversely affect the life of

the animals. For example, offspring may simply be observed for developmental milestones such as eye opening and the development of reflexes and as they grow they may be observed for evidence of sexual maturation, which may be precocious or delayed. Study animals are observed at least twice a day by highly trained technologists who monitor for any signs of discomfort. Other measures such as food consumption and bodyweight are used to closely monitor for treatment related effects. Veterinarysurgeons are employed on a full time basis and are available 24/7 to provide clinical treatment, guidance on animal welfare and the conduct of procedures including appropriate surgical technique, anaesthesia and analgesia.

Longer term studies are expected to have progressively fewer adverse effects. Many toxicological effects of test substances on developmental and reproductive parameters are not evident during the in-life phase of a study and do not impact the animals wellbeing (for example reduced numbers of maturing sperm and a reduced number of eggs). Only through microscopic examination of the tissues from each animal, can evidence of all toxicological changes be fully assessed and the scientific value of each animal maximised. In order to undertake these evaluations, the animals must be humanely euthanised at the end of a study, under terminal anaesthesia.

Surgery is performed using aeseptic technique the same as a patient would have in hospital.Veterinary surgeons advise on pain killers (always given) and antibiotics (if required).

Expected severity categories and the proportion of animals in each category, per species.
What are the expected severities and the proportion of animals in each category (per animaltype)?

On the last project, approximately 7% of animals were classified as having displayed moderate severity, the rest (approximately 93%) will have suffered only mild severity. This is because legally, allsurgical procedures carried out on an animal must be classified as moderate, and on occasions, therewere prolonged periods of dosing and sampling required to get the information we needed (also moderate). The rest of the animals were classified as having displayed mild severity.

It's impossible to predict the proportion of severities expected on a service licence, as this will bedependent on what study types we are asked to perform.

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

- Killed
- Kept alive
- Rehomed
- Used in other projects

Replacement

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

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

There are currently no scientific and legally acceptable evaluations of whole body, systemic toxicity thatwill satisfy regulatory requirements with respect to developmental and reproductive safety of pharmaceuticals, other than the use of animals.

Wherever possible, validated in vitro tests for specific organs are used and valuable information may also be obtained from alternative non-mammalian test species (e.g. fish, amphibians). Where available, review of scientific articles, non-animal methods and read-across to other animal data such as metabolism, pharmacology and general toxicology information is also utilised to reduce animal use.

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

There are some studies that can be carried out in vitro that can be used to support this work, includingtests that assess metabolism and absorption of substances, and how well they bind to key proteins in the blood. Predictive software can also be used.

These studies are often performed before the test substances reach us, for the testing detailed in thislicence.

Why were they not suitable?

None of these tests can yet model the complex and integrated mechanisms governing the effect ofpharmaceuticals on reproductive function and hence, animal testing is still a requirement by Globalregulatory authorities.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 on this project is estimated from those used under previous projects andafter consideration of regulatory trends.

Studies will be designed under this licence such that the minimum number of animals will be used inorder to obtain the maximum information, whilst the scientific objectives of each study are met, in accordance with regulatory requirements and agreed standard practices.

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

Studies are designed to provide maximal scientific value from the minimum number of animals, whilstusing sufficient animals to meet scientific objectives, and regulatory guidelines. Statistical input is sought, where appropriate, to strengthen the overall scientific quality and relevance of studies.

Where available, sensitive analytical techniques may be used to reduce animal numbers (for exampleby reducing blood volume requirements).

Wherever practicable, the re-use of suitable animals, and by looking across studies, the combination ofendpoints e.g. general toxicity, reproductive toxicology, safety pharmacology, mutagenicity etc. in studies is considered, to reduce overall animal usage.

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

For studies where a new substance is being tested in animals for the first time, we would often test thatin a small group of animals (usually 1-3) to give us confidence that the dose levels we chose are safe, and the substance affects the system its designed to, without making an animal ill. These are called pilot studies.

We will try and get as many outputs as we can from a single animal where possible, without adverselyaffecting its welfare. So if we need to get a blood sample, or if we need to find the levels of a substance in urine, for example, we will often do that in the same animal, rather than use separate ones, when possible.

Refinement

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

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

The majority of animals used during the course of this licence will be rodents. The rat is generally the most frequently used as it is tolerant of normal laboratory husbandry, amenable to the experimental procedures, fecund and has a modest level of neurophysiological sensitivity relative to non-rodent species. The database available as an aid to assessment of the biological significance of reproductiveparameters showing apparent treatment-related effects is heavily dependent on the rat. The usual availability of relevant range-finding data on the systemic toxicity of the test substance from complete or contemporaneous acute, subacute and chronic toxicity studies using the rat has also contributed tothe status of the rat as the preferred species for reproduction toxicity studies. The ICH has provided guidance on the advantages and disadvantages of a wide range of species considered for use on reproduction toxicity studies.

If for some reason, for example unusual metabolism patterns or sensitivity, the rat or rabbit are considered to be unsuitable for reproductive study work, then the mouse can usually be substituted.

Animals used under this Licence will be sourced from suppliers within the UK, EU or non-EU.We will use animals at all stages of development from those in utero to adult.

Animal welfare costs are minimised by the careful selection of dose levels to reduce the likelihood of unexpected toxicity, and the application of rigorous and comprehensive humane endpoints. Individualstudies are designed to cause the least possible suffering by

frequent review of practices, provision ofhighly skilled technical staff and veterinary support, purpose built facilities and a clear focus on animalwelfare. Any confinement or restraint is restricted to the minimum required to achieve the scientific objectives of the study and all study plans/protocols are reviewed for adherence to welfare guidelines and best practices by the site's Animal Welfare and Ethical Review Body (AWERB).

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

Rodents will be used in most of the studies conducted under this licence. Rodents are considered to be the species with a similar enough brain/nervous system and physiology, and fecund, that will allow us to achieve the study aims and are considered suitable for the predicting what's likely to happen inhumans.

The database available as an aid to assessment of the biological significance of reproductive parameters showing apparent treatment-related effects is heavily dependent on the rat. The usual availability of relevant range-finding data on the systemic toxicity of the Test substance from completeor contemporaneous acute, subacute and chronic toxicity studies using the rat has also contributed tothe status of the rat as the preferred species for reproduction toxicity studies.

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

Many of the procedures used in this licence are standardised, well defined and already well refined over many years. We will continue to assess any future possible refinements over the duration of thislicence.

Where animals do show adverse clinical signs after dosing, we will increase the frequency and length of observations, and provide supplementary interventions (like extra bedding/food/heat) where needed,until the signs resolve.

Similarly, if after discussing with a vet and senior technician, we decide an animal is not recoveringfrom procedures, and there is no prospect of them doing so in the near future, we will humanely killthem to prevent further suffering.

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

ICH Safety Guidelines

ICH guideline M3(R2) on Non-clinical Safety Studies for the Conduct of Human Clinical Trials andMarketing Authorisation for Pharmaceuticals (2009).

ICH S5 (R3) guideline on reproductive toxicology: Detection of Toxicity to Reproduction for HumanPharmaceuticals (2020).

ICH guideline S11 on nonclinical safety testing in support of development of paediatric pharmaceuticals (2020)

ICH Topic S3A: Toxicokinetics: A Guidance for Assessing Systemic Exposure in Toxicology Studies(1995)

ICH S6(R1) Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals

(2011). •ICH S9 Nonclinical Evaluation for Anticancer Pharmaceuticals S9 (2010). Guidelines of The European Agency for the Evaluation of Medicinal Products (EMEA): Evaluation ofMedicines for Human Use Notes for guidance on repeated dose toxicity. Committee for Human Medicinal Products (EMEA,2010). CPMP/SWP/1042/99.

Guideline on the evaluation of control samples in non-clinical safety studies: checking for contamination with a test substance. Committee for Medicinal Products for Human Use (EMEA, 2005).CPMP/SWP/1094/04.

Notes for guidance on non-clinical local tolerance testing of medicinal products. Committee forProprietary Medicinal Products (EMEA, 2015). EMA/CHMP/SWP/2145/2000 Rev. 1, Corr. 1*

LASA/NC3Rs: Guidance on dose level selection for regulatory general toxicology studies forpharmaceuticals.

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017)

Diehl et al. A good practice guide to the administration of substances and removal of blood, includingroutes and volumes. Journal of Applied Toxicology: 21, 15-23 (2001).

Foote RH and Carney EW. The rabbit as a model for reproductive and developmental toxicity studies,Reproductive Toxicology 14, 477-493, 2000.

Gad et al. Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routes inmultiple species with notes on methods to improve utility. International Journal of Toxicology: 1-84 (2016).

NC3Rs: Recommendations from a global cross-company data sharing initiative on the incorporation ofrecovery phase animals in safety assessment studies to support first-in-human clinical trials (Regulatory Toxicology & Pharmacology, 2014).

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

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

A PRACTICAL TRAINING COURSE IN MICROVASCULAR SURGERY

Project duration

5 years 0 months

Project purpose

- Higher education and training

Key words

Microvascular, Surgery

Animal types	Life stages
Rats	adult

Retrospective assessment

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

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Education and training licence

Objectives and benefits

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

What's the aim of this project?

This course has the sole aim and objective of providing suitably skilled surgeons with highest qualityinstruction and technical training to allow them to undertake (or support) microvascular surgery on patients in their clinical practice.

Microsurgery refers to surgery on blood vessels or structures (such as nerves) which is not possiblewith the naked eye, requiring and as such uses magnification to allow the surgeon to achieve a successful outcome.

A retrospective assessment of these aims will be due by 17 July 2028

The PPL holder will be required to disclose:

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

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 course is a vital component in complex Head and neck reconstruction training

Reconstruction following complex head and neck ablative surgery utilises transfer of required donor tissue(s) with its constituent vascular pedicle (feeding artery and draining venous network) with anastomosis of those vessels to recipient vessels to provide vascularised reconstructions; otherwise referred to as “Free tissue transfer with microvascular anastomosis:. This has become routine practicewithin reconstructive surgery over the last 30 years. In the anatomical area of the Head & Neck, in cases of cancer, congenital malformation and trauma, reconstruction with free tissue transfer has demonstrated far superior functional and cosmetic outcomes than other methods of treatment (Gabrysz-Forget 2019 J Oto HN Surg).

Comparable microvascular anastomoses are required for the repair of complex neurovascular structures as undertaken by Neurosurgeons. This highly specialised, demanding and exceptionally technique-sensitive skill is typically only acquired by experienced neurosurgeons with established clinical practices, frequently in tertiary referral centres. The superficial temporal artery to the middle cerebral artery (STA-MCA) bypass exemplifies a neurovascular/cerebrovascular anastomosis. STA-MCA bypass is a particularly useful technique for the management of complex or giant aneurysms where surgical treatment sometimes requires the sacrifice and revascularization of a main arterial trunk.

Microvascular anastomotic techniques (the surgical connection of arterial and venous vascular networks to provide viable free tissue transfer) are difficult to acquire and involve a graduated learningcurve of practice on prosthetic devices, on chicken/turkey leg blood vessels, on terminally anaesthetised rats and finally performed on human patients under the direct supervision of experienced surgeons/trainers. Acquisition of microvascular reconstruction skills is now considered anessential element of Higher Surgical Training in a number of surgical disciplines (Intercollegiate Surgical Curriculum Programme (ISCP) - OMFS Curriculum 2021; Plastic Surgery Curriculum 2021; ENT Curriculum 2021).

In the UK context, basic training can be obtained in two ways:

Attending practical microvascular courses such as the one (Practical Training Course in Microvascular Surgery) which has been delivered successfully in since 1993

Undertaking research projects that involve microsurgical techniques

Our course has been awarded 37 Continuing Professional Development (CPD) credits by the Royal College of Surgeons of England (representing a significant element of the necessary 50 CPD creditsrequired annually for practicing clinicians) and is an integral component of the a Masters (MSc) in OMFS (20 credits Masters-level credits). Provision of the microvascular training course will be reviewed by the Animal Welfare And Ethical Review Body (AWERB), including detailed course feedback is annually.

Maxillofacial Surgery is concerned with the surgical management of acquired and congenital disordersof the face, jaws and mouth. At present there are approximately 80 Maxillofacial Units in the UK, of which half routinely carry out free tissue transfer procedures. There are 90 career grade doctors training in these units, each of whom are either required to obtain the basic skills necessary for the performance of microvascular anastomosis, or, having previously obtained the skills, have a need to maintain these skills. In many cases, the local unit is not a position to provide either.

In spite of the advances in models that allow trainees to practice operative skills in a simulated surgicalenvironment, such as is the case in laparoscopic surgery or in arterial access proficiency training; thereare no suitable prosthetic devices that replicate vascular microsurgery. The acquisition, development and maintenance of such skills can only be achieved by instruction and practice. A variety of techniques have been used in the past to develop and maintain these skills with variable success (e.g. Chicken legs, placental blood vessels). None of them has provided completely satisfactory. A surgeon needs to practice the key steps, critical for operative success, for example, mobilisation of the artery and vein from surrounding tissues, preparation of the vessel wall with minimal handling and trauma, accurate positioning and tension of sutures, management of vascular leakage (haemostasis), an awareness and appreciation of the problems such as vessel spasm. One is forced to conclude that presently the only truly viable method of assessing the adequacy of microvascular anastomosis is to observe blood flow across the anastomotic site and this inevitably involves living tissue. Training on living tissues (with cardiac output and vascular perfusion) is necessary to acquire technical skills in combination with direct, robust feedback on the adequacy of those skills (microvascular patency).

The critical success of microvascular anastomosis to survival of free tissue transfer in the clinical environment precludes initial training of surgeons until the essential practical skills have already beenacquired – practice and training on animal model prior to commencing training in a clinical setting on patients.

There are no suitable alternatives (either in terms of the animal model or the expertise within the available faculty - all with prior capabilities in the teaching of microsurgery on rats and the clinicalexperience of H&N reconstructive surgery).

Previous experience of running this course for over 30 years supports our understanding that the vast majority of trainees, i.e. more than 95%, gain sufficient skills to be able to perform clinical microsurgicaltechniques under supervision and a number (15-20%) develop sufficient competence to perform them without supervision. 95% are able to achieve a good end-to-end and end-to-side arterial and venous anastomosis using the femoral vessels, and 70-80% are able to produce a successful (patent) interpositional vein graft (Vein graft sited into an arterial system) by the end of the course. This should enable (or support) several units to take on (or continue to employ) microvascular procedures which they could not otherwise consider; which if it were the case could potentially deprive patients of the consequent considerable benefit.

This course provides an essential component in the training of both UK and international surgeons to alevel necessary to sustain ongoing complex microvascular procedures in specialised units.

How will course attendees use their knowledge or skills in their future careers?

All the students are full-time higher trainees on various clinical training pathways. this course and theclinical training supports their development towards becoming competent

microvascular surgeons.

The course equips the higher surgical trainee (HST) with the underpinning theory of H&N microvascular surgery and associated reconstruction techniques based on robust evidence and practical demonstration. It facilitates HST development of complex specific surgical skills in Head andNeck Surgery in a safe environment with direct support and immediate guidance and critique.

Without the skills developed in a course such as this, it is unlikely that safe, successful microsurgerycould be practiced - in effect a course such as this will reduce the risk of harm to patients through development of understanding and technical proficiency.

It is worth noting that all trainees will be either registered in higher surgical training programmes in respective surgical specialties (OMFS, Plastic Surgery, ENT and/or neurosurgery) or be establishedconsultants (or equivalent) in those specialties. As detailed below, it is an “entry” requirement for thecourse that participants will provide a formal statement from their respective head of department/specialty indicating that they have the clinical need and ability to develop or maintain microvascular skills.

What are the principal learning outcomes from the course?

The ability to demonstrate critical understanding of the technical set up, vascular preparation, andcompletion of microvascular anastomoses as would be necessary for successful free flap reconstruction in humans.

To understand the sources for technical error in microsurgery and technical means to mitigate theiroccurance.

Each participant should, by the end of the course, have developed the capability to prepare and anastomose vessels with a diameter of less than 1mm. They should be able to critically assess thequality of the anastomosis and have a feel for their aptitude in this area of surgery

How are these learning outcomes important to the people on the course?

These learning outcomes are essential to successful microsurgery in humans.
They ensure the greatest liklihood of successful surgical procedures when undertaken in the clinicalsettting.

Who or what will benefit from the transfer of knowledge, or acquisition of skills that this coursewill deliver?

There are several beneficieries from the course;

- Delegates - as detailed above, the technical surgical abilities and repertoire of the delegates willbe enhanced in such a way as to facilitate successful microsurgery.

- Patients - the impact of failed microvascular surgery for Head & Neck patients is difficult to understate (function, cosmesis and ultimately survival all require successful microvascular freetissue transfer). without success in this area of surgery, outcomes for patients are consistently poor.

- Healthcare providers - successful microsurgery within Head & Neck departments in the UK andinternationally rely on the development of surgeons who possess the necessary understandingand technical abilities

How will you look to maximise the outputs of this work?
This course has now entered its 3rd decade of successful provision with an ever increasing cohort of surgeons worldwide who have benefitted from its education. This course in internationally renowned forits rigour, technical standards and educational benefit.

Each new delegate is able to return to their parent unit and further develop their skills whilst impartingknowedge to others (including their trainees who frequently return following recommendation to complete the course)

Species and numbers of animals expected to be used

- Rats: 65 animals per annum (325 animals in total)

Predicted harms

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

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

The rat femoral and epigastric vessels remain the standard animal microvascular model used widely inthe UK and beyond. They possess many of the features of human microvasculature and in addition, provide exceptional “feedback” as to quality of surgical technique such that errors (which might lead todelayed microvascular failure in patients) are immediately apparent to the operator. The rat femoral and epigastric system reflect comparable vessels in terms of size, calibre and quality to that which is utilised in the human microvascular setting.
Typically, what will be done to an animal used in your project?

Anaesthesia will be induced and maintained using suitable anaesthetic agents.

Blood vessels will be exposed, isolated, divided, and either repaired, anastomosed or grafted.(AC)

In some instance free tissue flaps may be raised on a vascular pedicle which is then divided andre-anastomosed. (AC)

At the end of the procedure, animals will be terminated by a Schedule 1 method. (AC)

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

Each procedure shall be carried out under general anaesthesia (with associated analgesia) which shallcontinue until termination of the animal by a Schedule 1 method. Anaesthesia shall be induced, monitored and maintained by experienced personal licencees. Termination of animals at the end of theprocedure shall be performed by Schedule 1 method by the licensees responsible for induction and maintenance of

anaesthesia.

There will be mild stress and discomfort associated with restraint and the initial injection of the anaesthetic agent. This will be minimised by using experienced licensees for anaesthetic induction. Itis obviously imperative that a suitable anaesthetic plane is maintained throughout the procedure and again those supporting/provising the anaesthetic for the animals will be experienced in such circumstances. The pedal withdrawal reflex, along with respiratory parameters will be assessed regularly to ensure adequacy of anaesthetic depth.

Hypothermia is a fundamental consideration in recovery anaesthesia in rodent surgery but these animals will not recovered. Previous extensive experience has demonstrated that we can comfortably maintain suitable anaesthetic depth without inadvertent loss of animals, without supplementary heatingfor a period of up to 9 hours from induction by providing additional support in the form of heat and fluids. Rats are maintained throughout the procedure on a heated mat with warmed fluids (such ascompound sodium lactate) administered subcutaneously.

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

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

The procedure is always conducted under terminal anaesthesia (animal does not recover from theanaesthetic).

End-to-end and end-to-side anastomosis (suturing of blood vessels to allow normal blood flow) will beperformed on the femoral artery and vein and on the epigastric artery and vein.

Not all anastomoses will be technically successful, however, as all animals are terminally anaesthetized, failure of an anastomosis will not cause animal welfare concerns.

Occasionally, due to operative error, haemorrhage will occur; under such circumstances if it is considered that blood loss is excessive the animal will be immediately euthanased. Under no circumstances do the rates ever experience pain or distress. The rats are killed humanely aftercompletion of the training exercise.

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

- Killed

A retrospective assessment of these predicted harms will be due by 17 July 2028

The PPL holder will be required to disclose:

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

Replacement

State what non-animal alternatives are available in this field, which alternatives you

haveconsidered and why they cannot be used for this purpose.

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

It would not be possible to achieve the objectives of the project without this animal model, as there isno alternative for a living vascular system with its dynatic nature and associated clotting profile(s).

A component of replacement has already been included in this course with the use of a prosthetic device (or synthetic blood vessel) to learn, consolidate and assess basic microsurgical skills/suturing.

Attendance and training on a course such as the one proposed here is considered a standard phase inthe learning of microvascular (surgical) techniques that, when applied, result in a successful anastomosis (ie allowing blood flow through anastomised vessels).

As the success of microvascular anastomosis is so important to the surgical patient there is a need foran animal model with flowing blood and natural technical complexities (akin to the human situation) to ensure the surgeons can practice in a situation that the success of the surgery can be fully assessed.

There is no adequate non-living substitute to living blood vessels for learning the critical aspects ofmicrovascular surgery.

Why can’t your aim be met by observing or by participating in ongoing research or clinicalprocedures?

The rat femoral and epigastric vessels remain the standard animal microvascular model used widely inthe UK and beyond. They possess many of the features of human microvasculature and in addition, provide exceptional “feedback” as to quality of surgical technique such that errors (which might lead todelayed microvascular failure in patients) are immediately apparent to the operator. The rat femoral and epigastric system reflect comparable vessels in terms of size, calibre and quality to that which is utilised in the human microvascular setting.

Attendance and training on a course such as the one proposed here is considered a standard phase inthe learning of microvascular (surgical) techniques that, when applied, result in a successful anastomosis (ie allowing blood flow through anastomised vessels). It would not be possible to achieve the objectives of the project without this animal model, as there is no alternative for a living vascular system with its dynamic nature and associated clotting profile(s).

Mere observation would fail to achieve the technical skill, proficiency and surgical capabilities of a microvascular surgeon. It is the physical undertaking of a complex (animal) anastomosis that a) teststechnical ability and b) provides immediate feedback of proficiency - this is witnessed by experiencedtrainers who can instruct where improvement should be made and thus maximise microvascular success.

A retrospective assessment of replacement will be due by 17 July 2028

The PPL holder will be required to disclose:

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

Reduction

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

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

The numbers of animals used is the minimum required for acquiring the desired surgical skills. Prior tocommencement of procedures on animals, a prosthetic device (synthetic vessel) is used.

A single animal is used per candidate per day ensuring complete utilisation of the resource prior totermination of the animal.
The procedures are carried out under non-recovery anaesthesia so that no animal will experienceadverse effects.

What in silico or ex vivo techniques will you use during training?

A component of replacement has already been included in this course with the use of a prosthetic device (or synthetic blood vessel) to learn, consolidate and assess basic microsurgical skills/suturing.

Will these techniques reduce animal numbers? If so, how?

The use of a synthetic vessel has reduced the number of animals used by one per delegate per course. It has the additional benefit of allowing trainers to witness the capabilities of delegates on thesynthetic construct BEFORE progressing to the animal model. If necessary delegates can continue using the synthetic construct until such time that they demonstrate suitable skills necessary to gain full/maximal benefit from the living model.

What other measures will you use to minimise the number of animals you plan to use in yourproject?

The design of each teaching day ensures that delegates make full use of each individual animal modeland gain maximal technical/microsurgical exposure and training. This includes using both the femoral arteries and both the femoral veins for anastomoses on each animal.

It is expected that trainees will utilise a single rat per day. There may be circumstances where a traineeexceeds training expectations and completes the technical proficiencies/is able to demonstrate outstanding abilities within the 5 day period and therefore would not require an animal for each of the 5days.

Where any trainee is demonstrably slower in their learning of surgical skills/techniques, this can besupported with a return to the prosthetic device (synthetic vessel) to consolidate

skills and learningbefore recommencing learning on the animal model.

Evolution in the course has reduced the number of animals used for direct demonstration purposes. Through the use of videos and simulation, animal numbers have been reduced in the last 3-4 years. Ata maximum, reduction would result in one less animal being required per day (n=5 over the course of the week)

A retrospective assessment of reduction will be due by 17 July 2028

The PPL holder will be required to disclose:

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

Refinement

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

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

Rat Femoral Vascular System;

The rat femoral and epigastric vessels remain the standard animal microvascular model used widely inthe UK and beyond. They possess many of the features of human microvasculature and in addition, provide exceptional “feedback” as to quality of surgical technique such that errors (which might lead todelayed microvascular failure in patients) are immediately apparent to the operator. The rat femoral and epigastric system reflect comparable vessels in terms of size, calibre and quality to that which is utilised in the human microvascular setting.

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

Animals utilised provide the optimal vascular model (rat femoral system) both in terms of calibre andcomparablity to human situation. The rat femoral and epigastric vessels are the standard animal model used widely in the UK and beyond.

All animals receive terminal anaesthesia

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

Over the course of 30+ years of running this course, the host BSU team have developed a structuredtechnique for the monitoring of animal welfare during the course. This is supplimented by a strict instruction regarding animal care at the outset of the course for all PIL holders and direct supervisionby experienced surgical trainers with many years of experience of animal (and human) microvascularsurgical instruction. Under no

circumstances do the rats ever experience pain or distress.

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

No evidence exists to support a non-live animal for microvascular training. All attempts to refine theproject and mitigate harm to animals are undertaken as part of the proposed protocols

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

The course directors (inc Home office project licence holder) undertake regular analyses of suitablealternatives for the animal model utilised.

This includes frequent interaction and conversation with similar course directors and a regular literaturereview.

A retrospective assessment of refinement will be due by 17 July 2028

The PPL holder will be required to disclose:

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

ADOPTIVE CELL TRANSFER FOR CANCER IMMUNOTHERAPY

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, ortheir effects, in man, animals or plants

Key words

Cancer, Immunotherapy, T cells, Chimeric antigen receptors

Animal types	Life stages
Mice	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To develop novel and more effective immunotherapies for the treatment of Cancer

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

Why is it important to undertake this work?

Cancer is a diverse and prevalent disease caused by the uncontrolled proliferation of cells within the body. Additionally, proliferating cells can break away from the main tumour mass, migrate through the body and establish at a distant site in a process called metastatsis. Proliferating and metastasizing tumour cells invade and destroy healthy tissues and organs within the patient disrupting their function and causing significant illness and ultimately death. Immunotherapy using chimeric antigen receptors (CARs) harnesses the innate potential of the patient's immune system by reprogramming immune cellsto recognize and attack cancer cells. Despite substantial advances in immunotherapies many patients fail to achieve complete control and clearance of the

tumour and for many tumours no appropriate immunotherapy yet exists. Thus the development of new immunotherapies and the improvement of existing ones through research leading to a greater understanding of their mechanism of action is vital for improving outcomes for the multitude of patients developing and suffering from cancer.

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

This project will generate multiple outputs important to the field of Immunotherapy. Primarily it will produce new chimeric antigen receptors (CARs) for the treatment of both blood-derived and solid cancers. Additionally, we will generate new technologies which enhance the overall effect of immunotherapies including CARs. The data generated on around these CARs and new technologies will contribute to pre-clinical packages which will support the initiation of phase I clinical trials aiming totest these immunotherapies in patients for which few to no alternative treatment options exist.

Additionally, the data will support new and ongoing collaborations with both academic institutions and the biotech industry in order to broaden their availability to patients. Data will also be published in peer-reviewed scientific journals and presented to medical professionals at Oncology and Immunotherapy conferences in the form of posters and oral presentations.

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

In the medium to long term the primary beneficiaries to this project will be patients suffering from cancers which have relapsed or proven resistant to existing treatments. The data generated from thiswork will allow the development of next-generation chimeric antigen receptors and form the basis for exploratory clinical trials in which new immunotherapies will be tested on patients who have run out ofalternative treatment options. This will address substantial unmet medical need and the long-lasting remissions achieved by such therapies will save lives and confer a significant economic benefit to healthcare providers.

Additionally, the work will benefit clinicians as it will result in them having access to new tools to use totreat their patients. Furthermore, the outcomes of these early clinical trials will be distributed to scientists working in the field of immunotherapy informing them on the development of additional therapies in their specialist disease areas. The outcomes from this project and the resultant trials will allow us to further improve and develop more effective immunotherapeutic treatments.

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

The data generated by this work - development of novel chimeric antigen receptors (CARs) and T celltechnologies, pre-clinical evaluations of CARs, improvements to cell and viral vector production processes and the results of early-phase clinical trials - will be disseminated to the field in the form of peer-reviewed publications in relevant, high-impact journals and presentation to clinicians and scientists at Immunotherapy and Oncology conferences through posters and oral presentations.
Additionally, the data will form the basis of collaborations with other Biotechnology companies and withacademic group in order to broaden the impact of the knowledge and technologies generated outside of the field of T cell immunotherapies and in disease indications other than cancer. We will also provide training to our collaborators in the new processes and techniques that we develop over the course of this project.

Species and numbers of animals expected to be used

- Mice: 8000

Predicted harms

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

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

Our aim is to investigate the complex mechanisms by which CAR T cells can mediate the rejection ofestablished tumours in order to understand and enhance these processes. Recreating and studying this complex interplay of these mechanisms and factors is not possible using lab-based models. Adultrodents are the simplest animal in which we can establish physiologically relevant models of cancer and the use of rodents allows us to establish models which more accurately recreate the conditions found in human patients and so more accurately assess the efficacies and benefits of our immunotherapies. Immunocompetent mice possess a functioning immune system allow us to assess the interaction of CAR T cells with tumour and endogenous immune systems and to investigate technologies which rely on the endogenous immune system to enhance cancer rejection. Converselygenetically-altered mice which lack all or parts of their immune system allow us to establish human tumour cell lines and primary cells and can engraft human CAR T cells without fear of rejection by endogenous immune cells.

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

Typically, an animal will be injected with cancer cells and the cancer will be allowed to form or establish. Subsequently the animal will be injected with an agent or cells, such as CAR T cells, in orderto measure their effect on the animal and the established cancer, possibly after treatment with irradiation or immunosuppressive drugs to suppress the animal's immune system. The growth of the tumour will be monitored over the course of the experiment through methodologies such as bioluminescence imaging or direct size measurement in order to measure its growth and localisation.

Throughout the course of the experiment the animals will be monitored for signs of pain and distress such as hunching, reduced activity, piloerection and will be humanely killed if these signs of distress are greater than transient and minor. At the end of the experiment animals are expected to be humanely killed. Experiments are expected to last an average of 6-8 weeks although infrequently the survival of the mice may be monitored for longer periods. Imaging is expected to occur up to 3 times per week over this period potentially under anasthaesia. The total number of imaging sessions including any modality will not exceed 20 sessions per animal with minimum downtime of 24 hours between sessions and animals will not be anaesthetised for more than 10h in any week. A further injection of tumour cells may occur during the course of the experiment in order to assess whether the animals have maintained or generated immunity against the respective cancer.

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

The growth of tumours within the animal may cause adverse effects due to the infiltration of organs anddisruption of their physiological function. This may result in pain, weight loss,

impairment of bodily function such as hind limb paralysis or decreases in feeding and activity, ulceration of the tumour massand ultimately death. Additionally, treatment of the mice with CAR T cells could result in xenoGvHD in which human T cells recognize and react against mouse tissues due to differences in proteins expressed by the two species. Treatment of animals with irradiation or immune suppressive drugs to ablate the immune system or with hormones to support growth of implanted tumours may also result in adverse effects. As above, these process are likely to result in pain, weight loss, impairment of bodily function and death. Animals which lose 15% of their body mass or develop GvHD or paralysis will be humanely killed. Additionally, animals which develop the other symptoms which do not abate with appropriate supportive care over a period of 24 hours 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 animaltype)?

The majority of animals used in this project are not expected to show signs of adverse effects howeveras a result of the cumulative impact of multiple steps in each protocol 100% of animals are expected toexperience 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 haveconsidered 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 investigate the complex mechanisms by which CAR T cells can mediate the rejection ofestablished tumours in order to understand and enhance these processes. Recreating and studying this complex interplay of these mechanisms and factors is not possible using lab-based models.
Immunocompetent rodents possess a functioning immune system which shares many relevant genes and proteins with the human immune system so allowing us to assess the interaction of CAR T cells with tumours and the endogenous immune system and to investigate technologies which rely on the endogenous immune system to enhance cancer rejection. Conversely, genetically altered mice which lack all or parts of their immune system allow us to establish human tumour cell lines and immune cellsand can engraft human CAR T cells without fear of rejection by endogenous immune cells.

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

Co-cultures with primary tumour cells freshly isolated from patient biopsies and with immortalisedtumour cell lines derived from the cancer of interest and which grow indefinitely in the lab.

“3D” tumour cell culture models in which cancer cells are grown in a gel in order to form

tumour-likemasses of cells more accurately recreating how the cancer would grow in the patient

Why were they not suitable?

Where possible we will utilize in vitro alternatives to animal models however there are no such models which are capable of accurately recreating the growth of cancer cells within a living organism. Primary tumour cells from the patient generally do not survive or grow well in the lab as they lack the conditionsthat they need to survive and thrive making it difficult to meaningfully assess the effect of any treatmentapplied to them. Even 3D tumour cell culture models are unable to reproduce the structure and growth of a tumour within a living organism as process such as the development of blood vessels within the tumour and the recruitment of accessory cells from the host animal are lacking.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 considered our strategic plan over the next 5 years regarding the Immunotherapy products that we are currently developing and those that we plan to develop in order to undertake clinical trialsin cancer patients. Based on these plans we have plotted out the in vivo models likely to be required and calculated, as accurately as possible, the numbers of animals required to generate the data to support the development of our planned new immunotherapy products and regulatory clinical trial applications taking into consideration our past experiences in this field and with previous products.

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

Where appropriate the NC3Rs Experimental Design Assistant will be used to assist in experimental design. We will use a variety of techniques to reduce the number of animals and will keep our work in adherence to PREPARE and ARRIVE guidelines. The sizes of experimental groups and the number ofrepeated experiments will be kept to a minimum while ensuring that reproducible results are obtained with clear biological significance. Power analysis will be used to determine the minimum numbers of animals and repeated experiments that are required to achieve statistical significance. In order to remove sources of variation efficacy studies will be randomised and tumour growth measured using unbiased methods such as bioluminescent imaging according to standardised methods or electronic calipers. For data analysis we will use appropriate statistical methods as recommended in current published literature.

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

New cell line transplantation models will be initially tested in small-scale pilot experiments to establish optimal tumour dose and growth kinetics required for meaningful, reproducible

data in subsequent experiments and removing the need for multiple tumour doses in our models. Non-invasive methods ofmonitoring tumour growth will enable sequential analysis of several biological timepoints within the same animal allowing us to define study endpoints without killing the animal and reducing the overall numbers of mice required for the models. Additionally, we will take advantage of all possible tissues ineach experiment and use multi-parametric flow cytometry to simultaneously measure multiple cell parameters and to analyse and fully characterise tumour cells, human cells and mouse cells from a single sample thus maximising the data obtained from each experiment.

Furthermore, we will us common cell lines engineered to express antigen of interest which reduces theneed for initial studies to measure the growth and establishment of novel cell lines for each new antigen under investigation

Our novel immunotherapies to be tested in mice will undergo extensive in vitro characterisation to understand which immunotherapy is likely to be the most optimal and most suited to testing in animalmodels so reducing the number of animals 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

We intend to use mice during this project as mice are the lowest vertebrate for which there exist the best developed and characterised models for the study of immunotherapies for the treatment of cancer. Mice that have been genetically altered to lack a functioning immune system are frequently used for the establishment of models of human tumour as they will not reject transferred tumour or human immune cells. The models and techniques used in our work are those which are accepted by regulatory authorities for Clinical Trials Applications and constitute the least invasive methods to obtainthe required data. Real-time bioluminescence imaging of systemic tumour growth or physical measurement of established sub-cutaneous tumour allow detailed assessment of effect of experimental treatments on tumour cell growth and allow us to assess when tumour growth is irreversible before causing adverse effects, suffering or distress to the animal. Where appropriate during procedures we will use anaesthetia to reduce stress or administer analgesics to relieve pain anddiscomfort. For models involving sub-cutaneous implantation of tumours sites will be chosen which will cause minimal discomfort such as the flank.

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

Species that are less sentient show a greater separation from human biology rendering the informationwe would obtain less relevant to the treatment of human cancer patients. Additionally, the establishment of tumours in mice is a process which occurs over a number of days or weeks making the use of more immature animals or terminally anaesthetised animals unsuitable

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

We continually review our protocols and procedures to ensure we are using the most refined methods through the applications of new techniques and reagents detailed in the scientific literature and developed by our collaborators and peers and by utilising the expertise of our academic collaborators in the establishment of new models. Additionally, animals used in our project are subject to close and frequent monitoring for signs of distress and suffering and animals experiencing such signs are humanely killed. Furthermore, we establish limits of tumour growth as measured by bioluminescence imaging to define levels of growth that are irreversible prior to any adverse effects experienced by the animals and where possible we will use standard tumour cell lines which follow predictable growth rates and which have been engineered to express the antigen of interest. Additionally, we use the mostnon-invasive methods of monitoring tumour growth and treatment efficacy and use doses of tumour that display a controlled rate of growth preventing the probability of the sudden and unexpected appearance of adverse effects.

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

We will plan and report experiments in accordance with Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines as well as follow relevant guidance publishedby the Laboratory Animal Science Association (LASA) and the NC3Rs.

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

We keep abreast in the relevant scientific publications in the field of Immunotherapy development utilising animal models and frequently present the models we have used and data generated with our peers in the field and consult sources of information such as those published by the National Centre forthe Replacement, Refinement and Reduction in Animals in Research (NC3Rs).

Furthermore, we maintain numerous collaborations with academic groups working in the field of Immunotherapy and routinely meet our academic colleagues to discuss new and refined animal models which have been developed within the groups and to share advice and expertise. Additionally,we will liaise with the local AWERB and animal care staff to seek advice regarding the 3Rs.

ANALYSIS OF EARLY EYE DEVELOPMENT IN FISH

Project duration

5 years 0 months

Project purpose

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

Key words

Zebrafish, Eye, Development, Genetics, Cell signalling

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This project aims to unravel the molecular, cellular, and genetic mechanisms that enable eyespecification and growth compensation.

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

Why is it important to undertake this work?

Sometimes eye development fails, and people/animals are born with smaller or no eyes. There is littleunderstanding on what are the eye developmental mechanisms that fail in such cases. Impairment of the growth compensation mechanism we discovered could explain the origin of such developmental abnormalities as the growth of eyes that start developing with fewer cells, as can occur because of mutations, will not be compensated. Understanding the mechanisms that enable eye growth compensation could open the door

to the development of new therapies.

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

The output of this project will be the publication of the research findings we achieve when studying the genes involved in eye formation in zebrafish. These will include the identification of new genes that work in defining the embryo cells that will become the eyes, and on the mechanisms that control the retina growth and differentiation. Our research will address their function and the cellular and molecularcontext they work in during embryo development. This will shed light on new cell-signalling and morphogenetic mechanisms that are required for vertebrate eye formation.

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

Our research project will validate the gene mutations we identify in patients with congenital eye defectsand study their function during early eye development. From a medical perspective, finding new diagnostic genes responsible for congenital eye defects will benefit doctors and genetic counsellors working with patients by enhancing their capacity to provide genetic counselling and patient care. It willalso set the grounds for translational research, opening the possibility of future therapies to prevent these kinds of pathologies.

My work on the genetic and molecular basis of eye development has connections to many different scientific disciplines in biology. Therefore, the mechanistic insights we generate on eye developmentand disease will impact the communities of scientists working on eye and embryo development, andalso colleagues working on genetics, gene networks, organogenesis and neurogenesis in general.

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

Our findings will be communicated to clinicians and scientists through peer-reviewed articles, conference presentations such as talks and posters, seminars and meetings. Ultimately, all cliniciansand genetic councillors dealing with eye globe defect patients will be able to access our findings fromthe open access articles we publish and seminar series and international conferences.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 10625

Predicted harms

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

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

The tissue interactions involved in eye development and the genetic experiments that will be performed in this project cannot be replicated in vitro, therefore, the studies in this research programme require the use of animal models. Furthermore, zebrafish is an excellent model to study the genetics of eye development because eye formation can be analysed from initial tissue specification to completion, and a diverse repertoire of powerful genetic tools is available. We will onlyuse zebrafish embryos up to 4 days post-fertilization,

one day before they become free-feeding larva and subject to Home Office regulation.

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

Adult fish will be bred for embryo collection. Experiments on embryos will only be performed before thestage zebrafish larvae are under regulated procedures.

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

Fish will be mainly used for breeding and will not experience any pain or stress in the process of pairing. Most genetic identification of animals in this project will be performed at 3 days post fertilisation(larval stage), two days before zebrafish larvae are subject to regulation, therefore this does not count as a regulated procedure. Only occasionally, adult fish will be briefly anaesthetised to reduce mobility and enable the cutting of a small fragment of the caudal fin or skin swab for genetic identification.
These methods are considered a mild regulated procedure and will only be performed occasionally (atmost 1000 fish in the course of this project license, approximetely 27%) to confirm the genetic identity of fish when necessary.

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

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

Only occasionally, caudal fin clipping or skin swabbing for adult animal identification will be performedunder anaesthesia. This is considered a mild regulated procedure and over the course of this project will be performed on less than 1000 animals.

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 haveconsidered and why they cannot be used for this purpose.

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

The tissue interactions that lead to eye development and the complex genetic experiments that will beperformed in this project cannot be replicated in vitro. Therefore, studying the genes involved in eye development required the use of animal models. Zebrafish is an excellent model for the study of the genetics of eye development because its external fertilisation allows studying eye formation from the very beginning of its specification. We will only use zebrafish embryos up to 4 days post fertilisation, this is one day before they become free-feeding larva and subject to Home Office regulation.

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

In vitro studies using induced pluripotent stem cell (iPSC) derived retina organoids can be used tocomplement studies like the one proposed in this project.

Why were they not suitable?

Studies on iPSC-derived eye organoids are not conclusive, as the organoids do not include all the tissues and cell types that arise during in vivo eye development. Also, the complex morphogenetic cellmovements and tissue interactions that happen during in vivo eye development cannot be replicated by eye organoids.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 adult zebrafish to be used over the course of this project was calculated based on the need for a maximum of 150 fish lines which include wildtype strains, transgenic lines, mutant lines anda combination of double transgenic/mutant lines. Each line will be kept in tanks that can harbour up to 15 adult fish at a time, which adds up to 2250 fish at a time. However, to minimise possible health issues, each line must be regenerated every 2 years and hence the total number of fish must be factored by 2.5x giving a total of 5625 fish over a 5-year period. On top of this, we will generate approximately 10 new genetically altered lines every year. This will involve raising 100 extra animals per line, adding a total of 1000 animals per year, 5000 during the course of the project.

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

To reduce the number of animals being used in this study we will only grow to adulthood those animalswe are sure are carrying mutations or transgenic insertion in their genome during larvae stage at 3 days post fertilisation, before fish are subject to Home Office regulation. No adult fish or larvae past 5 days post fertilisation will be used for experiments.

Although embryos or larvae used for experiments are not subject to regulation, we will keep their generation to an essential minimum such that the numbers used fulfil the statistical significance required for the experiments. Each fish pairing can generate over 200 embryos. The number of embryos required for experiments will inform the number of fish pairings required to avoid excessiveuse of animals.

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

The key measure to reduce animal use involves the genotyping identification of animals by caudal tail clipping or Zebrafish Embryonic Genotyper at larvae stage, before being subject to protected regulation. The expected line regeneration protocol considers a heterozygous

mutant or transgenic lineoutcross to a wildtype strain to ensure robustness and vigour in the next generation. In this breeding scheme, only half of the animals will carry the mutation or transgenic insertion. Therefore, in genotyping at the larvae stage we will optimise the number of fish raised to adulthood by only growing those animals carrying mutant and transgenic insertions and reducing to 50% the number of animals used in this project.

Refinement

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

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

This project will only use zebrafish embryos before they become free-feeding larva and are subject toHome Office regulation. Adult fish will only be used for breeding a collection of embryos.

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

Of all the vertebrate animal models suitable for genetic experiments, zebrafish is the one that has the least impact on animal welfare as the production of embryos does not involve the culling of the femalesas when using mice, or the culling of males and injection of hormones when using frogs.

The option of using less sentient animal models like flies is not an alternative as the eyes in flies havea completely different morphological organization and development compared to vertebrates.

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

Optimisation of efficiency for each genetic alteration experiment allows us to minimise the number of zebrafish used. Where possible we will use refinements such as Zebrafish Embryonic Genotyper or caudal fin clipping on larvae <5dpf, or fluorescent imaging of progeny. This will avoid the genotyping ofadult animals.

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

Our research involving animal use will follow RSPCA's (2011) "Guidance on the housing and care ofzebrafish" and the PREPARE guidelines (https://norecopa.no/prepare).

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

I get regular updates on the advances of the 3Rs via Biological Services emails and

newsletters andthe NC3R´s website.

ASSESSING THE HUMANENESS OF SPRING TRAPS

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 aimsmentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man oranimals

Key words

Spring traps, Humaneness, Spring Trap Approval Order, AIHTS, Wildlife

Animal types	Life stages
Mice	adult, juvenile
Rats	juvenile, adult
Rabbits	adult, juvenile
Grey squirrels	adult, juvenile

Retrospective assessment

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

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

To produce evidence necessary for the regulation of the Spring Traps Approval Order (STAO), alegislative instrument of the Pests Act (1954).

For any new combination of trap type, trap setting, or target species added to the STAO list, qualifyingspring traps must undergo a quantitative assessment to determine whether they meet the humannesscriteria required. Work under this licence provides the necessary evidence for regulation.

A retrospective assessment of these aims will be due by 31 July 2028

The PPL holder will be required to disclose:

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

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

Why is it important to undertake this work?

Ensuring that qualifying spring-traps deliver a rapid and reliable death to wild animal targets and meeta minimum standard of humaneness, avoids the sale and widespread use of relatively inhumane devices. Inhumane spring-traps might otherwise maim wild animals or produce prolonged periods of substantial suffering before death.

By supporting the regulation of spring traps, this work helps substantially reduce the suffering of verymany wild animals which are subject to wildlife management.

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

The principal benefit is robust evidence of the humanness of tested spring-traps, ensuring that trapsfailing the current regulatory standard are not permitted for use where they might cause excessive harm in specific settings or for specific target species.

A secondary benefit occurs by providing manufacturers with assurance that novel designs meet or exceed minimum standards of humaneness for qualifying spring-traps, fostering improvements in theirdesign.

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

Preventing the marketing, sale and use of relatively inhumane spring traps produces significant andimmediate benefits to government, society and the welfare of millions of wild animals which may besubject to wildlife management using spring-traps. These benefits accrue overtime to become substantial.

Wildlife subject to legal management using spring-traps benefit by being protected from the mostharmful and inefficient trap designs which are most likely to produce widespread and substantial unnecessary suffering where more efficient and humane devices are available.

Across all timescales this project supplies the trap licensing authority (Defra in England) and other authorities of the devolved nations of the UK with robust evidence of the humanness of tested spring-traps. In this way work ensures that traps failing the current regulatory standard are not permitted for use where they might cause excessive harm in specific settings or for specific target species. This enables the UK government to maintain environmental standards necessary for its international commitments, as well as permitting all devolved administrations to continue to protect wild animal welfare. These social and economic benefits are central to the establishment of trap testing standards(e.g.

Agreement on International Humane Trapping Standards: AIHTS).

By enabling commercial clients to assure the humaneness of novel products we foster innovation in the design of new and yet more humane wildlife management approaches. We achieve this in part by using the experience we acquire undertaking this work and providing feed-back to clients (where theseare trap designers, manufacturers, or suppliers) on how further improvements to welfare might be secured.

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

As a regulatory requirement this work achieves substantial impact, directly informing regulators in all devolved administrations and avoiding the listing of trap designs for species or in settings where theydo not meet the minimum standard of humaneness. As most work is delivered to commercial customers, dissemination of test results is subject to their commercial interests.

Species and numbers of animals expected to be used

- Mice: 50
- Rats: 100
- Other rodents: No answer provided

Predicted harms

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

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

Spring traps generally comprise two physical design elements; a trigger and a killing bar or piston (often powered by a spring) which is designed to strike the animal and produce catastrophic injury sufficient to cause rapid and irreversible insensitivity (unconsciousness) followed by death. Preferred anatomical targets for injury are the brain or neck to produce deaths equivalent to the Schedule 1 methods of cranial depression or cervical dislocation, both so damaging the brain that insensibility anddeath may be instantaneous.

The efficiency of the interaction between trigger position, trigger latency, and the arc and power of strike are complicated by the behaviour of the target animal as it moves through the trap mechanism. This produces variation in strike placement and strike outcome. In some cases this might produce injury but not insensibility or death, or eventual death but after a significant and unsatisfactory period. Whilst trap testing is tangentially concerned with the simple physical characteristics of the trap (trigger speed, striking speed, striking power, clamping force) it primarily measures how variation in wild animalbehaviour may produce ineffective strikes, and result in poor outcomes for target animals. This is why trap setting is important (baited, unbaited, unenclosed, single entrance enclosure, run through etc) as these variations in deployment substantially alter the behaviour of some wild species.

Measuring the consequences of variation in wild animal behaviour in spring trap tests currently requires the use of conscious/active wild animals of the type specified for each STAO listing, e.g. rat, grey squirrel, stoat. This includes the most commonly encountered and anatomically robust individualslikely to be found in deployments in the field, i.e. adults.

Thus the use of active adult wild animals is unavoidable.

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

Animals of a target species assessed as suitable for study will be brought in from the wild and held captive for a short period before their use in tests. This can be for as little as a day but may occasionally be longer. Animals are allowed to become familiar with an unarmed trap in its test setting(i.e. in a single entrance enclosure) placed with its enriched enclosure.

The trap is then armed and observed continuously for the duration of the trial. The animal will be allowed to enter the trap of its own accord and once the trap has been triggered, the observer will notethe success of the trap strike throughout the specified trap testing period. If the animal has not becomeirreversibly unconscious before the end of this specified period, it will be promptly euthanised. All euthanasia in this project will be using an approved humane methods.

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

Spring traps may kill instantaneously with successful strikes to the cranium or cervical vertebrae. Alternatively, less efficient strikes may kill within the specified period by other means (e.g. internal haemorrhage, asphyxiation, destruction of limbs or major organs) and in cases of a failed strike, the animal may suffer substantial pain and distress up to the end of the specified period. Animals injured such that they are considered unlikely to become irreversibly unconscious within the specified period (e.g. pinned within the trap) will be euthanised immediately. The trap testing standard currently complies with the Agreement on International Humane Trapping Standards (AIHTS) which specifies different periods for different species, from 45 seconds (stoat) to 300 seconds (a number of species). Animals still conscious at the specified time limit will be promptly euthanised, though most will suffer formuch shorter periods

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

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

Severe for all animals

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

- Killed

A retrospective assessment of these predicted harms will be due by 31 July 2028

The PPL holder will be required to disclose:

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

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered 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 tests qualifying spring traps on target species to ensure they meet the required minimum humaneness standards before they can be listed on the Spring Trap Approval Order and become legalto use.

As trap testing requires wild animals to behave naturally as they move through the trap we are requiredto use live animal targets.

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

In the UK tests on species listed in international agreements (e.g. AIHTS) require the use of live wildanimal subjects.

Current policy from the regulator is to extend this principle to non AIHTS species, and all trap testingfor qualifying spring traps is required to be done on live wild animal subjects. Computer (simulation)modelling has been discussed and evaluated for this project and is currently used in Canada.
However, it was considered unsuitable for use in the UK.

Why were they not suitable?

Computer (simulation) modelling requires the use of very many live animal tests in order to parameterise and validate useful predictions. The development of a similar models for the UK would require gathering data from a minimum of 35 animals per species for each trap design and setting. Recently in the UK most new traps are novel designs each of which would require the extensive use ofanimals to produce a computer model, and fewer animals are used in a direct test of its humaneness. In addition we note the Canadian computer simulation program does not account for the behaviour of the animal, which is a critical component in the assessment of trap humaneness.

A retrospective assessment of replacement will be due by 31 July 2028

The PPL holder will be required to disclose**:**

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

Reduction

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

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

The number of animals will depend upon which traps are submitted in the next 5 years and

for whichtarget species. Estimates of numbers used across predecessor PPLs suggest an average of 3 trialsper year although this maybe on a range of species.

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

Data from other trap test authorities maybe used as evidence to support the regulators requirement.We always enquire after the existence of such evidence. This may either avoid the use of animals altogether or on occasion reduce the numbers used for UK regulators.

Trials are undertaken in a sequential manner. Trials can be stopped as soon as sufficient dataidentifies a fail or pass. This may be as few as 3 trials to fail and 10 trials to pass a trap.

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

Where trap testing is undertaken to a defined standard (e.g. AIHTS) there is little opportunity to optimise the use of animals. However we note that the strict regulatory use of this project ensures thefate of every animal tested returns inferential value in either supporting the success of a relatively humane trap or ensuring the failure of a relatively inhumane trap.

A retrospective assessment of reduction will be due by 31 July 2028

The PPL holder will be required to disclose:

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

Refinement

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

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

This trap testing is for regulatory purposes where the traps are assessed against AIHTS standardswhich is to standardised model and method of testing.

Before any animals undergo any trials, the traps are initially given a visual assessment by the PPLh (as expert in trap testing) to identify any flaws that may affect trap success. If any potential design flawis found, then a follow-up discussion would be held with the customer on how to proceed, in order to make the trap more humane before animals are used.

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

The regulator requires us to test traps on the wild target species specified.

Trap testing includes the natural behaviour of the target wild animal species as it moves through the trap we are required to use conscious and active wild animal species. Further wild caught animals have a different muscle, fat distribution and bone density and therefore the power of the trap requiredto meet the standards may be different for wild animals as it would need to be for laboratory bred animals.

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

Wild animals will only be acquired when all preparations for trials are in place to minimise the period animals are help in captivity before being used. Acquisition of wild animals will always form part of thestudy plan and be approved by AWERB. This will include ensuring that methods of capture and transport are undertaken in the most refined manner possible and that all additional licenses and authorities required for the work are in place.

Wild animals will used in a prompt manner ensuring that they are not required to be kept in captivity forlonger than is necessary. Details of the approach used and controls on working will be specified in thestudy plan and agreed with the AWERB.

The substantial differences between wild species, seasonal availability of some species in the wild, differing requirements of traps to be tested, and the length of time wild animals can be kept in captivitywill be discussed with the NVS and NACWO on study by study basis and then specified in each studyplan and agreed with the AWERB.

Husbandry and environmental enrichment requirements will be discussed with the NVS/NACWO/species specialist and specified in each study plan and agreed with the AWERB.

For all trials the animals will be under constant observation from when the trials begin. If an animal receives a 'foul' strike and it is apparent that the animal will not become irreversibly unconscious withinthe specified time, then the trial will be stopped and the animal euthanised promptly.

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

There is no published best practice as to undertake these regulatory tests. As one of the only providers of regulatory trap testing we are conscious that we would define best practice and constantlyseek challenge from AWERB as to how to refine research.

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

I work closely with the establishment NIO, NACWO, AWERB and 3Rs champion to ensure that I stayup to date with advances which may provide refinement in this project, and will be applied where possible.

A retrospective assessment of refinement will be due by 31 July 2028

The PPL holder will be required to disclose:

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

BASIC RESEARCH AND PRE-CLINICAL ANALYSIS OF THERAPIES FOR NEUROMUSCULAR DISEASES

Project duration

5 years 0 months

Project purpose

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

Key words

Muscular Dystrophy, Therapy

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

Retrospective assessment

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

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

This project aims to assist in the development of better treatments for patients affected by musculardystrophy and other genetic muscle wasting diseases. We intend to primarily study pathogenic, diagnostic and therapeutic approaches in muscular dystrophies.

A retrospective assessment of these aims will be due by 18 July 2028

The PPL holder will be required to disclose:

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

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 overriding purpose of this programme of work is to assist in the development and refinement of therapies for neuromuscular diseases. The broad scope of this license reflects a wide range of needs within the neuromuscular scientific community and the diverse interests of the investigators within our institution which focuses on translational research with the aim to better understand the mechanisms ofthe many types of neuromuscular disorders and ways to improve treatment and quality of life for affected patients.

The likely benefits include improved understanding of the pathological processes occurring in genetic neuromuscular diseases and accelerated development of new therapies to treat these diseases. Thesebenefits will translate into more effective treatment for patients with neuromuscular conditions, improving their quality of life and assisting them and their families in dealing with these devastating conditions. In the longer term this will feed through into reduced dependence on state-funded care.

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

At the end of the project we think we will have a clearer idea on the mechanism leading to muscledegeneration in patients with muscular dystrophies and will have identified potential therapeutic compounds for these diseases that can be moved into clinical trials.

We are especially interested in the process of muscle fibrosis and adipogenesis. We have developed aprotocol to isolate fibroadipogenic progenitor cells (FAPs) from fresh muscles of mice and humans. Ourstudies will provide new information on how these cells are activated, how they proliferate and which mechanisms are responsible for their differentiation into fat and fibrotic cells. We think we will be able to identify the exact molecular mechanisms activated in human FAPs, which will help us to identify specific drugs that could potentially block these mechanisms.

We are also interested in testing new treatments for muscular dystrophies, such as genetic therapies, anti-sense oligonucleotides (AONs), nanoparticles, small molecules, pro-regenerative and anti-fibrotic drugs. All these approaches could have an effect on cardiac and skeletal muscle function as well as onthe brain. Our research will also lead to a better understanding of the pharmacokinetic and pharmakodynamic characteristics of these compounds in muscular dystrophies. Ultimately we aim to identify drugs that can be tested in a clinical setting.

We aim to share all our results with the scientific community. In order to do so, we will present our workat conferences and will publish scientific manuscripts. Moreover, we will share the results with patientsand advocacy groups at joint meetings.

The freezing reaction in response to scruffing is very relevant to the human disease, as some patients with DMD suffer from high levels of anxiety and autistic spectrum disorders. We are interested in the cognitive dysfunction seen in this disease and would like to explore this freezing response in more detail to see if this is down to anxiety or to a physiological response due to the firm restraint or possiblya combination of the two. This

work may well identify possible pathways for therapeutic intervention.
Moreover, we would expect this research to be of benefit to the research community working with dystrophin-deficient mice in helping researchers to understand the stress response and how to avoid it.

In summary, we want to contribute to a better understanding of the pathology and pathomechanisms ofneuromuscular diseases. We will do so by investigating the development of fibrosis in muscular dystrophies and by studying cardiac and behavioural changes in a dystrophin-deficient mouse model of Duchenne muscular dystrophy (DMD). Furthermore, we aim to test novel therapeutic compounds fortheir safety and efficacy profiles in mouse models before moving them to a clinical phase.

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

The likely benefits include an improved understanding of the pathological processes occurring in genetic neuromuscular disease and the accelerated development of therapeutic strategies to treat these diseases. We expect that our work will ultimately benefit both patients, their families and otherresearchers.

The results from our studies will also help to improve the experimental design of future investigations,as we are planning to share our results with the scientific community through presentations and publications. The project should also be of benefit for researchers outside our field who work on fibrosis in liver, kidney or lung disease.

We expect that the results from our work will translate into more effective treatments for patients withneuromuscular conditions, improving their quality of life and allowing them and their families to better
deal with these devastating conditions. In the longer term this would also reduce the necessity forstate-funded care.

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

We are well positioned to collaborate with both academic and industrial partners worldwide.

Myself and team members regularly travel to conferences to present their findings, to share ideas andlearn from each other’s positive and negative experiences of techniques.

Though not always easy to publish, it is important to share where possible with colleagues andcollaborators unsuccessful approaches and negative data.

Within our institution, we have many colleagues with varying experience in animal models and socommunication locally is also an essential part of our work.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 model system of choice in this field due to the high degree of homology in muscle structure and function between rodents and humans. Many aspects of muscle function, such as the role of individual proteins and the structure of the neuromuscular junction, can be effectively modelledin lower vertebrates (such as the zebrafish), invertebrates or even in cell culture. However, when it comes to drug metabolism and efficacy, rodent models are required to be confident that effects can betranslated back to patients. The mouse is the most amenable rodent available, with a large repository of existing control data and well established standard procedures for the measurement of relevant parameters. As such, the mouse is the only model system with both the necessary and sufficient characteristics for the project to meet its aims.

The protocols described for this project are designed to be applied to adult animals. We are not goingto use pups for any kind of experiments. The protocols described here will use animal models of specific muscular dystrophies that are 2 months of age or older. This is based on the fact that many ofthe drugs we will evaluate are more effective when used at early stages of the disease. We will treat 2month old animals for 2 months, expecting to see significant differences between treated and non- treated animals for effective drugs.

On some occasions, animals will be aged in order to obtain a better model of a muscular dystrophy. This is for instance the case for the dystrophin-deficient mdx mouse, the well-established mouse model
for Duchenne muscular dystrophy. These mice do not have major structural changes in its muscles until it is older than 9 or 10 months of age. Exercise regimens using a treadmill or running wheel mayalso be used to worsen the phenotype in order to mimic human muscular dystrophy pathology.
Voluntary wheel running can already lead to more prominent histological features of muscular dystrophy. In the case of treadmill exercise, we will use publicly available standardized operating procedures generated by TREAT-NMD, a global network supporting translational research in neuromuscular diseases, and accepted by the scientific community. We will ensure to apply the treadmill protocols according to the age and severity of the mouse models by adjusting speed and running distances. If necessary, minor encouragement methods such as a touch on the tail may be used for mice that stop running. Dystrophic mice may require more recovery time from treadmill exercise than controls, but based on our experience and the experience of others both the wheel andthe treadmill exercise are well tolerated by mice.

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

Mice will be bred, aged to meet specific experimental needs and may be used in studies where variouspharmaceutical agents are tested.

New drugs (or control substances) expected to modify the disease process in genetic muscle diseaseswill be administered at different doses and by different routes, limited by LASA. We will monitor the mice treated to be sure that the administered drugs don’t affect their wellbeing by using different scoresheets. For new drug studies, where there is no previous data for the application in mice, small pilot studies will initially be carried out to investigate that the drug at the specified dose is well tolerated. In these specific cases, we will check the wellbeing of the mice more often than in the standard protocols, to be certain that we pick up any potential adverse effect.

We are interested in better understanding the behavioural phenotype in mice lacking dystrophin. Theseanimals can develop an adverse effect in response to handling and scruffing. After scruffing, mice appear distressed and breathless and may be relatively motionless for an hour or more. It has been observed by many groups working with mdx mice that ten seconds of scruffing, a commonly used method of restraint for carrying out e.g. ear-clipping or intraperitoneal injections, can lead to this freezing response. Therefore, scruffing of these mice will only be carried out when absolutely necessary and for as short as possible. In order to avoid scruffing, we will use other handling methods,such as tunnel handling, as much as possible.

For behavioural testing such as the Barnes maze and novel object recognition test, tunnel handling orhand cupping will be used. These are not harmful tests, as the mice are being given a safe area to explore whilst we observe their behaviour.
We will use some additional tests to measure muscle strength and function. These tests include the grip strength meter or treadmill exercise. These tests will be performed following standard protocols already published (TREAT-NMD website). Mice can be fatigued after the exercise and may need somerest, but we do not expect animals to have any harm from these studies.

We are also going to analyze the structure of the brain using magnetic resonance imaging, which is a non-invasive technique that could be carried out more than once to study changes in brain over time.

The number of times and precise timepoints in the lifespan of the mice when these studies are going to be carried out is not predefined and will be established based on the information collected from the behavioural tests performed. We may therefore obtain brain images at baseline and again after some months, if we observe changes in the clinical parameters. Mice may undergo up to four MRI scans withat least 2 months in between, therefore mice will fully recover before the next scan. Other imaging methods, if more appropriate for a particular research question, may be explored. Such methods may include computed tomography (CT), positron emission tomography (PET) or in vivo imaging system (IVIS).

For all these procedures, mice will be anaesthetized using a general anaesthetic such as isoflurane and if a contrast agent is to be used, for example gadolinium, the tail vein may be cannulated. During MR imaging, mice will be placed on a sled connected to a warming system in order to maintain the mouse within an appropriate ambient temperature with maintenance anaesthesia. An air pillow placedunder the chest will be used to monitor respiration rate and a temperature probe used to monitor bodytemperature. Following scanning, the mouse will be removed from the scanner and either placed on aheated mat to recover or culled humanely via a schedule 1 method.

Imaging modalities are safe, non-invasive methods conducted under maintenance anaesthesia with fullrecovery. In rare cases, death can occur during the imaging process, which is believed to be caused byadverse reaction to anaesthesia. In order to prevent this reaction, mice undergoing MRI will be monitored for respiration rate and temperature. Anaesthetics will be adjusted when necessary to prevent adverse reactions. Mice will be placed on a heat mat and returned to their cage once they are awake and able to move. Mice will be checked 24 hours after MR imaging to confirm that they are completely recovered.

At the end of studies, mice will be culled and tissues may be collected.

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

Adverse effects will vary between mouse strains though the majority of our mice will be on a mild severity threshold. Our previous experience has shown that most of these mice will be assessed retrospectively to be sub-threshold. For example, despite the fact that dystrophin-deficient mdx micedevelop muscular dystrophy and cardiomyopathy, they generally do not show any severe symptomsand have a normal life expectancy.

There are certain symptoms that each specific strain may develop. This is based on the scientificliterature and our personal experience.

For instance some strains can develop cardiac problems.Other adverse events the mice may experience:

A prolonged freezing reaction has been observed in mdx mice after scruffing

Temporary pain and discomfort in young animals if a tail biopsy is required for genotyping. There are general adverse events related with ageing in mice: dermatitis, greying of hair, hair loss and scruffy fur. Over-grooming may also be observed. If any of these are observed, we will increasemonitoring and if this behaviour exceeds the severity threshold, the mice will be culled.

Aged mice with neuromuscular diseases have a more advanced clinical stage characterized by increased fibrosis in skeletal muscles and sometimes in the heart. Therefore, these animals are proneto develop symptoms related with these histologic changes such as cardiomyopathy, difficulties in running when placed on the treadmill or reduced movement during daily activities.

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

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

In our wild type mice, the expected severities will usually be sub-threshold (usually over 50%), these will include the mice which do not receive any treatment or undergo MRI. They may be used for the milder behavioural studies included in this licence, such as the Barnes maze and novel object recognition test. The remaining wild type mice, up to 50%, may be expected to be participating in mild(45%) to moderate (5%) protocols due to drug administration, muscle function tests or MRI where themice will be culled as has been described on each protocol.

Severity of the mice included in this research may vary according to the genetic mutation. The symptoms of the mice that we use are mild or even less, not identifiable after visual inspection (sub- threshold severity almost 50% of the animals). However, with age mice can develop clearer and moresevere muscle weakness and have problems to walk on the treadmill and consequently reduce their daily activity. This being considered, animals will experience mild to moderate severity, 45% of the animals will suffer mild severity and 5% of the animals moderate severity.

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

- Killed

A retrospective assessment of these predicted harms will be due by 18 July 2028

The PPL holder will be required to disclose:

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

Replacement

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

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

We have considered using non-sentient alternatives, such as cell cultures, echinoderms (echinodermsare a type of marine invertebrate such as sea urchins or starfish), sponges or hydra to our mutant mouse models, but at present this is not possible as none of these animals models reproduce the same histological and functional pathologies as patients with the muscular diseases. The main non-
animal alternative to these studies is to use cells in culture. This is unsatisfactory for a number ofreasons. Most cell culture systems consist of a single population of cells which are identical.

Specifically, we work with myoblasts, a type of muscle resident stem cell, that are in charge of regenerating damaged muscle fibers. Myoblasts in culture can be induced to fuse and form myotubes,the fusion of several myoblasts represent the in culture counterpart of muscle fibers. However the limited size and structure of muscle fibres in culture do not recapitulate the anatomical and functional structure of muscle fibers. Muscle is a complex syncytial organ consisting of multiple cell types including myoblasts, muscle stem cells, fibroblasts, endothelial cells and neurons. The complete arrangement of all these cell types and systems is required to form functional muscle and cell culture systems or tissue preparations are not capable of delivering this at present. Furthermore, skeletal muscle has several interactions, notably with motor neurons via the neuromuscular junction, with the blood vessels and with cells of the immune system, which cannot be modelled in culture systems. Forthese reasons, cell culture models are only suitable for initial pre-clinical drug screening and basic scientific questions regarding myoblast and cardiomyocyte function (cardiomyocytes are the cells of the heart muscle). We aim to use cell cultures for these purposes wherever possible but cannot envisage completely replacing animal experiments at the present time.

Where possible we utilise the least sentient model organism. For our purposes this equates to the zebrafish, where it is possible to examine the consequences for muscle of various genetic manipulations and pharmacological interventions. However, the structure of fish muscle differs markedly from mammalian muscle, limiting the applicability of this model. Furthermore, this model isnot acceptable for bio-distribution and pharmacodynamic studies (assessing the biochemical and physiological effects of drugs) which are required before novel treatments can be applied in patients.Since this is our direct goal, there is no choice but to use a mammalian model system. These experiments can be undertaken using mouse models based on the close similarity between mouse and human muscle in terms of biochemistry, physiology and pathological states.

Mouse models for neuromuscular diseases are well developed, with a large body of control data and well-established standard assays for pathology and muscle function testing. From a regulatory point of view, experiments in mouse models are sufficient for progression of therapies through the pre-clinical phase to translation into clinical use. As such, we conclude that mouse models are both necessary andsufficient for the purposes that we have outlined.

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

We are developing good cell culture models to test some of our hypotheses. We have already well established 2D culture models of myoblasts or fibro-adipogenic precursor cells (2 types of cells presentin skeletal muscles) and we are using them to test new therapeutic compounds. Moreover, we are setting up 3D cell culture models. We do not yet have co-culture models that could be used in research. We are also starting collaborations to develop software that could predict response to a certain drug in vitro.

Why were they not suitable?

For many or our research questions a single population of cells in isolation is not an appropriate modelto address the complex pathomechanisms of muscular dystrophies. We are nevertheless progressing in finding new better cell culture-based methods to replace animals, for example we are working on 3D cultures of muscle cells and have started testing drugs in these new models. However, these culturemodels are developed with one cell type, and we are starting to co-culture two cell types. Skeletal muscles contain several cell types, including muscle fibers, vascular cells, nerves, and fibroblasts.
Each of these cells release factors that influence other cells present in the muscles. This complex network of cells is something that cannot yet be reproduced in vitro. If we want to test the efficacy of anew treatment, we can perform some preliminary experiments in vitro, but need to move in vivo and test it in murine models to confirm safety and efficacy. So far there is no cell culture model or softwarethat can reproduce the complexity of a living organism.

A retrospective assessment of replacement will be due by 18 July 2028

The PPL holder will be required to disclose:

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

Reduction

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

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

We estimated the number of animals based on our planned experiments over the next 5 years. Basedon our experience and the results from previous studies using new

treatments or behavioural modifications in the same mouse strains, the minimum number of animals per group was 10. This is the number that we need to achieve statistical significance and to reduce the risk of background variation as an explanation of the findings obtained. For example, when we test a new treatment in murine models, we need almost 40 animals (10 healthy, 10 non-treated affected mice, 10 treated at lower doses and 10 at higher doses). We add 10 animals that could be used if animals die during the procedure. To arrive at 50 animals per experimental group, we need to breed almost 150 to 200 animals. Moreover, we need a minimum number of animals to maintain the colony.

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

Each experiment is designed with great care, reference to previous experiments and what we havelearnt from our successes and mistakes.

Literature reviews, advice from colleagues and the use of websites such as:

https://www.nc3rs.org.uk/experimental-design

https://www.nc3rs.org.uk/experimental-design will be utilized when planning experiments, specifically https://www.nc3rs.org.uk/experimental-design-assistant-eda

https://www.nc3rs.org.uk/experimental-design-assistant-eda may be used for deciding on appropriate group sizes.

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

We will undertake the following measures

Efficient breeding: We will work closely with the colony manager/team to establish the most efficient breeding program. We will follow the recommendations included in the document “Efficient Breeding of Genetically Altered Animals” published by the Home Office. We will perform breeding calculations before we plan our experiments and intend to only produce the numbers of animals that we need. The number of breeding pairs to be set up varies for one strain to another. Control and youngmice have a mean of 7 to 10 pups, mdx and GAA mice have 5 to 7 pups and sarcoglycan-deficient mice have 4 to 8 pups.

Use of cell cultures whenever possible: we are setting up new 3D culture models that could substitute animal experimentation in some circumstances and therefore reduce the number of animalsneeded for research.

Small pilot studies may be used to test the robustness of protocols before embarking on studieswith larger numbers of mice.

Tissue archive: When experiments are completed and mice are culled, tissues which are considered useful for current or future experiments will be collected. The maximum output from everyanimal will be considered on a case by case basis

A retrospective assessment of reduction will be due by 18 July 2028

The PPL holder will be required to disclose:

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

Refinement

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

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

We will use mouse models of neuromuscular diseases that are diseases involving the nerves and the muscles characterized by progressive muscle weakness. Some of our mouse models, for example themdx mouse, generally have a mild phenotype and do not suffer any adverse effects with a near normallifespan.

Methods to investigate these diseases will include grip strength measurements, a non-invasive technique where mice are given the opportunity to hold on to a wire mesh and their tail is pulled backwith slight force in order to measure the strength of muscles.

Mice may undergo non-invasive behavioural testing for example using the Barnes maze test. In this test, the mice are given a safe area to explore and their behaviour and memory are monitored for finding a hidden box to hide in. This is preferential as it is less stressful for the mice than the significantly more challenging Morris water maze, where mice are placed in an opaque bath of waterand their ability to locate a submerged platform to rest on is measured.

Other methods will include MRI, where mice are anaesthetised prior to undergoing preparation andimaging. If no further information is required from the mouse, scanning will be performed under terminal anaesthesia.

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

I have long-standing experience working with zebrafish and where possible, we will use zebrafish models (under separate licence authority) rather than mice. However, the structure of fish muscle differs markedly from mammalian muscle. In addition, this model is not suitable for many of our studieswith new drugs such as bio-distribution and pharmacodynamic studies which are crucial to find out the optimal doses to see an effect of a drug in a new organism. Such studies are required before novel pharmacological compounds can be taken to clinical trials.

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

Mice are checked daily by animal care staff. Concerns will be raised as necessary with PIL holders,NACWOs, NVSs and myself.

Where possible, i.e. in all maintenance and experimental handling (except in protocol 4,

where we specifically plan to scruff mice to study the stress response), mice will undergo low stress handling by using cupping or tunnel handling. Following experimental procedures, mice will be monitored using appropriate score sheets until sufficient recovery is made. Animal care staff will be notified to make anyadditional checks required. In procedures that may cause pain, any suffering will be assessed and if appropriate soaked diet, extra bedding, and advice for appropriate analgesia will be sought from the NACWO or NVS.

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

RSPCA and LASA, 2015, Guiding Principles on Good Practice for Animal Welfare and Ethical ReviewBodies. A report by the RSPCA Research Animals Department and LASA Education, Training and
Ethics Section. (M. Jennings ed.)

From the RSPCA guidance, we do not expect our animals to experence severe suffering: https://science.rspca.org.uk/-/severe-suffering

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

Our group maintains close links with the animal facility and members of my research team regularly attend their meetings. We find the NC3Rs website an excellent resource and liaise with colleagues toshare ideas. We hold regular meetings to discuss our animal work and how we can optimise experiments whilst following the 3Rs. We also follow the Home Office guidance and attend regularly, webinars organized by them or by other organizations about novelties in this field.

A retrospective assessment of refinement will be due by 18 July 2028

The PPL holder will be required to disclose:

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

BIOCHEMISTRY, GENETICS, VIRULENCE AND DRUG ACTION AGAINST TRYPANOSOMES AND LEISHMANIA

Project duration

5 years 0 months

Project purpose

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

Key words

Parasite, Infection, Treatment, Diagnosis, Host-pathogen interaction

Animal types	Life stages
Mice	adult
Rats	adult
Hamsters (Syrian) (Mesocricetus auratus)	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To identify and validate potential drug targets and anti-parasitic compounds for further development and assess the risk of anti-parasitic drug resistance.

To identify virulence factors, possible diagnostic biomarkers and parasite effects on host metabolism and immunity.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 overarching long term aim of this project is to intervene against diseases caused by parasitic protozoa, including trypanosomes and leishmania, that cause devastating disease in the developing world. This will be achieved through studies that enable us to better understand how the host respondsto the parasites as well as testing new drugs against the parasites and other ways to prevent them growing in people or animals.

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

We anticipate multiple open access publications outlining findings on efficacy of new potential drugs foruse against trypanosomes and Leishmania. Other work will also be presented to key stakeholders in scientific meetings and available through online abstract publications.

Some compounds that we identify as possessing significant anti-parasite activity may proceed throughdevelopment to clinical trials. If safe and active, further development as drugs to use against these
diseases may proceed.

We will also generate key knowledge on factors that influence parasite infection in human and animalhosts which will be made available via publications to assist in developing new strategies to interveneagainst these diseases. This may include identification of both parasite and host factors (metabolic orimmunological) that can be exploited towards novel diagnostic tests or ways of limiting infection.

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

SHORT TERM BENEFITS

This project aims to (a) learn about the fundamental biological processes that underpin infection of mammals by trypanosomes and Leishmania and (b) to test interventions (e.g. drugs, vaccines or changes to metabolism) that can cure rodents of infection. This will provide important new understanding about parasite biology that will lead to the longer term benefits of this project and alsoprovide information useful to other scientists addressing related questions in their research. We will always publish our research findings in Open Access journals in order to assure findings are disseminated appropriately. These benefits will accrue across the five years of the license.

MID-TERM BENEFITS

This work aims to provide the groundwork to enable development of new therapies to treat humans and animals infected with these parasites, and also identify new diagnostic tools that can be used to determine whether people and animals are infected by the parasites. We also aim to learn about parasite dependency on host metabolism with a view to change host metabolism to weaken the parasite, given how parasites depend on host metabolism for their survival. These benefits will accrue across the five years of this license and continue beyond the end as results are translated. Scientific, health and veterinary workers translating information towards new tools will be direct beneficiaries overthis time frame.

LONG TERM BENEFITS

The long term aim of this work is to underpin the development of new ways to treat the diseases caused by parasitic trypanosomes and Leishmania that currently afflict millions of people and animals each year. In addition to our own work, we also anticipate that our findings related to host and parasitebiochemistry during infection will be of use to others, elsewhere, seeking new interventions against these diseases too . Some compounds we have tested are approaching points of clinical developmentelsewhere. One new drug for veterinary trypanosomiasis could reach the marketplace over the duration of this project. Additional work, by others and independent of this licence is required to take other products identified here forward for human or animal use.

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

All of our work will be published in open access publications giving full access to our findings to anyonewho may benefit from taking them forward in their own research. We will endeavour to include results from experiments that do not fulfil predictions in hypotheses to help make sure unnecessary repetition does not occur. We will also present data at international meetings in Parasitology and Drug Development and we will, where appropriate, proactively bring findings to the attention of key stakeholders, for example drug development companies, diagnostics companies and national and international agencies that could benefit from these findings e.g. should we identify processes that would be useful in diagnostic tests we would liaise with colleagues at the Foundation for Innovative New Diagnostics (FIND) and the World Health Organisation (WHO). For new drugs we would make certain the Drugs for Neglected Diseases initiative (DNDi) and companies actively engaged in bringingcompounds forward for neglected tropical diseases are aware.

Species and numbers of animals expected to be used

- Mice: 4600
- Rats: 100
- Hamsters (Syrian) (Mesocricetus auratus): 100

Predicted harms

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

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

Research outlined in this project will use several different animal models. Most work will involve mice. All species of trypanosome and Leishmania that we will study infect mice and induce infections, clinicalsigns of which overlap significantly with those seen in man or domestic animals. *Leishmania mexicana*, for example, create skin infections in both mouse and in man, while *Leishmania infantum* create whole body infections, particularly in the liver and spleen in mice and in man This can also manifest itself in similar host responses to the parasite at the metabolic and immunological levels. In the case of trypanosome infections, for *T. brucei* parasites that are infectious to man, blood and lymphatic system infection precedes infection of the central nervous system. The same is true for mice. For the veterinary trypanosomes too, generally similar infection dynamics are observed betweenmice and the cattle and other domestic livestock animals they infect.

Drug responses are generally similar between mice and man too (albeit with caveats of which we are aware and for which we will monitor).

Research using mice has had a long history of making important contributions to the understanding ofhuman biology and many valuable resources (such as gene-deficient mice) are available.

On occasion, very large numbers of trypanosome are required for biological characterisation in the laboratory and the rat model will be used since trypanosomes are infectious to rats and a single animalcan provide yields of parasite that would require far more mice to achieve the same yield.

There are also instances where leishmania infections can produce the form that is pertinent to humaninfection and these forms are able to develop more rapidly in hamster models of disease and will be used for this purpose. Therefore, the models selected enable us to make scientific advances with maximum confidence of interpreting experimental outcomes in ways to most effectively support development of new drugs and other interventions in human and livestock health. Because immune systems are advanced in adults we will only work with adult rodents, mainly mice with some less frequent work requiring rats or hamsters.

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

Details vary depending on the different protocols used. Generally, however, rodents will be infected with trypanosome or Leishmania parasites (including genetically modified parasites in some instances). A single inoculation with parasites initiates an infection. Virulence will be determined through either measuring parasite numbers by counting in blood (by taking a small blood sample from the tail vein) or by *in vivo* imaging, under general anaesthesia for restraint purposes only, of bioluminescent signals produced by genetically modified parasites or by measuring lesions (cutaneousleishmaniasis). The response of the animal to infection may also be evaluated, for example by taking blood samples for biochemical and immunological analysis. In some protocols the parasites will be harvested from rodent blood in order to ascertain biochemical and molecular aspects of their makeup.

In many experiments, after parasite infections have been established, animals will be treated with chemicals known to kill parasites in *in vitro* culture conditions to assess whether these chemicals can cure the disease. Chemicals will be administered generally by intraperitoneal injection, but occasionallyby intravenous, sub-cutaneous or orally as determined by the physicochemical properties of the compounds. Administration may be on multiple days to reach a point where safe cure is achieved. *In vivo* imaging, under general anaesthesia, generally weekly for stage 2 trypanosome infections or twice weekly for leishmania or stage 1 trypanosome infections. Identification in blood or determination of lesion size may also be used to determine whether drugs do kill the parasites within the animal host.

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

Most of the animals used in the procedures proposed in this project show no outward signs of distressor suffering. Injections produce mild discomfort and some stress associated with handling. Most animals infected with trypanosomes will develop mild anaemia. In the stage 2 trypanosomiasis modelwith central nervous system involved

paresis may be evident. Paralysis has been noted in stage 2 trypanosome infections (an incidence of around one mouse in fifty). In the case of leishmania infections a swelling appears on the footpad, very rarely this may ulcerate (we have not had any incidence on our previous license). The lesion is painless in leishmaniasis. Anaesthesia is used for restraint where needed, for example during whole-body imaging, and recovery from anaesthesia occurs within minutes. Infections are monitored closely at least weekly by an experienced researcher and culled before any severe clinical signs occur (e.g. weight loss greater than 20%, continuous diarrhoea or unresponsive to provocation). Weight loss can occur over a period of several weeks but will not exceed 20% body-weight and in most cases is negligible. We do not expect animals to show abnormal behaviour because of drugs or other substances administered. Cutaneous leishmaniasis causes skin lesions at the site of injection that develop over several weeks, but these do not impede normal behaviour. Experiments can last from days to months, in the case of trypanosome infections, the infection itself can last up to 4 weeks followed by a “cured” period after treatment where the animals are monitored for infection relapse. **Expected severity categories and the proportion of animals in each category, per species.**

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

We do not expect that any procedure will exceed moderate severity and the majority of procedures performed under this license (>95%) will be of moderate severity in mice. We consider infection to induce moderate suffering. The 5% of cases that are mild are those mice used in drug toxicity testingwithout infection (or in cases where an infection has not established). 100% of rat infections will involve infection and thus be at moderate severity.

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

- Killed

Replacement

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

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

The main objective of our work is to understand and reduce the disease caused by protozoan parasites. To do this we need to understand the mechanisms that parasites use to survive and growand this includes necessary propagation of defined life-cycle stages through the mammalian host.

In some cases, parasite lines have recently been isolated from humans or animals and require adaptation to nutrient enriched laboratory culture medium. We continue to seek new ways to cultivateparasites in the lab and will always perform any work that can be successfully achieved through the culture medium route in this way. Currently some parasite species and individual strains, however, need to be continuously grown within an animal because laboratory culture medium capable of sustaining growth has not been developed yet.

In our Trypanosoma and Leishmania drug studies we often observe significant lethal effects in culturedparasites that don’t necessarily translate to a complex living mammal.

This can be attributed to drug availability in the host because parasites can be difficult to reach in these systems (for example Africantrypanosomes can invade the central nervous system which is protected by the blood brain barrier andLeishmania parasites can reside within difficult to reach granulomas that cannot be reproduced in culture systems). Drug assessment in the animal is the only way of evaluating whether a compound would reach and kill parasites in the mammalian host, as well as remain stable within the physiologically complex system of the mammalian host. There is currently much interest to better define the ways that existing and new drugs interact with the body in terms of whereabouts they accumulate and how long they remain stable so that we may more accurately predict which drugs are likely to be efficient in treating an infection within a mammal.

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

Culturing of parasites in medium and organoid systems capable of sustaining parasite growth.

Why were they not suitable?

While we can assess growth factors to a certain degree in the laboratory, this approach only allows investigation of cells in isolation and over a short period. In order to study the parasite interaction withvarious components of the host immune system and how this affects later disease progression we need to look at the host-parasite interaction as a whole within an animal.

Moreover, as the use of growing parasites in laboratory culture has increased, it has come to light that there are marked differences between strains grown in the lab and within an animal, with effects seen in, for example, harm caused to the host and drug sensitivity.

Since a large part of our work relates to drug treatment and virulence we need to assess these by infecting and treating animals where the dataproduced may be more relevant for future drug development for treatment of human disease.

Reduction

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

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

The number of animals estimated is based on the design of the experiments we wish to carry out and from past experience using the procedures in this application. We have discussed with a biostatisticianand also colleagues working on similar projects in other institutions the procedures used to enable calculation of sample sizes. We use the experimental design assistant (https://eda.nc3rs.org.uk) from NC3Rs for individual experiments following assessment of outcomes from any preliminary experimentsas outlined in each individual protocol.

We will always aspire to keep numbers of animal used at the lowest possible to obtain the

data required to allow us to fulfill our scientific objectives. For trypanosome and leishmania work, in assessing the activity of drugs against parasites compounds will be tested at the maximal tolerated dose which is first identified using any new compound in uninfected mice with an escalating dose protocol. It will only be necessary to determine a more precise ED50 value where that information is needed. For example, if we are testing the ability of parasites resistant to a drug that have been selected and resistance levels quantified in vitro, it is often sufficient to use a single discriminatory dosethat will kill wild type parasites in a mouse, such that failure to kill at that dose leads to the conclusion that the resistance phenotype quantified in vitro is retained in vivo. It is important to ascertain that resistance developed in vitro is retained in vivo since currently unknown physiological differences between the parasites cultivate in vitro and in vivo could prevent in vitro observations being replicatedin vivo.

When testing for new drugs, controls will include infected mice treated with a known effective trypanocidal/leishmanicdal drug (positive control) and also untreated mice (negative control). Control groups are also added in order to assess virulence of trypanosome/leishmania mutants where these are used. Controls are also added with non-bioluminescent parasites, or uninfected controls, in order to generate base-line imaging data where in vivo imaging protocols are used (and these protocols areused to minimise numbers of animals used in particular experiments where infections can be followedin the same animal over time

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

The experiments proposed in this application are already established and have been used on previouslicences. Experience and data from the previous work has allowed the refinement of protocols and the reduction of animals. This has included the use of whole body imaging to allow long term assessment of parasite burden (trypanosomes and leishmania). We have recently developed improved lab culture medium and drug testing systems for *Trypanosoma congolense* and continue to develop systems for cells that traditionally cannot be grown outside of an animal, to assure as much work as is possible canbe conducted without recourse to animal work. For drug testing we will only test compounds in animalswhere those compounds have suitable activity in the lab. For parasites that cannot be grown in the lab,this will extend to using parasites harvested from animals to pre-screen compounds in batches prior to consideration for work in animals. This is because we do not, yet, have continuous culture for all species or strains of trypanosomes, but can sustain some of these cells for up to one week outside a mammalian host and will use this as our pre-screening system until we have a suitable continuous culture system.

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

Non-invasive whole body imaging is used when possible. This allows long term assessment of parasitedistribution and the effects of compounds over the full course of infection. This approach reduces the number of animals compared to previous procedures where groups of animals are killed at different time points to assess parasite burden. We have recently established a novel imaging system for *T. congolense*.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative

care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

There are not yet models other than whole animals that enable precise understanding of the relationships between the host as a holistic physiological unit and the parasites. Nor are there models that can exactly predict the ability of a drug to interact with parasites that take into account all of the complexity of a mammalian system, i.e. a drug may kill a parasite in an in vitro culture system, but for efficacy in a disease situation the drug must retain stability within a mammalian host and also distributeto organs, tissues and cells where parasites may reside. Drugs must also be non-toxic to mammalian hosts. For these reasons it is necessary to use animals in order to perform experiments to progress our understanding of host-parasite relationships and also to ascertain the efficacy and safety of potential interventions.

Rodent models are well established and described for both Leishmania and Trypanosoma. Mice canbe used in most of the work. In addition, genetically altered mice are readily available for study of parasite virulence factors and immune responses. Rats are occasionally used where large parasite numbers are required for purification and biochemical analysis. This means that fewer rats could be used where a large number of mice would provide the same yield.

For Leishmania species such as *L. donovani* and *L. infantum* a good mouse model does not exist forall required analyses; and many studies have used a hamster model for these parasites. We will use the hamster model when necessary to achieve data comparable with those previous studies.

All personal licence holders working under this licence are trained to a high standard to minimise animal suffering. Newly trained personnel will be assisted by more senior staff members to ensure thatthis standard is maintained. For PK analysis we minimise samples required from individual mice by splitting into two groups and taking samples from mice in alternate groups (thus minimising blood letting from individual mice).

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

Mammals are the natural hosts for trypanosomes and leishmania and metabolism and immune responses are sufficiently conserved between rodents, domestic livestock animals and humans to provide meaningful comparisons. Parasite-host interaction as well as response to drug treatment fromboth host and parasite is of utmost importance, therefore mammals must be used.

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

All efforts will be in place to minimise discomfort for rodents. Mice are provided with nesting material and "fun" tunnels. Animals are generally group-housed, although males are monitored regularly to assure they do not engage in violence. Score sheets are used to follow body condition, weight and clinical score. In drug treatment tests, mice are

reviewed every day and if parasitaemia is noted as a relapse it is concluded the treatment failed and animals are euthanised by schedule 1 method. Whole body imaging is done on a heated stage and heat mats are available for use after imaging. We will alsoinvestigate whether using an analgesic e.g. oral paracetamol given as infections proceed can ameliorate clinical signs.

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

Latest guidance found at https://www.nc3rs.org.uk/ provides a resource on protocols, publications,guidelines, videos to guide best practise. The NTCO of biological services updates on new recommendations too. To report results we follow ARRIVE2 guidelines. Discussions with other scientists working in the same area also assures that improvements in local practices are quickly implemented.

In planning our research we follow PREPARE guidelines:

http://journals.sagepub.com/doi/full/10.1177/0023677217724823.

For frequency and volumes of administration we refer to numbers in: D. B. Morton *et al. Lab Anim.2001 Jan;35(1):1-41*. doi: 10.1258/0023677011911345, which reports the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement and also: Diehl, K-H et al. (2001) A good practice guide to the administration of substances and removal of blood, including routes and volumes. J Appl. Toxicol 21, 15-23. https://doi.org/10.1002/jat.727

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

Animals are housed in a designated building, run by staff who host regular meetings to inform and discuss advances in the 3Rs. They also send regular emails providing links to the latest news from theNC3Rs.

CALCIUM ALTERING MECHANISMS IN MALIGNANT HYPERTHERMIA

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Malignant Hyperthermia, Calcium, Muscle, Anaesthetics, Exertional Heat Illness

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 notrequired.

Objectives and benefits

Description 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 mechanisms causing human malignant hyperthermia (MH), and also exertional heatillness (EHI) (as it has clinical overlap with MH), and their functional consequences.

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

Why is it important to undertake this work?

Malignant hyperthermia (MH) can be fatal or cause significant morbidity, and is caused by exposure to volatile anaesthetics and certain neuromuscular blockers. Underlying changes in calcium ion regulationare important in MH. The only MH drug treatment is dantrolene, though it has limitations in its use.

Further study is important to better understand the causes of MH. This includes the events upstream and downstream of the calcium ion changes, and the mechanism of dantrolene and other potential therapeutic drugs. Understanding the basic mechanisms of disease are critical to be able to find bettertargets for treatment, and those that could

be important in prevention. Although MH and EHI are acuteconditions, there is also the potential for underlying myopathy that would benefit from better understanding and drug treatment. The genetic causes are not fully understood, and the relationship between genotype and phenotype is unclear. Understanding the genetic contribution to MH and EHI will aid development of genetic tests to identify potentially susceptible individuals to prior to an event.

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

The research findings will generate new information that will be available to the scientific communitythrough publication (peer-reviewed journals, conferences, seminars).

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

Scientists and medical doctors in the MH community will benefit in the short term through better understanding of the proteins/pathways involved in MH e.g., Ca2+ handling, mitochondrial dysfunction,development of a myopathy. The research data allows further hypothesis development and testing for the molecular, biochemical, and physiological events involved.

Scientists in the wider scientific community will also benefit as many of the same proteins (or theirsimilar protein isoforms) and same pathways are involved in other diseases e.g. other muscle conditions, heat stroke, heart (dysrhythmias and cardiomyopathy), hypertension and neurologic disorders (stroke and neurodegeneration), and ageing.

Scientists and medical doctors in the longer-term will benefit, as this may lead to clinical trials for betterand more specific targeted therapies/diagnostics in individuals with abnormalities of Ca2+ homeostasis.

Patients will ultimately benefit with the development of new drugs, and the development of animproved genetic diagnostic capability so reducing/replacing the current invasive test.

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

We will collaborate with others and share results and data, including negative data. This will include through publication of the research work (positive and negative), but also attendance at conferencesand meetings to share new knowledge.

Species and numbers of animals expected to be used

- Mice: A number of different relevant GM mice lines will be used, and some interbred. Overall amaximum number of 7200 is estimated.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 genetically altered mouse models that exactly mimic the human condition of MH and can show an MH-like phenotype in response to heat or exercise. During an MH reaction the body temperatures rises to a dangerous level, and patients have high carbon dioxide and breathing becomes rapid and shallow. In addition the heart rate can become irregular and fast, and the muscles can spasm and become rigid. If there is no intervention this can cause shutdown of organ systems anddeath.

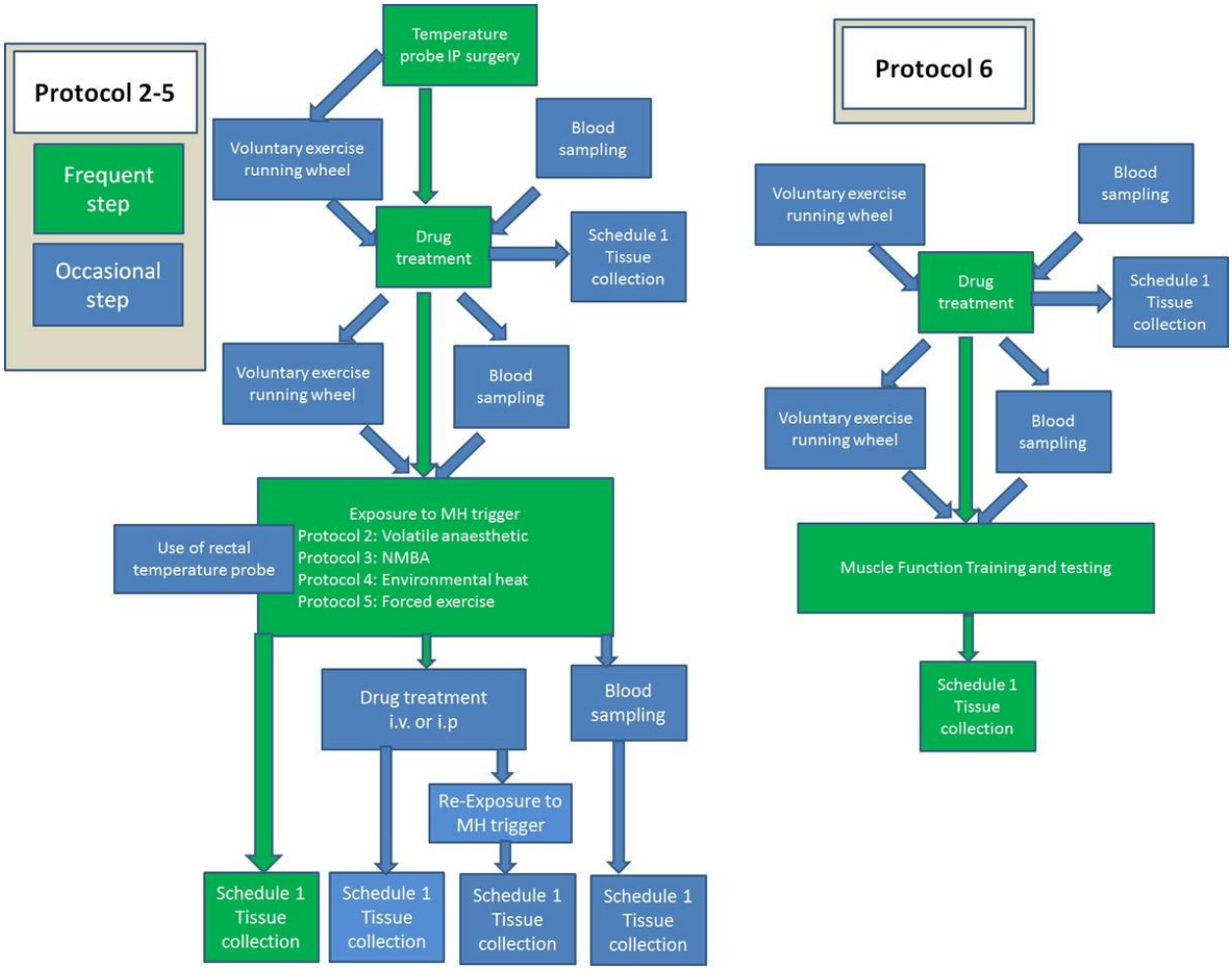
An MH reaction can occur in both adults and children upon the exposure to triggers e.g., volatile anaesthetic used during surgical operation. Therefore mouse models may use both adult and juvenileanimals. Breeding of genetically modified animals will generate all lifestages.

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

Animals will be mated to generate the correct gene changes in offspring to compare normal andgenetically altered mice **(Protocol 1)**.

Animals can be killed for collection of tissues for ex vivo functional studies e.g. study of muscle twitch after exposure to anaesthetic, e.g. isolation of cells for culture in vitro to study calcium ion changes dueto different substance treatment.

For the experimental Protocols, the figure below shows the key steps experienced:



In **Protocols 2-5** the animals will be exposed to one of the triggering agents that can cause an MH reaction in GAA. Monitoring core body temperature identifies the initial signs of the MH reaction, an increased body temperature. This will most frequently be done by surgical placement of a temperaturemeasuring probe intraperitoneal (IP) under anaesthetic. Less frequently a rectal thermometer will be used for those animals exposed to the anaesthetic and/or NMBA trigger that are killed under anaesthetic. A rectal thermometer with restrainer will infrequently be used on the increased environmental protocol.

For those that do not have an MH reaction or recover from an MH reaction, they may be re-exposed tothe triggering agent a single time.

Animals will also frequently be treated with drugs to test their effectiveness to prevent an MH reaction,or stop and recover from an MH reaction. Drugs can be administered through drinking water, but may involve injections, or gavage (if drugs cannot be injected). Limits are placed on frequency of drug administration (e.g. IP injection maximum twice daily for 10 days).

The animals may also undergo blood sampling procedures. Animals are killed at the end of these Protocols.

Protocol 2: MH trigger volatile anaesthesia. Experiments are limited for time under anaesthetic (120 mins). This may be interspersed with a recovery period (maximum 4 hours). These times are reducedfurther if they have the initial stages of an MH reaction.

Protocol 3: MH trigger volatile anaesthesisa and/or NMBA. Experiments are limited for time under anaesthetic (120 mins) with no-recovery. Whilst under anaesthesia dissection/surgery for placement ofelectrodes may occur. The time under anaesthesia is reduced further if they have the initial stages of an MH reaction.

Protocol 4: MH-like trigger increased environmental temperature. This involves placing the animals ina heated chamber (maximum of 40^0C) for 1 hour. This may be interspersed with a recovery period (maximum 4 hours). These times are reduced further if they have the initial stages of an MH reaction.

Protocol 5: MH-like trigger forced exercise. This involves training the animals initially to run on a treadmill for a maximum of 20 mins a day before the actual experiment. Experiments are time limited (45 mins). The experiment is a forced exercise assessment, as the animals are brushed with a "paintbrush" or a puff of air to encourage them to move if they stop. This may be interspersed with a recovery period (maximum 4 hours). These times is reduced further if they have the initial stages of anMH reaction.

The typical experience of the animals on **Protocols 2-5** is therefore surgery whilst under anaesthesia for temperature probe IP placement, followed by repeat drug treatment, followed by exposure to an MHtrigger once.

In **Protocol 6** animals will be assessed for any myopathy as a consequence of the genetic defect. Many of the mice will be allowed to age so that the myopathy develops, to a maximum of 2 years old. They will be tested for muscle strength (measuring how well the animal holds onto a grid (grip strength test), balance and motor coordination (how well the animal walks along a bean of various different thickness (balance beam), or how well

a mouse walks on a rotating rod (rotor rod). These three tests are standard phenotyping tests that have SOP's to minimise harm. For the grip strength the paws grip onto a mesh/bar and the animal is pulled steadily and gently by its tail. The maximum force and time prior to release is measured. This takes no more than 30 mins. For the balance beam the time is measured for the animal to cross a beam from a lighted end to a darkened chamber that they prefer, this takes no more than 15 mins. For the rotarod the ability of the animal to maintain its position on a rotating drum is measured. The animal must continuously walk to stop from falling off. The rotation ratecan increase if the initial rate is not found to differentiate between mouse groups. The maximum time expected is 60 mins. These animals can be acclimatised to the equipment by prior training for less than1 hour over several days.

Animals will also frequently be treated with drugs to test their effectiveness to prevent the myopathy. Drugs can be administered through drinking water, but may involve injections, or gavage (if drugs cannot be injected). Limits are placed on frequency of drug administration (e.g. IP injection maximumtwice daily for 10 days).

There may also be blood sampling occasions

On all the protocols, some animals will be assessed for their ability to undertake voluntary exercise, asrunning wheels can be placed in their home cage to record spontaneous running recorded over a 7- day period. This is not expected to have any associated harms.

Animals will usually be killed at the end of this protocol.

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

For animals used only for tissue collection there will be no expected impact or adverse effects.

Some of the animals may have an MH reaction- the early stages of increased core body temperatureraised to a level of a fever. This reaction can be stressful and painful.

Drug injection may cause transient distress and pain. Gavage is a difficult procedure that will cause stress to the animals but may also cause pain and damage to the oesophagus. This is time limited to less than 5 mins for a single dose. Multiple doses may be given over a 10-day period - maximum two doses a day, and this causes additional stress/pain/and discomfort from any tissue damage. Potentiallygavage itself and the drug may not be well tolerated and cause weight loss.

Where a drug is given through drinking water, measurement of an individuals drug intake will require animals to be single housed. Mice are social animals and single housing can cause additional stress. Itis expected this would be for no longer than 1 month.

A temperature probe implanted under anaesthetic may cause some limited pain and discomfortfollowing recovery from anaesthesia due to the wound.

If a rectal temperature probe is used for mice under anaesthetic this will not cause any pain or discomfort. For conscious animals a restrainer will be required to insert the rectal temperature probeand keep it in position during the heated chamber

experiment. This is because the restrainer with mouse is placed in the heated chamber. The amount of time the animal has a rectal probe can be stressful, and cause pain and discomfort as the animal moves around.

Mice may experience increased stress in an unusual environment - e.g., when grip strength/balancebeam/rotarod testing, when placed on a treadmill/in a heated chamber.

Experimental exposure to the treadmill is for a maximum of 45 mins per session, and can be stressful.If an MH reaction develops or there is a significant myopathy additional pain will be experienced.

Experimental exposure to a heated environment will cause stress for a total maximum time of 60 mins.If an MH reaction develops or there is a significant myopathy additional pain will be experienced.

Experimental rotarod - maximum expected time is 60 mins. Can induce stress and anxiety. Animalsmay also fall off causing distress/pain.

Experimental grip strength - less than 30 mins a day. Can induce stress and anxiety due to pulling onthe tail.

Experimental balance beam - less than 15 mins a day. Can induce stress and anxiety. The animalsmay also fall causing distress/pain.

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

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

Sub threshold: 50%

This is expected for wild type and GAA only used for tissue collection. It includes use of a runningwheel in any protocols is not expected to increase the severity as it is a voluntary exercise.

Mild: 25%

This is expected for wild type and GAA that have drug administration through drinking water or moreinvasive means that cause no cumulative effects. These animals may also be exposed to an MH trigger whilst under anaesthetic, but are killed under anaesthesia.

Moderate: 20%

This is expected for animals wild type and GAA that have surgery for IP placement of a temperature probe, and exposure to an MH trigger whilst conscious (heated environmental chamber and forced exercise). Some of the GAA animals will show the initial signs of an MH reaction whilst conscious, butthe reaction will not be allowed to progress. A proportion of animals will also have been treated with drugs (drinking water/injection/gavage): this is not expected to increase the overall severity rating.

Some animals may develop a myopathy that can cause discomfort/pain/distress. Our experience witha number of mouse lines is that the myopathy is most likely to have a subclinical level that will not cause a major departure from an animal's usual state of health and well-being. However, as it may bemore pronounced in aged animals a score sheet will be used to monitor the extent of any signs of myopathy, so limiting associated harms. Animals may also undergo muscle function testing (grip strength/balance beam/rotarod) that will cause more pain and distress. A moderate severity is expected for all animals that have repeated injections or gavage, muscle function testing (grip strength/balance beam/rotarod testing) if they are aged mice that have a myopathy.

Non-recovery: 5%

This is expected for animals on Protocols 2 and 3 where animals are only exposed to anaesthetic andare killed whilst anaesthetic.

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

- Killed
- Used in other projects

Replacement

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

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

There are no non-animal or non-protected animal models of this human disease. Primary cell lines (which are animal derived) and isolated muscle fibers (also animal derived) will be used for some experiments, but it is impossible to study whole animal physiology in vitro therefore live mice will beused for some experiments. In addition, sex differences cannot be effectively studied in vitro.

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

Immortalised cell lines e.g. HEK (human embryonic kidney cell) and C2C12 (mouse muscle cell). The HEK cell line was considered as they are a widely used in medical research including MH, as introducing and over-expressing proteins with genetic mutations in these cells is technically easy to do.The C2C12 cell line was considered as it has also been successfully used in MH research, and as a muscle cell line is more relevant than the HEK cells.

Patient derived cell lines (excess material from surgery). These were considered as they have specificgenetic mutations relevant to MH.

Why were they not suitable?

In vitro culture of immortalised cell lines e.g. HEK and C2C12 have been successful in establishing thepathogenic nature of some RYR1 rare variants, using over-expression constructs. But they have limitations for more physiologically relevant investigations: HEK cells contain limited components of theCa2+ handling proteins. C2C12 differentiate and

produce myotubes, however, the complex chromosomal ploidy makes this a poor tool for the genetic investigation of oligogenic malignant hyperthermia where dosage of genetic variants is important.

Patient derived cell lines can be used for in vitro myotube cell culture experiments. However, there arecomplex genetic differences between individuals that means the study of a more complex genetic disorder is very limited, as associations rather than causative genetic mutations will be identified.

In addition, myotubes derived from either immortalised or primary cells are not physiologically ideal.Their cell membrane is more depolarised (it has a different charge) than skeletal muscle in vivo.

Myotubes also have embryonic forms of some excitation contraction (EC) coupling components required for muscle contraction, and a limited lifespan that makes genetic manipulation in vitro achallenge.

Reduction

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

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

We have designed breeding strategies for maintenance of the different lines, and estimated number ofmice from these. For individual experiments animal number is based on previous data from a number of GA mouse lines of interest. We have information on the strength of the differences between differentcomparisons groups, and the variability.

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

The PREPARE guidelines

(https://norecopa.no/prepare) were used.NC3R's

ARRIVE guidelines (https://arriveguidelines.org/).

Statistical analysis also used G*power.

The PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines were developed to help planning of experiments. They aid implementation of the 3R’s (Replacement, Reduction, Refinement), and increase the reproducibility of research and testing. They cover three broad areas which determine the quality of the preparation for animal studies:Formulation of the study, Dialogue between scientists and the animal facility, and Methods.

The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) are a

checklist of recommendations to improve the reporting of research involving animals – maximising the quality andreliability of published research, and enabling others to better scrutinise, evaluate and reproduce it.
This also helps in the design of experiments as it helps ensuring the best data and detail is generated.The website has the guidelines and additional resources such a webinar, checklist and examples.

The G*power software package resource was used for estimating animal numbers needed for experiments using previous published and unpublished data, including pilot studies. This is importantto limit the number of animals used that are not necessary, but also to ensure sufficient animals are used in order to detect statistical differences that would otherwise be missed.

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

The breeding of animals will be closely monitored to ensure the minimum numbers of animals are used. Many of the GA lines have been previously used by the research team, therefore efficient breeding strategies are already established. We will also consult the animal facility dedicated staff todiscuss and update the breeding strategies.

Where possible, tissues samples for in vitro analysis will be obtained from animals culled at the end ofthe experimental protocols. These will be used in-house or shared with external researchers. Optimum dosage of some of the drugs have not been firmly established. In vitro experiments will initially show the drug can modify the target function before any in vivo work is undertaken. In vivo pilotstudies will be used to estimate optimum drug dosage.

Preliminary in vivo experiments with a small number of animals will also be used, for example in the heat experiments, to determine if heat exposure at a lower temperature is sufficient to generate an MH-like response, rather than using the highest environmental temperature.

Experimental variability will be minimised so reducing the number of mice required per group. This willbe done using appropriately trained staff and adhering to the S.O.P's, with an experimental protocol which includes: objective statements, experiment description, number and types of animals per group,output produced/measured (e.g. core temperature, animal behaviour), method of result analysis, and statistical tests. Experiments will be planned so they can be published in accordance with the NC3Rs' ARRIVE guidelines (https://arriveguidelines.org/).

Refinement

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

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

harm to the animals.

To minimise in vivo suffering the induction of MH due to volatile anaesthetic/NMBA will only occur whenanimals are maintained at sufficient anaesthetic depth to feel no pain. Animals will be continually monitored under anaesthetic and depth will be assessed by either tail/toe pinch response and/or heart rate/blood pressure changes. The time under balanced anaesthesia will be monitored and recorded.

As body temperature for mice is difficult to maintain under anaesthesia supplementary heat using heatpads will be provided to maintain body temperature throughout the procedure.

The assessment of core body temperature is important as its increase is the earliest measurable sign of an MH-like episode. Limits have been placed in the protocols to minimise suffering and distress whilst still gaining useful scientific data. If a core body temperature of 40^{o}C (conscious), or 40.5^{o}C (unconscious due to anaesthesia) is reached they will be killed immediately by Schedule 1. Previous data has shown that at this temperature the mice have not progressed to a full MH reaction where theyhave muscle rigidity so cannot move. Intervening before later clinical signs reduces the suffering and harm to the animal.

Drugs will be tested to see if they can stop/prevent the MH reaction. The drugs used may have been used on other animal models with available information on if there are side effects. Other drugs may not be as well characterised. A score sheet will be used to monitor for side effects of drugs given. Thisincludes measurement of drug intake through drinking water and weight measurement if given over a period of time. Animal appearance and behaviour will also be assessed. This will ensure intervention for a humane endpoint. If drugs are given during the initial stages of an MH reaction, these will be given before the mice have reached a temperature of 40^{o}C temperature. Again, if the core body temperature does reach 40^{o}C they will be killed immediately. Where a drug is given through drinking water, measurement of an individuals drug intake will require animals to be single housed. Mice are social animals and single housing can cause additional stress, however the potential harm caused by reduced liquid intake means single housing is an acceptable approach. This will be for minimal amountof time before animals used in additional steps of the Protocols and the experiment is completed. A **scoresheet for each Protocol** will be used daily whilst the animals are singly housed to identify and limit experienced severity.

As volatile anaesthetic is a trigger to induce an MH reaction, the mice will be unconscious when the body temperature rises. However if drug intervention does counter the reaction they may be allowed torecover from the anaesthetic. They will be monitored continuously for up to 4 hours and if the core body temperature does not return to the normal range they will be Schedule 1 killed.

Exposure to elevated heat/forced exercise can induce MH-like symptoms. Although animals cannot besedated, the continuous measurement of core body temperature allows detection and intervention during the early stages of an MH reaction. They will be monitored continuously for up to 4 hours and ifthe core body temperature does not return to the normal range they will be Schedule 1 killed.

All experiments using MH triggers have time limits, so limiting exposure to the trigger and the time the animals may experience an MH reaction. This reduces any suffering and stress experienced during thetrigger exposure. Furthermore this reduces the potential for further MH clinical signs to develop that could cause tissue damage e.g. myopathy resulting in further pain and distress during exposure to the trigger. If the animals are recovered there are time limits for the recovery period, and they are monitored continuously during this time for clinical signs of distress/pain/suffering. This will allow immediate intervention by Schedule 1 killing to limit the severity of experience.

As the forced exercise uses a treadmill, the animals may need to be encouraged to maintain running.Rather than an electrical shock, a soft paint brush/air puff system will be used, and no more than 3 times per minute. If this is not sufficient the animal is removed and observed for signs of distress andpain for up to one hour. The animal will not be returned to the treadmill.

The heat stressor experiments have a maximum limit of $40^{O}C$. Where there is no prior evaluation of temperature response lower temperatures will be tested, as they may be sufficient to generate an MH-like response. Previous experience with MH mouse models shows $40^{O}C$ may however sometimes be required as a heat stressor, as lower temperatures either failed to generate an MH-like reaction or thenumber of animals used was much higher as only a proportion had responded.

A few of the GM mouse models useful to study MH have an inherent increased mortality rate. These lines will be assessed twice daily, as this may help identify clinical signs or potential triggers causing this if not known. As the elevated mortality rate is expected to continue to increase with age, mice at risk will be used at the youngest age scientifically appropriate, to reduce risk of death. The mortality rates will be monitored continuously and revised, and advice sought from the NACWO and the NVS inregard to the actual mortality rate identified, and action to be taken. Advice will be sought from a HomeOffice Inspector if current estimated mortality rates are found to be exceeded. A few of the GM models are prone to develop a myopathy. These lines will be assessed twice daily,used at the youngest age scientifically possible, and advice will be sought from the NACWO and theNVS. Analgesics may be administered to less pain from the myopathy.

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

The main human gene that causes Malignant Hyperthermia (MH) is *RYR1*. Humans and mice have 3specialised *RYR* genes that are expressed in different tissues and have different functions, it is only *RYR1* mutations that cause MH. Lower life forms such as C.elegans and zebrafish do not have the same number of genes and tissue expression profile, and have substantial sequence divergence thatshows they would not be good model systems to use. There are a number of MH mice models available that have demonstrated that they are a good model system for investigating the human condition.

Many of the studies will be on tissue taken from killed mice. As MH is caused following exposure to anaesthetic drugs, some of the mice will be under anaesthetic and not conscious during experiments,some of which will be terminal. However some of the mice will be conscious when exposed to other triggering agents or functional assessment studies (e.g. increased environmental temperature/forced exercise/grip

strength). Animals need to be conscious to do physical exercise and perform the phenotyping tests. The animals also need to be conscious when exposed to increased environmentaltemperature. If anaesthetised the animals lose sufficient heat immediately prior to increased environmental temperature exposure due to their body weight to surface area ratio. This drop in core temperature could be protective and does not mimic the human situation, so would not be an appropriate model for the human condition.

Some of the mice will be recovered from an MH reaction from drug treatment. This is important to perform as this is the current method of patient treatment. **Score sheets for each Protocol** will identify clinical signs will be used in the Moderate Protocols to assess levels of distress/pain/suffering:

Score sheet for a moderate Protocol:

Total score =1: monitor twice a day
Total score =2: an increased level of monitoring. Provide additional support e.g. gel or soaked diet, analgesics. Seek advice from NACWO/NVS regarding further measures and timeframe for humane intervention
Total score =3: humane endpoint reached kill immediately

Appearance	**SCORE**
Coat slightly unkempt -partial piloerection	1
staring coat - marked piloerection	2
staring coat marked piloerection- no change after 48 hours	3
Mild bruising, slight redness	1
Bruising, redness, heat	2
Bruising, redness, heat no change after 48 hours	3
Body functions	
Diarrhoea up to 24 hours	2
Diarrhoea for 48 hours	3
body weight	
5-10% gain or loss	1
11-15% gain or loss	2
16-20% gain or loss	3
Breathing	
limited fast /slow/deep	1
intermittent level of fast /slow/deep	2
intermittent level of fast /slow/deep not improved after 24 hours	3
Behaviour	
hunched -transient	1
hunched -intermittently	2
hunched -intermittently not improved after 24 hours	3
subdued but responsive, normal if provoked, interacts with peers	1
Isolated, subdued when provoked, little peer interaction.	2
Isolated, subdued when provoked, little peer interaction - no change after 24 hours	3
Locomotion	
Slightly abnormal gait/posture	1
Markedly abnormal gait/posture	2
partial weakness of any limb -still mobile	1
weakness of any limb -partial reduced mobility	2
reduced mobility / reluctance to move	3

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

We are using continuous monitoring and recording of the key early symptom of MH (core body temperature) during exposure to the trigger. Additional monitoring of animal behaviour could identify additional signs during the exposure, as well as the recovery period, so allowing potentially earlier points of intervention for specific mice. The use of intraperitoneal (IP) placed probes monitoring core body temperature and additional physiological responses in real time could identify additional clinical signs that potentially limit the MH reaction and reduce further harm.

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

Use of neuromuscular blockers will be done under guidance of ASPA appendix H. Prior notification willbe given for intent to use and allow observation.

LASA guidelines will be followed for all administration of drugs.

Experiments will be planned using the PREPARE guidelines (https://norecopa.no/prepare) andARRIVE guidelines (https://arriveguidelines.org/).

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

Continued professional development during the project will include keeping up to date with the literature to be aware of improved methods, use of the NC3R website, and attending specific meetingsand conferences. Discussions will be had with NACWO, NCO, NVS in how to implement any changes.

CANCER AND ITS MICROENVIRONMENT: FROM MECHANISMS TO TRANSLATION

Project duration

5 years 0 months

Project purpose

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

Key words

Cancer, Tumour microenvironment, Cellular damage and senescence, Immune system, Ageing

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 notrequired.

Objectives and benefits

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

What's the aim of this project?

The central aim of this project is to investigate how damaged tissues are predisposed to progress to cancer. Evidence is accumulating that the tumour microenvironment (non-cancer cells around the tumour) is a crucial player in cancer initiation and progression, as it fuels tumour growth, and hence we are interested in the contribution of **(i) cellular types** (such as immune system and vascular cells; **(ii) cellular states** (such as a particular type damaged and dysfunctional cells that we know as “senescent” cells); **(iii) pathological process** (such as inflammation and fibrosis) and **(iv) ageing**, to cancer development. We will use this information to identify targetable biomarkers (alteredbiological molecules or factors) for the design, development and validation of novel detection and therapeutic tools.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 incidence rates rise significantly with age and has been attributed to the accumulation of mutations in particular cellular types, such as the cells of the surface of our organs as tissues, also known as epithelial cells, over time. Emerging evidence, however, suggests that the development of adamaged and inflammatory microenvironment also promotes the outgrowth of cells subjected to the activation of oncogenes (mutated genes that have the potential to cause cancer). Thus, it is likely that the combination of accumulated genetic alterations along with a tumour-promoting niche drives the development and outgrowth of early tumours. However, the environmental changes associated with early tumour development are incompletely understood.

To address the central aim of our project our laboratory largely focuses on preclinical models of lung cancer, while remains open to malignancies in other tissues to validate the universality of our findings. Lung cancer is the most common cause of cancer-related deaths worldwide. Although the efforts devoted to lung cancer research over the last decades have been formidable we have not yet deciphered the underlying mechanisms or processes promoting malignant transformation of some cellsinto cancer cells, and the specific microenvironmental conditions driving lung cancer initiation remain largely unknown. Consequently, the available tools to achieve lung cancer early diagnosis, as well as the existing therapeutic approaches, are not good enough to tackle this disease. Unfortunately, most ofthe cases are still diagnosed at advanced stages and ~85% of the patients die within a 5-year window from the first diagnosis.

In the proposed project, we will use a variety of mouse models to gain insight into how damaged and/or cancer-predisposed tissues influence tumour onset and cancer progression. More specifically, we will investigate the niche that fuels lung cancer onset and development, as well as its interplay withdifferent tissue stressors and pathological conditions, including:

1. **The tumour microenvironment (TME).** E.g. immune system and vascular cells.

2. **Cellular states.** E.g. Damaged cells (senescent cells).

3. **Chronic and inflammatory conditions upon injury/damage**. E.g. lung fibrosis

4. **Ageing.**

We will address how these processes and cellular states collectively, or independently, relate to cancerinitiation and progression. This information will allow us to gain insight into the fundamental processes and drivers leading to cancer development, and also to identify more specific targetable signalling pathways and altered molecules (biomarkers). Ultimately, we will use this information to design and validate novel tools for cancer early detection and more efficient therapeutic interventions. These may include new tracers and detection probes, and pharmacologically active compounds consisting in small molecules/inhibitors, activatable drugs (known as prodrugs), and nanotechnologies for cancer-targetedand tumour microenvironment-targeted therapies. This is therefore a high return translational project with commercialisation potential and we envisage that precision-targeted medicine is key to improving outcomes for patients with cancer.

Our ultimate goal following successful completion of this project is to immediately move and prioritiseour most promising innovative therapies into early-phase clinical trials to establish their safety and clinical efficacy.

What outputs do you think you will see at the end of this project?
Cancer is a common age-related disorder. Its connection with ageing stands from the fact that we are continuously submitted to constant and multiples sources of damage that, when accumulated or persistent in our tissues/organs, cannot be repaired. This damage results in mutations that can eventually be oncogenic (affecting genes that have the potential to cause cancer when become active). However, increasing evidence suggests that although cancer-promoting mutations are requiredfor tumour development they also need of a damaged, aged, and/or inflammatory “microenvironment” to initiate cancer. This environment is composed of multiple cellular types (e.g. immune system cells, vascular cells, structural cells, etc.) and connective tissue, which is like the concrete that glue the bricks (the cells) to make a wall (our tissues or organs). Importantly, this tumour-promoting microenvironment is currently incompletely and, in most of the cases, poorly understood. This knowledge is essential to develop more efficient and refined therapeutic strategies against cancer by targeting simultaneously (i) cancer cells and, also, (ii) the adjacent tissue to the tumour (tumour microenvironment or TME), but such an effective pharmacological combination therapies for precision medicine still remains a challenge.

Therefore, we expect to deliver our outputs at two levels:

1. **Fundamental Cancer Biology Research:**

a. Identification of crucial cellular types of the TME (adjacent tissue to the tumour) contributing tocancer initiation and progression. Analysis of the underlying molecular mechanisms.

b. Identification of key cellular states and processes contributing to cancer initiation and progression.Analysis of the underlying mechanistic insights.

c. Analysis of cancer-predisposed tissues upon injury/damage or inflammation. Analysis of theunderlying processes and molecular mechanisms.

d. Dissection of the contribution and impact of ageing to cancer initiation and progression.

2. **Preclinical Validation of Novel Detection and Therapeutic Strategies:**

e. .Development and validation of tracers and detection probes.

f. Development and validation of inhibitors and small molecules with therapeutic activity.

g. Development and validation of activatable drugs (prodrugs).

h. Development and validation of nantotechnologies and modular systems.

Our main outputs will be in form of scientific publications. We expect to gain insights into how damagedand/or aged tissues contribute to cancer initiation and progression, which is crucial for the development and validation of novel detection and therapeutic tools to tackle cancer. The principal route for dissemination will be through scientific literature and published on Open Access.

Communications at international conferences [e.g. AACR and EACR International Conference, orInternational Cell Senescence Association (ICSA) Conference], as well as external and internal seminars series, will enable us to communicate our findings in real

time with other scientists.
Publication of outcomes will be accompanied by press releases, which additionally has a wide networkof interactions with national and international journals. We will work proactively with the media to ensure that significant research findings reach the lay public. Although some discoveries may be subject to intellectual property protection and commercialisation we will prioritise public data sharing infree repositories to facilitate advances by other researchers.

In parallel to the publications and spread of our research, we also envisage the delivery of a translational toolkit consisting in novel detection probes and therapeutic tools for targeting the tumour microenvironment and cancer precision medicine. For the validation and delivery of this collection of novel therapeutics and detection tools for the management of cancer it is essential their validation in invivo models that recapitulate, as much as possible, the human pathological conditions. To ensure relevance (**1.** Fundamental cancer biology research) and translatability (**2.** Preclinical validation of novel detection and therapeutic strategies) of our findings we will use a variety of mouse models of lung cancer allowing us to manipulate the tumour microenvironment. These include models of transplantation of lung cancer cells (either under the skin or in the lungs) and inducible models of lung cancer (so-called genetically-engineered mouse models). We expect to complete the preclinical validation of our translational toolkit of therapeutics by the end of the 5-year project. This is therefore ahigh return translational project that, in the long-term, aims at prioritising our most efficient detection and therapeutic tools validated in mice to early phase clinical trials with patients.

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

The potential beneficiaries of this project are multiple reflecting the fact that it aims to deliver practicalknowledge at three fundamental levels:

1. **Molecular level: (i)** molecular biologists and **(ii)** oncologist consultants, which will have at hand new tools and datasets to detect alterations during cancer initiation resulting from the characterisationof molecular profiles of cancer precursor cells and tumour microenvironment cells; **(iii)** drug discoveryprogrammes, after the identification of new drugable targets at the origin and progression of lung cancer.

2. **Cellular level:** Our progress on better understanding the role and impact of cellular types and states operating in the tumour microenvironment will be useful for cell biologists (including immunologists) interested in cancer initiation and development, inflammation, cell damage (senescence) and plasticity, cancer precursor cells and cellular reprogramming. Satisfactory outputsmay result in the identification of key (targetable) cellular populations, including cells of the TME (adjacent tissue to the tumour), precancerous cells, and cancer cells.

3. **Pathological level:** On the pathological front, a better understanding of (lung) cancer development and how it relates to damaged lungs or pathological conditions (e.g. fibrosis) is crucial to develop novel tools for early detection and the design of more efficient therapeutic interventions. Satisfactory results attained within this proposal may therefore provide us with the grounds for the diversification of our own research towards the development of pharmacological interventions and newstrategies to target lung premalignant and cancer lesions, which in turn could eventually be of interest for translational researchers, engineers and trialists working in imaging, biomarkers and therapeutic approaches. In addition to cancer, damaged cells (senescent cells) are also associated with multiple chronic or aged-related pathologies making our proposal thus

of interest to a broader research community operating in basic and translational projects both in the UK and overseas. Ultimately, if successful, patients and the NHS would be most notable beneficiaries of our research.

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

We will maximize the outputs by a variety of action points:

1. During the last five years I have managed and been the holder of a **Home Office PPL licence**. The accumulated experience is giving me the opportunity to: **(i)** develop a preclinical programme including animal experimentation, **(ii)** acquire and consolidate the required personal and laboratory skills, and **(iii)** deliver significant research outputs in terms of scientific publications and presentationsin national and international conferences. Altogether, I will manage this new project supported by additional skills and with a more consolidated laboratory team with the appropriate expertise and training in mouse research, thereby helping us to increase the chances of achieving the aims and goals hereby stated.

2. After the transfer and rederivation of our mouse colonies to our new animal facility, we count nowwith additional state-of-the-art facilities for procedures and imaging, as well as the continuous supportof a strong team of experienced staff. These resources and specialised staff will ensure the practical, technical, and ethical feasibility of our experimental tasks.

3. Our research is truly multidisciplinary, as it encompasses from cancer fundamental biology to translational applications. I have therefore forged a potent network of collaborators, including biologists, chemists, physicists, engineers and clinicians. They all contribute in a very positive manner to provide our projects with a translational orientation and ensure their progression to the highest standards. Remarkably, our network of collaborators allowed us to identify other groups interested in our animal tissues to address their own experimental question and the support of their projects, thereby making them available for other scientists (e.g. groups focused on the study of cellular damagein multiple organs or other cancer stages, such as the invasion of other tissues in advanced cancer).

4. Our results will be submitted (and eventually published) to international open access scientificjournals, as requested by the University of Cambridge. These will include public or free online repositories. Our research articles largely contain positive results to support our hypotheses and central aims but also experimental results that fail to support our hypotheses or negative data, whenever possible. This also prevents duplication by ensuring that other laboratories do not spendresources, efforts and funding in ideas that have already been tested.

5. Our experimental activities will imply the generation and analysis of large-scale datasets. To maximize the outputs obtained from these complex datasets (e.g. genetic profiles), we collaborate andare supported by Core Units with experts in high-throughput analyses (genomic profiles, protein profiles, etc.), mathematical modelling, statisticians, and bioinformatics, who have the skills to developtheories explaining the results obtained in our experiments. Methods and datasets generated in these types of analyses (e.g. gene expresion profiles) will be publicly available as part of the relevant research articles enabling new collaborative projects.
6. We will spread our observations from early stages, for example via national and international conferences, symposia, and in internal and external events. It will allow us

to create new collaborationswith experts in research fields beyond our own scope, and also to increase the training and communication skills and network of our postdocs and students.

7. We will share our science with lay members via public engagement activities. This will allowpeople to learn about novel aspects of cancer, ageing and new therapies, and will provide the opportunity for a productive exchange of ideas between the public, patients and scientists.

Species and numbers of animals expected to be used

- Mice: 12195

Predicted harms

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

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

Mice are well used in cancer research and there are many genetic models that allow us to understandcancer, which is not feasible in other models. As mammalians, they are much closer evolutionarily to human disease compared to non-mammalian model organisms, making the protocols able to better recapitulate human disease, and the results gained more translatable into early-phase clinical trials that could improve patient outcome. Mouse tissue structure are much more comparable to humans than in other model systems. Zebrafish for example do not have lungs and so studying lung tumours and their microenvironment are more accurate and reliable in mouse models. Cancer, particularly the types that we focus on, is a disease of adulthood and old age, which is why we need to use some adultand aged mice in our research to understand these important interactions that affect human cancer incidence, progression and responsiveness to treatment.

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

We use various methods of inducing cancer in our mice for our research. Most of our experiments willuse either genetically modified animals to induce models of cancer in a highly optimised and refined manner.

To address our research questions, animals will typically undergo one (or a combination) of three maintypes of experiments:

1. **Tumour initiation:** Animals can have tumour-promoting genes activated in the tissues of interest at the time of interest, through exposure to modified viruses that activate inducible genetic alterations in the mice (e.g. activation of oncogenes, which are genes that cause cancer). Reagents altering cancer genes can be provided via the diet and supplemented with sweeteners to improve feeding behaviour. Alternatively, tumours can be formed through injection of carcinogens (cancer-causing compounds), or DNA containing tumour-promoting genes. In addition, tumour cells may be manipulated in a plate and then be engrafted into the mice, either subcutaneously (under the skin) or transplanted in the tissue where they usually grow (e.g. lungs). These mice are then monitored over time for tumour induction and growth through labelling and non-invasive imaging techniques (e.g. X-rayscans). Mice can be exposed to therapeutic compounds

with translational relevance to change the rateof tumour growth and the components of the microenvironment (adjacent tissue to the tumour). Samples are collected at different times to capture how tissue transforms into a pro-tumour state and then how tumours form and grow, further modifying the tissue environment. Blood samples may be collected to identify potential markers that allow earlier and more reliable detection of tumours, potentially improving cancer detection in humans.

2. **Tissue damage:** Animals are exposed to tissue injuries and damage from a variety of environmental insults (e.g. chemical exposure, irradiation, infections, etc.). This is because we wish to understand how damage alters the tissue microenvironment, which can in turn promote inflammatory processes and tumour initiation or growth. Where possible, this will be carried out specifically on the target tissue (e.g. by providing a chemical or damaging agent directly into the lungs). Cells can be labelled to track changes in the tissue microenvironment, such as the immune response and healing. Subsequently, tissue samples will be collected following the humane killing of the mice at different stages of the damage protocol. A portion of mice may also undergo tumour initiation after tissue damage (methods stated above), thereby allowing us to better understand how damage can contributeto cancer.

3. **Ageing:** Animals will be occasionally aged before the tumour initiation stages mentioned above.This is because cancer is a prevalent disease in the adulthood and old age in humans (e.g. lung cancer, which is a marginal disease in children or young individuals). Therefore, we find important to understand what are the crucial changes in aged tissues that can create a good niche or microenvironment to promote cancer initiation and progression. E.g. changes in the cellular types present such as immune system cells or the accumulation of damaged cells (senescent cells). After a certain age (e.g. 12 months, considering than mice can live for more than 2 years), mice will be monitored closely and scored to ensure that adverse effects are limited and well controlled. At differentstages, mice may undergo tissue damage protocols or tumour initiation protocols and then be monitored over time (methods stated above). Since we are predominantly interested in early stages ofcancer, the majority of animals will be humanely killed before showing any signs of suffering or distress. Samples will be collected to investigate how the tumour microenvironment changes with age and can lead to tumour initiation.

For any of these typical experiments, we may regularly treat some animals with drugs that help usunderstand the processes that govern tumour initiation and progression. This will also allow us to validate novel detection and therapeutic tools and modalities.

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

Most of the mice in our protocols will experience tumour growth or moderate tissue damage, over the course of days to weeks. Typically, we are interested in the early stages of cancer, and the tissue microenvironment that leads to and promotes cancer growth, and so the majority of mice are humanelykilled before they develop adverse effects from tumour growth. Further, due to our ability to non- invasively image tumours, allowing us to following the tumour, most cases of clinical signs are avoided.Adverse effects of longer-term tumour growth include weight loss, shortness of breath, changes in the colour of the skin, accumulation of fluids in the abdominal cavity, or diarrhoea. Mice are checked twice a day, and when a mouse presents with any of these symptoms, they will be culled asap (15% weight loss is typically the limit). As the number of mice that develop a tumour burden that leads to clinical signs are low, the majority of adverse effects for the animals in

the project will come from the cancer initiation techniques themselves (e.g. delivery of cancer-initiating compounds, or surgery).

Animals may be aged in our protocols, such that they reach a maximum of 30 months of age. Potentialclinical signs of ageing and frailty that impair normal mouse behaviour, movement or feeding include the skin (e.g. dermatitis or alopecia), physical/musculoskeletal (e.g. tumour growth or lameness), digestive/urogenital (rectal/vaginal prolapse or malocclusions, meaning teeth too large for the mouth), respiratory (e.g. altered breathing rate) and discomfort (e.g. pain). We have developed End of Life criteria and a Score System for ageing colonies based on previous studies (Ullman-Cullere, Lab Anim Sci. 1999 Jun;49(3):319-23). Green scoring usually requires no actions, whereas Amber signs will be assessed by specialised animal facility staff (e.g veterinary surgeon). If treatment can be provided to alleviate these signs, or if the signs are providing minimal suffering or distress, then they may be allowed to be monitored and culled only if signs worsen. Mice presenting with red signs will be killed via a schedule 1 method.

The adverse effects presented here are based on our previous experience using these methods and genetically-altered animals. Our technicians are extensively trained on the techniques before carryingthem out on live animals and understand monitoring procedures to ensure the welfare of the animals.Generally, the genetic modifications are not expected to show adverse effects themselves.

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

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

Mice:

Sub-threshold 5%.

Mild 64%.

Moderate 30%.

Severe 1%.

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 haveconsidered and why they cannot be used for this purpose.

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

Cancer initiation and progression is a complex multi-step phenomenon that is modulated

by different causal stimuli (damage, activation of cancer-initiating genes, inflammation, etc.), the intrinsic propertiesof malignant precursor cells, and the surrounding tissue. Tissue microenvironment may involve numerous cellular types and states (e.g. immune cells, senescent cells, pluripotent cells, etc.). Such triggers, mechanisms, processes, and interactions, that lie at the origin of cancer cannot be modelled accurately in vitro (cell cultures) at present. While we are studying in vitro the interplay between cellulardamage, ageing and cancer we need to extrapolate and to confirm our results in an in vivo model, thereby reproducing more realistically the microenvironment and complexity of events that contribute tocancer in humans.

Rodents have already been widely used in cancer research and provide unique opportunities to dissectout the precise role of cellular damage and ageing in cancer and to implement the use of novel diagnostic and therapeutic tools. Importantly, mouse cancer models (such as oncogene-driven genetic models and chemically- induced models) have been shown to recapitulate accurately the human disease so they constitute an ideal platform to develop our aims and to reach the proposed goals.

A key component of this project is to improve the diagnosis and treatment of human cancers; therefore,it is crucial to test and validate novel tools in preclinical models that involve animals. This is an essential step before moving to future early-phase clinical trials.

While we cannot fully recapitulate human cancer outside of animal models, we are continually attempting to, at least partially, replace animal models as technologies develop. Experiments will be only performed in vivo with solid functional or correlative data from in vitro systems (cell cultures withcancer cells) or clinical samples.

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

We have considered the following alternatives for replacing animals in cancer research:

1. **Cells grown in vitro** (cell cultures in plates, also known as petri dishes), including co-cultures(mixes) of cell types.

2. **Cells grown as organoids** (three-dimensional cultures of cells), these include tissue slices and in vitro growth of cells using commercially available matrices (scaffolds for the cells, thereby mimickingthe tissue architecture), and bioprinting.

3. **Using clinical samples** (including biopsies) from cancer patients.

Why were they not suitable?

(1) Cells can only grow well in petri dishes if they become immortalised, this causes genetic changes that cause them to not act as they would in the environment of the body. Important components of the tumour microenvironment such as damaged cells (senescent cells), immune cells, vasculature, pluripotent cells do not grow as readily in a dish due to space limits in one dimension. As well as thesegrowth limitations, cell types can often no longer act the way they would in an organism because of the absence of the normal tissue microenvironment (adjacent cells or cellular “ecosystem”). Fibroblasts (structural cells) are a reliable cell type for growing in a culture dish, but this is a minuscule snapshot ofthe myriad of cell types we need to study. Focusing on these cell types, though useful, is limiting and does not provide the full information required to develop understanding of how cancers grow in an organism and manipulate their environment.

Co-culturing is useful as this can allow us to study the interactions between two cell types grown together, but this still does not consider the cross talk that occurs between multiple cell types, bothproximal (close) and distal (far) from the cells of interest, and so will not recapitulate these vital components with co-culture alone.

(2) **Organoids** are useful as they are a step closer to modelling a living organism, they can more accurately show a more 3D picture - for example with vasculature and immune cells interweaving between tumour cells. But organoids are frequently challenging to be developed for some tissues or tumour stages (e.g. in particular for the lung) and they also cannot currently fully recapitulate the complexity of whole living organisms (e.g. lack of high-fidelity of cellular types, limited maturation, atypical physiology and structure, or lack of compartmentalisation). We are following this research fieldclosely and will replace animal research where we can as new technologies develop and are tested and established.

(3) **Using clinical samples** from human cancer patient's ex vivo (out of the body) is again of significant use and will prove supportive to this project. The reason they cannot fully replace animalmodels is that both fixed and fresh samples provide only a snapshot of the disease. This is of particularsignificance for studying early cancers, as there are very few early-disease samples available (they aredifficult to get by biopsies as they are usually small and when available they commonly have to be use for diagnostic purposes rather than research). Also, the cancers we are focused on are often diagnosed until much later when symptoms present (e.g. lung cancer), this is when the samples would usually be obtained.

While these approaches are not suitable to fully replace animal models yet, we are using thesemethods to reduce the numbers of animals used to the minimum required.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 the second project license being held by the lab. We estimate the number of animals based on our usage during the tenure of the previous license. Also, due to our new aims and objectives of this project, some protocols may be used more or less than in the previous license due to changes of focus, this is considered too. We have experienced statisticians with whom we consult to determine ourexperimental cohort sizes and ensure our results obtained have enough statistical power to draw meaningful biological conclusions. Most of our estimated usage (~60%) comes from the breeding of genetically altered mice. Our breeding strategies are deemed optimal, but we keep our approaches under review.

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

To the extent possible, we will use in vitro cellular models and human tissue samples to limit thenumber of animals required for in vivo studies.
To reduce the numbers of animals in our experimental **protocols (5-11)**, we take the following steps:

Carry out pilot studies (4-6 mice per group) where to optimise the experimental design through identification of uncontrolled variables. This information can be discussed with local statisticiansto help us appropriately power our experiments and use the correct number of animals to draw statistical conclusions.

The experimental approaches described have been used extensively within our Department and Animal Facility, so we have the necessary expertise to develop the proposed research effectively. If necessary, we will consult statisticians based in our Institution. Additionally, we use online toolssuch as the NC3R's Experimental Design Assistant (EDA).

Where there is published experimental data, we will use that to estimate the numbers of animalsrequired for our procedures.

Power calculations are used to determine sample sizes. For most of the quantitative experiments, power analyses will be generally set by using a significance level of 5%, a power of80%, and a minimum practicable difference between groups of 20-25%.

We will pay special attention to controlling sources of variability related to the environment, animals, animal handlers and the experimental procedures. Examples include using randomisedexperimental designs, assigning animals at random to a treatment.

Blinded operators will perform procedures and subsequent data analyses where possible toavoid biases.

Where possible, we split tissue for different experimental outputs (e.g. using different lobes of thelung). This reduces the number of animals used to answer these hypotheses.

Subcutaneous injections of cancer cells are carried out on each flank of the mouse, halving thenumber of mice required to carry tumours.

We use a Computerised Tomography (CT) X-ray scanner for non-invasive imaging and monitoring of internal tumours in the same animals over time, reducing the number of animalsused.

In vivo imaging system (IVIS) allows an alternative non-invasive imaging of labelled-tumourgrowth over time in the same animal.

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

Cryopreservation is used to preserve important mouse strains when they are not required, reducing the holding of live animals for extended periods.

Genetically altered animals are maintained with homozygous genetic alterations (two identicalmodifications of a particular gene) to reduce the number of offspring mice having unwanted genotypes. We only have one genetic allele (KRas mutated, a well-known cancer-initiating oncogene) where this is not possible due to embryonic lethality.

We are utilising methods for producing genetic alterations without the need for breeding genetically altered (GA) animals at all. This includes viral administration of DNA & injections ofchemicals.

Offspring with unwanted genotypes can be used for in vitro experiments (using tissue outside ofthe animal), training, validation and pilot studies where possible.

We share our animal lines with other researchers and will place in international repositories, where appropriate, in order to reduce the number of animals used globally to derive these lines.We are part of local email lists where we can share mice locally that are unused. Further, we share tissues with other researchers to reduce wastage.

Refinement

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

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

We will use mice for this project, including genetically modified mice. Our fundamental research is focused on the mechanisms and processes that lie at the origin of cancer and, consequently, tumours will not usually reach advanced malignant stages, so most of the protocols are categorised as ‘moderate’. Most genetic alterations in our mice are inducible, meaning they do not lead to adverse effects until they are activated in our experimental protocols. As most of our expected animal usage comes from breeding genetically altered animals (~60%) developing tumours in an inducible manner rather than constitutive (chronic) manner, then this reduces suffering for many mice as they live healthymost of their lives. We have refined our approaches based on our previous experience, the experienceof our colleagues and collaborators, as well as published data on these models. We can effectively monitor tumour growth and have well established humane end points that result in the least suffering both in terms of intensity and time spent with adverse effects.

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

Mice are evolutionary the most useful form of vertebrates that can be used to study cancer biology andthey are the mammals in which the gene manipulation technology (transgene technology) is more developed and works and works in a more reliable manner. Mice are widely considered as the model organism of choice for studying human diseases (such as cancer and other chronic disorders), with whom they share 99% of their genes (Rosenthal and Brown, 2007). Less sentient animals can developcancer, but there are questions as to how well these organisms recapitulate human disease. The proposed genetically.altered and chemically-induced mouse models recapitulate accurately human (lung) cancer and the effects of the manipulation (or mutation) of tumour promoting proteins (e.g. Ras) and tumour suppression proteins (e.g. p53, p16/p19ARF or p21) (for review see Hynds et al., 2022; Wang et al., 2022). Processes such as cellular damage (senescence), the immune

system and the tumour microenvironment (adjacent tissue to the tumour) have been extensively studied in mice (for review see Gonzalez et al., 2018; Fane et al., 2020; Di Micco et al. Nat Rev Mol Cell Biol 2021), and can differ substantially in organisms other than mammals and, especially, in invertebrates. The types ofcancer that we are focusing on are mainly diseases of adulthood and old age, therefore it is essential that we use adult stage and aged animals. The important interactions of the microenvironment with cancer development, such as ageing, is important in human disease but poorly understood. Consequently, conventional wild type, genetically-altered and immunodeficient mouse models will beused.

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

From our experience we have made multiple refinements, amending our previous license to minimisewelfare costs to the animals. Some examples are listed below.

General refinements

Side effects from procedures will be monitored by regularly weighing animals, daily health checks, and the use of scoring sheets, as required. This will prevent animal suffering. Animals showing any signs of suffering will be immediately killed. This is called the Humane Endpoint. Weare not expecting animals undergoing procedures under this licence to experience suffering. If ananimal does begin to look unhealthy, we will monitor it more frequently and provide pain relief if needed. If it does not ameliorate the animal will be killed by a humane method.

Humane endpoints are well described and are continually updated according to our experienceand guidelines to promote animal welfare.

Home cages are enriched with tubes, bedding, chew sticks and is monitored according to the animal's wellbeing. For example, to mitigate for possible aggressive encounters between mice,we include extra enrichment, such as tubes, in the home cage.

Non-aversive handling techniques are utilised wherever possible, such as cupping and tunnelling.

When mice are ordered from an external supplier, we allow at least 7-14 days for the animals tohabituate into their new environment.

Our animals are mostly genotyped by TransnetYX providing quicker and more reliable results toavoid unwanted phenotypes and genetic backgrounds. The TransnetYX miniMUGA genetic report facilitates fewer generational mouse crosses to be required.

We always try to house animals in groups, unless strictly necessary due to experimental reasons,in order to improve their quality of life.

Claws are clipped where necessary to avoid excessive scratching.

Welfare scoring has been set up for mice with subcutaneous tumours (see **Protocol 7**), and we have develop scoring systems for animals over 15 months of age to minimise potential suffering and distress, which we have been established using End of Life criteria for ageing colonies: Based on Ullman-Cullere, Lab Anim Sci. 1999 Jun;49(3):319-23. Amber signs in such scoring systems may be assessed on a per mouse basis together

with the Named Animal Care & Welfare Officer (NACWO) and/or Named Veterinary Surgeon (NVS). If treatment can be providedto alleviate these signs, or if the signs are providing minimal suffering or distress, then they may
be allowed to be monitored and culled only if signs worsen. When using the above or a similarscore system mice presenting with red signs will be killed via a Schedule 1 method.

Experimental refinements

Most of our protocols use well established and extensively described methods, minimising uncertainty of how regulating agents and compounds affect animal wellbeing. Users are extensively trained in these protocols before carrying out techniques on live animals.

The choice of the application route of the regulating agent will generally be the one that causesthe minimum stress and discomfort on the mice. For example, using the intratracheal route to activate cancer genes means that these genetic alterations are only in the target tissue (in this case in the lungs).

In some experiments diet composition may be altered. We will use commercially available high-fat and low-fat diets, avoiding food restriction when possible.

The use of appropriate anaesthetics and analgesia, in the context of surgical procedures, areused according to best practice guidelines.

The majority of our genetically modified animal models are activatable and so are not affecting animal welfare outside of essential experimental contexts. For instance, to regulate the expression of a lung cancer initiating oncogene (KRasG12V) we will provide the inducer (viral recombinase or AdenoCRE) by intratracheal administration, which will permit cancer onset in thetissue of interest (the lung).

Providing Nesquick (sweet substances) with Tamoxifen (a gene modulator) pellets has improvedthe feeding behaviour and limited weight loss from maladaptive eating.

Due to previous experience, cancer cell transplantation in the target tissue (orthotopic cell grafting) is now only carried out on 7 weeks old mice or older, weighing at least 18 g, which hasreduced adverse events from rapid tumour development such as weight loss.

To preserve the structure of the lung, mice are sometimes culled by perfusion fixation underterminal anaesthesia. We have found that, in some cases, animals can instead be culled bySchedule 1 before re-inflating the lung post-mortem to preserve the structure for histologicalanalysis.

Transplantation of lung cancer cells via the intratracheal route, rather than through tail vein injection, facilitates engraftment (take root) in the target tissue (lungs) and limits their spread inoff-target areas.

Non-invasive imaging techniques such as Computerised Tomography (CT) based on 3D X-rays have been developed to better assess internal tumour growth over time, such that we know whento increase monitoring of animals and minimise adverse events.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?
The scientific literature includes journals dedicated to the publication of protocols, which often represent best practice and include tips for refinements to techniques. We also are in

close communication with other labs that perform the same techniques as us, including discussing anyrefinements they have made and/or are currently making.
Unless otherwise specified, the work in this project will be undertaken in accordance with the principlesset out with in the following guidelines and published research:

Guidelines for the Welfare and Use of Animals in Cancer Research: British Journal of Cancer(2010) 102:1555-1577

Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T (2018) PREPARE: guidelines for planninganimal research and testing. Lab Animal 52(2): 135-141. doi:10.1177/0023677217724823.

Prescott MJ, Lidster K (2017) Improving quality of science through better animal welfare:theNC3Rs strategy. Lab Animal 46(4):152-156. doi:10.1038/laban.1217

ARRIVE Guidelines: https://arriveguidelines.org/

LASA Guidelines. Principles for Preparing for and Undertaking Aseptic Surgery (2010): https://www.lasa.co.uk/current_publications/

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

The UK has a national centre for the 3Rs (NC3Rs). They produce newsletters that we are subscribed to and organise seminars that we attend. Our technicians routinely meet with managers of the animal housing facility, including Named Animal Care & Welfare Officer (NACWO) and the Name Veterinary Surgeon (NVS), where they update on the NC3Rs guidelines and constantly review breeding strategies such that we are ensuring our approach is the most refined it can be. We receive regular updates via email and have our own 3Rs search tool with a multitude of resources for implementing and being updated on the 3Rs (https://www.ubs.admin.cam.ac.uk/3rs/3rs-search-tool). We also discusswith other groups doing similar research and have an open policy whereby new advances are disseminated with one another.

CELL DEATH AND INFLAMMATION IN TISSUE REPAIR AND CANCER - 2

Project duration

5 years 0 months

Project purpose

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

Key words

Cancer, Cell death, Inflammation, Anti-cancer immunity

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to define the mechanisms underlying cell death, inflammation and themaintenance of tissue equilibrium, and explain how alterations of these processes contribute to disease.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 research will assist the design of future clinical trials and the development of novel anti-cancer treatment combinations. We will identify new and more effective treatment combinations to treat solidtumours in humans.

Moreover, this work will benefit the basic research community by increasing our fundamentalknowledge of how our body defends itself from disease-causing microorganisms and cancer.

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

The intended output of this project is that the gain of knowledge on the involvement of the immune system in the development and/or treatment of cancer and diseases where the immune system fails torecognise the difference between self and external antigens (such as Crohn's Disease (CD), psoriasisor Rheumatoid Arthritis (RA), and on the generation of immunity to infection) is likely to result in the development of new therapies for cancer as well as chronic inflammatory, auto-immune and infectiousdiseases.

This project will lead to high impact factor scientific publications and presentations at scientific conferences that will directly influence the basic research field of immunity, inflammation and cancer byproviding mechanistic insight into the molecular pathways regulating key physiological functions such as cell death, cell survival and associated inflammation. This will help the research community to harness cell death mechanisms to improve anti-cancer therapies.

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

Our research will assist the design of future clinical trials and the development of novel anti-cancer treatment combinations. We will identify new and more effective treatment combinations to treat solidtumours in humans.

The findings of this project will provide fundamental insights for drug development and clinical trials, and will consequently impact the clinical field in the long term. Moreover, this work will benefit the basicresearch community by increasing our fundamental knowledge of how our body defends itself from pathogens and cancer.

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

We have several collaborations established with key groups in the cell death, cancer and immunity fields, which in turn provides us access to various genetically modified mice strains. This will help in creating novel mice strains by transferring mice to the respective research groups without having to create our own individual mouse colonies and also reduces the number of animals generated. Sharingdata and collaborative efforts between research groups will also help reduce duplication of data wherepossible, especially with in vivo work.

Species and numbers of animals expected to be used

- Mice: 27300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 our studies and 80-85% of the mice will be in the age range of 6 to 16

weeks for tumour studies, skin and intestinal inflammation experiments. The rationale is to mimic the human setting as close as possible to enable the development of effective treatments which can potentially beused in the clinic. The availability of various genetic modifications in mice provides a great global resource to study basic biology, cancer and other diseases and aid in development of future clinical trials.

15-20% of the mice will be employed in tumour development due to mutations which need a long time period for tumour growth and development (>16 weeks). In some studies, mice younger than 6 weeks,postnatal pups or embryos will be used to study the functional role of novel genes in development, inflammation models and cell death during the early stages.

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

- The animals will be used to develop tumour models for studying breast, lung and other cancertypes. Tumours may develop spontaneously due to genetic modifications or established by injecting tumour cells or cancer-causing agents in the organ of interest.

- These tumour models will be used to study the effectiveness of various drugs as single or combination treatment groups. These experiments will also provide an insight into the type of cell death occurring in these tumours, thereby resulting in tumour regression.

- Animals will also be employed in skin or intestinal inflammation studies to study the effects ofdrug combinations in inflammation and cell death.

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

Tumour burden will be limited to the minimum number of tumours required. Occasionally, tumours maybreak through the skin and may cause issues with movements of the animals.

Depending on the tumour model, some cancer cells may migrate to other organs from the primary tumour site (metastasis). Animals with metastatic tumours may exhibit symptoms such as weight loss, internal tumours or lymph nodes or compromised respiration. Animal suffering will be minimised by making every effort to keep the tumour models employed at the subclinical levels. Non-invasive imaging will play a key role in monitoring tumour growth and limiting adverse effects that may rise from extensive metastasis.

Other adverse effects associated to the experimental manipulations described in this project include risk of infection and minor pain or discomfort that will be dealt with using aseptic techniques, antibioticsand analgesics.

Toxicity may arise from the use of anticancer agents and radiation. These doses and modes of administration will be optimised in pilot studies with a small group of animals to obtain the maximum dose that can be tolerated by the animal. Data for dosing and treatment regimens can also be obtainedfrom published studies which eliminates the need of animals for optimisations. In order to minimise anypossible adverse effects, we will closely monitor animals undergoing experimental procedures and payattention to any signs of suffering.
Animals will be humanely killed at the end of each procedure. We have also indicated several guidelines that regulate when animals should be euthanised at an earlier time

point to minimisesuffering.

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

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

The genetic models will be mostly mild or subthreshold in severity (98%) and will include all stages ofthe animal, from neonates to adults. The key characteristic of mild procedures is that any pain or suffering experienced by an animal is, at worst, only slight or transitory and minor so that the animal returns to its normal state within a short period of time.

90% of the adult animals will develop tumours in the mammary glands and will be classified asexperiencing moderate severity due to tumour formation.

All the animals involved in tumour studies and skin or intestinal inflammation models will be moderatein severity (>98%) and will include animals older than 6 weeks.

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

- Killed

Replacement

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

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

The ability of tumours to grow depends on interactions with cells of the surrounding tissue. Therefore, itis essential to study tumour biology in animal models (*in vivo*), as only limited information can be obtained from culturing cancer cells in incubators (*in vitro*). Additionally, metastasis is a process that can only happen within the whole organism and no non-animal alternatives are available. However, weplan to continuously monitor the research in an attempt to replace sections of *in vivo* work with *ex vivo* or *in vitro* alternatives wherever possible.

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

We will be using 3D organoid cultures where we grow mouse-derived tumour organoids in the presence of immune cells. This will allow us to streamline our research and rapidly evaluate tumour-immune reactions *in vitro*.

We are also use the fruit fly as a model system to study certain aspects of tumour defence mechanisms. This will help us to focus our questions and refine our *in vivo* experiments in mice.

Why were they not suitable?

The absence of a functional immune system which will mimic tumour formation, progression and response to therapeutic intervention as observed in humans, makes the

other systems unsuitable for these studies. Since our research focus is on how cells communicate after they die and if they are ableto trigger an immune response, the use of animals is well-suited to answer these questions and designeffective therapeutic options for cancer treatments.

Reduction

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

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

- The proposed animal numbers are based on the usage from our previous project licence for the same protocols. We have requested for the minimum number of animals for each protocol for theduration of this project licence.

- Pilot studies will be carried out to assess tumour growth of re-implanted tumours and evaluatetheir metastatic potential, response to therapeutic interventions and in some cases in combination with radiotherapy. Initial treatment regimens will be tested on a small group of animals based on data available in publications or assessing treatment efficacy from *in vitro* models for novel substances.

- Breeding will require a large number of animals for establishment of new strains and based onratios of the required genotypes, the crosses will be set up. Majority of the protocols will utiliseanimals from commercial providers for experimental purposes and due to the use of inbred strains, lower animal numbers will be required.

- Experimental Design Assistant (EDA) from NC3Rs is a very useful tool to design experimental studies. It takes into account all sources of variability or conditions which may influence the outcome measure and this will determine the number of animals needed for the study to obtainmeaningful data.

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

Pilot studies will be carried out to test any new tumour models or toxicity testing if these methods havenot been established in the research group. Experimental Design Assistant (EDA) from NC3Rs is a very useful tool to design experimental studies. It utilises a step-wise approach in the experimental planning phase and provides feedback when needed to incorporate the correct animal numbers to generate statistically useful data, which further reduces number of animals employed in each study.

Literature reviews will help evaluate if similar experiments have already been done by other researchgroups to avoid duplication of data and thereby avoid unnecessary usage of animals for repetitive studies.

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

In some experiments, animals will only be used to generate primary tissue cell cultures that avoid invasive procedures and uses fewer mice. We will use well-characterised cancer models that minimises the requirement for pilot experiments to define animal numbers. We will employ statistical power calculations to help determine the most appropriate number of mice to be used to test an experimental hypothesis. The use of highly inbred, genetically altered animals will decrease the naturalvariation and improve signal to noise. Demand for genetically altered mice will be carefully assessed before breeding and crossing, and mouse numbers with unwanted genotypes will be kept to a minimum by optimising crossing designs. Moreover, we will make use of novel genetic engineering methodologies to generate one-step genetically modified cancer models. This will dramatically reduce the number of mice needed to breed until we obtain mice with knockout of specific genes.

The use of the proposed cancer models are designed to provide maximum information from the minimum number of animals compatible with statistical requirements. This will be achieved by (i) usingwell-characterised systems with predictable behaviour, (ii) obtaining multiple measurements of tumourdevelopment, (iii) analysing a number of parameters from individual 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

Cancer is analysed in the context of the host and, therefore, these studies need to be mainly performed in vivo. The reasons why mice are the best choice as cancer experimental models can be summarized as follow: (1) the physiology of cancer in mice is consistent with the human disease; (2) the need for working with genetic modification (knock out, transgenic models) to help understand the role played by individual genes in cancer treatment. In mice, many models are available, as are well- defined techniques for de novo production; (3) they are economic, easy to handle, produce multiple offspring and they have a very short gestation period as well as a functional survival time. (4) well-defined inbred mouse strains and mouse cancer models minimise variability in the responses betweenindividuals, thus ensuring fewer animals are required.

We will continuously refine our model such that we optimally power our experiments and use just the right numbers of animals to generate data that is reliable and robust, yet avoiding the need to repeat experiments beyond statistical significance. We will commit to working within the guidelines on tumourgrowth in animal models, as outlined by the NC3Rs, and in the guidelines for the welfare and use of animals (British Journal of Cancer. 2010 May 25; 102(11) 1555). All animal work will be performed in close collaboration with skilled animal technicians and trained research staff. Moreover, we are collaborating with leaders in the field so that we can use the respective mouse cancer models with utmost efficiency.

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

Our studies involve development of tumour models and assessing therapeutic interventions for tumourregression, followed by triggering the immune system to prevent tumour recurrences. This requires adult mice with a fully functional immune system and hence animals at embryonic or neonatal stages cannot be used for all our experimental purposes.

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

The use of the proposed cancer models are designed to provide maximum information from the minimum number of animals compatible with statistical requirements. This will be achieved by (i) usingwell-characterised systems with predictable behaviour, (ii) obtaining multiple measurements of tumourdevelopment, (iii) analysing a number of parameters from individual experiments.

We make every effort to ensure the optimal welfare of the mice by (a) only having experienced staff undertake the studies, (b) using non-invasive methods such as ultrasound, bioluminescence imaging to monitor tumour growth and response to therapy, (c) using statistical power analysis to ensure that optimal number of mice in each experiment is used, and (d) taking care to ensure that each experimentis analysed in depth (often by multiple researchers working on different parts of the project) and that the maximum amount of information is gathered.

Animals will be constantly monitored post procedures and therapeutic interventions will be administered with the minimal number of injections needed for an effective treatment option.

The use of genetic animal models which restrict expression of genes to a particular cell type reducesthe adverse effects systemically in an animal.

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

Tumour models published extensively include breast and lung cancer models which is our lab's primary focus and similar procedures will be carried out in our research group to maximise scientific output with minimal number of animals used along with refined procedures. We also follow guidelines outlined by NC3Rs and 'Guidelines for the welfare and use of animals in cancer research' published in2010.

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

Online resources provided by NC3Rs and other commercial services for in vivo work are valuablesources to learn new ways to improve experimental design, planning and execution with minimal adverse effects to the animals. Collaborative efforts between research groups will also provide information on refining techniques and adopting new procedures for similar experimental aims.

CELL MOVEMENTS AND CELL BEHAVIOURS DURING DEVELOPMENT AND IMMUNE RESPONSE

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cell migration, Development, Morphogenesis, Immune response, Signalling

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 notrequired.

Objectives and benefits

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

What's the aim of this project?

Cells move in response to attractant signals to form organs at the correct places, mediate healing of wounds, and during pathological conditions including cancer spreading. We use fish to model various cell movement processes and observe activities inside and around cells in real time in order to uncoverthe molecular underpinnings of how cells interpret changes in the environment and respond rapidly and appropriately.

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

Why is it important to undertake this work?

The project proposed in this application will contribute to a better understanding of how errors arise in cell movements and cell behaviours in human development, as well as during immune responses to injuries and infections. Our goal is to contribute research data that can be used in drug development orfor diagnostics in the health system for errors in developmental and immune response contexts, as well as during pathological contexts such as cancer.

In development, to form a tissue or organ properly requires both the specification of distinct cell typesand the coordination of cell movements in populations. Failures of these events can lead to developmental defects. We are therefore interested in studying the mechanisms underlying morphogenesis, how dynamically moving (migrating) cells coordinate to give rise to a reliable shape that an organism finally takes. Furthermore, how cells and tissues move from one location to anotherinside an animal is a research topic of significant biological and medical relevance; because this process not only plays a central role in shaping an organism during development, but also in immuneresponses and many pathologies including cancer.

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

The most significant short-term benefits of this research programme is a better understanding of the fundamental mechanisms that underlie collective cell motility and cell behaviours in development andwound immune response inside a normal embryo. Furthermore, our research will provide insights intomechanisms regulating the cell-cell and cell-microenvironment interactions that enable reliable cell migration in a whole organism. In the longer term, the benefits will be extended towards a better understanding of the mechanisms in pathological situations where the above mechanisms are derailed, e.g. developmental defects, errors in immune response, and cancer invasion. We have a track record of publishing our findings in high impact journals and so these studies are contributing towards the better understanding of these processes.

The specific benefits are listed below:

We will have identified proteins that are phosphorylated or ubiquitinated in response to changes occurring in the cellular and tissue environment as cells ensure that they migrate accurately during robust development. We will thus provide a list of “phosphotargets” or “ubiquitination targets” for clinical and pharmaceutical researchers. We will also identify genes that mediate the “phosphorylation” and “ubiquitination”, which may provide a mechanistic explanation to when reliable migration goes wrong incertain clinical conditions.

We will have a better understanding of the regulatory mechanisms by which immune cells reliablymigrate to sites of breach to enable wound-healing processes to occur promptly and at the right levelof activity intensity. This will enable to identify differences and similarities of regulated cell/tissue migration in development versus during immune wound healing response.

We will have a greater understanding of the intracellular, cellular and tissue scale processes (i.e.processes across biological scales) that regulate robust cell and tissue migration both during development and immune response, inside the whole organism. Zebrafish is the least sentient vertebrate that can inform us of such complex biological scale-bridging mechanisms that are likely conserved in humans.

We will have more knowledge on how cells integrate chemical and physical information from theircomplex natural environment inside a whole organism to regulate their own cellular behaviours, including cell signaling, cell motility, cell-cell interaction, cell-environment interaction.

We will disseminate knowledge generated from research performed under the PPL by publishingour work in peer-reviewed scientific journals and presenting at international conferences as well as research institutions.

Who or what will benefit from these outputs, and how?
Clinical research scientists will use our data to screen patients for genetic changes (mutations) ingenes that mediate phosphorylation and ubiquitination; and pharmaceutical industry researchers will develop our novel disease models and transgenic animals for drug (e.g. agonists and antagonists for phosphotargets or ubiquitination targets) screens.

The GA animals (both transgenics and mutants) we generate during this project will be useful to other zebrafish researchers. This is because even though our project focuses on two cell types (1. a type of sensory organ precursors and 2. macrophages), the genes involved are actually not exclusivein their action. Instead, they are expressed in additional cell types in the animal and have been implicated in broad biological processes. Thus, zebrafish researchers, irrespective of their area of interest, may be able to use the GA animals we generate.

In the longer term, our findings from this project will help to tease out specific regulators of reliablecell behaviour during changes in the cell/tissue microenvironment from the vast number of general regulators of normal cell locomotion. Targeting the adaptation-specific players that are specialised in coping with change is advantageous, because this will avoid undesirably disturbing normal cell behaviour. We anticipate that some of these players can serve as future drug targets for treatment of: errors in development and immune responses , as well as invasion of cancers in humans. Thus these knowledge will, like (1), benefit both clinical and pharmaceutical researchers.

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

Other than publishing our results in scientific journals, the data and analysis codes and methods we have generated will be made available online such that the whole scientific community can make useof them as research tools.

We will also communicate our findings through invited talks at conferences and research institutions, both virtually and in-person. In these ways we will be able to directly exchange information and optimise our approaches to a particular scientific goal. In addition to the collaborations I have initiatedduring my postdoctoral training abroad, I will actively set up additional collaborations with labs and institutions both in the UK and overseas. These will enable both ends to broaden their research horizons. Finally, we will send our lab members to visit other labs to learn new techniques (and vice versa), in order to broaden our technical skills.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 180,500

Predicted harms

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

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

Our research programme investigates complex cell-cell and cell-microenvironment interactions as dynamically moving cells (i) organize into tissues and thereby allow the organism to take its proper final shape during development and (ii) enable properly

regulated immune wound healing response. We cannot model the development of the posterior lateral line primordium (pLLP), an aquatic vertebrate-specific sensory organ system, in animals such as worms (e.g. Caenorhabditis elegans) or flies (e.g. Drosophila melanogaster). Similarly, the wound immune response in vertebrates is too complex to be adequately modelled in worms or flies. As for cultured cells or tissues in laboratory dishes, the current technology does not allow us to reconstitute a microenvironment with chemical andphysical properties that are sufficiently similar to the natural environment in which the cells and tissuesare migrating inside the whole organism.

Zebrafish embryos are optically translucent, ideal for directly visualising dynamic cell behaviours including how they rapidly respond to changes in their surroundings, e.g. change of neighbouring cells,differences in the properties of the migratory substrates, and other types of fluctuation in the chemical or physical migratory microenvironment. Many zebrafish genes, including those involved in embryonic development and immune response, are highly conserved with human genes. Therefore, through our work we can play a part in reducing research on mammalian model organisms.

For most of our studies we will use embryos <5dpf, younger than the regulated developmental stage.Older fish are used for maintenance and breeding.

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

The fish from regulated developmental stages we use will be for maintaining the fish stock and forbreeding to provide eggs and embryos to use in our experiments.

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

Animals grown beyond 5 days are used for breeding and maintenance, where we do not expect adverse effects through the normal maintenance of lines. The rest, i.e. experimental eggs/embryos, areall from unregulated stages of development (i.e. <5dpf). We do not expect weight loss, tumours or other gross abnormalities.
Expected severity categories and the proportion of animals in each category, per species.

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

Our adult fish are used for breeding and maintenance. We expect that the normal maintenance of lineswill fall under the mild level of severity. Eggs and embryos that are experimented on (all <5dpf) will be killed by Schedule 1 in accordance with guidelines.

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

- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered 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 examine complex cell-cell and cell-microenvironment interactions in the organism as cellsorganise into tissues and organs to enable the developing embryo to take its final proper shape. In particular, we focus on how the posterior lateral line primordium (pLLP) migrate and concurrently formsensory organ precursors at the right places during early development. Furthermore, we investigate how vertebrate immune cells migrate in a targeted and reliable manner to sites of breach to enable efficient wound healing. Finally, we compare different cell motility models in zebrafish to reveal common principles in how cells and tissues integrate complex signals from the microenvironment to result in a reliable migratory outcome, and how derailing of such processes may lead to defects in development, errors in immune response, as well as provide insights into what may have gone wrongin cancer invasion.

The pLLP is specific to aquatic vertebrates, expressing genes conserved with human hearing genes;as such it cannot be studied in less sentient animals such as worms or flies. Furthermore, with the current technology, analysis for cell behaviour underlying morphogenesis can still not be adequately replicated outside an organism (in vitro). Vertebrate immune response is also too complex to be studied in worms or flies. Cells grown in a laboratory culture dish are not able to sufficiently reconstitutethe context and environment where cells are naturally migrating in, and cannot replace whole organisms at this stage.

Zebrafish is an ideal model because its organs are a simpler version of that found in mammals such ashumans. By studying zebrafish, we are using an animal considered less conscious/aware than mammals to achieve our research goals and to answer our scientific questions.

We are thus trying to model multiple cell motility models in the context of a whole organism, which wecannot study in less complex animals, or in a tissue culture dish or in a computer simulation.

We have, however, started a pilot project using cultured cells and tissues in some of our analysis into the genetic and molecular interactions of proteins thought to be required for normal cell/tissue migration, as well as identifying novel players. Thus, our approach in the embryo is complementary to,and extends the in vitro approach. This will help replace some of our fish experiments.

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

In order to analyse our data generated from animal research in the most efficient way, we employbioinformatics, computational analysis and modelling.

We perform tissue culture experiments in parallel to animal research to study the less complex cellularinteractions of cell migration and related cellular processes during early development or immune response. Although cells/tissues cultured in a laboratory dish are useful, they are limited in that the chemical and physical environmental factors surrounding the migrating cells and tissues in the naturalsetting cannot be recreated yet.

Why were they not suitable?

Cellular processes that lead to vertebrate organ formation and body shaping are very complex. We cannot study the role that particular proteins play in the proper cell migration and concurrent development of the posterior lateral line primordium (pLLP) sensory organ

system, or what happens when they do not function properly except in the whole animal. Similarly, vertebrate immune responseis highly complex, and we study how zebrafish proteins (because they share similarity to those in humans) function to enable efficient recruitment of immune cells to the correct place to resolve injuriesdespite the complex migratory environment, similar to that what is seen in humans. These cannot be modelled adequately in cells on a dish or on a computer. Lastly, we study the cell-cell and cell-microenvironment interactions of an animal, thus these studies have to be done using whole animals and modelling can only give us suggestions of what to explore further.

Reduction

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

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

We have estimated the number of fish based on our experience on how many fish we need for breeding. The experiments we do are exclusive to early time points from unregulated developmental stages. We will thus not sacrifice larvae beyond 5 dpf for experiments. Fish from regulated developmental stages will be used for the generation of new genetic lines, breeding and maintenanceof stocks.

We are using a rigorous statistical approach to ensure that we only use the minimal number of animalsrequired to obtain a statistically significant result that is publishable. I have attended courses on codingand statistics. In addition, we will consult a statistician when needed.

We are also using cultured cells and tissues for some pilot studies of genetic interactions and screensfor molecules required during cell movements.

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

We use statistical design to determine the number of animals we need to use for each experiment toget a statistically significant result that is publishable. It is important to balance the need to have statistically significant results with the desire to reduce the number of animals we use as much as possible.

We are also using cultured cells and tissues for some pilot studies of molecular requirements and genetic interactions, instead of fish. In addition, bioinformatics and computer modelling that we performin our group will also contribute to narrowing down the experiments we need to perform in animals.
Furthermore, computational analysis of our research data will ensure that we make excellent use ofexperimental findings from animals, avoiding repeat experiments.

Finally, in addition to having completed a fully accredited PPL Module course (Royal Society of Biology) provided by Charles River, I will refer to the NC3R’s Experimental Design Assistant to improvemy experimental designs.

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

All fish grown beyond 5 days are for the generation of new fish lines. In addition, we will apply new techniques that allow us to sample fish that might be of scientific interest to us at the embryo stage. For example, by unregulated techniques such as fin clipping of embryos at <5 dpf where the injury isexpected to be fully healed by 5dpf. In this way, we can avoid having to wait until they have reachedsexual maturity to identify those of interest.

We will also use cultured cells and tissues in a laboratory dish instead of fish for some preliminarystudies to identify new proteins/new interactions between proteins involved in the normal directed migration of cells and tissues. Furthermore, we will use bioinformatics and mathematical modelling, i.e.employing computers to analyse scientific queries in silico to narrow down the appropriate direction(s) to explore in the whole animal. Finally, we will use computers to analyse our data to ensure that we make excellent use of all data we generated from our experiments. We will share these data and analysis methods (computer codes) online with the zebrafish community locally and internationally, which will contribute to avoiding duplication and thus minimise animal use.

Refinement

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

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

We are using the zebrafish to model multiple cell motility processes, (i) during early development of theposterior lateral line primordium (pLLP) sensory organ system and (ii) immune cell recruitment to sites of injury.

Because unlike mammals, zebrafish lay their eggs externally, we can simply collect them from their home tank without doing any harm to the females laying the eggs. The embryos are transparent whichallows us to gain much information by directly examining them under a microscope.

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

Our experiments exclusively employ animals at the immature, embryonic stage. Animals from moremature stages are for generation of new lines, breeding and maintenance of stock.

We are using the least sentient model animal that shows similar developmental, morphogenetic andimmune response processes as humans.

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

We will refer to methodologies described in published best practice guidance e.g. local AWERBguidelines to perform harm/benefit analysis to refine our methods and to use fewer animals in accordance with the 3Rs. We will also regularly review the literature, attend conferences and workshops to ensure that all procedures we perform are as refined as possible.

For example, advances in ‘reverse’ genetics now enable us to generate mutations in specific genes orestablish transgenes at specific places in the genome. We can thereby induce targeted mutations onlyin specified genes, and test for ‘rescue’ of a defect rather than generating a large number of randomly mutagenised fish to be screened for a specific mutation. This results in a substantial reduction in the number of animals needed to identify a specific mutation. In addition, we will generate transgenic lines that are required for answering our research question more specifically and noninvasively. For example, complementary to inducing a needle wound to locally recruit macrophages, we will engineer a photoactivatable transgenic line that is able to induce local macrophage recruitment by light (using a microscope laser) without the necessity of creating a wound. Laser intensity and area of illumination are more controllable and repeatable than a needle wound, thus allowing more robust data generation.These efforts will help to reduce the number of animals that have to be used to obtain valid data, as well as severity levels, in observance of the two of the 3R’s, reduction and refinement.

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

Local AWERB guidelines; PREPARE guidelines; Guidance on the Operation of the ASPA; Goodresearch practice guidelines from the Wellcome Trust; LASA and RSPCA guidelines.

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

I have very recently attended a fully accredited PPL module (Royal Society of Biology) provided byCharles River to learn about state of the art knowledge of the 3Rs.

In addition, I follow the information given on the websites of organisations involved setting policy and also in the advancement of the 3Rs (e.g. the National Centre for the Replacement, Refinement and Reduction of Animals in Research; Institutional guidelines, Home Office). Furthermore, I stay informedthrough active communication with our Fish Facility’s Named Animal Care & Welfare Officers (NACWOs), Named Information Officer (NIO) and Named Training and Competency Officer (NTCO).

Finally, I share research space with four long time senior fish labs, where by attending weekly jointgroup meetings with them, I ensure that I keep myself updated with advances in the 3Rs and new technologies that help implement these advances as well as align with the Home office guidelines.

CONSEQUENCES AND MECHANISMS OF RESPIRATORYEPITHELIAL AND IMMUNE PRIMING PRIOR TO AND FOLLOWING RESPIRATORY VIRAL INFECTION

Project duration

5 years 0 months

Project purpose

- Basic research

Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

respiratory virus infection, asthma, lung, local immune response, respiratory epithelial cell response

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 orscientific needs it's addressing.

What's the aim of this project?

The project aims to understand the immune and epithelial cell responses of the lining of the lung and nose (respiratory mucosa) that control (or enhance) viral load and inflammation, both prior to and aftera respiratory virus infection.

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

Why is it important to undertake this work?

Respiratory viral infections can cause a severe lung disease such as viral bronchiolitis in

infants and pneumonia in older children and adults. Furthermore, viral bronchiolitis in infancy can predispose to thedevelopment of asthma, and respiratory virus infections can trigger severe asthma attacks. There is noprevention or effective disease-modifying treatment available for bronchiolitis or virus-induced asthma attacks. Excessive inflammation and high amounts of virus (viral load) are thought to underlie both of these conditions. To find new approaches to prevention and therapy it is important to understand the early immune and epithelial cell responses to respiratory viruses in the lining of the lung and nose (respiratory mucosa) that control (or enhance) viral load and inflammation, and if and how the respiratory mucosa can be set up (primed) to develop or prevent such responses.

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

A better understanding the immune and epithelial mechanisms of the respiratory mucosa that lead toor control viral and asthmatic inflammation or control viral load, and their longevity will be a major scientific advance. This will enable the development of compounds that can prime the respiratory mucosa against viral and asthmatic inflammation. In the future such new compounds may become powerful treatments to prevent or reduce the severity of bronchiolitis and asthma attacks and may even prevent the development of asthma.

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

In the short term the scientific community, including the pharmaceutical industry, will benefit from outputs of this project, in particular from the identification of novel targets for prevention and therapy ofsevere respiratory viral infections. In the longer term, after the development of such targets into licenced therapies, patients at risk of or with severe respiratory infections of all age groups and peoplewith asthma will benefit directly. There will also be important economic and societal benefit through a reduced disease burden and lower associated costs (e.g. to the NHS, employers, families affected by severe respiratory viral infection) and through increased economic activity (e.g. for the pharmaceuticalindustry).

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

The findings from this work will be presented at scientific meetings, published in scientific journals, andalso disseminated via the press to the interested public. Once published the data obtained will be deposited according to FAIR principles and will be made available to other researchers for further analysis. Where possible unsuccessful approaches will be published and communicated on our website.

Species and numbers of animals expected to be used

- Mice: 7000

Predicted harms

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

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

Although the disease processes are not completely identical to those in man, mice offer the best available mammalian model system in which a variety of relevant immunological, genetic and molecular tools are available to study mucosal priming in respiratory viral infections and obtain proof ofboth concept and principle. Most of the work will be conducted in adult mice, for which most of the required models and methods are well established. In order to use the best model viral bronchiolitis in infants, neonatal mice will be used to validate important experimental observations from adult mice.

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

Typically, mice will undergo infection with a respiratory virus by intranasal inoculation under anaesthesia. Prior to or after infection the immune and/or epithelial response to infection may be altered by administration of substances or cells. Blood samples may be taken and lung function assessed. Most mice will only be exposed to one (~50%) or no (~40%) administration of substances. Less often (~10%), mice will have to undergo combinations of two of such procedures. The duration ofexperiments, including 7 days of acclimatisation, will typically not exceed 22 days. A small minority of mice may undergo bone marrow transplants or surgical removal of their spleen.

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

Most planned procedures will have no or only short lasting mild adverse effects. The wellbeing of micewill be closely monitored during experiments for clinical signs of distress which include reduced activity, reduced feeding, weight loss, hunched posture, ruffled fur and pallor. If any of these signs are presentor do not resolve, the animal will be humanely killed.

Many of the animals will be used in breeding programmes of GA (genetically altered) mice, for whichno adverse effects are expected.

Mice will be infected with respiratory viruses, typically a virus called respiratory syncytial virus (RSV), by applying droplets to the nose or the trachea under light anaesthesia. RSV infection of mice usually does not result in disease, but in some cases can cause transient weight loss from which mice recoverafter 2-3 days.

In the asthma model, mice will be sensitised to an allergen and receive allergen challenge to theairways by aerosol inhalation or application of droplets to the nose or the trachea under light anaesthesia. Allergen sensitisation and challenges usually do not cause disease.

Changes in lung function (without clinical disease) can be detected after RSV infection and in the asthma model. Lung function will be assessed in a chamber, in which the mouse can freely move, using minimal changes in chamber air pressure caused by breathing. Agents provoking short lasting deterioration of lung function will be aerosolised into the chamber, resulting in short lasting discomfort,as experienced by patients during similar lung function measurements.

In some mice lung function will also be measured invasively, with mice being mechanically ventilatedthrough a tube in their windpipe. This will only be done under deep surgical anaesthesia without recovery and mice will not experience pain or distress.

A small minority of mice will undergo irradiation or chemical conditioning for bone marrow

transplantwith transiently increased risk of infection.

At the end of each experiments the animals will be humanely killed.

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

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

Most animals will experience no or only mild transient adverse effects (80%). In a minority of animalstransient clinical signs of moderate severity will have to be tolerated (20%).

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

- Killed

Replacement

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

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

The work in animal models outlined here is an integral part of my research programme on epithelialand immune responses in the lung to respiratory viruses and allergen sensitisation.

There are currently no in-vitro systems available that allow us to study the complexity of cellular and molecular interactions, within and between different organs, and the lung function changes in inflammatory lung disease induced by respiratory viruses and allergen sensitisation. We therefore haveto use animal models of disease.

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

My research programme uses in-vitro experimentation and clinical studies in humans wherever possible. In infants with bronchiolitis, we have assessed broncho-alveolar lavages for immune cellsand mediators. In parallel, we study effects of viral and bacterial exposures on human and murine mucosal cells generated in vitro, including epithelial cells and immune cells.

Why were they not suitable?

My research seeks to dissect the mechanisms of epithelial cell and immune system priming prior to and after respiratory viral infections. Such work requires experimental interventions in living organisms,
e.g. the depletion of individual cell types through antibodies or genetic alteration, to define the roles ofindividual components of the immune system or of specific subsets of epithelial cells. Such experimental approaches would be unethical in human beings. Thus, our aims cannot be achieved with clinical studies.

In-vitro studies of human or animal cells are very useful, and we use them wherever

possible, but theycannot model the interactions between different organs during health or disease/ infection, which are essential to understand in our research.

Reduction

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

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

We estimate the given numbers of experimental animals based on our previous experience, usage statistics of previous publications as well as information provided from other license holders at our establishment. The numbers required for upkeep and desired out-breeding of lines are derived fromestimates provided by the animal facility. To stay updated on optimised experimental design, we willregularly seek guidance from biostatistical experts employed by our establishments.

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

All experiments are designed to use the minimum number of animals to give publishable (statistically significant) results or to obtain sufficient numbers of primed cells for ex vivo cultures or for cell transfersto other animals. To that end all groups and controls of an experiment will be run in parallel and all organs of interest will be used simultaneously in each individually identified animal. All experiments have appropriate control groups as required. In the experimental design stage of the project, we have made plans to perform pilot studies, where required, before using new models with larger amounts of animals. We will also use the NC3Rs EDA tool to inform experimental design. All experiments will be conducted according to the ARRIVE and PREPARE guidelines.

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

We will implement efficient breeding procedures and will additionally perform well-designed pilot studies for new experimental models to estimate animal numbers required for publishable results.

Each mouse is individually identified within the experimental group and we collect the maximumamount of data from each mouse by harvesting multiple organs, which are routinely shared withcollaborators

Refinement

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

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

We will use mouse models of respiratory viral infections and of allergic airway disease/ asthma,involving nasal application of viruses and allergens.

To minimise suffering, applications of substances to the nose or the windpipe will be done only under anaesthesia. Furthermore, we will use the lowest appropriate dose of infectious agents and mild strains where possible. For new pathogens that we have not worked with, we will work out the minimaladequate dose in pilot studies. In some experiments, infectious doses are required that induce clinicaldisease, i.e. transient weight loss during the peak of illness, which will only be tolerated up to pre- defined humane endpoints.

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

To study mechanisms and consequences of acute viral infections of the lung and of their clinical effectsincluding bronchiolitis, pneumonia, subsequent asthma and lung function changes, we require a modelthat has lungs similar to humans and for which extensive tools for immunological research are available. Given these requirements, the least sentient option are mice and 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 theanimals?

We have recently refined the work flow to isolate epithelial cells from mouse lungs, obtaining much higher yield and viability of cells, reducing the number of mice required for ex-vivo experiments. We willcontinue to optimise cell isolation methods.

When using neonatal mice we will use refinement measures to minimalise cannibalisation or neglect by the dam. These include olfactory conditioning of dams to the user by gentle handling and to inhalational anaesthetic for 7-10 days prior to birthing. After procedures, pups will be rubbed down withdirty bedding prior to return to dam, to minimise changes in smell. Entire litters will be used for experiments so that individual pups are not singled out and cannibalised/neglected.

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

We will follow the PREPARE guidelines: http://journals.sagepub.com/doi/full/10.1177/0023677217724823 and associated information at: https://norecopa.no/prepare. We will stay up to date on updated best practice recommendations andpublications available on the NC3Rs website (https://www.nc3rs.org.uk/) and from the Laboratory Animal Science Association.

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

We will stay informed about the advances in the 3Rs by attending informational events provided locallyand provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

CONTROL OF NEMATODES AND ANTHELMINTIC RESISTANCE IN 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, ortheir effects, in man, animals or plants

Key words

Livestock, Treatment failure, Roundworms, Sustainable control

Animal types	Life stages
Cattle	juvenile, adult
Sheep	juvenile, adult
Pigs	juvenile, adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of the research is to understand better and optimise control of parasite infections in livestock.Improved understanding and control will reduce the impact of these endemic pathogens on livestock health, welfare and productivity and enable the development of strategies that are sustainable for the future whilst minimising environmental impacts. The work will also look to improve our understandingof the mechanisms involved in treatment failure (anthelmintic resistance).

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

Why is it important to undertake this work?

Gastrointestinal and pulmonary nematodoses are arguably amongst the most important

diseases affecting livestock production world-wide. Livestock are affected by a number of pulmonary and gastrointestinal nematodes (GIN) with *Dictyocaulus, Teladorsagia circumcincta, Nematodirus battus, Trichostrongylus species, Haemonchus contortus, Ostertagia ostertagi, Cooperia species, Oesophagostomum* species and *Ascaris species* being the most important in the UK. The level of disease caused by these nematodes depends on the size of the challenge faced by the animals and their susceptibility to infection. All nematode species cause damage to the tissues in which they reside. In the case of the roundworms living in the stomach, small and large intestines, this can lead to reduction in the animals' ability to digest and absorb foodstuffs. Some of the species (*Teladorsagia* and *Trichostrongylus* in particular) also affect appetite, so that heavily infected sheep will tend to consume less food. Although roundworm infection can lead to overt clinical disease, particularly in young stock, usually these parasites result in sub-clinical infections in which livestock perform below their full productive potential. In the UK the cost implication of roundworm disease in sheep was estimated to be around £48-120 million per annum and account for a significant proportion of all production losses in sheep.

Although significant work has been conducted into non-chemotherapy based treatments such as pasture management, selective breeding, nutritional manipulation, vaccination and the use of biologicalcontrols, anthelmintics still remain the mainstay of helminth control globally. To date control of gastrointestinal nematodes in production animals has largely been achieved using anthelmintics either therapeutically or prophylactically and relies on a limited number of anthelmintic classes (currently five in sheep (1-5 below), three in cattle (1-3 below) and two in pigs (1 and 3 below)); benzimidazoles (1- BZ), levamisole (2-LV), macrocyclic lactones (ivermectin and moxidectin; 3-ML), amino-acetonitrile derivatives (4-AD) and the spiroindoles (5-SI) however, this is threatened by the emergence of nematode populations in all host species that are resistant/tolerant to one or more of the available classes of anthelmintics.

It is important to investigate the sensitivity of anthelmintic in livestock per se, obviously the long termprofitability and viability of enterprises relies on the efficient control of roundworms.

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

New information on i) anthelmintic resistance (AR) prevalence; ii) Mechanisms of anthelmintic resistance; iii) sustainable helminth control strategies; environmental impact of anthelmintic usage

Multiple scientific publications and lay articles on sustainable control of helminths and anthelminticresistance

Continued professional development training programs for vets and Registered Animal Medicines Advisors (RAMA)/Suitably Qualified Persons (SQP) and training for farmers, undergraduate students(e.g. vet/agricultural/biologists) and other scientists.

Advice for industry relevant groups such as Sustainable Control Of Parasites of Sheep (SCOPS),Control Of Worms Sustainably (COWS), Scottish Government, Veterinary Medicines Directorate (VMD), Animal Medicines Training Regulatory Authority (AMTRA), Animal Health Distributors Association (AHDA) and pharmaceutical industry

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

Roundworms are a major concern to livestock and humans alike, with anthelmintic medicines such asbenzimidazoles and macrocyclic lactones being used extensively in their control. As such, the resources generated over the course of the project will be of significant value to the wider community of scientists working on the biology, genetics and control of parasitic nematodes, as well as mathematical modelers and extension scientists.

Pure and applied parasitologists working on livestock and anthelmintic resistance (AR): The development of tools to assess AR development and spread will be of relevance to both veterinary andmedical researchers. Understanding the prevalence of particular roundworm species and the development and dissemination of AR will help researchers highlight areas of greatest need and provide tools that will be use in the study of AR in the wider community. Understanding farmers perception of stress factors will aid the development of targeted biosecurity guidance focusing on control strategies using multi-format materials to widen audience appeal.

Genetics: The project will provide tools to study AR surveillance, resistance development and improveour understanding of the molecular basis of AR. Gastro-intestinal nematodes are a major concern to producers and scientists globally. The proposed work will provide a theoretical framework to understand the development and evolution of anthelmintic resistance in helminths and generate tools to characterise and monitor variation in helminth populations over time. These results will be directly applicable to researchers focused on anthelmintic responses, both to understand the genetic mechanisms by which resistance arises, to manage the rate of resistance accumulation and to those investigating new dosing strategies in helminth populations targeted with anthelmintics.

Epidemiologists/mathematical modelers: Currently the epidemiology of cattle and pig parasites are poorly defined under current farming and climatic conditions. The proposed research will enable the improvement and utility of existing models by providing a better understanding of egg to larval development, species prevalence and whether benzimidazole resistance is acquired in one 'large' stepand spread around with animal movement or selected independently on many occasions. Modelling these parameters will provide quantitative predictions on their spread and evolution. Outputs will help those with an interest in animal health, welfare and food safety/security by helping to identify treatment/control strategies likely to have greatest impact and where empirical trials are costly/impractical to perform at scale. Modelling can be used to reduce animal experimentation by targeting resources in line with 3Rs.

The proposal adopts an interdisciplinary approach that identifies the best pathways to impact for scientific advances through collaboration with end-users. Beneficiaries will have access to findings through peer reviewed publications, presentations at national and international meetings, online through institute websites, blogs and social media accounts. Results will be further disseminated through continued professional development (CPD) programs, agricultural events and lay publications.

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

The host organisation has a long track record of collaboration and effective knowledge exchange andthe findings as a result of work undertaken on previous project licenses has informed government policy, national farming and veterinary practices and the development of industry recommendations. The findings have also been discussed and disseminated through national and international conferences and meetings. Outputs from this current project will be maximised through the followingapproaches:

Scientific Audiences: The results of our investigations will be published in open access journalsappropriate for subject matter, 3Rs and scientific impact. All manuscripts will follow the ARRIVE guidelines to promote accurate reporting appropriate for 3Rs initiatives.

During the course of the project we will attend national and international scientific conferences tocommunicate progress (successful and unsuccessful) and to emphasize the focus on scientific excellence in this project.

The licence holder interacts with a large number of researchers through formal networks which promote co-operation and multidisciplinary networking between scientists and stakeholders from EUmember states and globally. As objectives are successfully completed, we will directly communicatewith these groups to disseminate findings and methodologies.

Stakeholders and policymakers: Findings will be disseminated through industry led groups Sustainable Control Of Parasites of Sheep (SCOPS) and Control of Worms Sustainably (COWS) thatrepresents the interests of the sheep and cattle industries respectively.

The host organisation has an established Communications Team through which we will disseminate findings to all interested parties involved in livestock production (e.g. farmers, veterinarians, RegisteredAnimal Medicines Advisors (RAMAs), pharmaceutical industry) and attend UK agricultural events. UK-wide Animal Health Roadshows are held and run by the host organisation annually to report on our progress and to promote the principles to both stakeholders and policymakers. We have close interactions with many relevant stakeholders and lay updates on the research will be sent to these stakeholders through newsletters, website content and regular interactions at agricultural events androad shows.

Educational and Public: The licence holder teaches undergraduate veterinarians and zoology students in parasitology and will use these opportunities to promote the outputs and principles of this project. For dissemination to the wider public, we attend the Major and local agricultural shows where results can be communicated to members of the general public. The impact of the project will be madeavailable on specific pages of the host institute website as a resource for the public, policymakers and Government stakeholders in the food, animal health, environment and rural sectors.

Species and numbers of animals expected to be used

- Cattle: 200
- Sheep: 5500
- Pigs: 100

Predicted harms

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

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

The animals used in these protocols are the natural hosts for the parasites under study. Currently thereare no *in vitro* systems to enable the generation of such parasite material. Young life stages of ruminants/monogastric are generally the most susceptible to the

parasites and are also the stages for which the economic impact of infection is the highest, as such are most commonly used. Adult animalsare also affected by parasites and are useful to follow, particularly under field conditions, where pasturecontamination and subsequent infection of young stock may be an issue.

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

Animals in this project will be naturally or artificially infected with parasites which the animals would normally encounter in the field. Animals will be monitored for signs of disease e.g. weight gain and other indicators of wellbeing. Repeated faecal and/or blood samples will be collected from animals toassess parasite numbers, immunity and to provide biological material for lab studies.

Animals will be treated with anthelmintics or other anti-parasite medicines/compounds to check how well they work and identify if the parasites are developing resistance to them. Very occasionally theremay be a need to surgically transfer parasites in order to generate pure isolates or isolates of known origin. These animals will undergo general anaesthetic and subsequent surgical transfer of parasites.

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

Adverse effects on the animals may be loss of appetite, diarrhoea, respiratory distress, reduced weightgain. Typically these effects would be for 4-6 weeks in the field.

The blood sampling programme should cause neither anaemia nor hypovolaemia and the blood sampling programme should cause neither pain (other than transient from needle prick), anaemia norhypovolaemia. Faecal or swab sampling will cause no pain, distress or lastingharm. Blood volumes collected from each individual will adhere to Home office guidelines

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

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

For all animal types:

Moderate severity: <10% of animals infected with gastrointestinal and or pulmonary worms
Moderate severity (Sheep only): animals undergoing surgical procedures
Mild severity: ~90% of animals infected with gastrointestinal worms

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?

Since none of the ruminant nematodes life cycles can be maintained *in vitro* the studies need to be conducted using infective larvae obtained from donor animals and/or experimental animals. Nematodepopulations are inevitably over-dispersed, the field-based aspects of this research will necessitate the use large numbers of animals. However conservation of efficacy can usually be determined using *in vivo* (faecal egg count reduction) and *in vitro* (e.g. egg hatch and larval feeding) tests that do not require animals to be killed.

Serial passage of parasites is routinely required to preserve specific isolates and species but where possible organisms will be cryopreserved as a replacement to this animal intensive approach. Work isongoing to investigate new crypopreservation techniques to help facilitate improved long term storage of infective larvae.

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

Where suitable, parasite material will be archived and biobanked for future studies and bioassays willbe used to assess anthelmintic sensitivities.

Why were they not suitable?

Unfortunately no viable non-animal alternatives are available for the generation of parasite material,the organisms requires a definitive host to complete their dioecious life-cycle.

Reduction

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

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

The numbers are based on data generated form animals on trials that have been conducted under twoprevious project licenses and the experience of the applicant and associated personal license holders associated with the proposed license. These estimates have also been optimised through collaboration with statisticians reviewing data from our previous experiments.

Experimental groups sizes and dose level administrations are generally determined using industry goldstandard and licensing guidelines such as those published by the World Association for the Advancement of Veterinary Parasitology (WAAVP) and VICH. The guidelines also highlight numbers ofcontrol animals required to account for inherent variability in the model systems.

At all stages of the experimental design there is consideration to added value that may be generatedfrom a study whether this is actions such as the bio-archiving and preservation of tissue or parasitesfor future studies or collaborating with scientists from other fields such as mathematical modelers to help parameterise their statistical models through the supply of findings and results.

Group allocation and randomisation is achieved through use of random number generator or pickingnumbers out of a hat. Blinding of studies is achieved through using different individuals at different stages (where possible) or removing trial information from samples prior to processing and analysis.Statisticians are involved at all stages of the experiment from initial design, data analysis and resultsinterpretation to censure appropriate and statistically relevant comparisons can be made between groups or experimental situations and to ensure the maximum efficiency of animal use.

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

Prior to each experiment data is reviewed from previous similar experiments and we collaborate withexpert statisticians to determine the minimum numbers of animals required to accurately determine treatment efficacy.

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

All animal experiments are examined and approved by an Animal Welfare and Ethical Review Boardwhere they are also subject to statistical and ethical review prior to work being undertaken.
Statisticians provide guidance on the most appropriate number of animals to use in each trial, taking into account the risk of failing to achieve the experimental objective through the use of too few animals.

Material from the proposed project, both host and parasite, will be archived for future studies, therebyreducing the number of animals and studies that are required. Biological material generated from thisproject is supplied to internal and external colleagues/collaborators therefore reducing the requirements for animal usage in other institutes and research establishments.

Individual housing of animals will be undertaken where feasible to reduce the numbers used for the generation of parasite material. All individual animals will be housed in such a way as to be able to seeother animals of the same host species.

Refinement

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

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

<u>Animal models</u>

The nematodes to be investigated in this project are obligate parasites and are species-specific in theirhost therefore sheep, cattle and pigs are the most appropriate species for

this project as they are the target species. Infections rates are based on published guidance and personal experience and are selected due to them being expected to generate sub-clinical infections and therefore cause least pain, suffering and distress. Generally, the parasites are excreted from the host in faeces and as such this is the sample collected most frequently. Further details of methods are outline below

Methods

Infection route: Almost all artificial dosing occurs orally, with parasites administered in a small volumeof tap water. The process causes minimal stress beyond the animal being restrained for the few seconds it requires to be achieved. Generally this is done once but repeat administrations may be required (trickle infections) where we want to mimic grazing conditions more closely. Nautural infections are investigated with animals at pasture. Animals are routinely monitored by both scientific, animal facilities and veterinary staff at the institute and rigorous monitoring of animals will be performedto ensure adherence to guidelines. The experimental procedures are in routine use by Personal Licence holders. The PPL applicant will take responsibility for compliance and the overall implementation of the plan of work and ensure that all those working under the licence have the correct competencies. Infections rates are based on published guidance and personal experience and are selected due to them being expected to generate sub-clinical infections and therefore cause least pain,sufferings and distress.

Treatments: anti-parasitic treatments are administered either orally, via injection or topically. The process causes minimal stress and only transient pain beyond the animal being restrained for the fewseconds it requires to be achieved.

Sample collection: Include blood and faecal sampling, none of which cause anything more than transient pain or discomfort to the animal. Repeated faecal sampling may be required to follow the timecourse of infection, sampling is undertaken by licence holders that have the correct competencies.
Repeated sampling may also be achieved through the harnessing and bagging of animals to collect excreted faeces. The most accessible parasite lifestages are eggs and larvae that are excreted fromthe host in faeces and as such this is the sample collected most frequently.

Surgical transfer of worms: Small numbers of animals are used for the purification and crossing ofparasites. The use of surgery is often the only way to purify field infections to generate a mono- specific isolate or to generate genetic crosses for future downstream analysis. Post-operative administration of antibiotics and a suitable analgesic will be given

Housing: Animals are housed in groups where possible and all animals are able to see others. Where single housing is unavoidable, the time frame is kept to a minimum and all animals are able tosee others.

Day to day husbandry: Animals are routinely checked (twice daily) by both scientific, animal facilitiesand veterinary staff at the institute to monitor their health and welfare and to ensure appropriate intervention is undertaken if required. The MRI experimental facilities contain open span sheds and barrier accommodation suitable for sheep and cattle experimentation and have dedicated stockmen/animal technicians responsible for day-to-day husbandry. Records of husbandry and procedures are kept for all animals on study.

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

Many studies require the involvement of grazing animals, or animals that are of a developmentalmaturity, to enable the lifecycle of the parasite to be completed successfully.

These parasites are adapted to living in sheep, cattle and pigs have relatively long-lasting lifecycles(several weeks to months) so the use of terminally anaesthetised animals would not be practical.

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

The host organisation has a policy of reviewing trials via an end of study report which helps identifyareas of where refinement may be beneficial. The reports include identification of the need for increased monitoring for example.

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

Where appropriate, the World Association for the Advancement of Veterinary Parasitology (https://www.waavp.org) and VICH (https://vichsec.org/en/guidelines/pharmaceuticals/pharma-efficacy/anthelmintics.html) guidelines for best practise will be followed.

In addition, the ARRIVE guidelines 2.0 will be followed to allow the experiments to be performed in themost refined way and the publication of the data in the most appropriate form. https://arriveguidelines.org/.

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

The host Institute has an active 3Rs committee which provides advice to project and personal licence holders as well as liaising with our animal health and welfare colleagues. Any relevant advances in the3Rs will be disseminated to relevant parties and implemented into any of the protocols where appropriate.

ZEBRAFISH MODEL OF EMBRYOGENESIS AND DEVELOPMENTAL 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

Neuroscience, Embryo development, Brain disorders, Neurodegeneration, Organ and tissue integrity
Stem Cells, Hearing Loss, Auditory Neuropathy, Vestibular Hypofunction

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

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of the programme is to understand how the brain develops and interact with other partof the body, in order to better design ways to cure or prevent neurological defects, either during pregnancy or after birth/in adults, such as motor and cognitive neuro-developmental disorders (eg.
FOXG1 syndrome, Autism) and neurodegeneration (eg. Amyotrophic lateral sclerosis, spasticparaplegia, Alzheimer, fronto-temporal dementia).

The current project has the overarching aim of developing stem cell therapies for the treatment of innerear conditions.

Previous work has established the initial proof of concept that stem cells can be used to functionally replace damaged cells in the hearing organ, the cochlea. We have also explored the potential of cells to engraft into the vestibule, i.e. the part of the inner ear responsible for the control of balance. Now wewill expand on these studies, refining the identity of the cell populations to be transplanted and further exploring the combination of cells with cochlear implants.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 proposed programme will deliver a set of key findings that will open new avenues of therapies forthe most devastating neurological disorders, affecting together around 20% of the population (0.1% ofnewborn affected by neurodevelopmental disorders and 20% neurodegeneration associated to old age).

In the last years, the identity of all human genes (and all mice and all zebrafish genes) has been uncovered and numerous lists of genes implicated in specific human disorders have been produced.Yet we understand very little about the function of the vast majority of these genes. Our studies will contribute to the understanding of gene function during embryonic brain development. This includesneuronal fate specification, neuronal connectivity and establishment of neuronal circuits. It will also provide insight into fundamental processes such as cell migration and cell division. These finding willhave direct impact in understanding of human diseases such as neurodevelopmental disorders (eg. Autism Spectrum Disorders, intellectual disabilities, FoxG1 syndrome), neurodegeneration (eg. Alzheimer, ALS, dementia) and cancers.

The characterisation of more genes involved in fundamental processes during early vertebrate braindevelopment will give greater understanding of the molecular and genetic interactions that establish structures in the nervous system. It will also give insight in evolution of the forebrain in vertebrates.

A long-term outcome will be an increased understanding of the genes contributing to congenital andneurological defects in vertebrate brain, including humans and the development of therapeutic avenues. Our previous HO licence contributed to the identification of a new therapeutic target for FOXG1 syndrome.
Over 400 million people have disabling hearing loss (HL) worldwide, with this figure expected to nearlydouble by 2050. This epidemiology is only equalled by the global burden of diabetes (>400m) and outstrips conditions like rheumatoid- and osteoarthritis (~200m). HL has a devastating effect on patients' quality of life, causing significant direct and indirect socio-economic harm.

Despite the scale of the problem, there are no disease-modifying therapeutics for HL. Palliative medical devices like hearing aids (HAs) and cochlear implants (CIs) remain the only treatments. Thesedo not repair the damaged tissues, HAs simply amplify sound, while CIs bypass damage to stimulate neurons. This has resulted in considerable patient and healthcare system demand for disease- modifying therapies as this would transform the lives of millions of patients across the world.

The inner ear also harbours the vestibular organ that controls balance. The impact of vestibular failureon a patient's mobility and functional independence can be devastating.

Patients with bilateral vestibular failure rarely recover. Traditional vestibular rehabilitation benefits a limited number of patients and has no significant effect on number of falls. Impaired vestibular function is a major risk factor for wrist and hip fractures from falls.

A cell therapy for inner ear diseases would be the first of its kind, and would offer a biological,restorative solution to conditions that are currently intractable.

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

The applicant's team publishes regularly in international journals and will likely publish around 20 highimpact papers in the course of the coming 5 years. We envisage that at least 5 of those will lead to specific collaboration with clinicians.

The work proposed will open new avenues of research and will attract funding from internationalfunding agencies.

They will advance the understanding of brain development substantially and share the new knowledgethrough teaching and public engagement events.

Some of the findings may potentially lead to IP and development of therapeutic avenues. The initial benefit of this work will translate in the generation of new knowledge on how transplanted cells engraft and integrate in a recipient. Data to be gathered will advance our understanding on how torepair the inner ear, although some fundamental principles could be beneficial to the neurosciences in general, and to the field of regenerative medicine in particular.

Besides the scientific benefit, this project could have a direct impact in the treatment of hearing loss and vestibular pathologies. Data generated will support the development of a stem cell-based product for hearing loss and advance our understanding of its efficacy. The information will be included into scientific publications and reports that will support applications to the regulatory bodies to progress intohuman clinical trials.

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

The beneficiaries of this programme of work are primarily scientists, students, clinicians and generalpublic.

<u>Scientists and Clinicians:</u>

In the last years, the identity of all human genes (and all mice and all zebrafish genes) has been uncovered and numerous lists of genes implicated in specific human disorders have been produced.Yet we understand very little about the function of the vast majority of these genes. Our studies will contribute to the understanding of gene function during embryonic brain development. This includes,neuronal fate specification, neuronal connectivity and establishment of neuronal circuits. It will also provide insight into fundamental processes such as cell migration and cell division. These finding willhave direct impact in understanding of human diseases such as neurodevelopmental disorders (eg. Autism Spectrum Disorders, intellectual disabilities, FoxG1 syndrome), neurodegeneration (eg. Alzheimer, ALS, dementia) and cancers.

The characterisation of more genes involved in fundamental processes during early vertebrate braindevelopment will give greater understanding of the molecular and genetic interactions that establish structures in the nervous system. It will also give insight in

evolution of the forebrain in vertebrates.

A long-term outcome will be an increased understanding of the genes contributing to congenital andneurological defects in vertebrate brain, including humans and generate therapeutic avenues for common neurological disorders.

Students and trainees:

The work proposed will educate and train a series of undergraduate, postgraduate students and postdoctoral fellows (over 150 individuals at lab level and many hundreds through undergraduate lectures over 5 years). Their training will improve the quality and experience of individuals who willcontribute to the UK research and development locally and abroad.

General public:

The applicant contributed to key public engagement initiatives sharing knowledge and findings with thebroader public as well as developing awareness of research findings and taste for studying biological sciences in secondary schools (Dev Neuro Academy).
As described above, the population affected by inner ear diseases is vast. If a therapy is finallyachieved, the long term outcome of this project has the potential to benefit millions. In a more immediate time scale (3-4 years), the data to be generated here will support applications to theregulatory bodies (e.g. Medicines and Healthcare Products Regulatory Agency-MHRA) to seekauthorisation for clinical trials.

Moreover, the scientific community will benefit from advances presented at conferences andpublications in peer-reviewed journals.

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

The applicant's lab is sharing their findings with the community through multiple means.

The lab members are communicating findings at numerous international conferences.

They developed a series of international collaboration with scientists. Some examples: Harvard(USA), Pasteur Institute (Paris, France), Manchester University (UK), Bath Uni (UK), Australian Regenerative Medicine Institute (Melbourne, Au)

Lab members are participating to science dissemibation to the public (eg. Pint of Science, Green manfestival) and secondary school science events (Dev Neuro Academy https://devneuro.org/cdn/public- engagement-dna.php)

Datasets are all put on general repositories (including negative results, eg. ZFIN)
We are very actively driving the translational application of our work. We collaborate closely with industry and clinicians. In particular, we have close ties with a biotech company that is aiming to develop the stem cell strategy into a realistic, clinically applicable treatment. The output of this work ispart of a larger project, that includes developing the manufacturing of stem cells under industrial standards, overcoming the surgical challenges for the delivery of cells in humans, and aligning the advances with the appropriated regulatory bodies.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 70000
- Mice: Approximately 800 mice are to be used for the entire project, with 160 allocated to eachexperimental protocol.

- Rats: Approximately 800 rats are to be used for the entire project, with 160 allocated to eachexperimental protocol.

- Gerbils: Approximately 800 gerbils are to be used for the entire project, with 160 allocated toeach experimental protocol.

Predicted harms

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

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

We have the ambition to understand the cellular and genetic steps needed to development a normal brain. We chose the zebrafish as sole animal model because it is a vertebrate and therefore 'close' tohuman yet having their embryos spawn in the water. This is allowing collection without affecting adult mothers. The embryos are optically clear, allowing for observation of organ development with no invasive intervention needed. We study early development and therefore make most of our observations without any need for surgery or other intervention giving discomfort to the animal at any stage of their life.

All mutations and transgenes carried by adult fish are not affecting their health. We chose to work onembryos because we aim to understand the genetic and cellular mechanisms driving normal embryonic development.
We propose to transplant these cells into mature gerbils, rats and mice that have two different types ofdeafness and/or vestibular problems. In the first type of condition, the main deficit is generated by thedegeneration of their cochlear or vestibular neurons, the second will have primarily a loss of cochlear or vestibular hair cells. These resemble the more common mechanisms that produce deafness and vertigo in humans. In a third model, we will explore the interaction of stem cells with cochlear implants.Gerbils are a good experimental model with a human-like hearing range, while mice and rats allow us to study genetic defects that impact on the inner ear.

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

No surgical procedures, injection or drug treatments are done on adult animals.

Adults carry mutations or transgenes because they were injected with DNA or genome editing tools atembryonic stage (fertilised egg stage). We only put in nursery embryos developing normally, while carrying the genetic modification. These adults carriers are kept for breeding.
Cell transplantation and implantation will be done through a surgical procedure under general anaesthesia. Animals are expected to be deaf, or to have vestibular problems. We anticipate that thevestibular problems will be detected with behavioural tests (e.g. walking on a beam) but should not substantially disrupt their daily routine. The severity of the procedures is considered moderate. At theend of the procedure, the animals will be humanely euthanized.

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

Genetic identification of GM individuals is done on caudal fin clips under anaesthesia. A very small section of the fin is taken (less than 10%) and the fish is swimming normally as soon as out of anaesthesia. Fish undergoing this procedure are under observation for 30 min before being put back intheir tank. No other impact or adverse effects are generally expected on the adult fish.

Some GMs will affect motor behaviour of the embryos carrying two copies of the modified gene. Thesewill not be grown to adulthood.
Animals may be deaf. After the surgeries, they may walk in circles, roll or rotate, but these behaviours usually subside after a few days. They may also undergo some discomfort after the surgery, but this isalleviated by anti-inflammatories and analgesics. Moderate weight loss may occur (~10-15%). In theory, formation of tumours is a potential side effect, but it has not occurred in the studies so far.

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

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

All procedures are of mild severity and do not generate pain or distress.
Approximately 75% of the animals are expected to reach a moderate severity, ~20% will be mild withthe remaining ~5% being subthreshold. The spread of different severities is likely to be similar for thethree different species.

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

- Used in other projects
- Killed
- Kept alive
- Killed

Replacement

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

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

As the main goal of our research is the understanding of the cellular and molecular mechanisms required for the formation of the brain in the vertebrate embryo, alternatives not using animal at all arenot yet available. The originality and strength of our study is the ability to follow cell behaviour in vivo, using imaging of fluorescent transgenic zebrafish. In vivo studies are the only way to identify the cellular behaviour at the source of developmental defects.
The cells to be transplanted have been extensively studied in vitro, and we have evidence that they can differentiate into functional cells when treated with the appropriate conditions. We are performing more experiments in a test tube (in vitro), trying to understand their molecular and functional properties. However, interactions of cells with a live recipient are

too complex to be modelled in the cellculture lab. Before transplanting them into a human patient, we need to study them in an animal modelto analyse the responses they may trigger, as well as their therapeutic effects. Gerbils are a good experimental model with a human-like hearing range, while mice and rats would allow us to study genetic defects that impact on the inner ear. Furthermore, there are strains of rats and mice that have a modifed immune system that would minise the rejection and would help to study the engraftment of the cells. The three different species are needed to achieve the scientific goals, as they provide different advantages depending on the parameters under study (i.e. functional restoration, impact of genetic mutations, etc).

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

Some of the molecular candidates involved in formation of neuronal circuits will be assessed in neuronal primary culture. We are currently developing 3D cell culture technologies ("organoids"), starting to get these to be used for some of our scientific questions, instead of the fish. We did searchfor more alternatives, using FRAME and other sources suggested on the NC3Rs website, without success.
The interaction of cells with host tissues has been explored in vitro, using cell aggregates (known asorganoids) generated from human stem cells.

Why were they not suitable?

The alternatives such as organoids are only suitable to assess a limited number of questions as these 3D organised pieces of brain tissue are extremely variable in size and shape and only recapitulate some aspects of normal development. Because of the lack of information normally received by the brain tissue from blood vessels, surrounding skull tissues, immune cells etc. these cultures are only very partial representation of the embryonic brain and do not develop into mature structures. Moreover,they do not connect to different brain areas normally, therefore not allowing for assessment of effect of mutations or specific neurons on neuronal circuit development.
Although some useful information can be obtained from the in vitro models, the organoid systems arestill very rudimentary and do not provide the complexity that a whole organism possess. Critical parameters such as efficacy for functional restoration, biosafety and the interaction with the host immune system can only be studied in vivo.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 is made based on the zebrafish animals used in the previous four 5-year project the applicant has run in the past twenty years and the size of her lab/ambition of her project in the next 5years.
We have estimated the number of animals based on our previous experience of what is needed tomeasure a biologically and statistically relevant effect size. We have used these assumptions andestimates to determine the sample size using power calculations.

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

The principle of reduction is applied for all procedures used. Experimental design is aided by the use ofEDA as a very useful online tool to achieve best reduction. Number of embryos and adults used are defined following power calculation as the minimum required to ensure statistically-relevant results.
Most of our work will employ zebrafish larvae prior to independent feeding – effectively being a replacement of mouse use. We will employ the optical clarity of fish larvae to allow the use of new microscopic methods to image brain development or brain disorder models. The aim of much of our work is to use transgenes that do not disrupt normal processes to report on those processes as they happen. So, although the animals are transgenic and therefore fall under the Act, they will not suffer inany way compared to non-GA fish. Creating stable transgenic lines is by itself a way to reduce numberof animal used. The establishment of healthy viable adults carrying a transgene allows us to reduce thenumber of embryos studied compared to the quantity we would have to use in a transient system. Each experiment is carefully planned, considering minimising confounding variables such as allocatinganimals of comparable age and equal proportion of both sexes to each experimental group.

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

The zebrafish facility has been designed to optimise breeding as well as nursery output (eg. automaticfeeders, regular health and fertility checks). Moreover, it cater for all zebrafish labs on campus and anorganised system of sharing lines and embryos is in place across multiple labs.
We optimise the number of animals and aim to reduce variability by controlling other parameters thatcould impact on the experimental outcome. For example, each batch of cells to be transplanted is carefully assessed. We characterise them in vitro and capture batch numbers of reagents consideredto be relevant to the process. Pilot studies using small cohorts are implemented when a new experimental variable is introduced.

Refinement

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

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.
We chose the zebrafish as sole vertebrate animal model because the embryos are spawn in the water(allowing collection without affecting adult mothers) and are optically clear (allowing for observation of brain development with no intervention needed). We therefore make most of our observations without any need for surgery or other intervention giving discomfort to the adult animal. Our procedures are ofmild severity and do not generate pain or distress. When the fish need to be kept at the end of a procedure, it is first monitored for sign of pain/distress (lack of activity, appetite or sexual behaviour).

Any rare individual presenting any sign of pain or distress that is not rapidly curable will be killed underschedule 1.

We choose to produce and use transgenic animals expressing non-toxic fluorescent proteins in specificcell populations or sub-cellular organelles in order to avoid any invasive intervention while observing the behaviour of cell types or cellular organelles in vivo. This approach allows us to image embryos at high-resolution under normal fluorescence microscopes without any impact on the animal, with the exception of mild anaesthesia.

Mice, rats and gerbils are species widely used in hearing research. All of these species possess a cochlear and vestibular anatomy similar to that seen in humans. For this particular application we haveinitially chosen the gerbil, since their hearing resembles that of humans, with an ability to detect a similar range of frequencies. Models for pathology, primarily those that affect the neurons and nerve (neuropathy) are well established in this species and mimic the hearing impairments occurring in the human population. Several laboratories are now using the gerbil, creating a wealth of information that would help us to compare and put in context the functional data we would obtain from our transplants. Besides the gerbil, we are exploring the applicability of this potential therapy in other species such as mice and rats. Both these species can be manipulated genetically, and transgenic models of inner ear conditions as well as models with reduced immunity are available. These would greatly facilitate the validation of a stem cell transplant and treatment. Ototoxic models are also well established, for instance strains like the Gunn Rat offer an excellent model of early onset jaundice, one of most common causes of auditory neuropathy in humans.

All surgical procedures are performed under anaesthesia and suitable analgesia is providedafterwards.

Work performed during previous licences has allowed us to refine the model in which the hair cells andthe spiral ganglion neurons, are ablated (to be used in protocol 3). To mitigate the systemic effects of the ototoxic treatments (e.g. ouabain and Kanamycin/furosemide), we now spread the procedure over the course of a week, allowing full recovery from the ouabain surgery before the kanamycin/furosemidetreatment is given. This regime leads to better survival rates with fewer associated health issues.

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

As we aim to understand the development of the nervous system, the animal we need to use to do soare animal having sensory perception. However, we do all of our studies on embryos at stages beforetheir nervous system is mature enough to feel any pain or discomfort.

The only procedure on adult involving a very mild pain is the small caudal fin clipping we need to do toascertain the genetic identity of the animals and this is done under anaesthesia and is extremely well tolerated by the animal. We are exploring ways to improve skin swabbing for genotyping aiming to improve the existing protocol given on a guidance page from NC3R resource.
The nature of hearing loss and vestibular dysfunction entail that it is a condition which primarily affectsolder adults. Consequently, it is appropriate to use adult animals as a model for this. Likewise, in orderto see if a potential treatment is effective, it is necessary to follow the animals' progress over a longer period of time.

Similarly, hearing loss is mostly caused by dysfunction within the cochlea - this organ is

lacking in lesssentient vertebrates such as zebrafish.

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

The applicant already refined substantially her approaches. However, further exploration of animal caremethods by her fish facility team is undergoing to improve care, fertility, spawning efficiency and genotyping protocols.
Animals sedated for hearing tests (ABRs) are given a reversal agent to ensure a rapid recovery fromanaesthesia.

Animals undergoing surgery to induce neuropathy (by application of ouabain) are monitored regularlyin the days after the procedure to observe and log any incidences of temporary vestibular dysfunction
e.g. rolling, head tilt. Refinement strategies involve giving the animals a 'vet-bed' fleece lining to thecage, which is a better substrate for them post-surgery than sawdust. Hay is also provided as a distraction - an entertained animal recovers better than a bored one.

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

We are using both NC3R resources and our technical team is participating in international husbandryworkshops aiming to continuously improve husbandry and experimentation protocols.
Advice from in-house specialists (AWERB, NACWOs, NVS etc.) will be acted upon as and when newpractices are adopted. We regularly visit the NC3Rs online resource library.

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

There are regular opportunities to engage with in-house workshops run by the NC3Rs organisation. Any new, relevant information will be disseminated amongst the group and put into best practice wheresuitable.

DEVELOPMENT OF IMMUNE CELL THERAPIES FOR HUMAN CANCER

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir 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 aimsmentioned in paragraph (b)

Key words

cellular immunotherapy, T cell, cancer, chimeric antigen receptor, T cell receptor

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We aim to develop new treatments for human cancer that utilise immune white blood cells with intrinsicor genetically programmed attributes.

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

Why is it important to undertake this work?

Cellular immunotherapy consists of the treatment of human disease with immune cells derived from the patient or another source. Therapeutic immune cells may be genetically modified (“genetic engineering”) and/ or expanded outside the body prior to their administration to the patient, most commonly into the bloodstream or site of disease.

These treatments constitute living drugs that may continue to exert therapeutic benefit over a sustained period of time. Already, cellular immunotherapy isimpacting in the treatment of blood cancers and there are strong grounds to believe that innovative cellular immunotherapies will also benefit patients with solid tumours and other chronic disease states.

Although recent developments in cancer therapies have improved the survival rate of many patients, cancer is now overtaking heart disease as the biggest killer of adults in many countries in the Westernworld. In the UK, cancer kills more people each year than did covid 19 at the height of the pandemic. Simply put, currently available treatments are inadequate for most patients with advanced cancer.
Thus, there is an overwhelming need for new and more effective means of treatment. The overall aim of this project is to develop cancer immunotherapies that harness white blood cells to control the tumour. Within the patient, white blood cells are suppressed by the environment created by the cancer.However, if they are removed from the body and manipulated in the laboratory, they can acquire potentanti-cancer activity. Alternatively, these cells may be sourced from healthy donors. The approach we wish to test may involve the use of cells that naturally recognise and kill cancer cells. Alternatively, we will boost the ability of white blood cells to undertake this task by delivering new genes to these cells.

Testing of these treatments may be performed using a variety of laboratory model systems, which enable the selection of the most promising candidates. Prior to testing of these experimental medicinesin man, there is a regulatory requirement to demonstrate efficacy and safety of the approach in a living non-human host. For this reason, animal work is generally undertaken. As explained below, mice are most commonly used for this work. Efficacy (and sometimes safety) of human immune cells can be tested in mice that are immunodeficient. However, such models do not recreate the complexity of the ecosystem found in the cancer microenvironment. For this reason, studies in which mouse immune cells are infused into immune competent mice also provide useful information.

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

We envision that this project will lead to the development of new cellular immunotherapies which willbe described in patent filings, publications and presentations at scientific meetings. The most promising candidates will be advanced to clinical testing in patients with relevant disease.

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

The most effective treatment for chemotherapy unresponsive acute lymphocytic leukaemia involves theadministration of white blood cells that have been removed from patients, genetically engineered in thelaboratory and then re-infused back into the patients bloodstream. Terminally ill patients who are treated in this way achieve remission in 80-90% of cases – a level of effectiveness that has never beenseen before when a new cancer medicine is tested for the first time in man. Six such treatments are now approved for clinical use in patients with acute B-cell leukaemia, aggressive forms of B-cell lymphoma and multiple myeloma. We hope to develop similar treatments for other cancers that are based upon this principle. Currently however, solid cancers and other forms of leukaemia are much more difficult to treat using this approach. In the short term, we will be able to optimise new white bloodcell-based therapies, demonstrate their effectiveness in mice with cancer and obtain safety data required for initiating clinical trials in cancer patients. Promising treatment approaches will be advancedto clinical studies in patients

with advanced and otherwise untreatable cancer, while ineffective approaches will be discontinued. We have already advanced once such white blood cell-based treatment to a clinical trial in patients with advanced head and neck cancer, so we have the necessary experience to translate our experimental work to the patient setting. Currently, we are planning a second clinical trial involving a new cellular immunotherapy recently developed by our group. In the longer term, we will continue to develop the most promising candidates through late stage clinical studies.

Advancement and dissemination of scientific knowledge will benefit the scientific community, particularly those who work towards the development of cellular immunotherapies. Materials generatedin the course of this work will be made available to members of the scientific community under an appropriate material transfer agreement. Patients will benefit from new cellular immunotherapies advanced to clinical trials as a result of this project.

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

New technologies developed in this project will be protected with patent filings and then published inhigh impact peer reviewed journals. Key findings will be presented at scientific meetings. The projectwill benefit from ongoing and new collaborations that will ensure that state of the art solutions are employed to develop and test new cellular immunotherapies, including underpinning mechanisms ofaction.

Species and numbers of animals expected to be used

- Mice: 37,100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 only animal species to be used. Other than breeding colonies, only adult mice will be used. Mice are less sentient than higher mammals, but are still relevant to human biology. Moreover,there are well-established baselines available for mice in the literature and several useful and well- characterised strains are available.

Mouse models have already proven to be predictive of success of cellular immunotherapy in man. A good example of this is CD19-specific CAR T-cell immunotherapy, which is an approved treatment forB-cell malignancy in many territories. This clinical success was heralded by mouse studies that demonstrated the ability of CD19-specific CAR T-cells to control established murine B-cell tumour burdens.

In this project, we will mainly use immunodeficient mice (NOD SCID gamma null and SCID Beige) for the establishment of human cancer xenograft models and subsequent treatment with human cellular immunotherapies. We will also use immune competent mice (mainly Balb/c and C57/Bl6) to evaluate mouse immune cell derived cellular immunotherapies in a system where the tumour microenvironment is more representative of that found in spontaneous cancer. Such models are also informative in the likelihood of on-target off-tumour toxicity where a specific target is co-expressed by malignant cells andcells found in

one or more vital organs. Finally, spontaneous tumour models will be used in some experiments to avoid non-physiological manipulations such as tumour implantation while modelling thetumour microenvironment most closely. These models are of particular importance in identifying relevant immune checkpoints that operate in the spontaneously occurring tumour microenvironment and which may compromise the efficacy of cellular immunotherapy.

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

Experiments may last for up to 8 months but a typical duration would be 12 weeks. Typically, mice withan established tumour burden will receive one infusion of an experimental cellular immunotherapy.
Most typically, animals will have pre-existing cancer which has been induced by injection of tumour cells under the skin or at another site. To monitor the state of the disease and/ or the presence and location of infused cells, mice may undergo repeated calliper measurements and/ or imaging procedures under anaesthesia. Typically this occurs 6-10 times. Less commonly, additional drugs (such as immune checkpoint inhibitor) may be added but this would not be typical. Moreover, blood samples may be collected (for example to measure cytokines, which are a marker of toxicity of cellularimmunotherapy), but once again this would not be typical.

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

In most cases, mice will have established malignant disease (e.g. solid tumour or leukaemia) prior to receiving cellular immunotherapy. Typically the malignancy has been established for between 5 days to3 weeks depending on aggressiveness of the disease model, prior to intervention with cellular immunotherapy. Malignancy may persist for up to the duration of the life of the animal, especially in control treatment groups. While treatment may reduce disease burden, mice that receive ineffective treatments (e.g. control drug or cells) will develop increasing disease and these animals will be humanely killed immediately using a schedule 1 method once a humane endpoint has been reached. Humane end points associated with malignancy include weight loss >15%, limitation of normal behaviour, impeded vital organ function (e.g. impairment of locomotion, vision, mastication, excretion etc.), clinical signs such as jaundice, abdominal distension causing a clinically evident increase in bodygirth (similar to a pregnant mouse), dyspnoea, neurological signs, lameness, or general signs of ill- health (e.g. piloerection, hunched posture, inability to groom, inactivity, inappetence).

In therapeutic studies, we will endeavour to humanely kill mice at a time point which indicates that disease is advanced but remains asymptomatic. In asymptomatic mice with malignant disease that is amenable to non-invasive imaging (e.g. using bioluminescence imaging) or calliper measurement, animals with progressively increasing disease burden will be humanely killed when a durable and statistically significant difference in tumour burden between test and control groups is seen in at least 3sequential measurements.

Additional expected adverse effects during this project are infection (duration days), and features of cytokine release syndrome. Cytokine release syndrome is a common side effect of cellular immunotherapy in man and can also affect mice. Clinical features typically occur within 24 to 48 hours after administration of cellular immunotherapy and last for 48-72 hours. Signs consist of transient flu- like features, weight loss, roughening of fur, reduced mobility and/or diarrhoea. In the majority of cases,these signs are fully reversible. In the human setting, features of cytokine release syndrome are commonly associated

with improved clinical efficacy.

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

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

Breeding of animals is considered of mild severity, which is envisioned for all mice. All other procedures will have a maximum severity category of moderate, which is envisioned for up to 100% ofmice.

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

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered 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 the microenvironment established by cancers are both very complex and it is impossible to reproduce this fully in laboratory model systems. Moreover, we cannot measure how immune cells which kill cancer cells can control or prevent the spread of cancer without using animals. Consequently, we need to use living animals to investigate the activity and the safety of the experimental cellular immunotherapies to be developed in this project. *In vivo* testing of the active drug substance for both efficacy and safety is generally an essential pre-requisite prior to undertaking PhaseI clinical trials in man, unless (exceptionally) a suitable *in vivo* model does not exist for this purpose. Animal studies are only performed after every feasible test has been conducted on cancer cells in thelaboratory and where no alternative exists.

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

In all cases, experiments are performed using tumour cells grown in the laboratory and immune cells taken from healthy volunteers or cancer patients. Some aspects of the tumour microenvironment maybe recapitulated in three-dimensional models of cancer (e.g. organoids and organotypic cultures) which are established in the laboratory. In addition, *in silico* computational modelling can assist with predicting the likelihood of successful cellular immunotherapies, for example modelling those that target more than one antigen. In addition, serial tumour cell re-stimulation assays are also very usefulin selecting those cellular immunotherapies that are more likely to prove efficacious *in vivo*. Increasingly, these provide useful tools for the initial testing and modelling of our experimental cellular immunotherapies, allowing us to discard poorly effective cellular immunotherapies without the need foranimal experimentation. Only those cellular immunotherapies that perform well in these studies will beadvanced to animal studies using mice. Mice are the most established model for pre-clinical *in vivo* evaluation of the safety and efficacy of experimental cellular immunotherapies. We considered other model organisms such as zebrafish. However, these animals lack a functional adaptive immune system, and they do not recapitulate the trafficking of human CAR T-cells following

injection into the circulation, a point that is maintained in the mouse. There have been very few studies in which zebrafish have been used to model cellular immunotherapy and these have highlighted a number of additional important limitations of the model. For example, orthotopic tumour formation is technically very challenging and assay duration is very short, precluding studies of delayed therapeutic failure or tumour re-challenge. Moreover, there is little available information regarding cross-species reactivity ofhuman T-cell effector molecules and cytokines in this species.

Why were they not suitable?

As indicated above, testing experimental therapies for their safety and efficacy in animals is an essential regulatory pre-requisite prior to initiating early stage trials in patients. Although a considerableamount of information can be obtained from *in vitro* experiments performed in the laboratory, the interaction between a new treatment and the whole organism can only be defined in animals. The limitations of other model organisms such as zebrafish are highlighted above.

Reduction

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

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

We expect that we will use 37,100 mice over the course of the project. This is based on an estimated use of 5000 mice for breeding purposes, 5,200 to set up cancer models and 26,900 to test experimental cellular therapies. These numbers derive from our experience over the past 5 years, the anticipated expansion of the workforce over the next 5 years and the use of locally generated and online tools that will assist in the determination of numbers of mice required for individual experiments.

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

All new experimental cellular immunotherapies that are developed in this project will first undergo rigorous laboratory based testing so that ineffective approaches are not unnecessarily tested in animals. We will only proceed to test experimental cellular immunotherapies for *in vivo* activity once *in vitro* studies have been completed which support the therapeutic potential of the approach under study.

All tumour cell lines that are used in this project will be rigorously checked for their provenance, geneticidentity and negative mycoplasma status, thereby preventing the wasted use of mice in animal experiments that are unnecessary.

To minimise the number of mice, pilot experiments will be carried out to determine variability in tumour growth. Experiments will be designed and optimised with the aid of online resources such as the NC3R's Experimental Design Assistant. We will conduct careful power calculations using this and otherresources to ensure that the minimum number of animals is assigned to a particular experiment, while maintaining power to

detect meaningful differences between test and control groups. Whenever possible, tumour growth will be monitored using non-invasive imaging systems, greatly reducing the need to kill mice at defined time-points. Serial sampling will also be undertaken wherever possible for similar reasons.

Where tumours are discordant for target antigen and do not metastatise following subcutaneous implantation, implantation of antigen discordant tumours may be performed into either flank prior totherapeutic intervention, thereby halving the number of mice required for such studies. We do not envision that this will have additional welfare implications for animals compared to those bearing a single tumour. In this instance, the experimental unit becomes the tumour rather than the mouse.

The risk of surplus breeding will be minimised by use of equal numbers of male and female adult miceaged 6-10 weeks across protocols described here, except where sex-specific tumours are modelled.

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

Numbers of breeding pairs/ trios will be subject to ongoing review to ensure that excessive numbers ofmice are not produced for experimental work. To minimise the number of mice, pilot experiments and power calculations (including historical data) will be carried out to determine the lowest number of micerequired to give statistically valid results, thereby minimising the number of repeat experiments required. These will derive from online tools including the NC3Rs Experimental Design Assistant. Dataoutput from experiments conducted using the minimum essential number of mice will be potentiated through serial imaging, serial sampling and maximisation of tissue use post culling 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

Mice are the only animal species used in this programmer of work. Animals will be maintained underbarrier conditions to minimise infection risk.

Most commonly, immunodeficient mouse models will be used, which permit engraftment with human tumour cells and immune cells. Whenever possible, tumour cells will be administered SC into the flankas this causes least distress to the mouse. However, SC tumours are often poorly vascularised and frequently do not undergo metastasis, meaning that alternative models are required to model metastatic spread of disease. In some cases however, more aggressive tumours do reliably undergo metastatic spread from a primary subcutaneous or orthotopic tumour (e.g. mammary fat pad). In this instance, resection of the primary tumour will be performed on a single occasion to enable the modelling of spontaneous metastatic disease while minimising suffering to the mouse from excessive

growth of the primary tumour. The IP route may be used to mimic disseminated peritoneal carcinomatosis. The IV route may be used to establish pseudo-pulmonary metastasis or models of haematological malignancy. To mimic attributes of specific primary tumours (e.g. pancreas, mammary gland), it may be appropriate to introduce cancers cells by the orthotopic route. Intracardiac and intra- tibial/femoral injections will only be used when there is a requirement to study bone tumours. In these models, tumour cells will always be administered under general anaesthetic. The intra-tibial/femoral route may also be used to establish leukaemic models, but only where IV delivery is inadequate. To model spontaneously occurring tumours in which an immunosuppressive microenvironment is present,genetically engineered mouse models (GEMMs) may be used. Appropriate controls will be included in all experiments. Analgesia and anaesthesia will be used whenever appropriate, subject to review by the Named Veterinary Surgeon.

One important attribute of cellular immunotherapy is the generation of a memory immune cell population that affords long term protection against disease. To test this, mice that achieve complete remission of their disease following an experimental cellular immunotherapy may undergo tumour re-challenge, meaning that a second injection of tumour cells would be performed. Once again, humane endpoints will apply as per individual protocols to ensure that pain, suffering, distress, and lasting harmare minimised.

Use of pilot experiments (with smaller numbers of mice) and imaging (allowing monitoring of disease inthe same animal, without the need to kill the mouse) should ensure that, whenever possible, mice will be humanely killed using a schedule 1 method before or when only mild clinical symptoms are evident.In asymptomatic mice with tumour that is amenable to non-invasive imaging (e.g. using bioluminescence) or calliper measurement, animals with progressively increasing disease burden will be humanely killed using a schedule 1 method when a durable and statistically significant difference in tumour burden between test and control groups is seen in at least 3 sequential measurements. Mice will be regularly monitored and humanely killed immediately using a schedule 1 method if they developsigns of distress or if humane endpoints are met, as described in individual protocols.

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

Mice are less sentient than higher mammals. Immune competent animals allow scientific interrogation of the interaction between cancer, immunotherapy and the immune system. By contrast, immunodeficient mice allow testing of the safety and anti-tumour activity of experimental human cellular immunotherapies, two processes that are required by regulatory authorities prior to approval offirst in man clinical trials. Moreover, increasingly complex mouse models are available that closely mimic human cancer or which have specific defects in the immune system that allows us to ask questions about the role of that immune component in treatment response or failure.

As indicated above, we considered other model organisms such as zebrafish. However, these animalslack a functional adaptive immune system, and they do not recapitulate the trafficking of human CAR T-cells following injection into the circulation, a point that is maintained in the mouse. There have beenvery few studies in which zebrafish have been used to model cellular immunotherapy and these have highlighted a number of additional important limitations of the approach. For example, orthotopic tumour formation is technically very challenging and assay duration is very short, precluding studies ofdelayed therapeutic failure or tumour re-challenge. Moreover, there is little available information regarding cross-species reactivity of human T-cell effector molecules and cytokines in this species.

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

Mice are maintained in individually ventilated cages (IVCs) and are provided with an appropriate environment (e.g. nesting material, shelter, environmental enrichment etc.), including sufficient spaceand complexity to satisfy their normal behaviour, in compliance with the standard code of practice required by the Animals (Scientific) Procedures Act 1986. Animals are maintained under barrier conditions, to minimise infection risk. Animals are not housed in isolation when possible. We are required to use an identification method on mice for data collection. To minimise animal stress, tail marks using a pen will be used in preference to ear tags/notches. Moreover, refined animal handling techniques shall be implemented including picking up mice using a tunnel or with cupped hands.

Picking up mice by the tail shall be avoided wherever possible. Animals shall be handled using cleaned, gloved hands particularly when handling between cages to prevent possible transmission ofinfection.

Available knowledge will be used to predict and thereby minimise adverse effects associated with individual procedures, to ensure that appropriate humane endpoints are developed and that specialist care is provided. While the choice of animal model depends on the scientific question being investigated, the mildest possible procedure will be used wherever possible. Thus, where the scientific question can be addressed using subcutaneous or intraperitoneal tumour models, these will be used inpreference to more invasive models such as orthotopic tumour implantation, in which surgery is necessary.

Mice are monitored daily for adverse effects. If adverse effects occur, animals will be subject to increased monitoring and consideration given to interventions that may reduce suffering (e.g. mushy diet, increased analgesia etc). In the event of weight loss of 10%, mushy diet will be provided. If surgery is required (e.g. orthotopic tumour implantation, surgical resection of tumour etc.), animals willbe subject to additional post operative monitoring and will receive analgesia as specified in individual protocols. Wherever possible, pharmaceuticals/agents will be administered via drinking water/diet to reduce procedures/injections.

To minimise toxicity due to cytokine release syndrome, cellular immunotherapies will be infused at thelowest dose that is likely to achieve efficacy and discriminate between test and control groups. In general, the intravenous or intraperitoneal route of administration of cell therapies will be favoured.

Pilot studies will be performed with unfamiliar cells and procedures to establish experimental andhumane endpoints.

Use of imaging and serial sampling (allowing monitoring of disease in the same animal, without the need to kill the mouse) should ensure that, whenever possible, mice will be humanely killed before clinical symptoms are evident. In asymptomatic mice with tumour that is amenable to non-invasive imaging (e.g. using bioluminescence), or calliper measurement, animals with progressively increasing disease burden will be humanely killed using a schedule 1 method when a durable and statistically significant difference in tumour burden between test and control groups is seen in at least 3 sequential measurements.

General, protocol-specific and tumour-specific humane endpoints are indicated in each of the protocolscontained within this project licence. Animals that reach any humane endpoint

will be immediately humanely killed using a schedule 1 method.

Competence of staff to perform procedures will be reviewed and documented on a regular basis.

Post-mortem examinations will be undertaken as a routine part of all pilot studies and to investigateany unexpected deaths.

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

https://www.ema.europa.eu/en/review-update-ema-guidelines-implement-best-practice-regard-3rs- replacement-reduction-refinement,

Workman et al (2010) Guidelines for the welfare and use of animals in cancer research. Br J Cancer102: 1555-77.

NC3Rs website: https://www.nc3rs.org.uk/3rs-resources/search

Refining procedures for the administration of substances.
https://doi.org/10.1258/0023677011911345

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

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

The animal lead for the applicant group has recently completed module 5 (September 2021). We regularly review the literature (via PubMed) and useful websites to identify new resources available to enable Reduction, Replacement and/ or Refinement in animal use. Examples of such websites include the NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research - https://www.nc3rs.org.uk) and EURL ECVAM (European Union Reference Laboratory for alternatives to animal testing - https://joint-research-centre.ec.europa.eu/eu-reference-laboratory-alternatives- animal-testing_en). The NC3Rs also provides a monthly podcast which summarises the latest researchand updates that pertain to the 3Rs (see https://www.nc3rs.org.uk/news/new-monthly-3rs-podcast-co- produced-labanimal). Other useful websites can be accessed at https://www.hpra.ie/homepage/veterinary/scientific-animal-protection/3r%27s/3rs-information---useful- websites. The applicant group are encouraged to engage in these activities as well as discussions around the 3Rs that are included in weekly meetings in which animal work is planned and presented.

DEVELOPMENT OF MOLECULAR IMAGING AND THERANOSTIC AGENTS FOR CANCER AND INFLAMMATION

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, ortheir effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Cancer, Inflammation, In vivo imaging, Theranostics

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to develop and evaluate new ways to image and identify cancer and inflammation. Some of these methods are aimed at providing information to develop new therapies forthese diseases.

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

Why is it important to undertake this work?

Improving dedicated in vivo (performed in a living organism) imaging methods to better understand thebiology of cancer and inflammation is desirable as there are many areas where improvement is needed in the management of these disorders. We aim to develop

new imaging methods to improve our ability to detect disease and to determine if treatments are being effective. New therapeutic strategies are also expected to be derived from this work.

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

The end goal of this research is to develop and characterise imaging methods that may be of benefit tothe treatment of human cancers and inflammatory diseases. The imaging tools that will be developed will increase our understanding of disease biology, provide means of predicting the efficacy of therapeutic interventions and help establish the basis for new therapeutic clinical trials.

This programme of work is expected to provide the following benefits:

Development of imaging and theranostic (therapeutic approaches driven and tailored by diagnostic observations) agents as companion diagnostics to “fast-track” the process of drugdiscovery; and

Identify the best imaging and theranostic agents for translation into clinical use.

Publication in peer reviewed scientific journals, dissemination of findings, including unsuccessful approaches or non-significant data via open access and through platforms such as F1000 Research.

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

Short term increase in knowledge which will be public domain in the scientific community and will serveas reference for further research.

Useful imaging and theranostic tools generated from this programme of work may be used to fast-trackthe process of disease characterisation and drug discovery at the preclinical level.

This work is intended to provide the basis for clinical trials making use of the products of this research.These may benefit patients suffering from cancers and inflammatory disorders in the future.

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

The results generated from the research will be shared with the scientific community through internalmeetings, conferences at both local and international level and peer reviewed scientific publications.

Collaborations with laboratories and institutions with expertise in different scientific areas to include chemistry, radiochemistry, chemical engineering, pharmacy, physics, basic oncology and immunology,clinical imaging and clinical and veterinary medicine have been established to carry out the proposed work.

Species and numbers of animals expected to be used

- Mice: 1500
- Rats: 60

Predicted harms

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

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

We are developing new ways to image cancer and inflammation and find novel ways of treating thesediseases. While much of the preliminary work can be done in tissue culture and other in vitro systemstesting in rodents is the mainstay of preclinical development of these imaging methods as it provides the most sensible way to demonstrate whether the new methodology has potential for further application in humans. Adult mice and rats, some harbouring specific tumour models will be utilised. These are the most commonly utilised and most accepted animal models in this type of research.

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

The procedures carried out on these animals are standardised, generally accepted and will be conducted abiding to humane treatment conditions of animals involved. Where possible healthy controlanimals will be utilised for imaging. Animals will be administered imaging agents by injection or ingestion and their distribution in the body will be measured with dedicated imaging equipment and modalities. These types of procedures are mostly commonly utilised in humans but we intend to
develop new and/or improved agents in this arena. Depending on the specific experiment we will be utilising different types of molecules or other agents. These will range from tiny amounts of radioactively labelled small molecules, peptides or proteins to larger structures such as synthetic nanoparticles or radiolabelled cells to be administered in the same way. Similar agents designed to alter tissue density for computed tomography, provide fluorescent light or generate signal to capture with magnetic resonance imaging will also be utilised. All these agents are designed with the intent to not alter physiology of the animal and will in most cases be utilised in quantities that will have no effecton the animals' wellbeing.

The imaging procedures are carried out under anaesthesia and can last from a few minutes to a few hours. In healthy control animals the imaging experiment is likely to only be carried out once. Imaging agents such as cells or other substances showing promising features for further development will be utilised in appropriate models of disease. The experimental work will involve generating tumour models(to start with, animals injected with tumour cells reproducing targets normally seen in specific human tumours that we plan to image). We will routinely measure tumour size either in awake animals with calipers or through imaging while under anaesthesia. We will on occasion administer drugs with food orwater or more frequently use injection. Blood samples may be taken from the animals in order to measure levels of the imaging agents, drugs or for evaluating treatment effects. In some experiments tumours may be treated with radiation. In order to measure the effects of the imaging agent, some animals may need to be housed alone or food may be withdrawn temporarily on very rare occasions.

Particular attention will be given to limiting the number of disease model animals utilised to that strictly necessary and ensuring an adequate time window for the experiments is used to avoid exceeding severity estimates. Imaging with the new methods will be used at different stages of this process and will be the main objective of this research. In select

cases the agents developed may be tested for theirability to inhibit cancer growth in time course experiments. All these procedures will be carried out using standardised and most refined methods available

At the end of the experiments animals will be killed by a humane method such as overdose of anaesthetic and the organs and tissues collected for further experiments. Tissues may be frozen andstored for use in future studies.

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

Most imaging studies will be carried out in unconscious, anaesthetised animals and with carefulmonitoring there should be no side effects.

As cancer and inflammation are main focuses of the research, disease specific models will need to be utilised for some of the experiments. This will involve implanting tumours or inducing inflammation which can affect animal wellbeing and careful consideration will be given to minimise the impact of this.The tumours may take 1 week to 3 months to grow to a stage required for imaging and treatment studies. For most of this duration the animals will be unaffected by the tumour growth. When and if they do experience signs of distress these may include weight loss, ulceration of the tumour or impaired movement, whether due to the tumour or the treatment. If 10% weight loss is maintained over 3 days, then additional support will be provided such as easy access to soft and palatable feed and pain relief. In addition, animals showing signs of ill health e.g. hunched posture, piloerection or
subdued behaviour which persists for 24 h and cannot be ameliorated by minor veterinary interventions, will be humanely killed before the severity limits are 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 animaltype)?

Mice

Non-recovery: 16.6%
Mild: 50%
Moderate: 33.3%
Severe: 0%

Rats

Non-recovery: 50%
Mild: 50%
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?

Cancer and inflammatory disorders are dynamic processes that require assessment in living organisms. Use of non animal alternatives to these types of studies are very limited but will be utilised to all extent possible. It is necessary to conduct studies on animals as the in vivo behaviour of imagingagents, cancer cells and the immune system cannot be replicated in vitro or by computational methods.

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

Before considering experiments in living animals, the imaging agent candidates will be tested oncomputer models, cells or animal or human tissues (in vitro studies).

Imaging methods will initially be tested using objects called imaging phantoms that stand in for animals. Use of imaging phantoms will be applied where possible in order to minimise the number of animals required for imaging experiments for the purpose of optimisation of acquisition parameters andsetup of the imaging procedures.

Tissue culture experiments assessing the biological properties of imaging and therapeutic agents beingtested will also be carried out preliminarily to obtain information on the suitability of the procedures being developed.

Why were they not suitable?

In vitro and computational methods are not fully representative of the biological characteristics of cancer cells and the immune system within living organisms. These non-animal alternative methods can only provide preliminary information on the properties and target selectivity of the imaging and theranostic agents to allow preliminary screening and selection of the best agent for further evaluationin animals. Only animal models can reproduce the complex interactions between organ systems that may affect the behaviour of the imaging agents.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 each experiment are based on statistical consultation, referencesfrom published scientific papers and our previous research experience and published track record in conducting studies on small animal imaging and therapeutics. A minimum of 10 animals per group is often used in imaging studies, and each imaging study may involve 3 or more imaging and treatment groups.
In most experiments the same animal is imaged at different time points which will reduce

the overall animals necessary for the research (e.g., scan one group of animals from 0 min to 2 hours in order toobtain the time dependent distribution of imaging agents into various tissues rather than different groups of animals that are killed at various time points within that 2 hours).

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

Computer modelling and in vitro studies on human/rodent cell lines and tissues will be used to decidewhether the imaging agents can proceed on to animal testing.

All experiments will be designed in consultation with radiochemists, biologists, imaging scientists andanimal facility staff via study plans. Imaging data analysis plans will be developed under the guidanceof trained imaging scientists and statisticians consulted where required.

Pilot studies will first be conducted on small groups of terminally anaesthetised animals to select thebest imaging agent for further evaluation as well as to determine the number of animals required to obtain robust data.

Reduction in animal use is further achieved in longitudinal experiments where the same animal can beimaged over a refined timeframe and acts as its own control, without the need for a separate control group. In addition, tissues will be harvested from the animals after the last imaging timepoint for archival and further in vitro evaluation. This will reduce the number of animals required.

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

Where possible tissues obtained after the animals are killed will be utilised to generate pilot data for future experiments. Being a part of a large biomedical research campus with many research groups working on different aspects of cancer and inflammation, such tissue may be obtained from other groups within the university via internal mailing lists. Such expertise will also be consulted and used torefine our techniques.

Resources such as the below are consulted.https://norecopa.no/PREPARE
The Experimental Design Assistant - EDA | NC3Rs https://nc3rs.org.uk/3rs-advice-project-licence-applicants-reduction

Refinement

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

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

This project will utilise normal mice and rats as well as mouse models of tumour and

inflammation. Rodents are the most appropriate model for the preclinical development of imaging and theranostic agents. The biological characteristics of cancer and the immune system in rodents are similar to those of humans. Several well-defined genetically modified strains and protocols are available, and resources exist for the development of additional models. Most of the imaging methods used are eithernon-invasive or minimally invasive. Animal suffering will be minimised by careful observation of the animals undergoing procedures. Guidelines for the assessment of clinical signs will be strictly followed,and experiment on the particular animal or cohort will be immediately terminated and the animal will behumanely killed upon observation of clinical signs classified as above moderate to severe. Dedicated imaging equipment for use in mice and rats will be utilised in this study. These scanners are designed to closely mimic state of the art clinical scanners and have been developed with imaging of rodents in mind as these models are well recognised to be the ideal preclinical model for this type of setting.

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

Rodents are widely recognised as the least sentient species to carry out research of this type. Cancerand inflammation are complex diseases that many aspects of pathogenesis and response to therapy are widely approached utilising rodent models. Rodents are the best model to use instead of higher organisms such as higher mammals or non-human primates. There are well-defined protocols and regulated procedures for studying cancer and inflammation in rodents.

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

We will have regular discussions and reviews with our in house animal welfare team, including the NVS and NACWO, who have a wealth of knowledge on best practice for implementing refinements tooptimise animal welfare, (such as non-aversive handling techniques).

We will use scoring systems to monitor and record the clinical signs in animals. Monitoring will beincreased in later stages of disease models. Trained animal technicians will support in evaluatingwelfare and pain monitoring using animal grimace scales.

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

Refining procedures for the administration of substances.
https://journals.sagepub.com/doi/pdf/10.1258/0023677011911345

Research Animal Training: https://researchanimaltraining.com/

PREPARE guidelines and checklists will be consulted. https://norecopa.no/about-norecopa
NC3Rs website https://www.nc3rs.org.uk/.
Focus will be given to the updated ARRIVE guidelines
https://doi.org/10.1371/journal.pbio.3000410updated in 2020 to design experiments that can be properly executed and reported.

Other resources from these websites will be consulted.(LASA) Laboratory Animal Science Association (AALAS) American Association for Laboratory Animals Science

(FELASA) Federation of European Laboratory Animal Science Associations(ICLAS)

International Council for Laboratory Animal Sciences
(InterNICHE) International Network for Humane Education

Prescott MJ, Lidster K (2017) Improving quality of science through better animal welfare: the NC3Rsstrategy. Lab Animal 46(4):152-156. doi:10.1038/laban.1217

Workman et al (2010) Guidelines for the welfare and use of animals in cancer research | British Journalof Cancer (nature.com)

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

The local University Biomedical Services has extensive documentation regarding 3Rs including links topublications, portals and websites with information pertinent to all commonly used laboratory animals. This information is continuously updated. Staff involved in animal handling and experiments undergo rigorous and continued training on these issues and have all these resources available.

DEVELOPMENT OF NOVEL BIOMARKERS AND TREATMENTS IN PROSTATE CANCER

Project duration

5 years 0 months

Project purpose

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

Key words

Prostate, Cancer, Treatment, Resistance, Biomarker

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 notrequired.

Objectives and benefits

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

What's the aim of this project?

To identify novel targets and develop more effective treatments for men advanced prostate cancer.

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

Why is it important to undertake this work?

In the UK, prostate cancer affects 1 out of 8 men and kills more than 12,000 men each year. In advanced disease, hormonal treatments, which have been used for over 50 years, control the prostatecancer in a palliative (i.e. non-curative) manner. We urgently need better and more specific treatment options. Better knowledge of how prostate cancer

spreads and resists treatment will help researchers to develop new ideas and approaches to treat prostate cancer.

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

Work in this project licence is carefully developed to provide important knowledge of cancer biology ofincurable or advanced prostate cancer. The planned programme of work focuses on prostate cancersthat are resistant to treatment, including both hormone treatment and chemotherapies. Hence, data from our work will help develop new strategy to treat patients with relapsing and/or persisting cancer following currently available treatments.

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

We hope to discover critical biological processes responsible for treatment resistant prostate cancer and invasive (or metastatic) prostate cancer which will be of importance to the scientific community. We will generate data that will be of value to other researchers in the field. New mouse models of treatment resistant prostate cancer and of metastatic prostate cancer developed from this project can also be used by other scientists to answer specific questions arising from their own research. Within the collaborative network that we have already in place, there will be a very efficient route to ensure asmany research groups as possible will benefit from our discoveries.

Medium term benefits

In the medium term (3-6 years), we hope to be able to identify new molecules as potential targets for developing new treatments. Ideally, we will also identify other relevant molecules that will lead to improved diagnosis and help ensure the most appropriate treatment is delivered to patients with prostate cancer. New therapeutic targets will facilitate collaborations with both academic and industrialpartners.

Long term benefits

Our ultimate aim is to improve the way we treat patients with invasive and/or treatment resistant prostate cancer. We hope to meaningfully extend the survival of patients with aggressive prostate cancer while maintaining their quality of life. To achieve this, we will need to be able to carry out clinicaltrials, so clinical oncology and urology departments can benefit from findings from our project. There will also be the opportunity to explore the value of these novel strategies in other tumour types, particularly in other hormone related cancers such as breast and ovarian cancer.

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

- Generate datasets that will be of value to other researchers in the field
- New drug targets and prognostic molecules (biomarkers) will facilitate collaborations with bothacademic and industrial partners.
- Carry out clinical trials, so clinical oncology and urology departments can benefit from findingsfrom our project

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

- Mice: 6000

Predicted harms

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

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

Genetically engineered adult male mice (>12 weeks) develop prostate cancer which closely resemblesthe stages of human prostate cancer, from early disease, to invasive cancer and ultimately metastasis.This reflects the physiological nature of prostate cancer being prevalent in the ageing male population.Our genetic models typically develop prostate tumours between 9-12 months of age, which is why we use aged mice in some of our studies; the timeline of tumour development in our models closely mimics the clinical situation in human patients and increases the translatability of our findings.

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

Typically, 25% of genetically engineered animals will develop prostate tumours (usually between 9-12months of age), and will undergo regular monitoring of the tumour development, by palpation and/or non-invasive imaging, and once develop a clinical sign such as significant abdominal distention, altered gait/movement and hunching it will be humanely killed.

A proportion of these mice with tumours (5-10%) will undergo non-invasive imaging (e.g. ultrasound scanning) and/or treatment (5%)(injection or gavage) with novel drug agents. These experiments willlast up to 18 months.

Another (different) 5% of all the mice will undergo surgical procedures to inject human/murine tumourcells into their prostate/bones +/- surgical castration (20% of these mice). All these mice will undergonon-invasive imaging (100%). These experiments will last up to 12 months.

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

To recapitulate the human prostate cancer disease state, we will use experimental animals with similargenetic mutations. These animals will develop prostate cancer, with many developing abdominal swelling (50%) and/or urinary symptoms (10%). The mice will be closely monitored by palpation +/- non-invasive imaging. Once the mouse develops significant abdominal distention, altered gait/movement and hunching it will be humanely killed.

We have refined the required surgical procedures (to administer treatment and/or facilitate imaging studies) described in this project, typically using a small lower abdominal incision (mouse aged typically 12 weeks of age). This is then closed when the procedure is concluded and mouse recoverywill be closely observed for development of a prostate tumour. Approximately 10% of the study animals will be administered drugs or therapeutic agents. The potential harms of this include weight loss, bowel and blood disturbances. We have refined the technique to produce prostate cancer in its natural environment by implanting cancer cells directly into the mouse prostate. This is a very useful method to test the growth behaviour of cancer cells with certain genetic contents. We will also use

thistechnique to test the usefulness of new treatments in the project. By either implanting cancer cells in the prostate gland (10-15%) or injecting into the mice (1-2%)(using different routes to mimic clinical spread to bone), we hope to study the reason for prostate cancer to spread and/or resist treatment.

Injection into the bone will be monitored by observing for mobility issues.

All animals on treatment or anaesthesia will be carefully monitored for discomfort, recovery or development of relevant clinical symptoms (e.g. weight loss or tumour development), with the use ofregular monitoring forms. Animals will be humanely killed at the end of the experiments and tissuescollected at post-mortem to maximise data obtained.

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

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

The majority of animals used (~75%) will not have any observable clinical signs (mild severity), sincethey will only be used for the breeding programme and will be humanely culled once their genetic statuses are known.
Those that develop signs (prostate tumours) (25%) will have moderate severity signs.

Of those that have surgical procedures (implantation or castration), 100% will have clinical signs(100% moderate).

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

- **Killed**

Replacement

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

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

Prostate cancer is a hormone driven cancer and its progression is also regulated by host factors such as liver, fat and the immune system. Since cell culture systems using prostate cells do not adequatelyrecapitulate the complex "whole-body: interactions, we intend to adopt a 3D organoid and co-culture systems to better mimic the human disease condition. In addition, we will also generate primary cultures from clinical prostate tumours to limit the need for animal experiments.

We will use animals only when there is no appropriate alternative. However, improving our understanding of how tumours rewire the host metabolism and immune system is essential fordesigning improved treatment strategies.

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

3D organoid and co-culture systems.

Why were they not suitable?

Prostate cancer progression is regulated by numerous "whole-body" factors, and the above systemsdo not recapitulate the complex "whole-body" interactions.

Reduction

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

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

With genetically altered mice, the cohort size and study period required will vary with the type of genetic model used, the genetic background of the mouse and the time to disease onset. We have considerable experience of such experiments and the statistical approaches required to analyse timeto tumour development. Where possible, inbred strains of mice are used to reduce experimental variability. The ongoing monitoring of a given cohort permits these experiments to be terminated as soon as significant data has been obtained. Since prostate cancer is a disease of males, we will prioritise the generation of the male mice through our breeding strategies.

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

Analyses based on small pilot experiments help us to minimise the number of mice required. We canuse the expertise of in-house statisticians and/or the NC3Rs experimental design assistant. Our reliable models, including transplantation models, ensure minimal number of mice to acquire meaningful results. We are also able to share controls between experiments to reduce the overall number of mice required.

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

Where possible, imaging will be used to monitor tumour growth. We have applied ultrasound imagingto (serially) monitor tumour growth and response, eliminating animal to animal variability and decreasing the number of animals needed. We have generated, established and characterised genetically defined cell culture and organoid systems from mouse tumours for exploratory research.

We have extensive experience and support within our Institute to use the minimal number of mice toanswer specific 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

We use mouse models with the same genetic changes that are known to cause human prostate cancerto replicate the human disease condition. These pathological changes are restricted to the prostate gland so that unrelated effects in other tissues do not occur. All experimental animals are monitored regularly for signs of normal behaviour and are humanely killed if they exhibit moderate abnormal symptoms. All staff are trained to recognise these clinical signs. Regular monitoring of mouse welfare allows us to complete studies at the earliest endpoint in which we observe a significant result toprevent unnecessary suffering resulting from high tumour burden.

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

Prostate cancer is a disease of aged male humans and experimental mice, intricately linked to hormonal control. Thus the experimental mice will have to be aged, with intact hormonal (testosterone)axis, to recapitulate the disease process accurately.

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

We have developed expertise in non-invasive imaging using ultrasound scans to monitor tumour growth so animals can enter our studies at the optimal time to ensure robust comparison between miceand minimise suffering. We have SOP's for all our cancer models and new researchers are trained andsigned off by dedicated trainers.

Evaluation of new therapeutic agents will be tested using small number of mice (3-4) in the firstinstance.

Analgesia and anaesthesia will be given, when appropriate, to ensure the welfare of the animals.

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

Our experiments will be conducted in accordance with the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines), including the use of randomisation and blinding where appropriate to avoid biases and to ensure robust data from the experiments. We have been and willcontinue to follow the PREPARE guidelines. We will also use both the NC3Rs, and Workman guidelines.

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

I will regularly attend the monthly in-house biological services unit meetings, to keep myself updated on new developments and advances in the welfare of animals used for scientific experimentation. We have an established culture of care for these animals and I am in regular receipt of the newsletter from"understanding animal research" (https://www.understandinganimalresearch.org.uk) and NC3Rs. We will be in regular contact with our NTCO and NVS.

DEVELOPMENT OF NOVEL THERAPIES FOR AUTOSOMALDOMINANT POLYCYSTIC 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, ortheir effects, in man, animals or plants

Key words

Kidney, Polycystic, Therapuetics, Translation, Disease

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a very common cause of kidney failure but currently remains without a cure. Our main aims are to better understand polycystic kidney diseaseand to develop novel therapies to treat or cure 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?

There is a pressing clinical need for new strategies to treat ADPKD. This requires a detailed understanding of the way polycystic kidneys are formed and how best to reat affected patients. 50% ofpatients with polycystic kidneys will have their kidneys fail by the age of 55, relying on dialysis and/or kidney transplantation to maintain life. Polycystic

kidney disease is the most common genetic disorder affecting the kidneys (1:800 people worldwide). Over 55,000 patients in the UK are maintained by either transplant or dialysis. Currently there are insufficient kidneys to transplant all patients and 65% of patients starting dialysis die within 3 years. The quality of life of patients on dialysis is compromised significantly and also places an enormous burden on the NHS.

If successful, this project will be worthwhile because it will:

- provide new therapeutic targets for the treatment of patients suffering with ADPKD
- improve the quality of life of patients with ADPKD
- delay the requirement for dialysis and transplant

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

Outputs will include peer reviewed publications, presentations at scientific meetings (American Societyof Nephrology annual meeting, Renal Association annual meeting)

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

In the short term these outputs will mainly benefit the scientific community following the release of peer-reviewed papers. However, in the long term these outputs will be disseminated to kidney charitiesas well as patient advocate groups. The impact of these outputs in the longer term may lead to testing new treatments for ADPKD in pre-clinical and human clinical trials.

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

In addition to the publication of peer-reviewed papers and dissemination of results at national and international conferences to a scientific audience we will endeavour to make our data available to a wider audience. Results will be presented to patient organisations both locally and nationally. In the UK, the PKD Charity and the National Kidney Federation have regular meetings where patients can learn more about the project and progress along with how it may lead to new therapeutics in the future.Finally, research updates and relevant publications or presentations will be shared on social media for patients and patient groups can freely view and share.

Species and numbers of animals expected to be used

- **Mice: 2000**

Predicted harms

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

Explain why you are using these types of animals and your choice of life stages.
We study a renal disease with a known genetic cause; therefore, we only use mice relevant to this genetic disease. These animals develop polycystic kidney disease with accompanied decline in kidneyfunction. Disease develops rapidly in these genetic models of PKD, and therefore most protocols will be completed before the age of 6 months. Animals will not be kept beyond the age of 12 months.

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

Mice will be treated with drugs which we have identified as having potential therapeutic

benefit in polycystic kidney disease from our cell culture experiments. These drugs will either be injected daily for7 days or administered in drinking water for longer time periods up to 6 months. Following treatment, the animals will be monitored to determine their effectiveness in slowing the progression of PKD. The hope is that we will then be able to translate the findings from the animal experiments into human clinical trials. We design our experiment through discussion with our statistician colleagues to get a statistical estimation of minimum numbers of mice required to achieve a meaningful result (statistical significance). We are also in constant contact with animal technicians and have optimised our breedingstrategy to avoid unnecessary breeding of animals.

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

The PKD mice show no phenotype in the heterozygous state (one mutated gene). Homozygous mice(both genes mutated) develop PKD from birth. Clinical signs of disease are not obvious although sequential monitoring of kidney function is possible with biochemical measures (how adverse effects will be recognised below). In this protocol we will use both PKD mutated and associated wild type control mice. Control mice are not expected to develop any signs of kidney disease. We will use bothheterozygote and homozygote mice here.

Expected adverse effects and likely incidence

Polycystic kidney disease is very likely to develop in mice with a mutation in either Pkd1 or Pkd2 genes. Advanced polycystic kidney disease results in enlargement of kidneys and is associated with abdominal discomfort, due to the large kidney. One potential adverse effect of advancing polycystic kidney disease is associated infections of the kidneys; however, mice are kept in a barrier environmentand as such infections are kept to a minimum. Disease develops rapidly in these models of PKD and therefore most protocols will be completed before the age of 8 months. If polycystic kidney disease is left untreated beyond 9-12 months end stage renal disease can develop. However, the timing of the development of end stage disease depends on the genetic background used, the sex of the mice (males are more susceptible than females) and the gene mutated (e.g., Pkd2 mutations are milder when compared to Pkd1 mutations). We will monitor the mice for signs of deterioration of health and if they exhibit clinical signs of end stage renal disease, they will be humanely killed. Mice will not be keptunder this protocol beyond the age of 12 months, they will be humanely killed.

Administration of substances

Specified dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies. The minimum number, volumes and routes of administration will be used to achieve the scientific objectives, causing no more than transient discomfort and no lasting harm. Aseptic technique will be used for all injections. Sites of injection will alternate to minimize discomfort from repeated injections. Should inflammation occur at sites of injection which does not reduce when injection sites are alternated then animals will be killed by schedule 1. Animals may show an adverse reaction to one or more of the administered compounds. When a new agent is administered for the firsttime, mice will be checked hourly for up to 5 hours, then on a daily basis. Animals showing respiratory distress, pallor or diarrhoea (uncommon in our experience under previous PPL<1%) that is not quickly ameliorated by minor interventions for example application of a heat pad, will be humanely killed.

How the adverse effects will be recognised.

All animals will be examined at least daily to assess health and welfare and to detect signs of potentialrenal failure. Veterinary and animal care and welfare officer advice will be taken (prior to beginning experiments) in order to agree monitoring / recording systems that will assist in identifying end-stage disease / renal failure at the earliest opportunity, and animal care staff will also be informed of signs tolook for during their duties. Abdominal discomfort will be recognised by examining the animal behaviour and assessing its response to gentle palpation in the abdominal area. Animals with kidney infections will experience pain which may lead to loss of appetite. Animals will be examined daily, and any rapid weight loss will be recorded. Animals with approaching 15% loss of body weight, compared with littermate controls, will be humanely culled. When novel agents are tested, pilot studies with micewill be conducted with acute (24h) and chronic studies (5-7d) starting at minimal effective doses to exclude any unexpected side-effects of the compounds before increasing to therapeutic dosing. The animals will be monitored at least daily to detect any complications.

Humane end-points and limits of severity

Animals that develop clinical or biochemical signs of end-stage kidney disease /renal failure will be humanely culled. Veterinary / NACWO advice will be taken before beginning experiments as above,and will be promptly obtained if animals appear unwell during the experiments. Specifically, mice exhibiting: a) hunched appearance, b) lessening of mobility c) piloerection, d) accumulation of fluid under the skin and in the abdomen, leading to a flabby, swollen appearance will be humanely culled although due to the short timecourse anticipated for our experimental protocols we do not expect renalfailure to occur in our animals.

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

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

- Sub threshold 30%
- Mild 20%
- Moderate 50%

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?

We have previously carried out extensive work using cultured human cell lines, however,

in vitro experiments cannot mimic whole body or organ systems and the disease that affects them. Renal failure caused by PKD and the systemic consequences of renal failure cannot be modelled in vitro. Thekidney is an organ containing a heterogeneous cell population, organised in intricate structures based on multiples of its basic functioning unit, the nephron. Overall kidney function is the result of a complex series of processes which include glomerular filtration, tubular reabsorption and tubular secretion in thepresence of functioning endocrine and immune systems and, to a lesser extent, a functioning nervous system. PKD is associated with a number of pathological events including cell infiltration, inflammation,proliferation and apoptosis. Examining the link between genetic mutations and how they lead to kidney failure can only be studied in vivo. Ultimately, it is only possible to test our hypotheses about therapeutic interventions for polycystic kidney disease using rodent models recapitulating human disease. The protocols to be used are those which will cause least pain, suffering and distress, whilst still inducing disease with measurable effect, to determine the effectiveness of the tested compounds.

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

Non-animal alternatives were that were considered include both 2 dimensional (2D) and 3 dimensional(3D) culture systems. Generally, relevant cell lines e.g. derived from human ADPKD patients, rodent Pkd1/2 mutant animals and CrispR/Cas9 PKD1/2 knockout lines can be used to identify novel signalling pathways involved in ADPKD progression. In addition, we also considered the use of kidneyorganoid cultures. All of these cell models will be used in preliminary experiments to test the effectiveness of new drugs before testing in mice.

Why were they not suitable?

Ultimately, it is only possible to test our hypotheses about therapeutic interventions for polycystic kidney disease using rodent models recapitulating human disease. The protocols to be used are those which will cause least pain, suffering and distress, whilst still inducing disease with measurable effect, to determine the effectiveness of the tested compounds. However, the use of data from cell culture andkidney organoids will be used to determine the effectiveness of drugs on cyst growth before treating mice.

Reduction

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

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

We based our estimate on the required number of breeding animals required to obtain the required number of experimental animals for the procedures we have planned. For the experimental animals weconsulted with our statistician colleagues to get a statistical estimation of minimum numbers of mice required to achieve a meaningful result (statistical significance). This is based on our previous studies using these models of polycystic kidney disease taking into account the variation in disease progression between individual animals.

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

We have used the animal model in previous studies and therefore have a good understanding of the natural variation in disease progression. This has enabled us to predict with greater certainty the required number of animals to achieve a meaningful result. In addition I will utilise the NC3Rs experimental design assistant to help design our experiments to make them more likely to yield reliableand reproducible results

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

We have an optimised protocol for genotyping our breeding animals which we have developed in ourlab. This enables us to make sure that all the offspring from our breeders will be suitable for use as experimental animals in our procedures. This optimised breeding strategy enables us to avoid unnecessary breeding 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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using genetically modified mouse models of ADPKD. These models will be inducible conditional knockout models. This has the advantage that animals will not develop kidney disease untilgene deletion has been induced thus causing the least amount of harm during the lifespan of the animals. Experimental procedures will be carried out for short periods of time (not more than 6 months)therefore these animals will be culled before end stage renal failure occurs, again minimising the suffering that these animals experience.

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

Rodents are the species with the lowest degree of neurophysiological sensitivity which can be employed in studies associated with chronic kidney disease. Since the progression of renal disease in PKD is of a heterogenous nature, a number of different animal models are required to study the effectiveness of drug intervention. We are fortunate enough to study a renal disease with known genetic underlying causative mutations; as such mice with mutations or genetic alterations in either Pkd1, Pkd2 or other cystic genes will be used in this program of work and as such represent the most refined models to achieve the aims of this programme of work. Mutations in Pkd1 are the most common form of PKD in humans (>85% of all cases) therefore it is not suitable to use zebrafish modelsas they contain two alternative copies of the Pkd1 gene and knockout of these genes do not lead to formation of kidney cysts for reasons unknown.

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

Refinement techniques represent a key focus of animal welfare. A number of refinement techniques will be implemented in this programme of work. Animals will be closely monitored for signs of chronic kidney disease. However, mice have large spare capacity in renal function and any renal fibrosis is largely asymptomatic. Other examples of refinement include ensuring that unless specifically requiredanimals will be housed in appropriate social groups and caging will provide sufficient bedding and shelter to encourage species specific behaviour, using appropriate anaesthesia and analgesia to minimise pain, and training animals to cooperate with procedures to minimise any distress.

At all times during this programme of work animals that develop clinical or biochemical signs of end-stage kidney disease /renal failure will be humanely culled. Veterinary advice will be taken before beginning experiments as above and will be promptly obtained if animals appear unwell during the experiments.

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

I will use the NC3Rs resource library to get the best possible guidance on 3Rs relevant to our work.

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

By regularly visiting the NC3Rs website (https://www.nc3rs.org.uk/) I will be able to keep informed about any new developments. In addition I will attend the local seminar series where I have previously presented on the topic of 'Easy and rapid method of zygosity determination in transgenic mice by qPCR' which detailed how we had developed a novel qPCR based homozygosity assay which enabledus to optimise our breeding animals reducing unnecessary wastage of unsuitable offspring.

DEVELOPMENTAL AND REPRODUCTION SAFETY TESTING OF CHEMICALS, PLANT PROTECTION PRODUCTS, BIOCIDES AND SUBSTANCES ADDED TO FOOD OR FEED PRODUCTS USING SMALL ANIMAL SPECIES

Project duration

5 years 0 months

Project purpose

- (c) 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 aimsmentioned in paragraph (b)

Key words

Regulatory, Safety Assessment, Developmental, Reproduction, Chemicals

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

With specific focus on potential reproductive and developmental effects (how chemicals affect the reproductive organs and process and the development of the foetus in the womb, right through until they are ready to reproduce themselves), the overall aim of this project is to provide regulatory authorities (agencies who require information regarding the safety of chemicals that the public may be exposed to) with the information they need to make informed decisions regarding substance safety andauthorisation for use.

This project licence authorises the conduct of safety studies in laboratory small animal species (mouse, rat and rabbit) to evaluate candidate molecules and novel and currently registered substancesin terms of systemic toxicity, toxicokinetics and the impact on all stages of development from conception to sexual maturity through one or two generations

of animals.

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

Why is it important to undertake this work?

Governments require (and the public expects) that substances we are exposed to are safe or that theirpotential hazards are well understood and documented.

The data generated from the studies performed under this project will be used to inform decision- making processes on substances under development and, where appropriate, to satisfy governmentalregulatory requirements necessary to gain marketing authorisation or product registration.

This safety assessment is of immense importance along with other non-rodent and non-animal studiesin demonstrating to governments and the public the safety of these substances or highlighting their known hazards and safe handling.

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

This project licence authorises the conduct of in vivo safety studies in laboratory small animal speciesto evaluate candidate molecules and novel and currently registered substances in terms of systemic toxicity, toxicokinetics and the impact of chemicals on all stages of development from conception to sexual maturity through one or two generations.

The overall benefit of this project is that it generates high quality data that is acceptable to regulatory authorities and enables internal decision making within our clients' organisations. This project will alsoensure that chemicals and pesticides that the general population are exposed to are safe.

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

Our customers will benefit, as the data we generate will allow them to progress their substances underdevelopment and, where appropriate, to satisfy governmental regulatory requirements necessary to gain marketing authorisation.

The studies ensure that non-pharmaceuticals such as food additives, agrochemicals and industrial chemicals that the human population are exposed to during their lives are safe or that their hazards areknown so that they can be handled safely, from pregnancy through to adulthood, including sexual function.

The primary benefit of work carried out under this licence will be to allow regulatory authorities (who are totally independent from the commercial interests behind every marketing application) to come toinformed decisions, based upon safety data generated in these studies, regarding the risks to which humans are exposed when compounds are produced, transported or used.

For all categories of material covered in this Licence, studies conducted will help to remove unsuitablecandidates from development at an early stage, thus saving animals and resources. Successful studies will also allow identification of target organs and

systems and effects on reproduction and development of offspring through one or two generations. In addition, they may provide biomarkers to allow monitoring and management of human exposure.

In the case of industrial chemicals, plant protection products, biocides, food and feed additives, achievement of the objectives of the Licence will allow selection of appropriate candidate materials fordevelopment, allows an assessment of safety-in-use for materials or facilitates hazard classification and marketing authorisation. In the case of veterinary products achievement of the objectives of this Licence enables safe (to humans) development of candidates to progress into clinical testing and onwards to marketing authorisation.

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

Where confidentiality permits, data, study design and best practice will be openly shared at conferences, workshops, webinars, blogs and publications.

As 3R's benefits are also realised under this project licence, these will be shared more widely withother establishments.
Species and numbers of animals expected to be used

- Mice: 5100
- Rats: 20400
- Rabbits: 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.

Rodents (rats and mice) will be predominantly used in this project along with rabbits. All life forms of animals will be used (either direct dosing, usually to adults, or investigating any developmental effectsof test substances).

Species choice and use of specific animal models is determined by the need to generate regulatoryacceptable data. Where a choice of species is possible, care is taken to select the most biologicallyappropriate species, and the species which most closely relates to humans. Studies to assess the types of material covered by this licence are usually performed on small animal species.

Generally, the rat is the rodent species of choice in Developmental and Reproduction Safety Testing. There is wide knowledge of the response of rats to various substances and a wealth of background literature. Rats are large enough to provide repeated blood samples, and big enough litters of pups, thus requiring significantly fewer rats than mice to achieve the same objective. Mice may be used when considered a more appropriate species, for example, if they more readily absorb the test substance, are more relevant biologically or show improved tolerance depending upon objective of thestudy.

Rabbits may be used when considered a more appropriate species, for example non-pregnant rangefinding studies prior to conducting reproductive studies in pregnant rabbits.

The rabbit has achieved acceptance as a suitable secondary non-rodent species of choice and offers several marked advantages.

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

Animals will be given the “test substance” under investigation in a way which mimics possible humanexposure. As the most likely route of exposure is orally the majority of animals will receive the test substance either mixed in their food or directly by insertion of a flexible rubber catheter into the stomach, via the mouth.

For some test substances, the oral route of administration may not be appropriate; for example, if the material is more likely to come in to contact with skin or other body membranes. Most animals are treated once daily; occasionally studies may require several doses within 24 hours. The length of studyis dependent on the tonnage of the test substance produced each year as a higher tonnage increased the risk of repeated human exposure and ranges from a simple study to explore effects on reproduction with a small number of animals to a multigeneration study to explore effects ofgenerational exposure to a compound.

Blood and urine samples may be taken to measure the level of test substance or its metabolites withinan animal’s circulatory system. These may also be analysed to detect any changes in blood or urine chemistry, allowing in-vivo monitoring of body systems and organs for example liver or kidney function.Neurobehavioural assessments may be carried out to identify potential neurotoxicity by observing and describing behaviour.

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

Many of the endpoints measured on reproduction studies do not adversely affect the life of the animals. For example, offspring may simply be observed for developmental milestones such as eye opening and the development of reflexes and as they grow they may be observed for evidence of sexual maturation, which may be precocious or delayed. Study animals are observed at least twice a day by highly trained technologists who monitor for any signs of discomfort. Other measures such as food consumption and bodyweight are used to closely monitor for treatment related effects. Veterinarysurgeons are employed on a full time basis and are available 24/7 to provide clinical treatment, guidance on animal welfare and the conduct of procedures including appropriate surgical technique, anaesthesia and analgesia.

The majority of animals are expected to have mild adverse effects of treatment such as reduced weightgains or changes in appearance or behaviour. A small number of animals (usually limited to the highestdoses evaluated in early studies) may show more moderate adverse effects. The nature and type of effect varies dependant on the biological systems affected, however, these usually result in findings such as reduced food consumption, body weight loss and changes in behaviour. Humane endpoints will be adopted, or dose levels reduced if animals show excessive effects.

Longer term studies are expected to have progressively less adverse effects. Effects on reproduction and fertility of a test substance are not always evident during the in-life phase of a study and may not impact the animal’s wellbeing (for example reduced numbers of maturing sperm and a reduced numberof eggs). Only through microscopic examination of the tissues from each animal, can evidence of all toxicological changes be fully assessed and the scientific value of each animal maximised. In order to undertake these evaluations,

the animals must be put to sleep humanely at the end of a study, under terminal anaesthesia.

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

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

On the last project, about 2% of animals were classified as having displayed moderate severity, the rest (98%) will have suffered only mild severity. This is because legally, all surgical procedures carriedout on an animal must be classified as moderate, and on occasions, there were prolonged periods of dosing and sampling required to get the information we needed (also moderate). The rest of the animals were classified as having displayed mild severity.

It's impossible to predict the proportion of severities expected on a service licence, as this will bedependent on what study types we are asked to perform.

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

- Killed
- Kept alive
- Rehomed
- Used in other projects

Replacement

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

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

There are currently no scientific and legally acceptable evaluations of whole body, systemic toxicity thatwill satisfy regulatory requirements with respect to developmental and reproductive safety of medicinal products and other chemicals, other than the use of animals. Wherever possible, validated in vitro testsfor specific organs are used and valuable information may also be obtained from alternative non- mammalian test species (e.g. fish, amphibians). Where available, review of scientific articles, non- animal methods and read-across to other animal data such as metabolism, pharmacology and general toxicology information is also utilised to reduce animal use.

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

There are some studies that can be carried out in-vitro that can be used to support this work, includingtests that assess metabolism and absorption of substances, and how well they bind to key proteins in the blood. Predictive software can also be used.

These studies are often performed before the test substances reach us, for the testing detailed in thislicence.

Why were they not suitable?

None of these tests can yet model the complex and integrated mechanisms governing the effect ofnew chemicals on reproductive function and hence, animal testing is still a requirement by Global regulatory authorities.

Reduction

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

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

The number of animals used on this project is estimated from those used under previous projects andafter consideration of regulatory trends, and in review of future services being developed and offered for the selection of new chemicals for regulatory studies.

Studies will be designed under this licence such that the minimum number of animals will be used inorder to obtain the maximum information, whilst the scientific objectives of each study are met, in accordance with regulatory requirements and agreed standard practices.

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

Studies are designed to provide maximal scientific value from the minimum number of animals, whilstusing sufficient animals to meet scientific objectives, and regulatory guidelines. Statistical input is sought, where appropriate, to strengthen the overall scientific quality and relevance of studies.

Where available, sensitive analytical techniques may be used to reduce animal numbers (for exampleby reducing blood volume requirements).

Wherever practicable, the re-use of suitable animals, and by looking across studies, the combination ofendpoints e.g. general toxicity, reproductive toxicology, safety pharmacology, mutagenicity etc. in studies is considered, to reduce overall animal usage.

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

For studies where a new substance is being tested in animals for the first time, we would often test thatin a small group of animals (usually 1-3) to give us confidence that the dose levels we chose are safe, and the substance affects the system its designed to, without making an animal ill. These are called pilot studies.

We will try and get as many outputs as we can from a single animal where possible, without adverselyaffecting its welfare. So if we need to get a blood sample, or if we need to find the levels of a substance in urine, for example, we will often do that in the same animal, rather than use separate ones, when possible.

Refinement

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

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

Species choice and use of specific animal models is determined by the need to generate regulatory acceptable data. The rodent is the first choice for reproduction studies run using the regulatory guidelines. Rabbit provides a second species for evaluation of prenatal development of the offspring. The rabbit has been selected as the second species for embryo fetal development studies because it was found to be sensitive to Thalidomide, a notorious teratogen, whilst the rat was insensitive to this chemical. The rabbit is only used in prenatal development studies under this project licence. The rabbit may be sourced from non-EU recognised breeding establishments to ensure good pregnancy rates, therefore reducing the number of animals required on a study to meet the study objectives. If forsome reason, for example unusual metabolism patterns or sensitivity, the rat or rabbit are considered to be unsuitable for reproductive study work, then the mouse can usually be substituted.

Animals used under this Licence will be sourced from suppliers within the UK, EU or non-EU.We will use animals at all stages of development from those in utero to adult.

Animal studies are only considered where there is a direct legislative or regulatory requirement and after review of all other available information to ensure that no alternative is feasible. A step-wise approach is taken, with higher risk studies, when little may be known about the test substance, beingperformed early in a programme and using only small numbers of animals. As confidence in the dataand the level of information grows, longer term studies in larger numbers of animals may become necessary and the design of these studies, whist adhering to regulatory requirements, can be refinedand tailored to obtain the most relevant and valid scientific information from the fewest animals and with the lowest level of adverse effects possible.

Animal welfare costs are minimised by the careful selection of dose levels to reduce the likelihood of unexpected toxicity, and the application of rigorous and comprehensive humane endpoints. Individualstudies are designed to cause the least possible suffering by frequent review of practices, provision ofhighly skilled technical staff and veterinary support, purpose built facilities and a clear focus on animalwelfare. Any confinement or restraint is restricted to the minimum required to achieve the scientific objectives of the study and all study plans/protocols are reviewed for adherence to welfare guidelines and best practices by the site’s Animal Welfare and Ethical Review Body (AWERB).

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

Rodents will be used in most of the studies conducted under this licence. Rodents are considered to be the species with a similar enough brain/nervous system and physiology, and fecund, that will allowus to achieve the study aims and are considered suitable for the predicting what's likely to happen in humans.

The database available as an aid to assessment of the biological significance of reproductive parameters showing apparent treatment-related effects is heavily dependent on the rat. The usual availability of relevant range-finding data on the systemic toxicity of the Test substance from completeor contemporaneous acute, subacute and chronic toxicity studies using the rat has also contributed tothe status of the rat as the preferred species for reproduction toxicity studies.

The requirements for marketing authorisation for feed additives or agrochemicals/residues present infoodstuffs, chemicals, biocides, plant protection products or substances added to food require a second non-rodent species to be used in studies of embryo-fetal toxicity. The rabbit has achieved acceptance as the non-rodent species of choice and offers several marked advantages.

Studies of prenatal development following the OECD guidance document (OECD 414) will generally berun using time-mated female rats or rabbits supplied to the test facility by a regulated supplier.

Studies covering the reproductive cycle (mating, gestation, littering and development of the F1 offspring), i.e. OECD 421, 422, 443, 426 and 416, will require the purchase of young adult males andfemale rats or mice.

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

Many of the procedures used in this licence are standardised, well defined and already well refined over many years. We will continue to assess any future possible refinements over the duration of thislicence.

Where animals do show adverse clinical signs after dosing, we will increase the frequency and length of observations, and provide supplementary interventions (like extra bedding/food/heat) where needed,until the signs resolve.

Similarly, if after discussing with a vet and senior technician, we decide an animal is not recoveringfrom procedures, and there is no prospect of them doing so in the near future, we will humanely killthem to prevent further suffering.

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

OECD Guidelines – see www.oecd.org or www.oecd-ilibrary.org

OECD 414 – Prenatal Developmental Toxicity Study (2018)

OECD 415 – One-Generation Reproduction Toxicity Study (1983)

OECD 416 – Two-Generation Reproduction Toxicity study (2001)

OECD 421 – Reproduction/Developmental Toxicity Screening Test (2016)

OECD 422 – Combined Repeat Dose Toxicity Study with the Reproduction/Developmental ToxicityScreening Test (2016)
OECD 426 – Developmental Neurotoxicity Study (2007)

OECD 440 – Uterotrophic Bioassay in Rodents (2018)

OECD 441 – Hershberger Bioassay in Rats (2009)

OECD 443 – Extended One-Generation Reproduction Toxicity Study (2018)

Summary of Considerations in the Report from the OECD Expert Groups on Short Term and LongTerm Toxicology (2006)

Notes for guidance on repeated dose toxicity. Committee for Proprietary Medicinal Products(CPMP), 2010. CPMP/SWP/1042/99

Guideline on the evaluation of control samples in non-clinical safety studies: checking for contamination with a test substance. Committee for Medicinal Products for Human Use (CHMP), 2005.
CPMP/SWP/1094/04

LASA/NC3Rs: Guidance on dose selection for regulatory general toxicology studies for pharmaceuticals. http://www.nc3rs.org.uk/downloaddoc.asp?id=1108

EU Directive 91/414/EEC – evaluation, authorisation, approval of active substances at EU-level andnational authorisations of plant protection products (PPPs); EU Feed Hygiene Regulation (183/2005); EU Regulation 882/2004 on official controls for feed and food law (and animal health and animal welfare); EU Biocides Regulation 528/2012 and EC 1907/2006 REACH Regulations

Diehl et al. 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)

Foote RH and Carney EW. The rabbit as a model for reproductive and developmental toxicitystudies, Reproductive Toxicology 14, 477-493, 2000.

Gad et al. Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routesin multiple species with notes on methods to improve utility. International Journal of Toxicology: 1-84 (2016)

NC3Rs: Recommendations from a global cross-company data sharing initiative on the incorporationof recovery phase animals in safety assessment studies to support first-in-human clinical trials (Regulatory Toxicology & Pharmacology, 2014)

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

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

DEVISING NEW THERAPIES FOR MULTIPLE SCLEROSISAND SMALL VESSEL 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, ortheir effects, in man, animals or plants

Key words

Multiple sclerosis, Cerebral small vessel disease, Neuroinflammation, Hypoxia, Cerebral blood flow

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

Retrospective assessment

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

Reason for retrospective assessment

- This may include reasons from previous versions of this licence.
- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

Our aim is to reduce the suffering of people with diseases such as multiple sclerosis and small vesseldisease. We seek to understand the biology of these diseases in order to develop new, clinically relevant therapies to protect from these disorders.

A retrospective assessment of these aims will be due by 16 July 2028

The PPL holder will be required to disclose**:**

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

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

Why is it important to undertake this work?

The proposed programme of research addresses the pathophysiology (i.e. understanding the mechanisms that are responsible for causing structural damage (pathology)) and therapy of inflammatory diseases of the brain, spinal cord and nerves in the arms and legs. These diseases cause severe and often permanent neurological symptoms. Neuroinflammation is a broad term that includes not only the classical inflammatory diseases multiple sclerosis and Guillain-Barré syndrome,but also small vessel disease and the neurodegenerative diseases (e.g. Alzheimer's and Parkinson'sdiseases). Our research is relevant to all these disorders.

The disorders cause inflammation and reduced blood flow within the brain, and this causes severe symptoms (including blindness, paralysis and numbness) and damages brain tissue causing dementia,amongst other problems. The mechanisms responsible are not known, but our research aims to discover the mechanisms so that we can develop new therapies to protect patients.

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

In the short term, our output will be the introduction of a valuable new model of secondary progressivemultiple sclerosis that has revealed not only when the damage responsible for the relentless advance of disability occurs, but also how to prevent it. Our ongoing research also provides an increased understanding of how neurological disability arises in the major neurological diseases. In the medium
term, our understanding of the causes and consequences of prolonged energy deprivation will indicatenovel therapeutic strategies for protection that will be tested in the laboratory within the period of this licence. The research will add to our existing strategies new ways to tackle neuroprotection in multiplesclerosis (MS), and to extend these strategies to other neurological disorders, including dementia and neurodegenerative disease.

Our research is literally changing the neurological research community's understanding of multiple sclerosis (MS), and we are introducing new approaches to therapy that are likely to persist and become established adjuncts to existing therapies. This research will also build on our recent findingsthat indicate an effective strategy to reduce dementia due to prolonged high blood pressure. Our research findings are, of course, published in the scientific and medical literature.

Who or what will benefit from these outputs, and how?
In the short term, laboratory and clinical researchers will benefit from a greater understanding of howinflammation and cerebral blood vessel disease cause short and long term damage to the brain, andthrough analysis of novel therapeutic interventions we will learn how to prevent this damage.

We have created models that aid in the refinement of protocols, including the only model of the type ofmyelin (nerve fibre insulation) loss that occurs in early MS lesions, and the best model of slowly progressive neurodegeneration that occurs in MS. These advances reduce the number of animals experiencing the severe model of experimental autoimmune encephalomyelitis (EAE) typically used inMS research.

We will gather data using spatial transcriptomics (a method that reveals the expression of genes in allthe different cells in a tissue while retaining the spatial relationships between the cells) in our models,and these data will be uploaded to public repositories so that other researchers may benefit.

With our collaborators, the work in this project will be used to develop techniques to understand disease mechanisms in awake patients, using non-invasive methods that are entirely pain free. Thuswe are using methods (magnetic resonance imaging (MRI) and near infrared spectroscopy (NIRS)) that are translatable to investigations in patients, and that can aid diagnosis and monitoring of therapeutic regimens.

In the long term, patients will benefit from therapies that protect brain tissue from causing symptomsand suffering.

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

We have strong collaborations with engineers to make devices to measure brain oxygenation, with physicists to make a device (a new type of ophthalmoscope) that can detect even subtle deficits in theoxygenation of the retina, and with clinicians to translate these new developments to study disease in patients. The new methods allow us to monitor the value of existing and novel protective therapies in patients. Data obtained from our spatial transcriptomic studies, and similar studies, are shared publicly, as noted above. Our findings are presented at specialised and major conferences, and published in the open access scientific literature. Pathology specimens are shared upon reasonable request. We also communicate our findings to patients at local and national conferences organised forpatient participation.

Species and numbers of animals expected to be used:

- Rats: 4000

Predicted harms

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

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

The diseases that we study are experienced by adult humans. Several selectively bred rat strains have been developed over the last 10 years that recapitulate and mimic different pathologies of neuroinflammation and small vessel disease with accuracy. Our research uses the most appropriateanimals for the research question we are studying. Individual experiments are usually completed within a month. We only use rats of a developmental age that is reflective of the onset and development of the relevant disease in humans.

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

For models of MS we mainly use a model where the disease is induced by immunisation (via subcutaneous injection) and autoimmunity rather than by surgical methods, and so these experimentsdo not involve surgery. Many people believe that MS is caused by a similar autoimmune activation of the immune system. Immunised animals are observed

over the following weeks (typically 2-3 weeks) while walking around to detect any deficits in their hindlimb or tail function. These deficits usually start10 days after immunisation, and since we are mainly interested in stopping further advance of the deficits, the animals typically do not experience any deficits for more than a few days. In the early days of deficits the animals usually only experience a slightly floppy tail and weakness in their hindlimbs, but if the disease advances to impair their ability to reach food and water, they are fed wet mash and water (e.g. hydrogel) on the floor, and by hand if necessary. Experiments involving progressive MS employ a lesion induced by microinjection into the spinal cord under general anaesthesia. This model only involves tail and hindlimb weakness. The therapies we use are mainly administered by adding them to the food, or by raising the amount of oxygen the animals breathe.

Rarely, therapies are administered by injection, typically subcutaneous or less commonly byintraperitoneal or intravenous injection.

Animals with a model of small vessel disease have the disease as a consequence of inherently raisedblood pressure, which is also the main cause of the disease in humans. Nothing is done to promote the high blood pressure, but the animals have previously been selectively bred for this characteristic. High blood pressure in rats, like high blood pressure in humans, does not cause any symptoms, at least at the early to medium stages.

All animals are typically housed in groups in standard cages. At the end of experiments all animals arekilled under general anaesthesia, because we typically want to take tissues after perfusion fixation under general anaesthesia.

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

Animals with a model of MS are likely to have hindlimb and/or tail weakness or paralysis, with deficitsstarting around 10 days after immunisation and usually expressed for only a few days before they improve. We are mainly interested in the very early consequences of inflammation in the nervous system, and at this stage the animals either do not show any symptoms, or only very mild symptoms.Therapeutic intervention is expected to reduce symptoms, and certainly not expected to cause furthersymptoms.

Any painful procedures (e.g. surgery if required) are, of course, performed under general anaesthetic. Demyelinating lesions, as in MS, are not usually painful in patients, and animals do not appear to be in pain either. Animals are more likely to lose sensation, resulting in numbness of parts of their hind limbsand tail, rather than experience pain.

Raised blood pressure does not cause any symptoms or discomfort in the animals, just as it causes nosymptoms or discomfort in humans, at least in the early and moderate stages. After a long time (e.g. 6months), the high blood pressure can weaken blood vessels and reduce blood flow and then animals can get cognitive (dementia-like) deficits, as occurs in older people, and they might get a stroke, again like humans. Animals that experience such severe symptoms are promptly 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 animaltype)?

Study 1. Breeding and maintenance of selectively bred animals. 95% Mild, <5% moderate.

Study 2: Terminal examination. 100% non-recovery as rats will only undergo terminal anaesthesia.

Study 3: CNS lesion (excluding EAE). 100% moderate as animals experience an intraspinal microinjection under general anaesthesia, with subsequent tail and hindlimb weakness. Weakness is expressed on days 2 and 3 after lesion induction, after which the animals recover for a month or so, and then the symptoms slowly recur over the lifetime. The hindlimb weakness is never severe, and thehindlimb strength is always sufficient to support the body weight and allow walking. In a typical experiment, hindlimb weakness that is visible to an observer is mainly only experienced by lesioned byplacebo-treated ('control') animals that are used to demonstrate disease, as a comparison to show the beneficial effects of animals we treat with our experimental therapies.

Study 4: Experimental autoimmune encephalomyelitis (EAE). 10% Mild, 60% moderate, 30% severe. EAE can cause severe symptoms as it progresses, but we aim to terminate our experiments before severe symptoms develop. Thus, most animals (70%) under this study will experience only mild or moderate symptoms, such as hindlimb and tail weakness. The weakness/paralysis usually only lasts for a few days, partly because the animals spontaneously recover after a few days, and partly because
it is only the onset of disease that we are mostly interested in studying, and so most animals are terminated within 2-3 days of disease onset. The weakness is expected to be painless (and animalsshow no signs of being in pain), as it typically is in humans with MS. Some animals (less than 10%)may experience more severe symptoms such as hindlimb and tail paralysis. Such animals are promptly terminated.

Study 5: Spontaneously hypertensive rats (SHR). 80% Sub-threshold, 20% moderate. Animals typically do not show any symptoms or discomfort, in common with humans with hypertension. After along time (e.g. 6-12 months), animals can get cognitive (dementia-like) deficits, as occurs in older people, and they might experience a stroke. Strokes in both animals and humans frequently cause either no symptoms, or only mild symptoms: animals with moderate symptoms are promptly terminated. Typically, half the animals under this study are on neuroprotective therapy (a therapy thatwe can directly apply to patients) and such treated animals have never experienced any neurological deficit from strokes in our experience over 7 months of age.

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

- **Killed**

A retrospective assessment of these predicted harms will be due by 16 July 2028

The PPL holder will be required to disclose:

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

Replacement

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

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

Our research has shown that understanding the pathophysiology of the inflamed nervous system, particularly with regard to oxygenation and mitochondrial function, absolutely requires a functioningimmune system, cardiovascular system, and pulmonary system. With current technology and understanding it is impossible to replicate the interaction of these systems in a physiologically meaningful way outside the body.

In particular, our experiments indicate that a key factor in causing symptoms is an inadequate supply ofoxygen to the inflamed tissue, and this can only be studied in the brain with a functioning blood system. Our aim is also to test novel therapies, and to do so we need a functioning blood supply to deliver the therapies, just as they would be delivered in patients. We also aim to improve function in the animals and patients, and to assess this we need to be able to watch how well the animals can walk along, before and after the medicine being tested has been given. It is therefore unfortunately necessary to use animals in the proposed research.

Other experiments study the consequences of a lifetime of raised blood pressure. It is not currentlypossible to achieve these requirements in cultures or computers.

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

We have considered the use of computer simulation, tissue culture, and ex vivo preparations.

Computer simulations of neuroinflammatory lesions are not yet realistic because we remain ignorant ofmost of the key values necessary to mimic the environment within such lesions. We use tissue culture to study the properties and behaviour of certain immune cell types (e.g. T cells and microglia), but suchpreparations cannot mimic the complex environment of a neuroinflammatory lesion because they lack the crucial ingredient of an intact blood supply. We employ ex vivo preparations when possible (e.g. excised peripheral nerves), but mitochondrial function is strongly affected by the concentrations of oxygen and nitric oxide, and it is impossible to reproduce meaningful concentrations of these agents invitro because their concentrations within inflamed lesions are not known.

We also need to see how the ability of animals to walk around is improved by the drug therapy. It istherefore unfortunately necessary to use animals in the proposed research.

Why were they not suitable?

Non-animal alternatives do not have a functioning vasculature and immune system, and models of neuroinflammatory lesions in culture are not closely comparable to those in humans. It is not realisticto study the effects of medicines intended to improve disability in humans using non-animal systems.

A retrospective assessment of replacement will be due by 16 July 2028

The PPL holder will be required to disclose:

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

Reduction

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

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

We use statistical power analysis to calculate the minimal number of animals required to achieve experimental aims. Professional statisticians are at hand within our department and within the Institute, and they are consulted as necessary to ensure that our research employs sufficient animalsto make statistically significant findings, but no more animals are used than required. Animals are randomly assigned to experimental groups, and 'blinding' of investigators performing judgement tasks,to avoid bias, is of course routine.

Numbers chosen reflect the minimum numbers required for initial pilot studies and subsequent largerstudies, for individual projects, in order to achieve statistical significance. Our individual experimentstypically employ 8-20 animals per group.

We optimize our breeding strategy to produce minimal numbers of animals.

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

We ensure our study design employs the best technical approaches to maximize the scientific outcomeof our work while ensuring the least number of animals are used and that experimental bias is minimized. Experiments are designed with reference to the ARRIVE guidelines.

We routinely employ the NC3R's Experimental Design Assistant to assist us in designing experiments and in calculating the number of animals required for statistically significant findings. In each experiment we obtain the maximal observations from each animal, such as studying them behaviourally during the experiment, correlating these findings with serial physiological observations using, for example, near infrared spectroscopy to measure brain oxygenation, blood pressure, multispectral imaging to measure retinal oxygenation), and correlating all these recordings with detailed microscopic examination of the affected tissues after death. These processes are reflective ofthe clinical scenario.

In some experiments we require histological examination of the tissues, in which case serial observations of the same animals are not possible, but mostly we employ serial observations to reducethe numbers of animals involved. The electrophysiological examinations are minimally invasive (temporary insertion of hypodermic needle electrodes under anaesthesia) so animals can be examinedserially. With retinal examination it is possible for repeated examination, as the process is non- invasive.

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

We routinely use pilot studies to assess the feasibility of new study protocols or new hypotheses. These pilot studies usually employ very small numbers of animals, and allow

us to examine and testmultiple parameters within individual test subjects. We find such studies invaluable to our research.

We have significant experience in the induction of experimental lesions, minimising experimental variation, and will employ this experience to ensure that minimal numbers will be used. We also employ small animal MRI using a 9.4T scanner, which allows the same lesions to be monitored over time, rather than in different animals. We are also able to ensure that animals with experimental autoimmune encephalomyelitis (EAE), for example, can not only be used for in vivo or MRI imaging, but following perfusion fixation their tissues can be used in the several histological studies ongoing inour laboratory. In addition, high volumes of tissue are generated from individual experiments as we harvest as much tissue as possible (e.g. spinal cord, brain, optic nerve, liver, kidney, sciatic nerve, muscle etc), allowing us to build up an indispensable bank of tissue. This bank of tissue is sharedbetween projects within the group, and also with internal and external collaborators.

Our ability to make and correlate multiple observations from each animal is a powerful strategy toreduce the number of animals used.

A retrospective assessment of reduction will be due by 16 July 2028

The PPL holder will be required to disclose:

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

Refinement

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

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

Animal suffering will be limited in our studies by our strict monitoring of animals to ensure that they remain within the severity limits of their associated protocols, which have been designed to minimizetrauma and suffering.

We apply well-established protocols that have been repeated over many years of use, ensuringstandard operating procedures and technical competence and standardisation.

We also apply state of the art approaches that take into account relevant developments in the literatureand the knowledge of competent researchers, with guidance from the NVS to ensure most refined models and methods are used.

To minimise stress whenever possible animals will receive environmental enrichment and be housed ingroups. Anaesthetic and analgesic regimes will be used to minimize pain wherever appropriate.

We will employ rats in the proposed research, and there is substantial evidence that findings made inthese species can be meaningfully translated to the care of patients in the clinic. Much of our preliminary data have also been obtained in rats.

We employ several animal models of multiple sclerosis and small vessel disease. The most commonlyused model of multiple sclerosis around the world is known as experimental autoimmune encephalomyelitis (EAE), and we sometimes use this model. EAE in rats, particularly in DA rats, has been described as closely reflecting the spectrum of pathology in MS (Storch et al., Brain Pathology, 1998). It is necessary to use EAE because regulatory authorities require demonstration of the efficacyof therapies in this model: EAE is arguably the closest model to MS. Indeed, clinical trials directly based on data obtained in rats with EAE have proven to be a successful translation to human disease,providing very significant protection of vision, establishing the value of rats in MS research. The modelis also widely used and a potential therapy has to be demonstrated in this model to gain scientific credibility.

Multiple sclerosis is typically not painful, especially in younger adults, and rats with EAE similarly show no signs of pain. Rather, multiple sclerosis and EAE tend to remove sensation, resulting in numbness. We are interested in developing medicines to treat symptoms at their immediate onset, and so we haveobtained all our observations within a few days of the onset of any symptoms, and the experiment is then terminated.

Inflammation within the CNS is normally hidden from view, but inflammation of the retina (CNS tissue)can be monitored in the intact eye via the pupil, just as in humans, and we employ retinal examinationas a non-invasive avenue to events hidden within the brain.

Another model has been developed in our laboratory and at its most severe the animals can have weak hindlimbs and tail, but the hindlimb weakness is typically so slight that it requires study of videosto detect it.

Our model of small vessel disease involves rats with inherently raised blood pressure (hypertension).High blood pressure in rats does not cause any symptoms, just as high blood pressure causes no symptoms in humans, at least in early and moderate ages. In late life the raised blood pressure can cause strokes, and these can cause symptoms, although many strokes in rats (and humans) do not cause symptoms. In fact, at least a third of people over 70 have had at least one silent stroke (i.e. thepatient is unaware and the stroke is detected upon routine scanning later in life). If strokes occur that do cause symptoms, the animals are promptly euthanised.

There is a range of animal models available for research into small vessel disease, and the main one chosen for our studies is the spontaneously hypertensive rat. This rat is one of the best existing modelsof human hypertension and cerebral small vessel disease. Examination of these rats allows us to study cerebral blood flow and tissue oxygenation, together with therapeutic studies to prevent cerebral pathology. We typically administer medicines by adding them to the diet, to avoid stress due to injections. Use of this model is consistent with published guidelines to improve translation from pre- clinical to clinical studies by using a model with many existing co-morbidities.

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

We use adult animals because most experiments last for weeks while the lesions (which mimic the lesions in multiple sclerosis) develop and are treated. Immature animals would not be representative as their brains are not fully developed and thus they do not have an

adult vasculature and oxygen consumption, which are important considerations for our experiments. The brains of neonatal animalsare much more tolerant of inadequate oxygenation than adult animals, and so they do not provide meaningful observations. Multiple sclerosis affects adult humans, and it is necessary to study adult animals for comparable observations.

Rats have a brain with a metabolism comparable with that of humans, and we study the changingenergy balance of the brain as it undergoes inflammation, demyelination and degeneration. Non-mammalian animals do not reflect this biology. Mammals are also required because we are developingmedicines for humans, and the medicines require testing in mammals.

We are unable to use terminally anaesthetised animals because the experiments last for weeks, and itis not feasible to study anaesthetised animals for such periods.

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

Our experiments aim to develop medicines to prevent symptoms, and so treated animals typically haveonly mild, if any, symptoms. Untreated animals typically only show any symptoms for a few days at most, as they are terminated once the beneficial effects of the treatments in the treated animals have been demonstrated by comparison. Experiments in our MS research that employ EAE do not involve any recovery surgery, and similar lesions in patients are typically not painful. In other experiments, if recovery surgery is involved, pain resulting from the surgery is controlled with analgesics. The animalsdo not display evidence of suffering, and similar lesions are typically not painful in patients. In experiments where we induce inflammation in a specific region of the brain and spinal cord, we choose“clinically silent” locations that do not cause any symptoms.

General anaesthesia is used for all procedures that may be painful, such as surgery, and electrophysiological examination (although such examinations are conducted without anaesthesia in patients). We employ state-of-the-art methods for our investigations to ensure the optimal data, just as are employed in the clinic, and we also closely monitor the physiological condition of the animals underanaesthesia. Notably, where possible we employ non-invasive methods similar to those employed in the clinic, such as small animal MRI, to examine lesions over the course of disease. This not only reduces animal numbers but it also allows lesions to be examined using the same technology as is used in patients, providing findings that are often directly applicable to findings in patients examined in the same way.

All experimental animals are examined at least daily. Animals with EAE are examined twice a day between days 8-14 post-immunisation, when symptoms can advance rapidly. Examination of animals includes study of not only whether there is evidence of a neurological deficit (e.g. limb weakness), but also whether there is evidence of more systemic problems such as weight loss and signs of discomfortor abnormal behaviour (listless, aggressive, sedated, hunched, fearful, piloerect, ‘hiding’ in corners).

Examination for pain includes evidence of spontaneous vocalisation, aggressive upon handling, hunched, piloerect, or scores on the grimace scale (Sotocinal SG et al. (2011). The Rat Grimace Scale. Molecular Pain 7: 55.)). Animals giving cause for concern may be discussed with the named veterinary surgeon (NVS), and will be killed if they exceed the severity limits assigned for their protocolstep.

Animals will always be able to reach water and food, so these will be provided within reach, includingmashed food, if indicated. Animals will also be fed and watered by hand if indicated.

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

All our experiments follow the ARRIVE 2.0 guidelines.

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

We keep a close eye on the NC3Rs website (https://www.nc3rs.org.uk/) and are also routinely updatedabout changes and best practice by the Biological Unit at our institution. We also attend seminars/workshops hosted by NC3Rs.

A retrospective assessment of refinement will be due by 16 July 2028

The PPL holder will be required to disclose:

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

DISEASE MODELS OF EMERGING AND RE-EMERGING VIRUSES

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir 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 aimsmentioned in paragraph (b**)**

Key words

Arbovirus, Robovirus, Therapy, Pathogenesis, Modelling

Animal types	Life stages
ice	adult, juvenile
Rats	adult, juvenile
Guinea pigs	adult, juvenile
Hamsters (Syrian) (Mesocricetus auratus)	adult, juvenile
Ferrets	Adult, juvenile

Retrospective assessment

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

Reason for retrospective assessment

- This may include reasons from previous versions of this licence.
- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

To develop specialised animal models of arthropod-borne viruses (arboviruses), rodent-

borne viruses(roboviruses) and other emerging and re-emerging zoonotic viruses of consequence.

A retrospective assessment of these aims will be due by 20 August 2028

The PPL holder will be required to disclose:

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

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

Why is it important to undertake this work?

Transmission and pathogenesis of viruses that cause severe disease in humans is often poorlydefined, largely due to the difficulty of studying pathogens of high disease consequence in the laboratory setting.

Development of robust animal models that directly compare to human disease will enhance our understanding of human pathogenesis and can be used for intervention (therapeutic and prophylactic)studies.

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

Information generated from infection models will determine the stages and situations where other susceptible animals - including humans - may be at risk of infection. The data will inform public healthbodies and provide advice on how to avoid exposure to zoonotic pathogens.

By understanding whether exotic arboviruses can be maintained and transmitted by UK arthropod vectors (ticks and mosquitoes), information for future UK government policy and resilience planningcan be provided.

A successful model for intervention testing could lead to the prevention of severe disease or reduction/delay in the onset of clinical signs in susceptible animals including humans. This informationcan be taken forward for subsequent human clinical trials.

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

Public health bodies will be better informed to provide advice on how to avoid exposure to zoonoticpathogens. Understanding of new viruses transmitted by UK ticks and mosquitoes can allow futureresilience planning.
Progression of therapeutics and interventions (vaccines) towards clinical trials and licensing will benefitclinicians, patients and animals.

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

All collaborations (either new or maintained from previous licensed studies) will ensure we keep pacewith relevant scientific developments, avoid duplication and use current best

practice in experimentaldesign of animal studies.

Importantly this work also underpins a UK capability and maintains a unique level of expertise that canbe rapidly called upon in times of emergency, for example during the COVID pandemic and more recently the monkeypox outbreak which were each declared as Public Health Emergencies of International Concern (PHEICs).

Our group actively publishes and disseminates its work in open access journals and scientific meetings. Dissemination of all data - both successful or less so, (For example, when the WHO declared the Zika virus outbreak a Public Health Emergency of International Concern (PHEIC), our group published the first data on Zika virus (ZIKV) susceptibility of IFN-a/bR-/- mice through a publiclyaccessible website before it had been accepted for print publication. This ensured that groups across the world could use an appropriate murine strain to facilitate intervention testing. It also contributed tominimising the numbers of mice used). will allow for refinements in further projects which may in turn lead to a reduction of animals used.

Species and numbers of animals expected to be used

- Mice: 3500
- Rats: 600
- Guinea pigs: 800
- Hamsters (Syrian) (Mesocricetus auratus): 900
- Ferrets: 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.

Use of resources such as literature and our knowledge gained in previous studies and previous licences, will indicate which species will be most relevant for the studies in this project. The preferred models will use a natural host and reservoir where possible. When this is not possible or is unknown, pilot studies will be conducted using a hierarchy of species that will include mice, rats, hamsters, guinea pigs and ferrets to provide insight into the most clinically relevant model. Prior to any studies using therapeutic agents and/or pathogens, prior knowledge will be researched for information on variables such as determination of dosage. In addition, pilot studies will be performed when evaluatinga novel virus.

Mice with genetic alterations in their immune pathways will also be utilised. For the majority of virusesthat will be used in this project it is our experience that genetically altered mice are likely to provide a more useful model of disease then wild-type mice because of a more consistent susceptibility to infection and progression to a defined humane endpoint. Studies will be designed using the most applicable species.

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

Typically, animals will have their temperature monitored and recorded during the study from an implanted chip. This may be repeated in a different area if the implanted chip fails,

but the likelihood ofthis happening is low.

During intervention testing animals will typically experience mild, transient pain and no lasting harm from administration of substances using standard routes (intravenous, subcutaneous, intraperitoneal, intranasal, intramuscular, intradermal, oral). Vaccines may need to be given up to four times. Antivirals(and some less stable therapies) may need to be given up to four times a day but for no more than three weeks, depending on the pharmacokinetic properties of the compound concerned. Most other therapies will be administered once before and/or up to three times after challenge. Regular pre-and post-interventions saliva and/or blood may be taken. Total volumes of blood in one sample and/or across multiple sampling points will adhere to guidance from Wolfensohn and Lloyd (4th edition).

Animals will be monitored for clinical signs to ensure minimal pain, suffering and distress.

Animals will be culled by a Schedule 1 method or exsanguinated under general anaesthesia without recovery at specified time points in general, no longer than six weeks post-final treatment but some may be longer,or once humane endpoints have been met.

During infection modelling, animals will be challenged directly by intradermal, subcutaneous, intramuscular, intraperitoneal, intravenous injection, by attachment of infected vectors (including but not limited to ticks and mosquitoes), intranasal or aerosol route, or indirectly by introduction of naïve animals to groups of infected animals and allow to interact. To allow vector attachment, a small sectionof fur may be removed to allow feeding or the attachment of a patch or holding device to be sealed onto the skin of the animal to contain the vectors. Sedation may be used to allow exposure of animal and mosquito feeding. Regular pre-and post-interventions saliva and/or blood may be taken. Total volumes of blood in one sample and/or across multiple sampling points will adhere to guidance from Wolfensohn and Lloyd (4th edition). Animals will be culled by a Schedule 1 method or exsanguinated under general anaesthesia without recovery at specified time points in general, no longer than six weeks post-final treatment but some may be longer, or once humane endpoints have been met.

Clinical signs will be monitored to ensure minimal pain, suffering or distress and humane endpoints willbe clearly defined by a set of clinical signs (significant weight loss, temperature deviations, neurological signs).

During efficacy testing all procedures from intervention testing and infection modelling may beperformed.

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

The adverse effects of novel interventions are largely unknown but would not be expected to cause continued pain nor negative effects. The route of administration (injection, oral, inhalation) may causeshort term discomfort and/or local inflammatory responses and as such are likely to cause adverse effects. These will be counteracted by the use of suitable analgesics where necessary.

Disease and challenge models whereby the animals are administered with a virus by different routes (injection, oral, inhalation) will have aspects of severe disease side effects as these animals are likely to develop disease over the course of the study. However, due to the nature of these studies all clinicalsigns observed will be recorded on a clinical score

sheet with a set of pre-defined criteria of what is and is not acceptable during the studies. Any clinical scores that exceed the criteria – or specific clinical signs that may cause enhanced pain/distress - will result in the animal reaching a humane endpoint with that animal removed from the study and euthanised using a Schedule 1 method.

During studies when the severity is likely to increase, or when there is a likelihood of a humane end point being reached, the frequency of observations and recordings will be increased to ensure pain and distress is kept as low as possible and animals are removed from the study to ensure no animalsuffers any unnecessary pain or distress.

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

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

- Infection models: 80% moderate, 20% severe for all species (mice, rat, guinea pig, hamster, ferret).
- Intervention characteristic testing: 80% mild, 20% moderate for all species (mice, rat, guinea pig,hamster, ferret).
- Intervention testing: 80% moderate, 20% severe for all species (mice, rat, guinea pig, hamster, ferret).Vector competence: 80% moderate, 20% severe for all species (mice, rat, guinea pig, hamster, ferret).

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

- **Killed**

A retrospective assessment of these predicted harms will be due by 20 August 2028

The PPL holder will be required to disclose:

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

Replacement

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

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

Computer models and laboratory cell-based models are not useable in these studies due to the complexity of the studies and the therapeutic/vaccine licencing regulation requirements for animalstudies prior to human clinical trials.

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

Published data on similar models, human or epidemiological data will be sought to replace potentiallyunnecessary early pilot studies.

Certain aspects of how therapeutics may act chemically can be assessed using computer

modelling, which may enable unsuccessful candidates to be removed from studies prior to animal modelling. Thiswill be employed wherever possible. It cannot be employed for all candidates but will replace the needfor some animals.

In early studies it may be possible to use lab-based cell assays to test novel therapeutics and vaccinesprior to using animal models. Where these are available, they will be used to remove early non- successful candidates prior to any animal modelling.

Why were they not suitable?

Cell based laboratory assays are not complex enough to align closely with animal models. Due to regulatory requirements for use of therapeutics/vaccines in humans, animal models are still needed.

Many of the viruses in these studies are novel and cause severe disease in humans. There is limiteddata available and therefore limited to no computer models nor lab-based cell assays for these pathogens.

A retrospective assessment of replacement will be due by 20 August 2028

The PPL holder will be required to disclose:

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

Reduction

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

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

Animal numbers have been estimated based on information from previous licences and published dataalluding to numbers of animals required per test group. In the past group sizes of no less than 6 have provided the quality of results required. In early modelling studies, the levels of dose for each virus will need to be assessed for novel pathogens prior to use in intervention testing. Where models/data are available for similar viruses, these will be used to potentially reduce the numbers of groups required.

Where possible genetically modified animals will be used to reduce variability within group and reducethe sample size required.

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

Use of an experimental design tool (e.g.: The NC3R's experimental design assistant) for each studywill be employed to ensure the minimal numbers of animals may be used in each procedure.

Pilot studies will be performed to assess variability and use of a particular species,

especially when emerging viruses with limited animal model data are being assessed. This will ensure that follow on studies are only performed once a suitable model is established. Data from pilot studies will inform on suitable group and experimental unit size in subsequent studies. Factorial experimental designs will beused to allow the most information from a single study with minimum numbers of animals used.

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

Lab based studies using cell culture or ex vivo studies using material from tissue banks will be performed wherever possible to reduce animal numbers. Where vaccines/therapeutics show little effectin the lab it may be possible to eliminate them from animal studies.

A further way to reduce animal numbers will be in study sampling. Providing a procedure (ie: bloodtaking), does not cause prolonged or unnecessary pain and distress, and it is beneficial to do so, sampling one animal multiple times over the duration of a study will reduce numbers, rather than culling a separate animal at each required time point.

Immunocompromised mice will be used in several studies as wild-type rodent reservoirs will not alwaysexhibit significant pathogenicity to viral infection. Where possible, wild-type mice will be used, but where immunocompromised/modified mice are to be used, they will be sourced from specific accredited breeders. The animals will have undergone minimal adaptation to maintain the clinical relevance of the models.

Study details are made available to other groups such that tissues not required in our study can bemade available to other groups for use in similar research.

A retrospective assessment of reduction will be due by 20 August 2028

The PPL holder will be required to disclose:

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

Refinement

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

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

The most relevant animal model that will provide the most robust scientific data will be used throughoutthe studies to ensure repeated experiments and increased numbers of animals will not be required.
Where possible genetically modified animals will be used to reduce variability within group and reducethe sample size required.

The use of clinical score sheets and increased observations during the studies ensures the

severity isreduced as far as is possible and potential pain/distress is limited.
If a procedure would benefit from anaesthesia beforehand or subsequent pain relief (which won't affectscientific results nor cause the animal more distress than the procedure itself), this will be performed. The potential welfare effects of the anaesthesia itself will be accounted for (regular checks to prevent hypothermia and dehydration).

Where possible, the routes of administration and the equipment used for administration will be altered to ensure the minimal impact on the animal (eg: some treatments will be better tolerated when injectedrather than orally administered and this will be accounted for when planning specific treatment studies).

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

Mammalian species are required due to the complexity of their immune systems and the ability to produce scientifically relevant results. Animals at an immature life stage do not directly compare tohuman models and are therefore inappropriate models to use.

Where possible anaesthesia will be used to relieve any discomfort that might arise, however, due tothe nature of the viruses being studied, it is not possible to alleviate all levels of discomfort.

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

Some viruses covered in the proposal may never previously have been used in a specific model or infectious route. Many viruses can cause severe disease in humans and so may also have severe effects in animal models. However, whilst the clinical signs seen in humans may be clearly defined there is a possibility that disease progression in an animal model may be different and that treatment regimes may also affect the expected course of disease. Thus for these studies it may be impossible to eliminate all potential severe aspects but every effort will be made to refine end points by increasingmonitoring frequency and by continuous refinement of the clinical scoring system such that humane intervention can be applied at the earliest possible stage. The clinical score system implemented is constantly refined based on previous experience and knowledge in prior studies. It has been shown, that by increasing the observations up to six times per day, animal suffering can be limited and, in many cases, where humane endpoints can be applied, adverse effects can be considered as moderate. The euthanasia criteria in adult animals used in this licence for all scientific procedures using infectious organisms includes immobility and neurological indicators (e.g., repetitive or unusual movement). Immediate euthanasia criteria also include 20% baseline weight loss for more than 24 hours.

If a procedure would benefit from anaesthesia beforehand or subsequent pain relief (which won't affectscientific results nor cause the animal more distress than the procedure itself), this will be performed. The potential welfare effects of the anaesthesia itself will be accounted for (regular checks to prevent hypothermia and dehydration).

Refinement of procedures to reduce the number of animals and the pain/distress will be applied wherethey can be. Suitable analgesics will be sought following procedures where the likelihood of pain is increased. All methods will be discussed with the NACWO and NVS to ensure the best approach is taken.

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

conducted inthe most refined way?

Publications from the NC3R's and the Laboratory Animal Science Association will be used tocontinually refine and enhance animal models.

The PREPARE guidelines will also be followed when planning new and updating existing models.

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

I am signed up to the NC3R's newsletter to keep me regularly updated on any changes. We regularlycheck information on NC3Rs website. We also have regular updates and meeting with our animal support team, NACWO's and NVS to ensure we have the latest information and are following new advances.

A retrospective assessment of refinement will be due by 20 August 2028

The PPL holder will be required to disclose:

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

The applicant receives regular update on 3Rs via the NC3Rs resource updates and web tools(https://nc3rs.org.uk/3rs-resources)

VACCINE DEVELOPMENT AND FUNDAMENTAL RESEARCH ON VIRUSES OF MEDICAL IMPORTANCE

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir 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 animalsreared 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 aimsmentioned in paragraph (b)

Key words

Vaccine, Immunology, Pandemic preparedness, Virus, Therapy

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

Animal types	Life stages
Hamsters (Syrian) (Mesocricetus auratus)	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To develop vaccines to viruses in animals that can cause pandemics in humans, specifically againsthaemorrhagic fever viruses (Ebola, Marburg, Lassa fever, CCHV) and respiratory viruses (Influenza and coronaviruses).

To carry out research on the above viruses in animals to discover how they interact with our immunesystems. This aim supports aim 1, but will result in research that stands on its own.

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

Why is it important to undertake this work?

Vaccines are widely accepted as the best method for reducing the impact of infectious diseases, even more so than antivirals or antibiotics that treat people after they have contracted a disease. It is therefore important to future human health that we produce safe and effective vaccines to existing andespecially future diseases before it's too late, and make our best efforts to exterminate them, or reducethe impact of the disease that they cause.

It is important that these vaccines work well, and work on future virus strains (future proof), ideally notrequiring yearly updates or 'boosters shots' as is seen for most respiratory virus infections (influenza, COVID-19). Future proof vaccines are designed based on our prediction of how viruses will evolve over time, and to vaccinate against these predicted viruses. This also takes into account other strainsof the virus that already exist, whether in humans or animals.

Basic research on viruses is also essential in animals, as viruses react differently in animals than in humans. Understanding these differences enables us to understand a lot from animal experiments and compare this to understanding virus infections in humans. This basic research can also be termed'enabling science', that provides us with the knowledge and tools to make vaccines.

All of this work will finish in the need for studies in animals, due to the nature of viruses needing a 'host'to infect. This research is essential as to properly study a virus, one must study it in the context of a whole organism (animal) with an immune system or parts of that immune system that allow us to predict the same results in humans.

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

The primary outputs will be the generation of new, better vaccines to existing viruses, as well asbroader vaccines that cover multiple viruses, or multiple strains of the same virus.

The work will produce vaccines that will be used in future grant applications that will in turn lead to more funding to expand this work. Scientific publications, patents and intellectual property will alsoresult from this work.
Enabling science (such as supporting work on how viruses work in animal models) will generateoutputs relevant to virology and viral vaccinology that are stand-alone and useful to the researchcommunity working on viruses in animals.

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

The global community will benefit from vaccine outputs, where successful. We are nearing the end ofthe primary part of the SARS-CoV-2 (COVID-19) pandemic, and this virus will now become seasonal,in line with other viruses such as Influenza or common cold viruses.

Future pandemics are inevitableas the global population expands - our vaccines aim to reduce the risk of such pandemics happening again, by protecting people through vaccination, against viruses of pandemic potential that we have identified as circulating in animal reservoirs (similar to SARS-CoV-2). A great part of our work falls under a 'pandemic preparedness' global initiative.

Benefits from these outputs are both short and long term. Short term benefits include the developmentand bringing to market of vaccines within the next 5 years, such as certain of our candidates that are inphase 1 clinical trial, or other less advanced candidates that are moving towards phase 1 clinical trial.

Long term benefits include advances to the design, testing and in vivo work with vaccines that will benefit the whole field of vaccinology, and contribute to the long term improvement of vaccines over thenext decades. These also include vaccines that will be brought to market after the end of this 5 year project.

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

We are extensive collaborators, and part of international consortia aimed at delivering safe and effective, broadly protective vaccines. Our grant funders are globally active charities with links worldwide and resources that will help us achieve our aims. New knowledge will be disseminated in the form of scientific publications showcasing our work and the data generated as a result of work carried out in animals. Where possible, negative data will be published or shared with the research community, to prevent other groups from trying the same techniques and vaccines that do not work inour hands.

Species and numbers of animals expected to be used

- Mice: 7100
- Guinea pigs: 1000
- Hamsters (Syrian) (Mesocricetus auratus): 300

Predicted harms

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

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

We primarily use adult female mice, hamsters and guinea pigs to test our vaccines. Each model hasits own benefits

adult female mice are used as the model is extremely well established, and we are experts in measuring immune responses in mice. Adult females are used to prevent fighting and to enable lowstress social groups for the benefit of the animals. In some cases males will be used, and single housed where necessary to avoid fighting.

adult hamsters are the gold standard model for many respiratory viruses, showing similar disease tohumans, and are an excellent non-lethal model of infection. Adult females are used to prevent fighting and to enable low stress social groups for the benefit of the animals. In some cases males will be used, and single housed where necessary to avoid fighting.

adult guinea pigs are excellent 'next step' models for testing vaccines after mice, and immune responses generated are more similar to those generated in a human. Adult females are used for thesame reason as with mice, to prevent fighting and to enable low stress social groups for the benefit ofthe animals.

For basic virology research, we will use only mice in established or new models to measure infection, disease and innate immune parameters in these animals. For the majority of experiments adult animals are preferred, but in some cases younger animals, immune altered animals or both, in order tostudy viruses that establish chronic (long lasting) infections.

Animals will typically spend several months on this protocol while they build up antibody responses.This will involve several immunisation steps, and multiple bleeds to measure immune responses.

Once immune responses have been measured, mice will then be moved to containment facilities depending on the virus (ACL2 or ACL3) and infected, then weighed and observed for a period of up to 14 days. Mice will then be placed under deep anaesthesia and a final blood sample taken (for finalvaccine performance analysis) before they are humanely killed.

The typical experiment will involve two administrations of vaccine, usually by I.M route. Mice will be bled by superficial vein 3 times before administration of virus by I.N route. Mice will then be monitoreddaily and weighed, until termination of the study.

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

There are three main themes in this project license. 1. Vaccine immunogenicity evaluation (testing theimmune response generated by administering a vaccine), 2. Basic Virus Research and 3. Breeding and Maintenance.

Vaccine immunogenicity evaluation: Testing how good our vaccines are at making immune responses in animals. Animals will be injected with 1-5 doses of vaccine (DNA, mRNA, protein orviral vector) over a study period of around 9 weeks, and serial bleeds taken at various time points(e.g. every 3 weeks) in order to measure the immune response from vaccination at those different time points. Animals may be infected with virus at the end of these studies to determinewhether the vaccines protect from infection or disease.

For example, an animal will be given two doses of 50ul mRNA vaccine, by intramuscular (I.M) route with a 3 week interval (W0 and W3). Blood will be taken from the animals at weeks W0, W3, W6 and W9 by a superficial vessel to obtain serum that we can analyse forantibodies, and how they mature over the 9 week study.

Basic Virus Research: Animals will be infected with viruses in order to measure immune responses, and host-virus interactions. Animals may be given antiviral drugs or antibody cocktails to prevent or impede virus infection progression.

For example, an animal will be infected with a virus via intranasal route. Animals will be weighed daily and monitored twice daily until symptoms appear, usually between days 5-7 after infection. Depending on the clinical symptoms and % weight loss from pre-infection weight, mice will be humanely killed, and organs dissected - so that we can measure how the virus grew in those organs. This protocol tends to last up to 14 days after infection at which point all surviving animals are humanely killed.

At the end of experiments, animals will be humanely killed, or in the case of guinea pigs or hamsters,re homed if a suitable home is available.

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

We test a range of different vaccines and delivery methods, that themselves may cause adverse effects in animals. As vaccines are designed to generate immune responses, sometimes these can have mild or moderate effects in animals while that animal generates an immune response because ofthe vaccine. This is similar to how humans feel unwell when infected with a virus, for example ‘flu like symptoms’.

In our experience only transient and mild discomfort is a result of immunisation with our vaccines in99% of our animals. In some cases animals do experience mild and short lived symptoms for up to24h after vaccine administration.

intramuscular injection can cause pain at the site of injection (especially in mice), which will almostalways be the rear thigh.

Protein vaccine injection with an adjuvant can sometimes cause piloerection (raising of the hair/fur)and slight hunching for 24h after injection in mice. This is a sign that the mice are feeling unwell, but normally they get better after a few hours.

Infection (challenge) of naïve animals (those that haven't encountered the virus, or vaccine) with a virus can cause a range of symptoms depending on the virus. These symptoms can include discharge from the eyes, problems breathing and sickness that will cause lack of appetite - leading to weight lossin the mice. Low doses of influenza virus can cause temporary weight loss within a 10 day period, including symptoms such as hunching and piloerection. High doses of influenza virus can cause symptoms such as laboured breathing, and loss of weight leading to death. For high doses mice will beclosely monitored and humanely killed after weight loss and clinical symptoms start. Any dose of SARS-CoV-2 in human ACE-2 transgenic mice will cause a rapid weight loss and moderate symptoms (such as laboured breathing, infection of the brain leading to inactivity and inappetence) within 5-7 days. There is also a chance for otherwise healthy mice to rapidly deteriorate overnight and die – due to infection of the brain by the virus, causing a stroke. In these cases mice will be humanely killed aftershowing moderate clinical signs or significant weight loss. In the majority of cases, mice reach humaneendpoint (the point at which they should be euthanised to prevent further suffering) within 5-10 days after challenge.

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

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

Mice:

Mild: 95%

Moderate: 5%

Severe: 0%

Guinea Pigs:

Mild: 100%

Moderate: 0%

Severe: 0%

Hamsters:

Mild: 100%

Moderate: 0%

Severe: 0%

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 haveconsidered and why they cannot be used for this purpose.

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

Unfortunately, there is only so much that can be done in the laboratory. We employ extensive experimentation to characterise each vaccine candidate that we design, and ensure its maximum chance of success before moving into animals. Before reaching humans, a vaccine typically must betested in multiple animal models, a very high bar that unfortunately requires quite extensive use of animals.

For basic virus research, complex in vitro (laboratory work) systems do not yet exist that match the invivo model, and therefore once all in vitro work has been exhausted for a certain virus or project, the final steps must be carried out in animals with a view to translating results to humans. For animal viruses, the animal model may be the final or preferred model to study the virus itself.

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

There are no alternatives to animals in the field of vaccine testing, or basic virology work. Ultimately these need to be tested in an animal that has a working system of organs, immune system, microbiota(normal bacteria, fungi and viruses that make up part of our body systems). As viruses are obligate intracellular pathogens (they can only survive inside our cells), they require a host, and while cell based systems are useful for very basic research, the whole organism is where the majority of scientific findings are discovered. This is especially true of viruses that infect animals with incredibly complex immune systems such as mammals.

We work extensively with a range of cell culture lines in the laboratory, as well as using biochemical and biophysics experiments to evaluate our vaccine candidates. These enable us to whittle down ourvast libraries of candidate vaccines until a select few candidates that fit the expected or desired immune profile are used.

For basic virus research, only cell culture based systems can be used, as viruses can only function inside living cells. We employ these in our day to day experiments working on these viruses. These are employed to grow and measure virus, and in some cases to image parts of the cell that the virusinteracts with.

Why were they not suitable?

Cell based systems are suitable for a range of experiments, but are very different to in vivo work, where a whole animal has various cell types, organs and a working immune system. It is unfortunatethat cell based systems are currently not at an advanced stage to replace animal work, and this will remain the case for the next century or longer.

They are not suitable as they provide a small amount of information, but not information on theimmunogenicity or characteristics of a vaccine or viral infection in a whole body organism.

Reduction

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

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

In the past two years, we have successfully executed three major vaccine projects involving largeamounts of animal work.

- Influenza universal vaccine project (£4m): Total of ~1500 mice
- Coronavirus vaccine projects (£2m): Total of ~1500 mice and 130 Guinea pigs
- Haemorrhagic fever virus vaccine projects (£500k): 28 Guinea pigs and 100s of mice

Based on these figures for two years, and taking into account that we have just been awarded £44m todesign a pan coronavirus vaccine, the projected animal numbers for a 5 year project, continuing at ourcurrent rate of animal work, would be ~7200 mice and ~300 Guinea pigs.

We have decided on 7100 mice, 1000 guinea pigs and 300 hamsters due to our pan coronavirus vaccine project requiring more Guinea pig work. This factors in a slightly lower number of mice thanpredicted, but also includes mice for breeding and maintenance protocols. Hamster numbers are forexploratory studies and can remain low at 300. This also takes into account non-vaccine work to develop animal models for newly discovered beta-coronaviruses that pose pandemic threats. This typically requires much fewer animals per project.

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

The vast majority of our studies are pilot studies. We do not know how the vaccines will perform, and the reagents to test how they have performed are not regulated or well defined in the research community. Therefore we cannot carry out power calculations that need a defined effectand set of hypotheses and we must use other means to reduce our group sizes.

All vaccine candidates are tested in vitro, and quality controlled (tested in the laboratory to makesure they are correct), enabling us to select the best candidates to take forwards to in vivo work.As there are very many variables in vaccine studies, it is only possible to perform power calculations in retrospect, when building on a previous study. Therefore it is not possible to do this for pilot studies where the variance and interplay between groups, controls in experimental assays - is unknown. The majority of our vaccine studies fit into this group of pilot studies. We use the below formula to ensure that we do not use too few animals per group and per study:

E = (number of animals per group x number of groups) - number of groups

An E number of between 10 and 25 is within the correct range for statistical analysis, below 10 beingtoo few animals and above 25 being too many animals. We will always aim to be on the lower side where possible.

Power calculations are carried out where possible to guide animal numbers for vaccine and basicvirology research experiments. Where possible, animal numbers are minimised in this respect.

All efforts are made to make each animal study fully controlled and publishable in its own right,with the minimum number of control animals required to allow us to have confidence in our results.

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

As this project license builds on a previous similar license, we are able to utilise our expertise in the field of in vivo vaccine and virus work to optimise the number of animals used in our projects. Our studies flow through an exhaustively detailed and planned pipeline from the design stage until endpointin humans.

The majority of our vaccine studies can be considered "pilot studies" as we are measuring immune responses to new vaccines. They are computationally designed and quality checked, then go throughseveral rounds of in vitro quality control. After this, the refined list of candidates is much reduced and can progress into animals.

When infecting mice with a new virus, or immune altered mice with an existing virus, there are very many unknown factors, which forces us to carry out pilot studies with the fewest animals possible inorder to build up knowledge about the viruses and how they work in animals. This builds on our existing knowledge, and helps us refine future studies with similar viruses or vaccines.
Through our use of standardised protocols developed over the past 5 years, we are able to reduce bias and account for variation when vaccinating animals. We have tested these

standardised protocolsand shown that results are reproducible over multiple studies. This gives us certainty that the small numbers of animals used in our groups is sufficient.

Refinement

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

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

We use a very simple immunogenicity protocol for the majority of our animal work, involving 1-5 injections of vaccine, and up to 8 bleeds at various time points in the vaccination schedule. These aremild protocols that cause very limited pain, suffering or distress - and no lasting harm to the animals. Our guinea pigs can and have been rehomed after being on such protocols with permission from the HO.

For basic virus research, we use wild type, and sometimes modified mice to interrogate different aspects of virus-host interactions. The most we can do in this case is administer a low dose of virus and be especially vigilant to humanely kill mice well before any humane limit is reached. This is an unpredictable aspect of virology, in the few cases when mice react badly to infection or animal checksfail to identify sick animals, they may breach the severity limit of mild and we will be required to submita SC18 report. We never have a scientific need to keep an animal alive after breaching the severity limit and this will be avoided at all costs.

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

We require animals with mature mammalian immune systems that are capable of mounting an immuneresponse to an infection or vaccination that can take months to manifest. Only mammals are suitable as they are models for vaccines in humans. There exists no cell-based model or less sentient model totest vaccines.

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

Our establishment and associated animal houses provide state of the art, and the most modernhousing for our animals, with an emphasis on their wellbeing.

While females are used in most cases to allow larger social groups and prevent fighting, males are housed separately where needed and in the case of fighting. Bullies are also separated on welfare grounds. Animals are allowed 7 days to acclimatise before procedures, and our best efforts are madeto reduce the number of procedures carried out in total, or to restrict these procedures to a minimum number of time points (or stress points) to reduce long term stress. Increased monitoring is carried outon moderate protocols, or those including virus infections, with animals monitored at least twice daily, weighed and a clinical scoring sheet used to aid assessment of the animal’s health. In human ACE-2 transgenic mice, any clinical signs that deviate from normal will lead to

animals being humanely killed.

For other mice where sickness is more predictable, animals will be allowed the opportunity to recover from infection, and only humanely killed when it is clear that symptoms are not going to resolve in shortperiod of time.

The procedures we use are always re-evaluated based on the expertise available, we have recently moved to using dark plastic restraining tubes and seen a marked decrease in stress in mice being sampled. We have also moved to more streamlined protocols, meaning that the majority of our studyplans will include only three bleeds and two immunisations, rather than 5-7 bleeds and 4 immunisations. However, some studies may require the full set of bleeds and immunisations depending on the research outputs and vaccines used.

As GM strains of desired mice become available from commercial suppliers, we will opt to order cohorts in directly rather than breed these mice ourselves - this should refine our ability to produce data at the least cost possible for the animals, as companies are better placed to breed in an efficientmanner and make full use of the resulting animals.

For monitoring purposes, we have refined how we deal with mice that are expected to develop clinicalsymptoms (e.g control unvaccinated, infected animals), in liaison with animal staff at our facilities. In such cases, a researcher will be ready to humanely kill animals on all of the expected symptom days, rather than waiting to be called in by animal staff after a monitoring session has raised concerns aboutthe symptoms shown by an animal. This refinement reduces the amount of time spent suffering by ananimal to a minimum.

Where possible, guinea pigs and hamsters will be rehomed where a suitable home is available.

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

All of our animal work is carried out within a framework provided by our establishment, ensuring that we have the most up to date information to plan our studies, and that our experiments are planned withthe latest guidance in mind. We will keep up to date on the latest LASA publications, NC3Rs 'ARRIVE'guidelines, NORECOPA 'PREPARE' guidelines. We will also keep up to date with latest RSPCA studies (e.g. https://science.rspca.org.uk/-/refining-procedures) to refine our protocols and animal use.

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

Our establishment provides regular updates on the 3Rs. We also receive regular updates on the 3Rsthrough the NC3Rs website/NC3Rs Gateway (https://f1000research.com/nc3rs).

We regularly monitor the scientific literature for advances or breakthroughs in the animal work relevantto virology or viral vaccinology, in particular paying attention to the development of in vitro models thatmay allow us to reduce or refine our animal work. We will also review journals such as the ATLA (Alternatives to Laboratory Animals) Journal: https://journals.sagepub.com/home/atla, and refer to the LASA Guidelines: https://www.lasa.co.uk/current_publications/

UNDERSTANDING MAMMALIAN VOCAL LEARNING USING BATS

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Speech, vocal learning, genetics, neurobiology, bats

Animal types	Life stages
Bats	juvenile, adult

Retrospective assessment

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

Objectives and benefits

Description 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 shed light on the biological origins of speech and language. Vocal learning is a key part of learning to speak and by revealing the genetic and neural mechanisms underlying vocal learning we aim to discover biology underlying speech. Exploring vocal learning in animals will help us reveal this biology and shed light on the evolution of vocal learning and of speech.Vocal learning is a rare trait, shared by only a small number of mammals. We aim to determine the genetic mechanisms and neural circuitry that support vocal learning behaviour in mammals and compare this with other animals to reveal evolutionary mechanisms important for speech in humans.

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

Why is it important to undertake this work?

Vocal learning is a fundamental building block of human spoken language and is a trait we share with few other animals. It has only been identified in 4 non-human mammal groups, of which small mammals are the most feasible model system in which the molecular and neural mechanisms can be addressed thus providing a unique window onto the biological

foundations of vocal learning and complex communication. Understanding the bases of vocal learning in mammals will shed light on thebiology underlying vocal communication in animals and reveal fundamental mechanisms of gene and brain function underlying complex cognition. In addition, understanding vocal learning in mammals willshed light on how speech and language are genetically and neurobiologically encoded and the substrates from which they evolved. In the long term, this work has translational implications for understanding and treating speech and language related disorders in children.

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

This work will generate a large amount of knowledge as to the genetic, genomic, neurobiological andbehavioural aspects of mammalian vocal learning. In addition, the projects will establish appropriate mammal models for vocal learning and speech disorders and will create tools and paradigms for assessing these factors in these species, which will be employed by other labs.

The outputs will take the following forms:

-Datasets (eg. genome and transcriptome sequencing data, histology data, electrophysiology data)published as data notes and/or deposited in public, open-access databases.

-Publications in open-access, international, peer reviewed journals reporting the findings of thesestudies. In addition, work will be disseminated via conference proceedings.

-Protocols and paradigms reported in open access publications, online and/or in methods papers(separately or together with the above)

- Cross-species comparison of brain architecture and functionality, shedding light on brain evolution

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

The wider academic community will benefit from these outputs, both short term and long term. We expect a range of fields to benefit from the data and knowledge generated from this project as well asthe techniques established including biologists, geneticists, genomicists, neurobiologists, researchersstudying animal behaviour and cognition (particularly social or vocal behaviour), echolocation or auditory neuroscientists, speech and language researchers. Long term this work could lead to the useof appropriate mammal models to understand the causes of childhood speech and/or language disorder, and drug-based therapeutics for alleviating the symptoms thereof.

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

We will maximise the outputs of this work by sharing our work widely and rapidly via a number ofroutes:

-Collaboration is a key way to maximise the benefit and output of research and we have a long historyof wide and interdisciplinary collaborations. We will continue this pattern, maintaining existing collaborations and forming new collaborations that this new work will facilitate. We will share knowledge with interdisciplinary collaborators addressing related questions across a range of other animal models, as well as human speech and language

researchers, and computer scientists using modelling approaches.
-Rapid dissemination of knowledge will be another cornerstone of the project to seek critical assessment of the work and maximise outputs. Dissemination will take place prior to publication viainvited talks, conference presentations and posters and article pre-print servers. We have routinely made use of these avenues in the past for this purpose.

-Wide and timely dissemination of outputs will be performed via journal articles in international peer reviewed journals. We aim to publish successful work, but also include null results to prevent redundancy by others. Where possible, we aim for the greatest impact and audience for our work by publishing in general interest journals. In all cases we aim to publish all work open access to maximise the reach and audience of the articles. These measures ensure the widest specialist and non-specialistaudience reach.

Species and numbers of animals expected to be used

- : 530

Predicted harms

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

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

This proposal studies the neurogenetics of vocal learning in mammals and therefore a mammalian system in which we can explore the role of genetic and neurobiological mechanisms in a living system is essential. Mammalian vocal learners are limited and we have chosen most suitable mammals that are the least sentient of the vocal learning species, and the only that are feasible for genetic and neurobiological manipulations. This research question cannot be answered without animals, as we will link neurogenetic mechanisms to behaviour in living animals using genetic manipulations. We chose toutilise juvenile and adult animals as the underlying genetics and neural architecture of mammalian brain change significantly during ontogeny of the animals, especially during the stages of vocal development. However wherever possible we use cell based and in silico approaches to reduce animalnumbers. We have set up a number of tools and paradigms in previous studies and pilot experiments to guide our experimental design and refine our experimental plans.

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

Animals will be investigated for vocal learning behaviour and the genetic and neural mechanisms relating to this behaviour. To do this, most animals will undergo cranial surgery that allows introductionof substances in the brain, and/or direct recordings of brain activity. Substances introduced will includegenetic constructs that allow manipulation of gene expression in the brain and tracing compounds thatallow us to observe connectivity between brain regions. A small number of animals (~6%) will also receive an injection into the laryngeal muscles to trace connectivity between the brain and the voice box. After surgery all animals will receive post-operative care including pain relief in consultation with the Named Veterinary Surgeon.

Recording of brain activity will be undertaken once animals have recovered from surgery via direct electrophysiological recordings or calcium imaging, where recordings are made

multiple times a weekover a period of months.
About 30% of animals will take part in behavioural training where they will learn new vocalisations (vocal learning). Some of these animals will have received molecular manipulations prior to behavioural training which will allow us to determine the molecular mechanisms of complex vocal learning behaviour. Training will involve food rewards that are provided when the correct vocalisationsare produced. Training is undertaken during weekdays for a period of months.

At the conclusion of the experimental paradigms, tissue will be harvested to assess gene expressionpatterns and neural connectivity.

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

Approximately 70% of animals will undergo surgical procedures. Any surgical method has the potentialto cause pain, however we will perform surgery under aseptic conditions and ensure best peri- and post-operative care including provision of pain relief to ensure best possible welfare for the animal during and post- surgery. Some animals might temporarily lose some weight directly post-surgery.

Repeated anaesthesia used for restraint to facilitate recording of brain activity has not shown anyadverse effects on the wellbeing of the animals.

Behavioural training is not expected to produce anything beyond mild and transient effects on theanimal. Some mild weight loss may occur in these animals in the early learning phases but this isexpected to be mild and transient.

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

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

Moderate – Protocol 1 – Animal #380Mild – Protocol 2 – Animal #150

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 haveconsidered and why they cannot be used for this purpose.

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

Little is known regarding the genetic or neural mechanisms underlying vocal learning behaviour in mammals including humans. In order to define these mechanisms, it is essential to explore them in aliving, behaving animal with a nervous system that can be interrogated and the ability to perform the relevant behaviour (vocal learning).

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

In silico and in vitro (e.g. cell lines).
Why were they not suitable?

In silico: Given the lack of knowledge on mammalian vocal learning mechanisms currently it is not possible to model them in silico. However, wherever possible we employ in silico phylogenetic analyses (eg. evolutionary selection tests) to reveal possible contributing factors to the evolution ofvocal learning. However, these are hypothesis generating tools and cannot reveal or confirm directlinks with the mechanisms underlying the behaviours. For this, exploration of the mechanisms in animals is essential.

In vitro: We are using in vitro, cell-based assays wherever possible to test methods or perform initialscreening of tools (such as genetic manipulation constructs), which will help to minimise animal numbers. For the experimental work herein, cell systems do not present appropriate complexity in tissue or the opportunity to link mechanisms to behaviour, again making animal systems essential.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 proposed in the project are estimated from our extensive prior expertise withthese experimental methods. We have had prior studies and pilot work (carried out in EU) that was approved for more than 5 years that has informed this work. We have also taken into account the published work of our collaborators and colleagues that employ similar or related techniques.

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

We have performed extensive in vitro testing of compounds and genetic alteration constructs that will be employed and will continue to use this in vitro validation method before moving to in vivo stages to minimise testing in animals. We also employ tracing dyes that are well established to reduce the risk ofexperimental failure and need for replication or for testing new dyes. We always aim to make the most use of each experimental animal and all tissue harvested will be placed in an in-house ‘tissue bank’ that can be used for repeated measures and in some cases new experiments, to reduce animal numbers. We use previous work and pilot studies to estimate the minimum number of animals necessary to obtain robust results for the methods being applied.

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

New techniques and methods will be tested, evaluated and optimised in pilot studies and with externalinput from expert collaborators. When harvesting tissue, wherever possible

we will harvest multiple tissues and multiple samples per tissues which will be placed in our tissue bank for future use. This should mean that fewer animals need to be used, as in some cases we will be able to obtain samples from the tissue bank rather than collecting new animals. Where possible we will also use both sexes inexperimental work.

Refinement

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

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

We utilise small mammals as a mammalian system in which we can interrogate the neurobiology andgenetics of vocal learning. The model is well suited for this research area.

Surgeries are required as we aim to investigate the biological mechanisms underlying vocal learning.Direct causative links between genetic mechanisms and behaviour, or the structure and function of neural circuitry cannot be assessed without invasive techniques that involve direct manipulation or observation of the brain and its activity and therefore require surgery. The surgical methods used arecarried out following strict peri-operative care measures including pain management and welfare monitoring.

None of the substances we will introduce into the brain during surgery (eg. dyes, viral vectors, calciumsensors) will cause adverse effects for the animals, as proven in extensive prior experimental work.
The genetic alterations we will induce will be localised within the brain and will all occur in young and adult animals. Because these are localised genetic alterations, they are highly unlikely to result in anyadverse physical consequences for the animal that would cause pain, suffering, distress or lasting harm.

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

We have selected the least sentient mammalian species that displays vocal learning behaviour. We must use animals that are able to perform this behaviour to determine the role of neural and genetic mechanisms in the behaviour. Learning occurs in early life stages and in adulthood, and to understandthe development of this behaviour, and how the neural circuitry established via genetic programs and learning influences the behaviour we must use awake behaving animals of both juvenile and adult animals.

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

We will use prior studies that we and our collaborators have conducted using these methodologies, aswell as further pilot studies to validate new approaches, to determine the variability in experimental outcomes and therefore refine the total number of animals needed to provide valid results. We will continually assess the output of experiments to

optimise and refine approaches and maximise the amount of data obtained from each animal. Additionally, we will implement appropriate peri-operative care measures including pain relief and aseptic technique and carefully monitor health and welfare of experimental animals. We will consult closely with the NVS and NACWO on health and welfare issues.

Our staff will receive training from an expert in bat husbandry and experimental procedures and will attend refresher courses as required. We will consult with internal and external experts for professional advice when implementing new methods.

Animal welfare will be carefully monitored to safeguard welfare Criteria include monitoring ofbehaviour, body weight/condition and wound healing.

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

We will utilise relevant guidance from the published Joint Working Group on Refinement, and follow allrelevant guidelines from NC3R including Prepare guidelines (https://norecopa.no/prepare) and LASA guidelines on aseptic technique (https://www.lasa.co.uk/current_publications/).

In addition, we will utilise the best practice guidance for small mammal research including the official German guidance for best practice (https://www.las-interactive.de). These guides are required training for German researchers and form the best practice guidelines for specialised small mammal handling, experimentation, and the 3Rs. They cover both the theoretical and practical aspects of experimentationand are a decisive factor for obtaining a personal license in Germany for these specialised small mammals. The course consists of an extensive theoretical background (handling, animal welfare, legislation, anaesthesia, surgery, pain management, 3Rs, euthanasia, sample extraction) and a practical demonstration of the applied theoretical aspects on a total of 3 animals.

We will also follow the best practice for specific techniques from scientific publications and most relevant experimental methods. We have close collaborations with other researchers and will regularlyupdate on best practice with our network of research collaborators.

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

We will continuously liaise with the named persons and experienced animal care technicians at the establishment to discuss possible improvements. I am also a member of the School Ethics Committee,where I am part of ongoing discussions about animal welfare and 3R advances. We will also be kept up to date about 3R advances by attending NC3R seminars, events and workshops, by receiving monthly updates via subscription to the NC3R newsletter, Norecopa (https://norecopa.no/) and attending institutional 3R symposia.

UNDERSTANDING IMMUNE RESPONSES IN ZEBRAFISH

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

white blood cells, inflammation, thrombosis, imaging, zebra fish

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Inflammation is a key driver in a number of diseases, including heart disease, dementia, stroke, and cancer. This PPL aims to establish zebrafish models of inflammation to further understand the complexeimmune cell dynamics and determine whether targeting immune cells with anti-inflammatory/pro- resolving agents (either alone or in combination) affords protection.

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

Why is it important to undertake this work?

Reducing, and ideally eliminating inflammation and thrombosis is critical for recovery from and management in certain diseases (including heart disease, stroke, dementia, sickle cell disease, sepsisand cancer), this process is called ‘resolution’. Understanding the mechanisms of action that drive these processes and understanding how and why they fail is vitally important to help provide possible therapeutic strategies for the treatment and management of these aforementioned diseases.

What outputs do you think you will see at the end of this project?
Data generated with this PPL will enable us to further understand how immune system functions undernormal (i.e. physiological) settings and when things go wrong (i.e. pathophysiological settings as seen in heart disease, stroke, cancer etc). This knowledge will help us to explore new and existing mechanisms of action that could provide therapeutic strategies to ultimately help fight inflammation andthrombosis in human disease.

The zebrafish model will enable us to investigate inflammatory and thrombosis dynamics from a completely novel perspective, which cannot be replicated using mammalian models or patients in theclinic. If successful, the zebrafish protocols and assays within this PPL could potentially be applied toreduce the number of higher vertebrate models (e.g. rodents) used in inflammation and thrombosis biology.

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

There will be a number of beneficiaries for our research project, including:Short term
In the short term, researchers locally will learn more about chronic inflammation and thrombosis andthe mechanisms of action involved in zebrafish. Additionally, considering the high interest in inflammation and health maintenance, we anticipate a considerable interest in our data.

Medium term

If successful, the zebrafish protocols and assays within this PPL could potentially be applied to reducethe number of higher vertebrate models (e.g. rodents) used in inflammation and thrombosis research. Specifically, here we propose that the zebrafish model will enable us to investigate immune cell dynamics in inflammation and thrombosis from a completely novel perspective, which cannot be replicated using mammalian models or patients in the clinic. Zebrafish will offer these important advantages: 1) In-vivo visualization of inflammation in transparent genetically engineered larvae; 2) The capability to perform sophisticated immune cell profiling and phenotypic anchoring by using specific software linked to a microscope, along with behavioural assessment box. These techniques will enable us to quantify a high number of functional endpoints in the same animal in a non-invasive manner (e.g. immune, cardiovascular, gastrointestinal, behavioural responses). If successful, the zebrafish tests developed in this project could potentially be applied to reduce the number of higher vertebrate models (e.g. rodents) used in inflammation and thrombosis research.

Long term

The translational impact and potential drug discovery implications of our findings open a number ofopportunities, including pharmaceutical industries and biotechs that are highly interested in the development of treatments for inflammatory and thrombotic diseases. Our zebrafish work will enable us to test some of the hypothesis that cannot be tested in humans,and to generate mechanistic knowledge that can guide the identification of the most effective and safedrug combination that will be administered to patients with inflammatory and thrombotic diseases.

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

Throughout this PPL and beyond, collaborations will be instrumental in driving science forward. The knowledge obtained through the data collected in the PPL will be

disseminated at scientific meetings and will be published in peer reviewed journals. Data will be deposited into repositories such as Figshare and tissues may be available upon request. Our research findings will be showcased on theinstitution's website, and on social networks such as LinkedIN, ZFIN, Research Gate, etc.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 7350

Predicted harms

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

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

The biology of inflammation and thrombosis are similar in zebrafish, rodents and humans, thus zebrafish (as the lower species) will be used. In particular, a major strength for the use of the zebrafishmodel is the ability to perform in vivo imaging to visualise inflammatory and thrombotic events in real time. During the course of this licence, most of the in vivo tests will be carried out using fish between 5and 14 days post-fertilisation (dpf). Although adult zebrafish will be used less frequently, they will provide a set of complementary and highly valuable data including the effect of the immune response over time.

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

Our main objective is to understand the mechanisms behind immune responses such as inflammationand thrombosis (termed thromboinflammation). Thus, in order to study thromboinflammation, we will use zebrafish that are genetically engineered to express fluorescent markers in immune cells involvedin this process. Typically, inflammation will be induced in zebrafish by via chemical treatment in the water or microinjection of an inflammogen or tail cut injury. Fish may experience mild to moderate painwhich will be treated with analgesics where applicable. We will test the effect of different anti-inflammatory compounds to determine their effect on e.g. behaviour and immune cell trafficking.

Throughout the project, we will use a tiered approach that involves first the use of non-protected zebrafish i.e. those <5 days post-fertilisation (dpf). As the timeline of the resolution of inflammation is acritical aspect to study, longer experimental observations will be required, involving the use of protected larvae. Finally, a small number of studies may require the use of adult animals to investigatethe role played by the immune system during ageing.

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

The genetically altered strains used in this PPL themselves do not generally display any adverse effects, as they only express fluorescent markers in specific cells/tissues. However, within this PPL wedescribe models of inflammation and thrombosis in which we assess the effects of immune cell functions and the effect of anti-inflammatory and anti-thrombotic drugs. Inflammation and thrombosis may be induced by the administration of pro-thrombotic agents, pro-inflammation agents, tail cut, or altered diets. Although unlikely,

it is possible that adverse effects such as brief increase of heart rate, mild alteration of the immune system, partial alteration of hormonal levels, histological abnormalities indifferent organs may occur. Diet enriched with fat/cholesterol may induce nutritional imbalance that may result in weight gain/loss, and injections and tail cuts may lead to effects around the injection site causing adverse effects and ill health.

Concentration ranges will be taken from the literature for the majority of the drugs we intend to test in this project. Where data are not available, we will perform maximum tolerated concentration experiments using non-protected zebrafish larvae younger than 5 dpf, or pilot studies using older fish.

We will keep detailed records of each strain used, recording any unpredicted adverse phenotypes andany useful husbandry techniques and maintenance measures so that the information is available for others using those fish.

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

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

The breeding and generation of genetically altered zebrafish (protocols 1 and 2. Both 10% mild, 90% sub-threshold) are both mild. Protocol 3 is moderate (~30% mild, ~70% moderate) due to the cumulative effect of procedures to the zebrafish e.g. blood sample taken, anti-inflammatory drug administration, inflammogen administration, thrombosis. In some cases, animals will be treated with aninflammogen e.g. LPS, to increase inflammation prior to thrombosis (to mimic the clinical situation).

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 haveconsidered and why they cannot be used for this purpose.

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

In order to understand and investigate what immune events happen during inflammation and thrombosis, we need to perform experiments in-vivo since some of the experiments described are notpossible either in humans or in-vitro models. While I have previously used cell culture models to studysome aspects of the cell-cell interactions elicited by inflammation and thrombosis, it is not possible to mimic all the complex, multicellular interactions of the human immune system. Therefore, zebrafish have been chosen as they are the lowest species in which thrombosis and inflammation can be effectively modelled.

This PPL will enable us to expand our studies to investigate the cellular and molecular mechanismsinvolved in thrombosis and inflammation in zebrafish, which will represent an invaluable bridge between our in-vitro and in-vivo rodent and mammalian studies, resulting in the replacement of a number of higher vertebrate models (e.g. rodents) used in research.

Throughout this programme of research I will ensure that I continue to adhere to the principles of reduction, refinement and replacement. I will share tissue samples with other researchers where possible and I will continually reassess possibilities for alternative or complementary experiments.

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

Human cells, in vitro tests and non-protected zebrafish.

Why were they not suitable?

In humans and in-vitro tests it is not possible to perform in depth analysis of all aspects of inflammationand thrombosis, such as the complex immune interactions that occur in these processes.Therefore, zebrafish have been chosen as they are the lowest species in which thromboinflammation can be effectively modelled. I will throughout the course of this PPL seek to review and incorporate any alternatives and other R's should they arise.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 specific statistical approach for each experiment will be agreed during the study design phase withthe support of an expert biostatistician and it will take into account both the aim of the experiment and the number of independent factors able to influence the dependant variables. In our previous work e.g.on drug-mediated behavioural phenotypes we have developed a data analysis pipeline including a diverse set of approaches, such as ANOVA, ANCOVA, Bayesian generalised linear mixed modelling. Atypical experiment will involve 3-6 experimental groups. Calculations informed by data generated from previous research in our laboratory or elsewhere, indicates that group sizes of between n=15-32 are needed to achieve the quality of results we need.

The numbers of fish requested in this PPL have been calculated on the basis that all experiments will take place. However, if, for example, an anti-inflammatory drug does not work at the concentrations chosen (based on in vitro data and EC50 values), then there will be no necessity in determining the mechanism of action e.g. co-administering the drug with a drug that blocks receptors known to mediatethe drugs' action. Thus, the number of fish will ultimately be less than that requested.

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

Advice from local statisticians was sought, along with adopting helpful tips and advice from the NC3Rs,including use of their “Experimental Design Assistant” (EDA. A tool which supports researchers in planning animal experiments in a robust way).

What measures, apart from good experimental design, will you use to optimise the

number ofanimals you plan to use in your project?

I will work with more experienced zebra fish breeders with respect to new and innovative methods of efficient breeding. Where known, the dosing regime will be based on the concentration of the drug that causes half of the maximum possible effect, and drugs will be given at sub-maximal doses. Where dataare not available, we will perform maximum tolerated concentration experiments using non-protected zebrafish larvae younger than 5 dpf, or pilot studies using older fish (less than 18 months of age). The most non-invasive route possible that is clinically relevant will be chosen, with the smallest volume to initially determine the effect of an unknown drug in a small number of zebrafish (such as five).

I will share tissue samples with other researchers where possible and I will continually reassess possibilities for alternative or complementary experiments. In general studies will be performed blinded and randomised, with a key system to identify which fish/sample has undergone which treatment. Furthermore, compounds administered will be made by laboratory personnel other than the one performing the experiment. Controls will comprise of a vehicle (often saline) for the respective drug to be tested (to reduce animal numbers, only one vehicle group will be needed for a set of drugs with thesame vehicle, being used in the same experiment).

Refinement

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

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

We will use the zebrafish, Danio rerio, as the experimental species. We will use transgenic zebrafish that we have bred on this licence as well as transgenic lines obtained from established sources. Theseinclude transgenic zebrafish that express fluorescent proteins in their immune cells e.g. neutrophils expressing green-fluorescent protein). We believe that to study immune cell dynamics during inflammation and thrombosis, transgenic zebrafish allow for a more refined model and method than using wild type strains injected with fluorescently labelled antibodies directed at immune cells.

Throughout the PPL and beyond, we will continue to seek to try to use the most refined models andmethods e.g. the ZEG (Zebrafish Embryo Genotyper).

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

To recapitulate the immune response seen in humans, we need to be able to use a species that enables us to investigate these complex immune events in a physiological setting. Zebrafish will enable us to understand the effects of the immune response in inflammation and thrombosis, and themechanisms involved. This will help us to find possible targets to help the immune system to be moreeffective. In addition, it will enable us to test compounds that may induce inflammation resolution thatcould ultimately lead to the clinic.

Studies using non-protected zebrafish larvae younger than 5 dpf, or pilot studies using older fish (<18 months old) will be used in cases where drug concentrations are unknown. Many of our studies can (and will) be performed in non-protected zebrafish larvae younger than 5 dpf. However, we need to useprotected stages of zebrafish to quantify the immune response across different stages of life, to recapitulate the clinical situation and increase the translational relevance of our findings.

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

We do not expect any more than moderate suffering of zebrafish in this project. During the raising of larvae to adulthood, especially in the first four weeks of growth, we expect about 20% of natural loss. We do not expect that our experimental treatments will lead to deaths exceeding these natural losses.We will ensure this by:

Compounds administered will be research grade and will have been tested in in vitro models ofinflammation.

concentration ranges will be taken from the literature for the majority of the drugs we intend to test inthis project. Where data are not available, we will perform maximum tolerated concentration experiments using non-protected zebrafish larvae younger than 5 dpf, or pilot studies using older fish.

where possible refined and less invasive methods will be implemented for genotyping e.g. ZEG

For genotyping by fin clipping, prior to anaesthetising adult fish or larvae (the least invasive and most appropriate choice of genotyping method will be used for each life stage), an evaluation is conducted to determine if fish are fit enough to undergo this procedure. This will be done by checking for any signal of illness or erratic swimming behaviour. Any animal that does not look well will be excluded from the procedure. The anaesthesia will be administered through immersion. Sampling only a small portion of the fin will minimise any risk of causing alteration of swimming behaviour, and amputated finswill regenerate within a few weeks. Following fin clipping, post-operative analgesia will be provided (such as lidocaine) as it has been demonstrated that this procedure improves welfare.

We will also minimise any potential animal suffering by using the best husbandry practices possible which are well established in our Animal Facility. All environmental conditions, such as water temperature, water quality, day length and light levels will be optimised. Daily visual inspection of the fish by our highly trained staff, usually when they are being fed, will allow us to identify any possible welfare problems. Loss of appetite can also be a sign of welfare problems. We will ensure that whenever possible minimising handling (and invasive procedures) occurs, and when anaesthetic is used, care will be taken to adequately buffer it (if necessary), the temperature and water parameters (e.g. dissolved oxygen) of the anaesthetic solution will be as close as possible to the home tank to prevent stress in the fish. Anaesthesia will be maintained at the appropriate depth for the procedure, bytesting withdrawal reflex before applying any stimuli and by observing opercular movement.

In general, studies will be performed blinded and randomised, with a key system to identify which animal/sample has undergone which treatment. Furthermore, compounds administered will be madeby laboratory personnel other than the one performing the experiment in our animal unit.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?
I will adhere to the Home Office guidance of the Animals (Scientific Procedures) Act 1986 (and revisedlegislation) and the NC3Rs guidelines.

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

New updates will be sought from the NVS, NCIO and AWERB at the University and from otheragencies e.g. the NC3Rs, RSPCA, norecopa. Updates that are applicable to the PPL will be implemented where possible at the institution.

UNDERSTANDING HOW MEMORIES ARE FORMED

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Learning, memory, plasticity, anatomy, behaviour

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to advance understanding of how the brain acquires and stores memory information. We will investigate the cellular processes that occur during learning, and how memoryinformation is communicated between brain regions.

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

Why is it important to undertake this work?

The work will advance understanding of how learning and memory occurs in the healthy adult brain,which in turn will enhance understanding of how learning and memory fails during ageing and in neurological and psychiatric disorders such as dementia, depression and schizophrenia.

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

The primary output of the project will be new information on the cellular and molecular processes thatoccur within the brain during learning, memory formation and the subsequent retrieval of information stored as short and long term memories. This

information will be published in relevant journals and presented at scientific conferences.

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

The immediate beneficiaries of this work will be researchers in the field of cellular physiology and cognitive neuroscience. In the longer term (>10 years) it is expected that there will be improved understanding of how the brain processes and stores memory information that might ultimately revealnew targets for the treatment of memory loss, and will therefore be of benefit to patient groups.
Typically the time between identification of a 'druggable' target and an available treatment is 10 years.

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

The outputs of the work will be maximised through collaborative engagement with other scientists andclinical colleagues. In addition, results will be presented at scientific conferences and published in peer reviewed journals.

Species and numbers of animals expected to be used

- Mice: 5000
- Rats: 2500

Predicted harms

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

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

In order for the findings of the work to be relevant to humans, it is necessary to use an animal with a brain known to function in a similar manner to that of the human brain. The findings of previous studieshave confirmed that the cellular and molecular processes involved in learning and memory formation inrats and mice share a close similarity to that of humans and that it is possible to study these mechanisms using these species. Consequently, rats and mice have been chosen for these studies.
Mature adult animals will be used as the aim of the project is to advance understanding of theseprocesses in the adult human brain.

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

The outlined studies will involve the manipulation of specific neural processes within the brain of animals and the assessment of how this influences memory and learning using behavioural tasks. To achieve this aim, some animals will either be bred with specific genetic alterations or will undergo an aseptic surgery procedure, under general anaesthesia, involving either the implantation of indwelling cannulae (to enable drugs to be delivered directly into the brain), or neural probes (to stimulate specificneuronal networks) or to enable viral vectors to be delivered to modulate neuronal activity. Thereafter,the performance of the animals will be assessed in a range of behavioural tasks to determine the effectof the manipulation on memory and learning. At the end of the study the animals will be humanely killed and analysis of their brain's undertaken.

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

Animals bred with specific genetic modification are not expected to experience any adverse effects. All surgical procedures will be conducted under general anaesthesia. Animals undergoing surgery will experience transitory distress during anaesthetic induction and mild pain upon recovery. To minimise these adverse effects, the animals will be habituated to handling prior to the start of the study and given drugs to minimise the effects of pain both during and post surgery. Animals are expected to makean uneventful recovery from surgery and to resume normal behaviour within a few hours. Some animals may incur a slight weight loss following surgery but are expected to regain this within a few days. During some behavioural tasks, animals receive food rewards to encourage them to take part.
Wherever possible, the motivation to engage with such tasks will be achieved by rewarding the animal with highly palatable food treats. However, where necessary, animals may be maintained under mild food or water restriction to increase their motivation to engage with the task. In all cases, the level of restriction will be kept to a minimum and will not result in a reduction in their growth or development rate or affect their general wellbeing. Some animals may receive drugs delivered directly into the brainvia implanted cannulae. However, though these drugs may temporarily modulate the activity of brain cells, they do not have any long-term impact on the animals' wellbeing and the animals general patternof motor and sensory behaviour is expected to remain normal. At the end of the study the animals 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 animaltype)?

For Protocol 1 Breeding and Maintenance of GA animals the severity is mild for 100% of the animals.

For Protocol 2 Neuroanatomical and cellular mechanisms of learning and memory the severity ismoderate for 80% of the animals.

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

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered 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 chemical and cellular processes involved in learning and memory. Since these biological processes only occur in the brain ofa live animal, it is not possible to undertake this work without the use of living animals. Furthermore, inorder to be relevant to an understanding of these processes in humans, it is essential that the species used has a brain that is functionally

representative of the human brain. Consequently, lower animal species, such as the fruit fly, are unsuitable as their brains are too primitive to be representative.

Which non-animal alternatives did you consider for use in this project?
Cultured neurons and computer simulations

Why were they not suitable?

Cultured neurons do not form complex networks, nor do they contain the variety of neuronal and non-neuronal subtypes to enable us to reproduce all of the processes that exist in the living brain.

It is not yet possible to effectively simulate the complex process involved in the acquisition and storageof different types of memory information using computers since present knowledge of such mechanisms is limited.

Reduction

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

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

The studies under this licence will expand on our on-going studies in this research field. We have estimated the numbers of animals through our extensive experience in the design and execution ofexperiments, and in the breeding of transgenic mice for the outlined experimental purpose.

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

A within-subjects experimental design will be used to minimise animal numbers while maximising statistical power. A cross-over design will be used, where possible, to ensure that each animal receives a control treatment to establish baseline measurement. In all experiments, animals will berandomly assigned to experimental groups.

To refine experimental design we will use the online NC3Rs Experimental Design Assist program.

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

The number of animals required to generate the transgenic models necessary to address the objectives, will be kept to the minimum by careful colony management matching to the demands of theexperiments. Colony management will be achieved through liaison between a dedicated research technician and specialist technical staff in the breeding unit.

We will test the same group of animals (which include both male and female animals) across a numberof established memory tasks.

To maximize the data obtained, *ex vivo* experiments will be undertaken at the end of the study usingslices taken from the brains of some animals after they have been killed.

Refinement

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

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

In order to ensure that the findings of the study are relevant to the processes involved in learning and memory in humans, it is essential to use an animal species with a brain that shares the same biologicalprocesses and has the capacity to learn and form memories. Consequently, the study will be conducted using rodents (rats and mice), which are the least neurophysiological sensitive laboratory species able to perform the memory tasks upon which the work depends. The rodents will be housed in pairs or in groups, in cages containing environmental enrichment. All animals will be handled extensively before the start of each study to habituate them to human contact.

For all surgical procedures both local and general anaesthesia with accompanying analgesia, will be used to minimise pain and distress. During surgery, the animal's body temperature will be monitored and maintained using a heat pad. Post-surgery, the animals will be given pain killers until they show nodetectable signs of pain.

To assess learning and memory we will use tasks which are the least severe needed to meet the objectives. The main test will be the object recognition task. This task exploits the natural tendency of rats and mice to spontaneously explore novel items and consequently, does not require the animals toeither undergo prior training or be motivated to take part. For dry land tests, that use either food or water as a reward to motivate the animals, highly palatable rewards will be used, and food or water restriction will only be used when this is absolutely necessary i.e., if the animals fail to engage in the behavioural task. Whenever food or water restriction is used, the animal’s weight and body condition will be monitored weekly and weight loss will be limited to a maximum of 15% of free feeding weight. For water-maze studies, a shallow water paddling variant of the task will be used, rather than the standard swimming verion to reduce stress in the animals. Immediately following the water maze task,each animal will be dried using a towel before it is returned to its home cage.

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

To ensure that the findings of the study are relevant to the processes involved in learning and memory in humans, it is essential to use an animal species with a brain that shares the same biological processes and has the capacity to learn and form memories. Consequently the studies will be undertaken using rodents, (rats and mice), as they are the least neurophysiological sensitive laboratoryspecies that meet these criteria. It is not feasible to use less sentient, non-protected species (e.g. fruit flies) as their brain anatomy and physiology differs markedly from humans and is incapable of undertaking the complex

processes that underpin learning and memory formation. It is also not possible to perform the investigations in terminally anaesthetised animals as the anaesthetic, by its very nature, prevents the sensory input that is essential to learning and memory formation.

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

Prior to the start of the studies, the animals will be habituated to human contact by regular handling. Allsurgical procedures will be performed under general anaesthesia using strict aseptic precautions. Bothduring and following surgery the animals will be given pain killers and these will be maintained until theanimal is showing no detectable signs of pain. Animals are expected to make a rapid and unremarkable recovery from surgery and to resume normal behaviour within a few hours. Apart from the immediate post-operative period the animals will be kept in social groups.

The animals will be group-housed in cages with environmental enrichment and cognition will be tested predominantly using spontaneous behavioural tasks that provide additional stimulation and/or for whichthey will receive treats. They will also receive additional attention from the research staff when they areon task. The animals will receive stepped habituation sessions to familiarise them to the testing apparatus.

Throughout the study the health of the animals will be monitored by trained research staff and experienced animal technicians to ensure any distress or discomfort is identified early and appropriatemeasures taken to mitigate suffering.

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

LASA: Guiding principles for preparing for and undertaking aseptic surgery

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

Information regarding advances in the 3Rs will be obtained through attendance of

3Rs events e.g. conferences, organised within the University

NC3Rs events organised by regional programme managers

On-line 3Rs webinars

and regular reviews of the literature on related studies

Discussions of relevant 3Rs advances are also undertaken at lab meeting whilst discussing thefindings and design of experiments.

UNDERSTANDING THE MECHANISMS UNDERLYING THE AGEING PROCESS

Project duration

5 years 0 months

Project purpose

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

Key words

Lifespan, Healthspan, Metabolism, Multimorbidity, Genetics

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to identify shared molecular and cellular processes that act to alter therate of ageing in mice. We will alter the rate of ageing in mice through genetic, dietary or pharmacological means and examine the impact of these treatments on lifespan and the period of life free from age-related pathology (healthspan).

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

Why is it important to undertake this work?

Ageing is defined as a decrease in physiological function associated with an increase in the probability of death with advancing age. Over the last two centuries there has been a

rapid increase in life expectancy in the developed world, leading to a greater proportion of our society made up of individuals over 65 years of age. Consequently, these changing population demographics will present profound medical, social and economic challenges for future generations, because age is the primary risk factor for many diseases including most cancers, dementia, cardiovascular disease, stroke and type 2 diabetes. Most people over 65 years of age suffer from more than one age-related disease, termed multimorbidity. While the economic and social impacts of our ageing society is evident, the fundamental processes underlying ageing are currently unclear. Consequently, understanding the general mechanisms of ageing is a major scientific challenge, but given that the changes associated with ageing appear highly similar from microscopic roundworms to humans, it is hoped that by studyingageing in model organisms, such as mice, this will provide insights into human ageing and hopefully identify ways in which to improve late-life health and wellbeing in humans.

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

This work will help advance fundamental scientific knowledge of the ageing process, help identify potential new genetic pathways that modulate both ageing and disease, and may also help to identifyrealistic intervention strategies to extend healthy lifespan in mammals, including humans. Our work may also help to identify potential ways in which to improve the health and wellbeing of people suffering from accelerated ageing (progeroid) diseases, for which there are no suitable treatments currently. Research findings will be made available to other scientists through publication in peer- reviewed journals and presentations at scientific conferences and meetings. The likely benefits of thisproject will be that it will generate high quality, publishable data that will generate new knowledge and a better understanding of what the key processes are that underlie the ageing process in mice, and that this knowledge has the potential, in the longer-term, to help find new ways to improve late-life health in humans. We would also hope to provide biological material to our collaborators for additionalstudies.

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

This work will help advance fundamental scientific knowledge of the ageing process, help identify potential new pathways that modulate ageing and disease, and may help identify realistic intervention strategies to extend healthy lifespan in mammals, including humans. Understanding the ageing process is important because ageing itself is the major risk factor for many common diseases, including type 2 diabetes, cardiovascular diseases, stroke, dementia and most cancers. Consequently,if we can 'treat' ageing then we should be able to reduce the severity of these diseases in older people.This would be a major benefit to the research group, UK science and to elderly people and potentially also companion animals. The scientific community will benefit from the publication of research and through presentations throughout this project. I would envisage the generation of publications throughout the duration of this project and would look to maintain current collaborations and build new collaborations during this project. In the short term our project will test whether specific genes or dietary interventions can affect the ageing rate and in the longer term may identify new treatments capable of slowing the ageing rate and improving late-life health both in individuals during normal ageing and in individuals suffering genetic diseases that lead to accelerated ageing (progeroid syndromes).

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

I would aim to maximise the outputs of this work through collaborative research and the dissemination of new knowledge and approaches to ageing research both through open access publications and presentations. The publication of any unsuccessful approaches

will be made through publication in preprint servers such as bioRxiv. Findings will be made available to other scientists through publicationin peer-reviewed journals and presentations at scientific conferences and meetings. We will also maximise our outputs by making any data and tissues available to other researchers.

Species and numbers of animals expected to be used

- Mice: 4800

Predicted harms

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

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

Mice are mammals that have many fundamental physiological similarities to humans. We know for example that dietary interventions that extend lifespan and healthspan in rodents also elicit many beneficial metabolic effects in humans. In addition, we know that specific genes that increase (or decrease) lifespan and affect healthspan in mice similarly associate with human longevity. The rapid generation time and typical lifespan of mice allows us to explicitly test and examine ways in which to modulate ageing rate within typical research cycles. We typically choose adults and aged animals, as in order to test how a particular intervention affects ageing and age-related health requires the animalsto age. We will also utilise accelerated ageing models, as their shortened lifespan allows us to more rapidly investigate ageing processes and test anti-ageing interventions.

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

Typically, a mouse will be group housed with same-sexed, age-matched litter mates. The mice may vary in their genetic background and/or be exposed to diets that are known to slow (e.g. dietary restriction) or accelerate (e.g. high fat diet) the ageing rate. Our ageing studies are typically split by design into longevity studies where individual lifespan is determined (following our humane endpoints) and where non-invasive measures, such as frailty, are collected across that individual's life. In addition,we typically have cross-sectional studies where animals experience procedures at specific times across their lifespan, for example for long-lived mice these are typically undertaken at young (e.g. 6 months), middle aged (12 months) and old age (18 months) and the mice subsequently humanely killed to collect biological materials. The timing of these cross-sectional studies will be dependent on whether the intervention slows or accelerates the ageing process. Typically, mice will have their metabolism measured (using dedicated metabolic chambers to measure parameters such as oxygen consumption, activity, food intake). Mice may be exposed to different diets (e.g. dietary restriction or high fat diet) across the life-course and aspects of metabolic health (e.g. glucose tolerance/insulin sensitivity) may be determined. Blood may also be collected across the life-course in order to measure,for example, hormone levels. To provide insights into age-related frailty we may measure motor strength and coordination in our mice as they age using a rotarod. This apparatus consists of a slowly rotating wheel, and the ability of a mouse to remain on this wheel over a defined period- typically 300 secs- correlates closely with motor strength and coordination, both of which are known to decline during ageing. To gain a better understanding of the processes underlying ageing, we may use molecular probes to measure compounds known to affect the ageing process (e.g. hydrogen peroxide, nitric oxide, hydrogen

sulphide), or compounds that can be used to provide insights into overall metabolism of that animal or compounds known to modulate ageing itself (e.g. ES-62).

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

Mice may be injected with substances that may cause transient pain and discomfort. The collection of blood from the tail vein (venesection) may also cause short-term (<24 hours (hrs)) pain and discomfort.

Mice with mutations that lead to accelerated ageing mutations typically become frail, develop metabolicand pathological disease and die younger than typical wild-type mice - all ageing studies and cross- sectional studies (for middle and aged mice) therein will be scaled as appropriate for that specific genetic model using previously published in depth studies on mortality rates.

Long term high fat diets (e.g. 20 weeks) can cause overt diabetes which is associated with increasedurine production, rapid weight loss and eventual organ failure. Any mice experiencing these adverse effects will be humanely culled before severe symptoms manifest.

Normal ageing itself leads to frailty and increased pathology- which can include hair loss, curvature ofthe spine, hearing loss, increased tumour incidence, weight loss- and so all our ageing studies will follow our monitoring system and humane end-points. In our most recent ageing study, 12 mice out ofa total of 210 mice (5.7%) were found dead as oppose to the rest (198 mice or 94.3%) that reached our humane end-points and were humanely culled.

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

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

The maximal severity is moderate.

Protocol 1- 2000 mice (100% of this total mild).

Protocol 2- 400 mice (70% (280 mice) of this total mild/30% (120 mice) of this total moderate). Protocol 3- 800 mice (60% (480 mice) of this total mild/40% (320 mice) of this total moderate). Protocol 4- 1000 mice (60% (600 mice) of this total mild/40% (400 mice) of this total moderate).Protocol 5- 600 mice (50% (300 mice) of this total mild/50% (300 mice) of this total 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

haveconsidered and why they cannot be used for this purpose.

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

Ageing acts at the whole-animal level and thus its effects depend upon coordinated interactions acrossmultiple organ systems within live animals. Consequently, these fundamental biological processes, which can impact differently depending on the specific tissue under study, cannot be fully captured in any other manner, such as through cell culture, organoid culture or computer modelling, although theseapproaches can help to inform.

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

Wherever possible we will identify appropriate alternatives to supplement our animal studies. We willaugment our findings in whole animals using primary cell culture (e.g. primary skin fibroblasts or muscle cells), commercially available cell lines or organoid culture (e.g. intestinal organoid systems).For example, we and others have demonstrated that cultured skin fibroblasts from long-lived mice retain similarities when exposed to various chemical insults as that seen in whole-animal studies. We will also support our findings through computer simulations, modelling approaches and machinelearning approaches wherever appropriate.

Why were they not suitable?

These alternatives can help inform but none of them fully capture systemic ageing and how exactly itimpacts on physiological, cellular and molecular changes within individual animals. We also need to understand how any new intervention strategy affects the whole organism rather than a cell-specific approach in culture.

Reduction

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

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

I have extensive experience in mouse ageing and metabolism studies. Consequently, I have an understanding of what numbers will be required to undertake the work described within this project. I keep up to date with the current literature and will carry out periodic literature searches throughout the timescale of this project to continually improve and refine our experimental approaches in order to maintain scientific rigour and power whilst always using the lowest number of animals. We routinely collaborate with other researchers and provide post-mortem animal tissues to inform on their research,thus helping to reduce animal use whenever possible.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?
I have extensive prior data on the variability in relevant experimental parameters related to mammalianageing and will use these data to inform power analyses to establish the

minimum numbers of animals required to obtain a reasonable effect size for any intervention. For our previous studies, we have generally shown that a sample size of 8 is sufficient for many of our physiological assays, assuming a maximal standard deviation of 10%, an effect size of 15% between groups at a power of 0.8 (at alpha=0.05) using one-way ANOVA. The sample size for the longevity studies will typically be 50 male and 50 female animals per genotype (total 200 mice). This will generate sufficient power to detect a 10% lifespan difference between control and experimental mice. It is essential to study both sexes as many ageing interventions have different effects depending on the mouse sex. If no sexual dimorphismis observed, then we will combine sexes which will provide even greater statistical power to detect a 10% difference in lifespan. Kaplan-Meier survival curves will be constructed using birth and known death dates and the log-rank test and Cox regression used to evaluate any genetic and/or sex differences.

We will liaise with statisticians in order that our studies are always undertaken using the minimal number of animals but retaining appropriate statistical rigour throughout. I will utilise the NC3R's onlineExperimental Design Assistant to help inform on best practice on experimental design throughout the project. Experiments will be planned so they can be published in accordance with the NC3Rs' ARRIVEguidelines. In addition, we are using advanced statistical (machine learning) approaches in our research to help identify meaningful features within data sets containing an increasing number of variables.

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

Any pilot studies will be run in such a way that they will be rolled into the main study wherever possible, so that they are not additional to the numbers ultimately required for the main experiment. The genetically altered animals and all suitable lines will be obtained from existing colonies, from collaborators or from relevant suppliers. In every case we will measure production and breeding performance and ensure the minimum numbers of animals are used in the programme through efficientbreeding and maintenance. Typically, we will study both male and female mice in order to minimise usage of one specific sex. We always maximise the amount of biological material we collect from our mice and we have initiated several successful collaborations over the years through the sharing of mouse tissues from our tissue biobanks. Our genotyping (required to identify mice harbouring the relevant genotypes) is outsourced to a cost-effective professional company. This helps provide optimal control of colony management, minimising the wastage of mice across large breeding programmes.

Refinement

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

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.
We use animal models best suited to address the biological hypotheses we want to test. Many of our studies will typically involve non-genetically altered (wild type) mice which are used extensively in ageing and obesity research and so provide optimal cross-centre

replication of outcomes. Any compounds will be administered at non-toxic dosages and following previously published data. Our ageing models may include genetic mutants that either slow down or accelerate the ageing process, orinvolve diets or pharmacological interventions that modulate the ageing process. Consequently, our established ageing monitoring systems will be tailored to each model and our strict humane endpoints will be applied to minimise suffering. To induce gene expression in animals or deplete specific cells, some animals will be given substances by mouth, injection or through food. Oral gavage or injection can be necessary to induce a rapid change in gene expression. This will let us to study processes that happen within short time periods of a few hours. Our metabolic cages allow us to determine metabolism in real-time without interfering with the animal (Indirect calorimetry measures oxygen used/carbon dioxide respired). Our metabolic cages do not have grid floors like most systems that allow the collection of urine/faeces but have been reported to cause foot lesions in rodents and increase both heart rate and blood pressure. Our system has solid floors that are more comfortable for rodents. At all times training and good practice will be to the fore of our research, and this will involve regular discussions with our named veterinary surgeons and key animal staff. We will follow a path of progressive method development and experimental refinement at all times.

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

Rodents are the lowest sentient animal model suitable for the study of mammalian ageing and the work described in this project builds on my previous research characterising the responses of mice to experimental interventions that modulate ageing rate and late-life health. Mice have been shown to behighly effective model organisms to study the basic mechanisms of ageing and have generated seminal findings on the physiological and genetic factors governing longevity. However, to do this effectively we need adult mice to age as we cannot capture the effects of ageing in young mice or neonates.

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

We will always look to identify more effective ways to improve our experimental approaches in line withbest practice. We will regularly send members of our team to dedicated courses on up to date methodsand approaches that include refinement of procedures. We have recently introduced a non-invasive clinical frailty index in our lifespan studies. This approach is a relatively simple approach to quantify frailty through clinical assessment of a large number of phenotypic parameters in mice, and a relativelylow frailty score has been shown by others to correlate with greater longevity. At all times we strive to improve the quality of our methods, thus generating better quality, less variable data and more optimal study power.

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

We follow the ARRIVE guidelines and will continually look for any updates over the duration of theproject.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?
We will attend NC3R webinars and will organise to meet out NC3Rs Regional Programme Managerover the duration of this project.

We will follow and check for updates in good practice on the NC3Rs web site and liaise with our namedtraining and competence officer.

THE CONTROL OF IMMUNE RESPONSES IN THE INTESTINE

Project duration

5 years 0 months

Project purpose

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

Key words

Immunology, Infectious Diseases, Inflammatory Diseases, Microbiota, Intestine

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 notrequired.

Objectives and benefits

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

What's the aim of this project?

This project aims to understand how immune responses in the intestine are controlled. Tight control of the immune response is important for the health of people and animals, and we aim to understand howthis control is achieved.

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

Why is it important to undertake this work?

Immune responses in the intestine are required to protect mammals from infections. Our intestines are colonised by billions of micro-organisms. Most of these organisms are harmless, or even useful, but some can cause infections and ill-health. The immune

system maintains health despite the presence ofthese micro-organisms and also prevents unwanted immune responses against our food.
Understanding the ways in which the intestinal immune system operates may enable us to improveresponses to infections, to manipulate or utilise our harmless micro-organisms, and to prevent damaging immune over-reactions. This is important for understanding a range of common human conditions, from intestinal bacterial infections to inflammatory bowel disease. We hope that this improved understanding will contribute to the development of new treatments for these conditions.

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

Work carried out in this project will identify the important cellular and molecular interactions that controlimmune responses in the intestine. The work includes investigations into the healthy gut, responses toinfections, and studies of inflammation.

This information is of fundamental scientific importance, leading to scientific presentations and publications. It will also reveal new targets for drug development, and new strategies for manipulatingthe intestinal immune response (e.g. through changing diet).

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

This work will continue to generate scientific data, presented in peer-reviewed publications, at scientificmeetings, and incorporated into immunology teaching. This will be of benefit to other scientists, to interested students, and to pharmaceutical companies with an interest in intestinal immunology.

Our animal experiments complement our ongoing work investigating the mechanisms underlying human inflammatory disease. Our understanding of how diet can influence intestinal inflammation andthe intestinal microbiota in rodent models has contributed recently to a clinical trial where a dietary therapy is now being used in a clinical trial to treat people with inflammatory bowel disease.

The aim of this programme of work, to which the animal experiments make an important contribution,is to improve treatments for people with inflammatory diseases that involve the intestine, including ankylosing spondylitis, alopecia, and inflammatory bowel disease. We aim to contribute to the generation of new medicines, and new methods to identify individuals who will benefit from specific treatments. This work will therefore be of benefit to patients, the doctors that treat them, to pharmaceutical companies, and to health policy makers.

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

Our work will primarily be published in the scientific literature, as primary papers, methods papers, orreviews. Experiments will be carefully designed to maximise their generation of publishable material.While these publications may focus on ‘successful’ experiments we will continue to include relevant negative data in publications; such data often enhance understanding and clarify scientific mechanisms.

We will continue to share our data via presentations and posters at meetings. These meetings will include scientific conferences, as well as meetings with patient groups and public engagement events.

We will provide detailed methods and training in methodological approaches to colleagues

nationallyand internationally, and host visits from interested colleagues with interests in our approaches.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

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

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

The work in this licence will be performed in adult mice.

The immune system of the mouse has been well-characterised, and many similar immune mechanisms are also found in the human immune system. Mice are also very widely used for immunecharacterisation, so that we can design our experiments and interpret our data in direct comparison toan existing and rapidly-developing body of research. This helps to ensure the quality of our experiments.

Mice also provide a unique range of genetically modified animals, many of which have been developedto enable investigations into mechanisms controlling immune responses. This includes mice expressing light-emitting molecules that can be used to identify the locations of specific immune cells, as well as mice with defined deletions or additions to their immune system to help understand the role of particular molecules and cells. This allows investigations of exactly how, a particular molecule or cellis involved in an immunological mechanism, and is not possible in other species.

Some of our experiments involve surgery (removal of lymph nodes, or insertion of a cannula (collectiontube) into a lymph vessel). These surgeries are used to collect specific cells, particularly cells that migrate from the intestine to other tissues (e.g. lymph nodes). We have developed and refined techniques for removing lymph nodes and for collecting intestinal lymph, which are also used in larger animals. In combination with the advantages described above, this provides a unique and powerful way to investigate the functions of cells that control intestinal immune responses.

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

We will typically use animals in one of the following three ways:

Some genetically modified animals will be bred to produce cells for experiments designed to test specific immune functions. These animals will be bred using normal procedures. A small sample of tissue or blood may be taken to check their genetic or immunological status, and they will be humanelykilled.

Other animals will be used in experiments where they are given substances that may affect their intestinal immune system. Substances will sometimes be given mixed with food or water, injected, or delivered by oral gavage. Occasionally they will be injected into specific intestinal tissues during surgery. Animals will then be monitored carefully (daily or more frequently where necessary), and mayprovide samples (e.g. blood or stool) for

analysis. At the end of the experiment they will be humanely killed and their tissues used for additional analyses. A wide range of substances may be used, and some may be used in combination. Examples include antibiotic treatments, changes in diet, chemicalsto induce inflammation, live or killed micro-organisms, or inert substances used to measure physiological functions.

Some animals will undergo surgical procedures designed to deliver substances to precise anatomical locations, or to collect cell migrating from the intestine. Surgery will usually involve openingthe animal's abdomen, a procedure called a laparotomy, on one or two occasions. Where two surgeries are performed, for instance to collect cells migrating in thoracic duct lymph, animals will be given sufficient time to recover between the laparotomy procedures. Animals undergoing surgery may also receive substances to manipulate their intestinal immune system.

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

The impacts and adverse effects will be different for each of the groups.

Genetically modified animals providing tissues may experience transient discomfort for <6 hourswhen providing a sample for genetic analysis. They will be humanely killed when needed for tissues.

The nature of their genetic modification may also impact their health, for instance by affecting their immune system and occasionally leading to ill health and weight loss. If this should occur animals willbe humanely killed.

Animals with affected intestinal immune responses may also experience a range of effects. Someanimals will be fed with diets designed to change their intestinal microbiota, or will be treated with inertsubstances, and will experience no adverse effects. Animals receiving antibiotics or infectious micro- organisms will typically experience some weight loss, and may also experience intestinal inflammation.These effects will not usually persist for more than a week, after which time the animal will either recover or be humanely killed.

Animals undergoing surgery will experience pain and discomfort associated with the surgical procedure. This will be minimised by the use of anaesthesia and analgesia. Animals will be humanelykilled if they do not make a full recovery from the surgical procedures. Animals undergoing a second surgery will generally recover from the procedure and will typically be humanely killed 1-2 days after this second procedure. Some animals that receive surgery will also be treated with substances to manipulate their intestinal immune system, but surgical procedures will not be performed on animals that show signs of ill health.

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

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

Mild 90%, Moderate 10%
Mild 50%, Moderate 50%

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 haveconsidered 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 is highly complex, involving interactions between multiple different cell types. At present while it is possible to model many immune interactions using cells obtained from animals without performing regulated procedures, these experiments cannot recapitulate the full range of interactions that determine the outcome of an immune response. For example, the cells of the immunesystem that we have studied are highly responsive to changes in their local environments; this sensitivity is crucial for their ability to detect infection or inflammation. Even removing immune cells from their natural environment can rapidly change their functions. Thus, while we will continue to makethe maximum possible use of cells form animals that do not undergo regulated procedures, and cells prepared from human tissue samples, it is currently not possible to investigate the questions describedin this application without performing procedures on living animals.

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

As a result of our previous animal research, we have developed a tissue culture model alternative to study how the intestinal environment affects the functions of dendritic cells. While this model represents an improvement on existing replacement models and a step forward in reducing animal usein the field, the cells we produce still do not replicate the full range of functions performed by cells collected directly from animals.

The information gained from the use of animal tissues contributes directly to our studies using humansamples from consenting individuals with inflammatory disorders and healthy controls. These human studies form a substantial proportion of our work; over half of the people in my research team do not perform any experiments using animals.

Why were they not suitable?

The dendritic cell culture model we developed does not allow analysis of immune cell migration in intact tissues, in inflammatory or infectious diseases, or model interactions where dendritic cells are able to inhibit potentially damaging auto-immune or inflammatory responses. For the field to move forward, we need to perform further mechanism of action studies, which in turn will develop improvedtissue culture systems to replace animal models.

Reduction

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

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

This estimate is based on the numbers of animals used under my previous license, with adjustments toaccount for both the effects of COVID (reduced animal use from 2020 onwards) and the focus of our experiments as we rebuild and progress. We will likely be increasing numbers of animals used for dietary and microbiota studies, and using fewer animals for surgeries.

Pre-COVID we used approximately 500 animals per year. So I estimate we will use 2500 over the next5 years. These estimates are developed with a number of factors in mind, including: the numbers of experiments that can be practically performed by my team; the group sizes and numbers of required control groups; and variations in these in depending on the purpose of the experiment and thevariability inherent in each type of model.

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

Each protocol is developed from our previous experience and uses the minimum number of animals required to generate statistically robust conclusions. When deciding on controls and group sizes we take account of expected effect sizes and the expected level of heterogeneity in responses in the particular model. Before starting an experiment, we use power calculations and randomisation approaches where appropriate. These allow us to avoid using excess animals, while enabling us to identify differences (e.g. in response to an infection or change in diet). In designing experiments we adapt our experimental design to take account of the characteristics of the output data and the necessary statistical analyses, and take advice on statistical approaches and experimental design. We frequently consult with colleagues with statistical and analytical expertise (Biostatistics / Bioinformatics)and use the NC3Rs Experimental Design Assistant (EDA).

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

We do not routinely generate new transgenic strains or maintain colonies with complex crosses, sogenetically modified animals can be efficiently maintained with simple breeding programmes.

We perform pilot experiments when using new interventions or approaches to generate robust estimates of effect sizes and variability. These enable us to more accurately calculate necessary numbers of experimental units required to test our hypothesis. They also help with decision making; experiments may need further refinement or may need to be discontinued if effect sizes are small andvariability is too large.

Tissues from experimental mice are often shared between researchers. Projects within the lab are designed, for instance, to focus on different anatomical or cellular responses to a core set of infections.Thus, three researchers focussing on epithelial cells, T cells and dendritic cells, can perform independent analyses on tissues collected from the same groups of infected and control mice. In somecases, these researchers may all work in my laboratory, but our close associations with other local intestinal immunologists frequently also allows sharing of tissues between staff from different 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

We will use three broad methods to understand the functions of the intestinal immune response, allinvolving mice.

Genetically modified mice will be bred to generate cells used in assays of immune functions. Themajority of these mice will generally only experience mild and transient discomfort. We do not plan to use animals with genetic modifications that themselves cause pain, suffering, distress, or lasting harm(PSDLH).

Mice will be treated with substances to manipulate their intestinal immune responses. In these experiments each experiment will be designed to test a specific hypothesis, and will be designed to cause the minimum possible level of suffering. We will, for instance, use attenuated strains of bacterialpathogens, and shorter durations of infection, to cause the lowest possible levels of PSDLH. Many of our manipulations have been refined to cause minimal effects on animals, while still enabling robust modulation of the immune response.

Our procedures involving surgery will cause some short-term suffering. We continue to refine these procedures to minimise PSDLH, including through the use of analgesia, and refinements to ourapproach to sterile surgery.

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

Mice are the least sentient species that can be used for the studies we propose on the functions of theimmune system. Being mammals, their immune system has many similarities, at both the cellular and molecular levels, to the human immune system. In addition, there are many mouse specific tools and reagents available for our work, enabling the maximum amount of useful information can be gained from each experiment.

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

In this license we describe three types of experiment protocols, with differing levels of severity. Whendesigning every experiment, we will use the procedures that cause the least possible level of harm.

When investigating immune responses to intestinal pathogens (e.g. *Salmonella*), animals will be infected with attenuated bacteria that cause only mild effects. In every case, and especially on rare occasions where more virulent strains may need to be used, experiments will be terminated as soon asthe required immune responses have begun, rather than waiting for overt signs of infection to occur.
Infection status is then monitored through microbial analysis of stool samples. In this way we aim to understand the effects of infection on immune cells without the confounding effects of illness, and alsoto minimise harms to our experimental animals.

On occasion, we will perform invasive surgical procedures which cause some short-term suffering. Thisis alleviated with appropriate analgesia to manage pain, as recommended by the NVS. This approach is necessary because it permits the study of dendritic cells and other migratory immune cells under near-physiological conditions. Since beginning to work with these techniques twenty years ago, we have made significant refinements to our procedures and have, for instance, developed techniques that remove the need to restrain animals post-cannulation. This process of refinement of our techniques continues to evolve. We are, for instance, continuing to improve standards for aseptic surgery, and to improve our prediction of the potential adverse effects of blood loss before animals experience suffering. We have made refinements in the last few months to the procedures we use for measuring blood loss, and for preventing complications during surgical procedures. This surgical workis done by experienced and trained members from my lab group, who have experience in all the techniques described in the license. They are assisted by animal care staff who administer analgesia/anaesthesia and monitor animal health during the procedures and during the animals' recovery.

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

We follow guidance from NC3Rs and the ARRIVE and PREPARE guidelines.

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

Up to date guidance will be monitored via the NC3Rs website (http://www.nc3rs.org.uk/) and throughupdates from our Animal Welfare and Ethical Review Board and Culture of Care subgroup.

We keep up to date on relevant scientific literature around immunomodulation and intestinal immunology. Where we find relevant and feasible improvements to our current methods that wouldenable us to improve our approach to the 3Rs we adapt these modifications. This approach has enabled us, for instance, to dramatically reduce our reliance on virulent *Salmonella* strains in our current work.

UNDERSTANDING HETEROGENEITY IN NEUROMODULATION

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neuromodulation, Neurotransmission, Brain disorders, Neuroanatomy

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aims of this project are to gain a better understanding of how the brain controls functions like mood, sleep, movements and senses. These functions are controlled by particular groups of brain cells which have certain things in common but also differ in important ways. We want to find out how these individual cells differ from each other in the proteins they contain, the way that they work, and theother cells in the brain they affect. Knowing all this will help us to understand how these brain cells control certain functions.

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

Why is it important to undertake this work?

Diseases and disorders which affect the brain include depression, anxiety, sensory disorders (e.g. visual impairment and hearing loss), dementias, and Parkinson's disease. Depression and anxiety affect around one in 10 adults and can recur throughout the lifetime. As the numbers of older people increases, the numbers of people suffering from age-related conditions like hearing loss, visual impairment, dementias, and Parkinson's

disease is increasing. At the moment, treatments for illnesseswhich involve the brain are not very successful and we need to develop improved medicines and othertreatments. To be able to develop better treatments, we need to understand how the healthy brain works. We need to know about individual groups of brain cells, how they work and how they affect other cells, which parts of the brain they connect to and how they affect movement, senses and behaviour.

If we can understand the differences between individual brain cells, we may be able to design bettermedicines or other treatments for brain disorders.

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

At the end of the project we will have a much better understanding of differences between individual brain cells. We will know what different chemicals they contain, how their activity differs, which braincells in which different parts of the brain they connect with, and how all of these differences influencetheir effects on mood, movement, senses, and behaviour. Knowing these things, we will be able to design studies to develop new treatments for brain disorders. We will share our findings with other scientists by publishing them in the scientific journals, giving talks to other scientists, and presenting our results at scientific conferences. We will also share our findings with the general public through seminars and press releases.

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

In the short term (3-5 years), our findings will help us and other researchers to understand how the healthy brain works normally, as well as how brain function might go wrong and so cause brain diseases or disorders. Beyond the term of this project, we expect that our findings will help scientists and medical doctors to improve the treatment of brain disorders. Our findings are likely to influence thedevelopment of new drugs and other (non-drug) treatments, such as electrical implants, to treat these disorders. Our findings will also help to improve the use of established treatments by allowing scientists and medical doctors to understand which patients are most likely to benefit from which treatments.

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

We will aim to maximise the outputs of this work by using the very latest technologies and methods in our experiments. We will work together with other researchers within our University as well as in otherUniversities, both in the UK and abroad. We will publish our findings (both positive and negative) quickly in science journals which are free for everyone to read, as well as more widely on the internet. We will also use social media to share our findings with people who are interested. We will allow otherresearchers to see our data in case they want to compare it with (or add it to) their own data or look atit using a different type of analysis. We will take part in conferences and workshops to share our technical findings including any improvements we have been able to make to our methods, as well as any unsuccessful approaches.

Species and numbers of animals expected to be used

- Mice: 600
- Rats: 320

Predicted harms

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

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

The project will use young adult rats and mice. Ultimately, we are interested in the human brain and how it works in healthy people and in people with brain disorders. The brains of rodents are very similar to human brains in their structure and the way they work. Our experiments will involve putting electrodes/probes and/or injections into specific parts of the brain. We will mainly use rats as their brains are big enough to accurately place electrodes/probes and injections into small brain regions. Some types of rats with genetic modifications are also available and we may use these where they willhelp answer our questions.

In some experiments, we will use mice. There are many genetically modified types of mice available inwhich certain groups of brain cells contain markers or proteins which make them easier to study.
However, the mouse brain is very small and it may not be possible to accurately place our electrodesand probes or to target specific regions. We will assess the advantages of these genetically modified mice, against the disadvantages of the fact that mouse brains are very small. If our questions can bestbe answered by using genetically modified mice rather than genetically normal rats, we will use GA mice.

We will use young adult animals. Although some of the brain disorders that we are interested in (suchas dementias and sensory loss) are more common in older people, this project is focussed on understanding how the healthy brain works rather than how it may be affected by diseases.

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

Most of the animals to be used in the project will be genetically normal but some will be geneticallyaltered (GA) animals.

Genetically modified animals

Genetically modified (GA) animals will be bought from commercial suppliers or obtained from researchers in our University or other research centres. In some cases the GA animals will be used inthe studies described below, in other cases we will breed GA animals to produce more GA animals which will then be used in the studies described below. These genetic modifications will not cause theanimals any ill effects or suffering.

Animals will be used in three types of study.

<u>Study 1. Behavioural testing (optional)/collection of tissues</u>

Some of the animals will have their behaviour tested. Before behavioural testing, we may need to limit when or how much the animals eat so that they will be hungry and will complete tasks to get food rewards. We will only restrict food for up to 16h and, overall, the animals will be given a normal amount of food and should not lose weight. During the behavioural tests animals may be watched by a researcher or be filmed. They may be in

their normal cage, or they may be moved to a special box or apparatus so that we can measure how they interact with objects, press levers, dig for food, or respondto sounds, lights, smells, or the presence of other animals. As part of the behavioural testing, we mightgive a small injection of a drug so we can test the function of a particular part of the brain we are interested in. The behavioural testing will take up to 2 hours and an animal might be tested once or twice (on two separate days).

We will collect the brain tissue from all of the animals. In order to make sure the tissue is in the right condition for our different types of measurement, we may need to euthanise the animals by perfusion. For this we will first inject them with anaesthetic and, when they are deeply anaesthetised, we will flushthe blood out of their blood system by perfusing a salt or sugar solution around the blood system via their heart. Alternatively, we may euthanise the animals with an overdose of anaesthetic or by an alternative regulated method performed by trained and competent individuals.

Study 2. Behavioural testing (optional), in vivo electrophysiology, collection of tissues (optional)

Some of the animals will undergo behavioural testing as described above (Study 1). To see how the brain cells work in living animals we will do in vivo electrophysiological recording. Forthese studies, we will anaesthetise the animals with an injection, cut open the scalp and drill small hole(s) in the skull so that we can place a recording electrode and/or stimulating electrode, glass fibreand/or probe directly into the brain region we are interested in. We will use the recording electrode tomeasure the activity of brain cells and will activate or inhibit the brain cells with the stimulating electrode or by shining light down the glass fibre. We may also give drugs that affect brain cells, directly into the brain (via a probe) or into the whole body by an injection. Animals will be anaesthetised throughout these experiments.

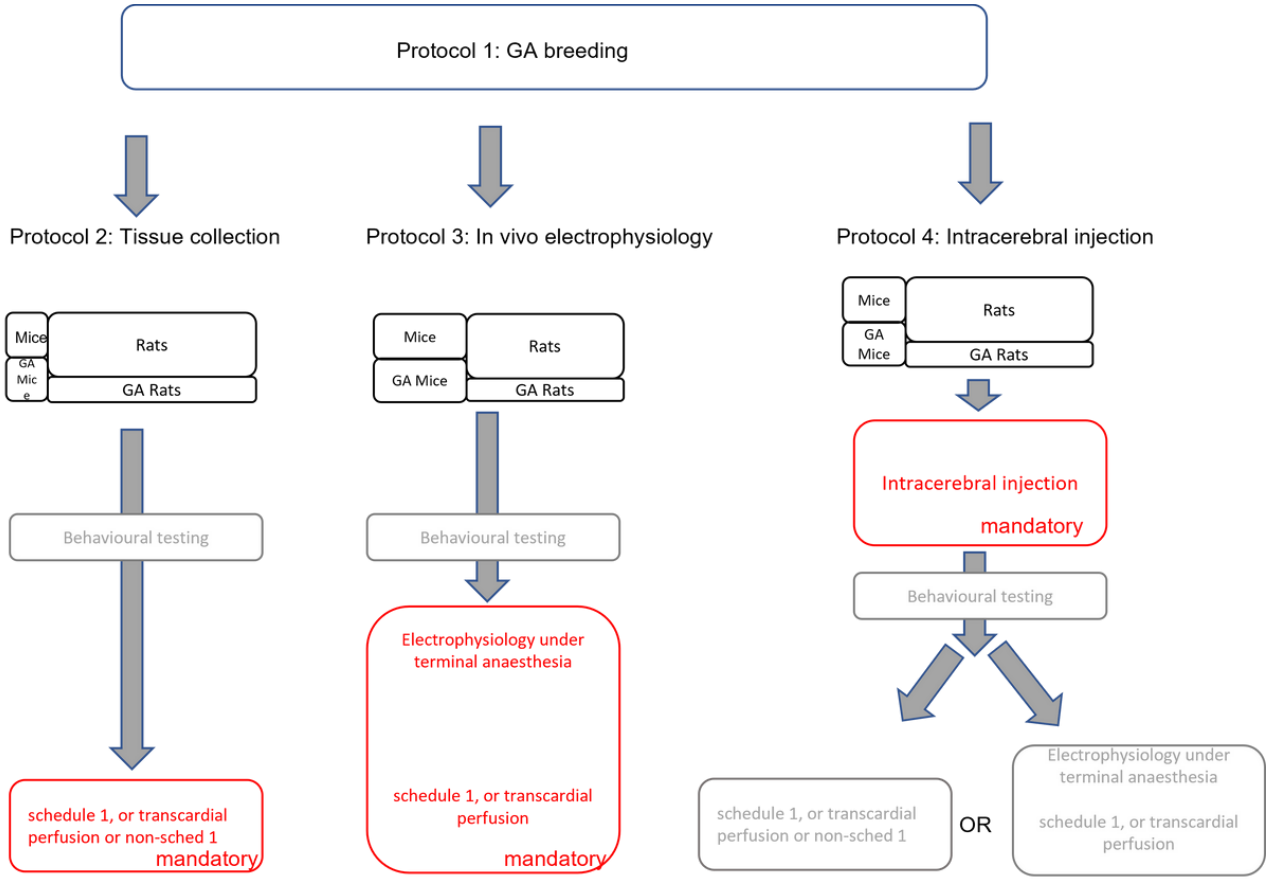
At the end of the recording, we may collect brain tissue from these animals. While they are still anaesthetised we will flush the blood out of their system as described above (Study 1). Alternatively, we may euthanise the animals with an overdose of anaesthetic or by an alternative regulated method.

Study 3. Injection of tracers/viruses, behavioural testing (optional), in vivo electrophysiology(optional), collection of tissues

In these experiments we will inject a tracer or virus which will allow us to see the connections the braincells make with different brain regions and/or will allow us to activate or inhibit a group of brain cells selectively. We will anaesthetise the animals, make a cut in the scalp, drill a small hole(s) in the skull, and inject a very small volume of a tracer or a virus which can go into the nerve cells and produce a protein. The wound will be stitched up and the animals will be given a pain killer and be allowed to recover from the surgery. Depending on the tracer or virus, we will keep the animals for up to 6 weeksbefore we use them in an experiment.

In some of these animals we will do behavioural testing as described above (Study 1) and/or we will doin vivo electrophysiology recording as described above (Study 2).

In all cases we will collect brain tissue from these animals. So that the brain is in the correct state for further study, we will flush the blood out of their system as described above (Study 1). Alternatively, wemay euthanise the animals with an overdose of anaesthetic or by an alternative regulated method.



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

Some animals we use will have been genetically altered (GA) so that some of their brain cells containa protein which will allow us to selectively identify and/or activate or inhibit those cells. These genetic modifications will not cause the animals any ill effects or suffering.

For some of the behavioural experiments, we might have to restrict animals access to food for a few hours (up to 16 h) before the test, but animals will be allowed a normal amount of food overall and theyare not expected to lose any weight. In some cases, we will inject the animals with a small amount of drug before behavioural testing. The dose, volume, and the way in which we give the drug will be chosen so that they would not be expected to cause any adverse effects apart from brief discomfort associated with the injection.

For collection of tissues, animals will be anaesthetised and will not suffer any adverse effects apart from brief discomfort associated with injection of the anaesthetic. Other animals will be euthanised byother regulated method and will lose consciousness instantaneously.

In the experiments which will be done with the animals under terminal anaesthesia, the animals will notsuffer any adverse effects apart from brief discomfort associated with injection of the anaesthetic.

Animals which have surgery for injection of tracers or viruses may experience some mild pain in the days after surgery but we will control this as far as possible by giving them a pain killer. For studies inwhich we are interested in hearing, we have to hold the head still

during the surgery by holding the animals cheeks to avoid damaging the ears. This may bruise the face and may make it difficult for theanimals to eat hard foods for the next few days. We will provide soft food until the animals are able toeat harder food comfortably.

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

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

Non-recovery: (50% rats, 20% mice)

- naïve genetically normal animals, terminal anaesthesia onlyMild: (20% rats, 50% mice)
- GA animals, terminal anaesthesia only

- GA or naïve genetically normal animals behavioural testing followed by terminal anaesthesiaModerate: (30% rats, 30% mice)
- GA and normal animals recovery surgery followed by behavioural testing and/or terminalanaesthesia

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

- Killed

Replacement

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

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

We need to use animals to achieve our aims because we are interested in the overall anatomy and thefunction of particular brain cells. The brain cells need to be intact and making their normal connectionswith other cells in the brain. They need to be alive and working normally for us to investigate how they work, how they are controlled, and how they affect other cells in the brain. We can only understand theroles that these nerves play in mood, movement, senses and behaviours in a whole animal. Ultimatelywe are interested in the human brain and so we need to use mammals which have similar brain structure and function to those of humans.

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

Cell cultureModelling

Why were they not suitable?

Cell culture may be suitable to answer some questions about nerve cell function. However there are many draw-backs of nerve cell cultures which mean that they would not be useful to answer our questions: i) normal nerve cells are very difficult to culture and can only be kept alive for short periods (days or weeks); ii) nerve cells in culture may not develop normally- in the brain, cells rely on signals from other cells to direct their growth and regulate their development, this cannot happen in culture; iii)brain cells in culture

cannot make the normal connections they would in the living brain and so cannot affect function in the normal way; iv) the overall effects of brain cells on movement, behaviour and complex functions cannot be expressed in cell culture.

Modelling: we can employ computer modelling to study how nerve cells might function in particular circumstances. However, in order to model what would happen in the normal brain, we need the detailed information about the brain cells and their function and connections which we can only get bystudying the brain cells in the brain.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 make measurements in relatively large numbers of individual brain cells in each animal. However it is important to be sure that any differences between in brain cells that we see are not specific to just one animal. To be certain of this, we will use 3-5 animals per measure. In many caseswe will be able to measure several different things in an individual animal.

With these factors in mind, we have estimated the numbers of animals to be used on the basis of the numbers of animals used under previous licences, the number of researchers working on this project,and the duration of the licence.

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

In designing the experiments we have reduced the number of animals needed to answer our questionsby making measurements from large numbers of brain cells in each individual animal. For example, when we measure proteins in different cells, we will take photographs of lots of cells and measure the amount of the protein in all of them; when we measure how brain cells work, we will use electrophysiological methods that allow us to measure tens or even hundreds of individual brain cells at once. As well as making measures in many cells at once, we can reduce the numbers of animals used by measuring several things in each animal. For example we can cut the brain into very thin slices and measure different proteins in different slices.

So that we know that differences between in brain cells that we see are not specific to just one animal,we will use 3-5 animals per measure.

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

We will breed only the numbers of GA mice and rats that we nee for our studies.

To optimise the number of animals to be used we will use brain tissue across several studies. Many ofthe studies will be carried out in brain tissue post mortem- this includes

both live tissue (used for studies of function), and fixed tissue (used for biochemical measures). In both cases, we will use the tissue to measure multiple outcomes. For example, in the case of live tissue, we will study brain slicescontaining several different brain regions from the same animal and/or record responses to several different drugs affecting function. In the case of fixed tissue, we will store the brain tissue in an antifreeze solution which allows it to be used for many months so that we have a 'bank' of tissue available for our studies which we can use to measure multiple genes or proteins in multiple brain regions of interest. In this way, small numbers of animals can contribute large amounts of data.

Where we make recordings of brain cell function both in live animals and in brain slices, we will measure multiple cells in a single animals. In some cases we will use single electrodes and measure activity in one nerve cell after another, but in some cases we will be able to use multielectrode probes(with up to 32 channels) or high density microelectrode arrays (MEAs) arrays (with up to 4096 channels) to examine tens, or even hundreds, of cells at the same time. In vitro recording will allow usto examine responses to several drugs in the same piece of tissue and in some cases in the same nerves. Coupled with computer analysis, this will maximise the data we get from individual animals.

Where appropriate and practical we will also be able to measure several behaviours (e.g. sleep,locomotion, food intake) in the same 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

Many of our experiments will be conducted in tissue collected post-mortem. These methods cause theleast suffering for the animals which will be terminally anaesthetised and never regain consciousness.The only pain or distress the animals will experience will be due to injection of anaesthetic. Pain and distress will be minimised by good practice and low stress handling techniques.

We will also make some recordings from the brain in anaesthetised animals. We need to do this in living animals because we need the connections between the brain cells and other cells to be intact.For these studies, the animals will be anaesthetised throughout and at the end will be killed by an overdose of anaesthetic without being allowed to regain consciousness. Recording in terminally anaesthetised animals ensures that they endure the least suffering. The only pain or distress the animals will experience will be due to injection of anaesthetic. Pain and distress will be minimised bygood practice, low stress handling techniques, and the choice of appropriate anaesthetics and analgesics. Refinements we have applied to these in vivo studies include the use of multielectrode probes to allow many cells to be measured at once.

For some studies we will need to inject a tracer or virus into the brain and must wait for

several days orweeks for the tracer to be transported or the virus to make the protein. This is the only way in which we can see the connections that brain cells make, or be able to cause the cells to make proteins whichallow us to activate or inhibit them. When the injections are made, the animals will be under anaesthesia. All procedures will be carried out using sterile instruments etc so that the risk of infectionis minimized. The animals will be kept warm during the operation and will be monitored continually to ensure the level of anaesthetic is optimal. At the end of the procedure the animals will be allowed to recover.

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

Ultimately we want our studies to be able to inform medical science and so we want to use animals which have similar brains to those of humans. Rats and mice have similar brains to humans. Less sentient beings such as fish and nematodes are not sufficiently similar to give us the information weneed.

In order to study the impact of the neuromodulator nerve cells on behaviour and other functions, weneed to have the whole brain intact and for the animal to be awake and able to respond to stimuli. However, many of our studies are in animals (or tissue from animals) which have been terminally anaesthetised.

In order to trace the connections between nerve cells and to be able to activate or inhibit particular nerve cells, we will inject viruses or tracers. The tracers/viruses take time to be transported along thenerve cells and the viruses take time to make proteins. For this reason, animals injected with tracersmust be kept alive for several days or weeks after the injection. They would not survive under anaesthetic for such a long period, so we must allow them to recover from the anaesthetic.

We are interested in how brain cells affect the functions and behaviours that are often affected in braindisorders (motor function, sleep, feeding, social behaviour, memory, complex tasks). In order to study these behaviour, we need to do experiments in animals which are capable of such behaviours. Whilefish, insects, and other invertebrates may express some of these behaviours, the fact that they do nothave the same brain structure and types of nerves as mammals like rats, mice, and humans, means that we cannot use these models to study the roles of nerves in these functions and behaviours.

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

During the 1, 3, and 5 year reviews of the licence I will update on implementation and consideration ofthe 3Rs that have occurred during the previous period. I will also review training plans and score sheets in collaboration with NACWO, NTCO and NVS team with a particular focus on refinements.

We have already refined our recovery surgery procedure to use a volatile anaesthetic delivered via a nasal cannula which means that the anaesthetic is easily adjusted and can be reversed very quickly ensuring rapid recovery. We use a specialised injector fitted with a fine glass tube to make our injections which minimises damage to the brain. We also administer analgesia as directed by the NVSto control peri-operative pain. We will monitor the animals' weight, wound healing, behaviour, and general appearance after the operation using a score sheet (attached) to ensure consistency of monitoring and actions.

Where drugs are to be administered in our behavioural studies in awake animals, we will take advice from NAWCO/NVS to ensure doses and volumes and routes of administration

are chosen to minimisepain and suffering.

We will continue to look for ways in which harms can be minimized by collaboration with colleagues,monitoring of the relevant literature, and online resources.

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

Alongside the guidelines listed below, I will adhere to local AWERB standards for research animals and, where appropriate, support the development of new local standards for refinements developedduring the term of this licence:

Code of practice for housing and care of animals bred, supplied or used for scientific purposesLASA Guidelines

RSPCA Animals in Science guidelinesUFAW Guidelines and publications NC3Rs and procedures with care

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

The local Animal Welfare and Ethical Review Body, Named Information Officer, Named Animal Careand Welfare Officers, Named Training and Competency Officer, and veterinary team regularly informand disseminate improvements and recent studies involving reduction, replacement and refinement. Recent examples include implementation of tunnel handling for mice, score sheets for post-operativerat well-being, and the development and recording of competency training for researchers.

We will make use of internal and external resources including collaborators, peers (including Researchgate), conferences, literature (including the Journal of Video experimentation (JoVE), andwebinars available on the NC3Rs website and other lab animal and animal welfare bodies.

In the process of the 1, 3 and 5 year reviews of this licence, I will update on implementation and consideration of the 3Rs taking into account any advances that have occurred during the previousperiod and will review and update where necessary the linked training plan, score sheets etc.

UNDERSTANDING GENETIC EYE DISEASE AND DEVELOPING 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, ortheir 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 aimsmentioned in paragraph (b)

Key words

eye, genes, therapy, genetic eye disease, blindness

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to help us understand how changes in genes cause eye defects and disease.By learning more about the disease mechanism we can then identify potential targets to develop a treatment which may help prevent or halt the disease process.

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

Why is it important to undertake this work?

Genetic eye disease affects 1 in 1000 people, it's the most common cause of blindness

amongst working age adults in the UK. These conditions are lifelong and have a significant impact on the individual and their families. For the majority of patients there are no treatments available, so this workwill advance our knowledge and help to develop potential therapies.

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

We will increase our knowledge of how defective genes cause eye disease in humans.We will hopefully lead to new potential therapies that we can translate to our patients.
It will lead to publications that will share information amongst our scientific and clinical community toadvance the care we give patients.

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

Short-term: Genetic eye diseases are rare, so quite commonly we may discover a new gene or geneticchange in a family but do not have enough evidence that it causes the disease (because the same change has not been found in any other families). To gain this evidence we need to disrupt a gene in amodel system to prove it causes the same clinical features, then a family can potentially get confirmation of their cause. Hence, patients will benefit in the short-term as it can support diagnoses, and aid with access to genetic counselling, family planning, and clinical trials/any treatments.

Over the course of the project and long-term clinical teams and scientists will benefit from these outputs as it will help advance the field of ophthalmology through improved diagnostics and futuretherapies.

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

We always strive to collaborate with other researchers to expedite the outputs to patients. We will disseminate our findings through presentations at patient charity meetings, scientific and medical conferences, publish in high quality peer-reviewed journals and where appropriate through public engagement events. We will also publish our unsuccessful results so that the community understandthe experiments and do not therefore need to duplicate the work or can modify to reach a more successful outcome.

Species and numbers of animals expected to be used

- Mice: 2500
- Zebra fish (Danio rerio): 20,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 zebrafish because they share 70% of their genes with humans, their eyes arevery similar to humans with colour vision and the same composition of tissues. This means that whenyou want to study a gene, it can be manipulated in the zebrafish and it shows similar clinical features.The conditions we are studying range from birth eye defects through to adult onset conditions where the light sensing layer of the eye

(the retina) degenerates and leads to blindness. Hence we will studythe zebrafish from birth through to 12-24 months of age. We can study disease mechanisms in the zebrafish and provide insights into human disease, and also screen drugs that may help prevent or treat the condition.

We will also use mouse to help us with testing therapies. The zebrafish eye is very small and we cannot deliver drugs or treatments in the same way as in our patients e.g. when we want to reach the human retina, we have to give an injection under this layer into the subretinal space, the mouse eye islarger and has a similar structure thus allowing us to deliver treatments as we do in humans.

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

When we work on zebrafish we will be modifying certain genes that contribute to eye formation andfunction. This is done by a small micro- injection into the zebrafish when it is only one single cell, before it has taken any shape or form. We then allow the fish to develop normally and monitor their eyes and overall health. We will study if the fish develops the same kind of disease as a human patientto allow us to understand the disease in more detail.

Once we have a zebrafish with an eye disorder of interest, we may test treatments on them to see if we can help make the condition better or eliminate it completely. This may be done by adding drugs to the water in which the fish are swimming or by a very small injection into the eye itself once the fish is an adult. We will monitor the fish to ensure they are responding positively to the treatment, overall healthy and not suffering in anyway. We may undertake investigations that allow us to examine the eyein detail such as scans which patients also have to monitor their eye health and structure, these are non-invasive and do not hurt the fish.

For mice, we plan to study mice with genetic changes, in a similar way to the fish, to better understandthe disease we see in our patients. We will purchase existing genetic models rather than create them, but will be able to undertake non-invasive imaging techniques similar to those used in humans, such as optical coherence tomography and fundus autofluorescence imaging. If we find a drug works well inour zebrafish, we will look to test this in our mice and this may be through an injection into the eye.

This is similar to how we dose patients and the injection is well established and practised procedure, very quick and causes little discomfort to the mouse. However we will ensure we monitor the mice giving them lubricants and antibiotic eye ointment to ensure we minimise any complications. We will observe these mice for up to 2 years in some cases, especially if we are testing a gene therapy and wewant to ensure that it is working long-term.

We will keep our experimental numbers to a minimum, in general we require 5-6 animals per timepointper experimental group in order to allow for statistical analysis i.e. to determine an effect. Over two years, this could be up to 50 animals per experimental group.

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

For most of our procedures we do not expect any adverse effects especially if undertaken at the single-cell stage of zebrafish development. But if a genetically modified zebrafish

grew up and developed physical features of the disease e.g. a bent back (also known as scoliosis) then we would stop raising these fish to avoid this outcome. Also, when we have to give an injection into the eye of a mouse, despite this being done under general anaesthesia, they may feel some discomfort or grittiness after waking up from the procedure. However, we do give the mouse lubricating drops throughout and apply a Vaseline-based antibiotic to help soothe and lubricate the eye, whilst also reducing the risk of any infection and prevents scratches as its greasy. This symptom would only last for 12-24 hours. We do not expect any abnormal behaviour, tumours or weight loss.

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

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

For both mice and zebrafish, the majority of animals will experience a mild severity (>50%). For zebrafish, we expect approximately 5% moderate and <5% non-recovery. For mice, we expect ~10%moderate severity and less than 2% non-recovery. The remaining animals will likely experience sub- threshold severity.

The overall workflow in this project involves breeding genetically altered fish, the majority of which are not expected to develop a harmful phenotype. None of the genetically altered mice we will be using areexpected to develop harmful phenotypes. Drugs, administered in concentrations not expected to causeadverse effects, will be administered to the water (for embryos) or directly into the eye under anaesthesia (for adults). Toxicity will be assessed by studying behaviour, including swimming, balanceand feeding. Non-invasive behavioural studies and imaging under anaesthesia will be performed to assess for response to treatment, and then fish will be humanely killed. If a harmful phenotype is seen,the animal will be humanely killed. Anaesthetics and/or analgesics will be used to minimise suffering. Animals will be humanely killed if they show adverse effects that are more than transient.

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 haveconsidered and why they cannot be used for this purpose.

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

We do our best to utilise cell models in our work to understand disease processes and test potential treatments. However, cells do not represent the whole eye and so the way tissues interact and influence disease cannot be studied fully and the ways we deliver treatments is not realistic, nor does itallow us to assess the impact on the whole eye and the rest of the body. Hence, we need to use animals as they allow us to parallel our findings to our patients.

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

We utilise cell models as an alternative e.g. we can take a skin sample from a patient and use this to study the disease and test drugs. We can convert these skin cells in to stem cells and then encouragethe cells to develop into parts of the eye such as cornea (front surface of the eye) cells or retina (the light sensitive layer at the back of the eye).

Why were they not suitable?

The human retina has multiple cell layers and support from an extensive blood vessel network, we cannot yet mimic this multi-layer structure with cells, so although we can get an idea if a drug works ora disease process, it is not realistic as it lacks the true anatomical structures and function that enable you to see. We need to use an eye that has proper visual function and structure for assessment of theinfluence of a defective gene on the whole eye and the safety of a drug, which may be translated to patients in the future.

Reduction

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

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

I have held a Home Office animal license for the past 10 years, and each year we report back the numbers used. Hence the numbers are based on our experience of the research undertaken and how many animals are required to achieve significant results. It is also based on considering future work and experiments, replacing animals where possible with cell model systems and undertaking statisticaltests to determine the correct numbers. It is worth noting that with zebrafish, they lay very large clutches of eggs, and so 100-200 may be used for drug dosing, but out of that only 25% may be a mutant fish. So numbers are larger for the zebrafish.

For therapeutic testing using novel drugs, small-scale pilot studies will be used first in wild-type zebrafish or mice. Experimental groups will be controls (untreated or treated with saline/vehicle used todissolve the drugs alone) and then other groups treated with different concentrations of drugs or different drugs. These groups are essential to be able to determine the therapeutic effects of any drugson phenotype. We will be guided by PREPARE guidelines for randomization and blinding. When possible, the investigator will be made unaware of the treatments performed on animals, or an investigator with no prior knowledge of the hypothesis or expected outcome will assist in data collection and analysis. We will use appropriate randomization methods like blocking and will take advantage of software like Graphpad.

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

I attended a course run by NC3Rs on experimental design. I have actively thought about how toreduce numbers by using the NC3R's Experimental Design Assistant and have based initial experiments on cell models, then the zebrafish model if required, and then only when absolutelyneeded to confirm safety and efficacy of a potential treatment, have we looked at mouse usage.

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

We will plan all experiments carefully and undertake a sample size calculation to ensure we have thecorrect n number of fish and mice at the relevant timepoints. We will also consider a pilot phase first e.g. we are keen to understand how our retinal cells of the eye respond to foreign DNA delivered as a gene therapy. We initially tested this on human cells and found there was response, but as this was a single cell layer we needed to do this on a live model with a multi-layered retina, so we injected 3 miceand then harvested the retina at 24 hours and identified key changes to highlight that the DNA was being degraded. So now we know that our cells are trying to block gene therapies, we can plan longerexperiments with the appropriate number of animals. If these pathways were not activated, we would not have proceeded any further, hence reducing the number of animals involved.

Efficient breeding of fish and mice is a further way we will optimise our animal numbers. All animals arekept in the state-of-the-art facilities with experienced staff to ensure optimal health for breeding, and wewill only maintain zebrafish breeding colonies that consistently produce healthy clutches of eggs.

In addition, we also always check open access databases for data that can be used for analysis, henceavoiding using animals. We believe in open science and as such always make our data available so other researchers can access this and hence avoids duplication. Furthermore, we are willing to share any animal tissues that may be of use to other lab groups or collaborators.

Refinement

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

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

We use the zebrafish model for the majority of our research, as these embryos develop ex utero andcan be manipulated genetically and are transparent, and thus ideal for imaging.

We will minimize suffering by paying attention to the fish population's general health, by paying attention to water quality, feeding regimes, and fish population density in each tank. We will check allbreeding stock daily and cull any that show signs of significant illness or deformity. Where surgical orother potentially distressing procedures are required, e.g. fin clipping, we will perform them under general anaesthesia with analgesia both pre and post fin clip, or where possible we will use a less invasive procedure e.g. skin swabbing. To generate genetically altered founder fish, we use gene editing techniques (e.g. CRISPR/Cas9), which are less harmful and more efficient than chemical mutagenesis. Any fish or fish larvae showing signs of distress on recovery from a surgical or other procedure will be killed promptly by an approved standard (schedule 1) method.

Only for validation of treatments that are effective in the zebrafish, we will consider further applicationin the mouse as this model allows delivery of therapeutics in a similar way to humans. Injections into the eye will be performed under general anaesthesia, lubricating drops will be applied and antibiotic ointment. This is a mild procedure and does not cause any discomfort on recovery.

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

We mostly use zebrafish embryos less than 5 days old for our research, which are deemed non- sentient and before the onset of independent feeding. To understand the later stages of retinal disease,we will have to work on juveniles and adults. In this case, we will mostly use non-invasive imaging and where possible, we will imply terminal anaesthesia and analgesics. For mice, we will use adults due tothe need for 1) fully developed retinas 2) suitable eye sizes for subretinal injection of drugs and 3) later timepoints to observe the development of retinal disease and/or long term efficacy of therapies.

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

We will achieve most of our objectives using non-invasive imaging techniques, which does not cause any pain or stress. Surgical procedures like fin clipping or injections into the eye will be performed withsuitable anaesthesia and animals will be monitored post-surgery to ensure that they recover well. We will also use suitable analgesia for all surgery. Any novel chemical substance will be tested in a small- scale pilot study for toxicity. We will standardize our imaging experiments on small-scale to first identifythe optimal condition for imaging to ensure minimum suffering.

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

We are aware of NC3Rs and their resources. The Norecopa website also has numerous informative resources, including the PREPARE guidelines which we will consult for experimental planning and implementing the 3Rs. In addition, the Laboratory Animal Science Association (LASA) have published several guiding principles that we can use, such as guidelines for preparing for and undertaking aseptic surgery. We also discuss with colleagues in other research groups new improvements that leadto refinement.

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

We will stay up to date via regular communication with the biological services at the institutions wherewe are based, other scientists in the field and regular visits to the following website https://www.nc3rs.org.uk/3rs-resources

UNDERSTANDING CARDIAC DEVELOPMENT AND DISEASE USING ZEBRAFISH

Project duration

5 years 0 months

Project purpose

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

Key words

Heart development, cardiomyopathy, aortic valve, congenital heart disease

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 notrequired.

Objectives and benefits

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

What's the aim of this project?

This project continues research that uses zebrafish to understand how genetic processes are involvedin the development of the heart and how disturbances of these can lead to structural defects (congenital heart disease) or diseases of the heart muscle cells (cardiomyopathy). We will be examining how variations in genes found in patients affect heart development and how environmental factors impact on these.

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

Why is it important to undertake this work?

Heart defects (congenital heart disease) affect almost 1% of births. Many of these are serious, requiring major surgery almost immediately after birth. Other heart abnormalities can affect specific types of cells such as muscle or specific structures such valves within the heart. These conditions affect a greater number of individuals, for example bicuspid

aortic valves are found in approximately 1in 40 adults. Although many will only have symptoms in later life, if left untreated, heart failure and sudden death are common. Again, these individuals require major surgery. Importantly, these abnormalities have their origins during the earliest stages of heart development, which occurs during pregnancy, long before symptoms appear.

All of these conditions are believed to have genetic causes, in which faulty genes disturb heartdevelopment. It is also possible these defects can be worsened by environmental factors.

We are continuing to learn much about how the heart develops using human and mouse. Heart development in zebrafish is very similar to that in humans and mice. In the last 25 years the zebrafishhas become a well-established, model to examine the early stages of heart development that are difficult to study in mice or human patients. We can also use the developmental similarities between humans and zebrafish to investigate how alterations found in the genes of patients impacts on heart development in an animal model.

By understanding how the heart normally develops, the effect of genetic changes found in patients andinteractions with environmental factors, we hope to help find new ways to help prevent and treat these conditions.

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

Short term out puts

Whilst overall appearances and studies carried out so far indicate that the zebrafish is a suitable modelfor human development and disease, these studies will provide immediate information by identifying details of the underlying processes by which the zebrafish heart develops, thus allowing comparison with what is known in humans and other models of heart development. This will be presented throughpublications and presentations at scientific meetings.

Medium term outputs

Building on the understanding of how the zebrafish heart develops, this project will then allow an examination of genetic variants found in patients. This will help understand the importance of differentgenetic variants and what non-genetic factors in the environment might be important. This informationwill be presented through publications and presentations at scientific meetings.

Long term outputs

Information may be obtained indicating how genetic pathways might be treated to prevent suchdisorders or reduce the long-term complications. This information will be presented through publications and presentations at scientific meetings.

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

Research community

These studies will provide immediate information for scientists and other specialists working on the heart, which cumulatively may lead to new ideas or disprove established

theories of heart developmentin the long term.
Industry

These studies will be important in advancing the overall understanding of genetic abnormalities and will provide basic information for others, for example, the pharmaceutical industry, about the relevanceof animal models to human disease and the biological relevance of genetic variants found in patients. Together with other research, this fundamental research may have an effect on the direction of productdevelopment in the long term.

Clinicians

As these studies examine genetic variants from human patients they will be of importance in the overall understanding of genetic sequencing information and the potential impact of particular variantson disease progression. This functional evaluation of variants is important in understanding their relevance when talking to patients. This research may provide initial information on how genetic pathways might be influenced to possibly prevent such disorders or reduce the long term complications.

Patients

Patients will ultimately benefit from the results of these studies through patient based scientific studiesand applications carried out by clinical scientists, and industry. In addition patients and the public will benefit from a greater understanding of why heart malformations happen, through dissemination of findings in media and to patient groups.

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

We will maximise the outputs of this work by integrating them with other human and mouse-based studies to ensure they are of human relevance and have impact. This will be through integration withfindings from other researchers and our own studies, that includes both mouse based developmentalstudies as well as NHS Research Ethic committee approved human studies which have completed patient recruitment and genetic sequencing phases.

Where possible we will share our data with other researchers in collaborative projects and theimplications of these findings will be explained and discussed in presentations at internationalmeetings, in scientific journal review articles and book chapters.

Importantly, we will publish studies that although have been correctly carried out, produced unexpectedor negative results.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 60,000

Predicted harms

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

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

The genes and developmental processes involved in zebrafish heart development are extremely similar to those in humans and almost all aspects of heart development that we are currently studyingare completed in early zebrafish embryos prior to the time when licence regulations come into action.

We need this licence to keep genetically modified adult fish that, although are clinically normal, areused to provide embryos for experiments.

Some healthy genetically modified fish may be humanely culled during adult life to understand how heart development progresses during later life. Others may have abnormalities of the heart that are notclinically apparent, but may be studied after the animal is humanely culled in order to understand if changes affect the heart at the earliest stages before there are any symptoms.

This will also allow reduction in the use of mice for studies in this area.

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

Genetically altered zebrafish that are otherwise healthy will be created on this licence, transferred from the previous licence or imported from other appropriate establishments. They will be maintained as eggproducing colonies and used to provide embryos for experiments at non-regulated stages of embryonic development by natural spawning. Most research into heart development is carried out in embryos atnon-regulated stages of development (protocol 2).

Breeding zebrafish will be ideally culled at approximately one year of age and no older than 18 monthsof age. On occasion, some of these healthy adult zebrafish may be humanely culled and then tissues used to study normal development of the heart. In some cases drugs may be provided in the water before death, for example to activate an inserted artificial gene or to establish levels of cell replication.

Sperm may be collected and frozen for storage. Eggs will be collected and used to check sperm arestill capable of producing healthy embryos after freezing or in order to regenerate the genetically modified line at a later time (protocol 1).

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

We do not expect any adverse effects on the zebrafish maintained on this project licence and from our20 year experience carrying out such experiments we expect their health and behaviour to be no different from normal laboratory zebrafish. The expected severity for the majority of animals used as breeding stock is mild, however around 5% will experience subthreshold as they will experience nothing beyond standard breeding practices.

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

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

As above - 5% subthreshold, 95% 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 haveconsidered and why they cannot be used for this purpose.

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

Normal heart development requires interaction of several different cell types and a flowing circulation.Whilst it is not possible to carry out experiments on human embryos, other animals with backbones, such as mice and zebrafish, which develop using similar genes and processes and can be used to investigate how the heart forms instead.

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

Human and mouse derived stem cells and cell culture of cells that line the surface of blood vessels.

Why were they not suitable?

The development of the heart involves the interaction of several different cell types and a flowing circulation. It is not possible to study our questions of heart development and disease in cultured cells.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 listed in this licence relate to the generation and maintenance of genetically altered zebrafish and are estimated from current experience. The vast majority of actual experiments to understand cardiac development and disease will be performed on embryonic forms prior to the stageof regulation by this licence and are informed by pilot studies and power calculations.

Over the course of this licence, we will analyse up to 20 genes of relevance to cardiac development and disease in detail. These will be genes that exist in zebrafish that are associated with human cardiovascular developmental abnormalities: either those that affect heart development or are associated with genetic heart disease. To do this we will need to generate and establish two geneticallyaltered zebrafish lines for each gene. One that completely removes the gene function (promoterless- null mutation) and another that reproduces the mutation found in human patients (knock-in mutation).
Thus, in total we will establish up to 40 new mutant lines.

We will then begin to uncover the molecular mechanisms by which these genes are required for normal heart development and function with the aid of transgenic reporter lines, in embryonic formsprior to regulated stages.

Protocol 1: Obtaining zebrafish gametes – up to 3000 animals required

We will preserve zebrafish lines with genetic modifications by freezing sperm. We will extract sperm from up to 15 healthy adult males between 8-12 months of age freeze them and immediately check thesperm can be defrosted to produce healthy embryos.

Genetically altered zebrafish for this project will be created in batches to make efficient use of researcher time and their immediate storage as frozen sperm will allow analysis of mutants one at a time later on. Over the course of the licence we will freeze sperm from up to two distinct generations for each of our possible 40 novel lines. Initially this will be after creation of the genetic alteration to ensure it is not lost and later once we are sure that any other inadvertent genetic alterations have beenremoved through repeated breeding and genotyping.

To revive a genetically altered zebrafish line, we use mixed frozen sperm from the different males andup to three different females.

Extraction of sperm from 40, novel mutant lines:

15 fish (males) x 40 (specific allele) x 2 (generations over 5yrs) = 1,200 Extraction of eggs for checking cryopreserved sperm quality of 40, novel mutant lines:
2-3 fish (females) x 40 (specific allele) x 2 (generations over 5yrs).
several samples will be checked at the same time reducing number of females required = 600Extraction of eggs for IVF of 40, novel mutant lines:
30 fish (females) x 40 (specific allele) = 1,200

Total animals required under Protocol 1: 3000

Protocol 2: Breeding and maintenance of genetically altered zebrafish – up to 56,400 animals required

Generation of the promoterless-null mutations and knock-in mutations use similar techniques. Specificchemicals are injected into fertilised eggs to create genetic alterations and approximately 120 zebrafishraised to adulthood. Promoterless-null mutations occur at a rate of 10-20% and knock-in mutations occur at a rate of 1-3%. At breeding age the fish are allowed to breed and the mutations sought in embryos prior to regulated stages. These parents will then be used to generate breeding lines which will be stored as frozen sperm. For analysis of our novel mutant lines, we will cross the genetically altered zebrafish with up to 6 different zebrafish reporter lines.

Genetically modified lines will be maintained as breeding colonies during analysis and until acceptanceof publications.

Generation and establishment of 40 novel, mutant lines, 18,000:

300 fish (120 @F0, 120 @F1, 60 @F2) x 40 (specific allele) = 12,000

Importantly, there may be other events during this stage of the licence that complicates the approach

e.g. failure of the process at a late stage. We therefore factor in contingency planning of a further 6,000animals, totalling 18,000.
Maintenance and use of reporter lines, 38,400:

50 fish x 12 (reporter/mutant lines) x 4 (generations over 5 yrs.) = 2, 400Analysis and maintenance of novel mutant lines:
50 fish x 6 (reporters for pipeline) x 3 (generations over 5 yrs.) x 40 (specific allele) = 36, 000

Total animals required under Protocol 2: 56,400

Protocol 3: Pharmacological treatments – up to 600 animals required.

Although almost all experiments examining heart development and disease will be carried out in embryonic forms at non-regulated stages, it is possible that some tissues will be harvested from zebrafish at adult stages. Application of drugs at regulated stages will be required in a small number ofgenetically altered fish lines to measure cell division or switch on an artificial gene . From previous studies we know that between 6-12 samples will be needed for each analysis.

Total animals required under Protocol 3: 600

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

There are very few experiments indicated within this licence as the majority are carried out onembryonic forms at stages prior to regulation.

In making genetically altered zebrafish we have previously sought out published methods and reagentsthat have the highest efficiency of producing genetically altered fish but that also minimise the steps that need to be carried out at regulated stages of life. We have experience of this process and know that whilst generation of 50 potentially genetically altered fish is sufficient to ensure success at efficiency of 10-20%, generation of approximately 100 potentially genetically modified fish might be required to ensure success at 2-3% efficiency levels. By carrying out production of several genetically altered lines at the same time we are able to guarantee a least some of the alterations will be successful at the first attempt allowing research work to continue and further genetic alterations to be repeated later on. We have used this pragmatic approach before in creating genetically altered zebrafish.

Where we need to use thymidine analogues to evaluate cell replication, we know that between 6-12 experimental animals and 6-12 controls are required. This is based on statistics already carried out andpresented in our published studies on cell replication in the zebrafish heart throughout life.

The numbers of animals used for sperm freezing are based on personal experience and the protocolwe use that was obtained from a major UK University zebrafish facility.

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

We employ optimal stocking density and high quality varied nutrition to ensure that the developing zebrafish have high survival rates and grow rapidly to sexual maturity. This

includes varied diet and water flow during development. This maximises numbers of viable eggs and allows us to complete experiments within fewer generations of adult fish. We ensure that females do not become blocked upwith eggs that have not been laid through regular pair mating and use of marble traps within tanks.

We have prior experience of almost all experiments carried out and also carry out pilot experiments todetermine power calculations for ones that are new.

Where possible we hold genetically altered zebrafish lines as frozen sperm, which greatly reducesexcess stock.

Refinement

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

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

The genetically modified fish we keep are healthy and indistinguishable from normal laboratory zebrafish. Thus they have no increase in pain, suffering or harm compared to normal fish. We ensurethat females are able to lay eggs by providing pair mating or marble traps in holding tanks approximately weekly once egg laying has begun.

Where zebrafish are housed singly or in low numbers, environmental enrichment is provided to reducestress.

The majority of experiments are carried out at immature life stages prior to regulation by this licence oron tissues obtained after humane culling of the zebrafish.

Cryopreservation of genetically altered zebrafish lines has been introduced and animals used to produce eggs and sperm for this are briefly anesthetised and only gently stroked to release the cells.

A new aquarium has been installed which provides high levels of cleanliness and biosecurity.

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

This project uses zebrafish as they are the least sentient vertebrates available for heart research. In addition, the majority of studies are carried out on immature life stages before the zebrafish embryosare capable of feeding or on tissues from mature zebrafish obtained after humane culling.

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

Although we have already minimised all welfare costs to the animals we take advice from

NACWO andthe veterinarian on improvements on husbandry and anaesthesia. All PIL holders also attend meetingswhere welfare is discussed.

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

We follow the advice on animals in scientific experiments provided by:

The UK Home OfficeThe European Union
RSPCA Animals in Science guidelinesUFAW Guidelines and Publications NC3R's and Procedures with Care
We also follow advice provided by the local Animal Welfare Ethical Review Board, Named InformationOfficer, Named Animal Care Welfare Officer, Named Training Competency Officer and Veterinary team

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

The local Animal Welfare Ethical Review Board (AWERB), Named Information Officer (NIO), NamedAnimal Care Welfare Officer (NACWO), Named Training Competency Officer (NTCO) and Veterinaryteam regularly inform, and disseminate improvements and recent studies involving reduction, replacement and refinement to the researchers in our institution.

We are also able to access external resources including, but not limited to, collaborators, peers,scientific conferences, and laboratory animal and animal welfare bodies.

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

TISSUE-ENGINEERED ELECTRODES FOR NEURAL IMPLANTS

Project duration

5 years 0 months

Project purpose

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

Key words

Neural implants, Bioelectronics, Biointegration, Neuroengineering, Brain-computer interfaces

Animal types	Life stages
Rats	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We will investigate the ability of electrodes coated with a soft gel-like material embedded with liveneural cells to form functional connections with the brain.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 implants (NIs) are devices that are surgically placed inside the body to interface directly with thenervous system. To achieve this, these devices rely on electrodes that record or stimulate the activity of neurons, which are electrically excitable cells that make up the brain and the nervous system. NIs have been used for the treatment of neurological disorders such as multiple sclerosis, epilepsy, Parkinson’s, and Alzheimer’s disease. They have also been used to drive neural prostheses that substitute motor, sensory, or cognitive

functions that have been affected by injury or disease. However,inflammatory responses triggered by the host immune system often induce the formation of a fibrotic scar around the implant, which increases the risk of device failure and the need for corrective surgeries. Given the increasing burden of neurological disorders driven by global population growth and ageing, there is a strong clinical need for strategies that can help circumvent this limitation to achieve longer-lasting neural intervention. Furthermore, as the potential applications of NIs continue to expand, this could not only bring about significant benefits to patient quality of life but also enable the widespread adoption of neurotechnologies beyond the clinic.

The stiffness mismatch between rigid electrodes and soft neural tissues constitutes one of the main contributing factors to the inflammatory and fibrotic responses that lead to the long-term failure of NIs. One of the most promising strategies to overcome this limitation is the use of soft and conformal electrode coatings composed of biologically compatible materials. Although several biomaterial systems have been developed, the ability to create a long-lasting and seamless interface between biological tissues and implantable devices remains challenging. In this project, we will explore a new approach to engineer this "bioelectronic interface", which is based on the use of electrode coatings composed of live neural cells embedded in soft polymer matrices called hydrogels. We anticipate that the cells in these "living electrodes" (LEs) will form functional connections with the host nervous systemto provide a more natural mode of tissue interfacing, while the soft and biocompatible hydrogel coating will minimise inflammatory and fibrotic responses after implantation. In turn, this approach will lead to safer and longer lasting NIs with improved clinical efficacy, which could be implemented in a broad range of current and emerging applications.

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

The overarching aim of this project is to increase our understanding on how tissues respond and interact with NIs and how these responses can be modulated to develop safer, more efficient, and longer lasting neurotechnologies. For this, we will engineer bioactive electrode coatings composed of live neural cells embedded in a soft hydrogel matrix that imitates the properties of neural tissues. This strategy will provide insight on how tissue engineering could aid the development of NIs that are better tolerated by the body and able to functionally integrate into the host tissues. In addition, we will incorporate this new knowledge into the design of tissue engineered NIs based on the LE concept, which will then be delivered to the animal subjects for preclinical evaluation. The successful completionof this project will generate actionable data that will inform the development of tailorable biomaterials for applications in neural engineering, bioactive coatings for tissue engineered implants, and bioelectronic interfaces that make use of natural mechanisms of synaptic neuromodulation. These findings will be published in relevant scientific journals and presented at national and international conferences.

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

In the short term, LE prototypes will be first produced to assess different aspects of biomaterial and device design and thus, the initial recipients of the data will be the researchers within our own group. Different LE constructs will be fabricated with varying formats to evaluate electrode performance and tostandardise the manufacture and implantation procedures. This approach will allow a thorough and systematic optimisation of the prototypes to produce the iterations that will be evaluated in the animal models.

In the medium term (< 5 years) we anticipate that this research strategy will yield fully

functional LEs that can be safely and efficiently delivered to the animal subjects. At this stage, the influence of different device parameters on the induction of chronic tissue responses that could hinder long-term device performance will be evaluated. This information will not only be used to further refine the designof the LE platform, but also to inform the development of other implantable neurotechnologies developed by our group. In addition, we will approach different industry and academic partners to explore the development of LE iterations with enhanced functionality or with different device formats fornew applications.

In the long-term (5 – 10 years), neurotechnologies will continue to increase our understanding of neural function and provide therapeutic solutions for a broad range of conditions brought about by trauma or disease. Moreover, as NIs become increasingly more sophisticated, safe, and available, their implementation beyond the clinic holds unprecedented potential to transform the way in which humans interact with machines and the outside world. However, realising the full potential of NIs is largely contingent upon whether long-term stability at the device-tissue interface can be achieved. Therefore, we anticipate that the successful development of the LE platform will be instrumental to the advancement of current and emerging neurotechnologies, and for the field of neuroengineering as a whole.

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

New knowledge generated throughout this project will be readily made available to the scientific community through publication in open-access journals and presentations at national and internationalconferences. Collaborations with industry and academic partners will be explored in the short and medium term. In addition, we will communicate our research activities and findings via our social mediaplatforms, and through outreach events held by our institution and scientific networks to maximise public engagement.

Species and numbers of animals expected to be used

- Rats: 180

Predicted harms

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

Explain why you are using these types of animals and your choice of life stages.
Rodents are the least sentient and most relevant species that could be used to evaluate immune and tissue responses elicited by devices implanted into the brain. In contrast to smaller rodents such as mice, the larger anatomical features from adult rats can greatly facilitate surgical procedures and handling. Adult rats weighing approximately 250 – 300 g have been widely used as models for electrode implantation into the brain and thus, they have been selected for this study. Moreover, there is an established body of literature on the use of rats to study tissue responses to penetrating neural probes, such as those being investigated in this project. Therefore, data from these previous works canbe used as reference for comparison and benchmarking. In addition, there is a wide range of commercial surgical products and devices that are specifically designed for rats, including penetrating electrodes, headstage units, wireless monitoring modules, electrical stimulators, and equipment for intraoperative monitoring.

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

The animals will be housed for at least 1 week after arrival to monitor their health status. During this time, all animals will be habituated to experimenter handling, and they will also be accustomed to the use of bespoke rodent backpacks for wireless interfacing, if needed. On the day of the procedure, a microelectrode array will be surgically implanted into the brain of the animals. A headstage unit will alsobe placed to seal the wound site and to allow access to the array throughout the study. The expected duration of the surgery is approximately 4 – 6 hours. After at least 3 days of post-operative recovery, recording or stimulation of brain activity will be performed via the headstage unit using either a tethered or a wireless connection. Recording may be performed for up to 2 hours per day for the entire study, with a maximum of 5 consecutive days per week. For the subset of animals that undergo electrical stimulation, this may be performed for up to 6 hours per day and for up to 20 days in total, with a maximum of 5 consecutive days per week. Animals may be singly housed temporarily during these sessions to prevent any issues that could compromise the welfare of the animals. Two study endpointswill be used to assess sub-chronic (4 weeks) and chronic (12 weeks) responses to the implants. At the allocated endpoints, animals will be humanely killed to retrieve the samples for analysis.

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

This project will involve the surgical implantation of a penetrating probe electrode into the brain of the animal. All surgical procedures will be performed under general anaesthesia. Analgesics will be administered via injection prior to the procedure and routinely during recovery to minimise pain and discomfort. We anticipate that any potential adverse effects associated with the surgery will be effectively mitigated using adequate surgical technique and proper postoperative monitoring and care.

After recovery, the electrode arrays will be used to record or stimulate electrical activity at the implant site. The threshold for electrical stimulation will be determined during implantation under general anaesthesia, and all subsequent stimulation sessions will be carried out below that level. Similar experiments carried out previously by our group have shown that stimulation at sub-threshold levels does not cause pain or distress to the animals. Animals are also likely to undergo acute weight loss immediately after surgery. Supportive measures will be provided to minimise post-operative weight lossfor all animals, but they generally resume weight gain 1 – 3 days after the surgery. At the end of the experiments, all animals will be humanely killed by an approved humane method and under general anaesthesia.

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

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

100% of the animals in this study will undergo an overall cumulative experience 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 haveconsidered and why they cannot be used for this purpose.

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

The main goal of this project is to evaluate the potential of bioactive coatings to enhance the biologicalintegration of NIs by minimising inflammation and establishing functional connections with the brain.

We have thoroughly assessed the ability of the biomaterials to support the development of isolated neural cells and neural tissue ingrowth under laboratory conditions (*in vitro*). Although extensive in vitrotesting has been carried out to optimise several aspects of biomaterial development, the study of complex physiological phenomena such as inflammation and fibrosis requires assessment in an animalmodel (*in vivo*). This is mainly because in vitro models cannot reproduce the multitude of interactions that occur between different cells and tissues in a living organism, which are key determinants of device efficacy and performance. In addition, implantation into an animal model will allow the study of long-term tissue responses, as well as any potential impacts on the surrounding tissues or in animal physiology.

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

We have performed extensive in vitro testing using isolated cell cultures to optimise the physical and biological properties of the biomaterials used in this project. Several biological assays have been conducted to assess the safety and the capacity of the hydrogels to support neural cell growth and development. We have investigated how neural cells interact with the hydrogels, and how scaffold properties and biochemical cues influence the ability of these cells to generate functional neural networks in vitro. We have also established models based on brain slices from humanely killed animalsthat are a few hundred microns thick and that can be maintained in culture for several weeks. This strategy allows us to generate multiple experimental samples from a single animal, and we have used these to study tissue-biomaterial interactions that occur over chronic time scales. Lastly, we have begun to fabricate device prototypes that will allow us to optimise the manufacture of the LE constructsand the methodology used for surgical implantation.

Why were they not suitable?

In vitro experiments have allowed us to rule out any biomaterial variants that could negatively impact the health of the animals, and to select a formulation with optimal biological performance. These experiments will also allow us to optimise the fabrication of the electrode constructs, and the methodology to deliver them safely and efficiently to the animals. However, there is currently no experimental model that can reproduce the complex series of events that take place after surgical implantation. Physiological processes such as inflammation and fibrosis involve extensive interplay between cells from the nervous system and the immune system, as well as intricate homeostatic mechanisms that are only present in a living organism. Therefore, animal implantation remains the onlyway to assess the chronic tissue and material responses that ultimately determine the longevity and the efficacy of NIs *in vivo*.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 is based on similar implantation studies carried out previously by our group and by others in the literature. This estimation considers the number of experiments, experimental groups, and animals per group that are needed to detect significant differences in the variables of interest, consistent with the aims of our study. Apart from the animals needed for the experiments to test our research hypothesis, this number also accounts for animals needed to carry out initial pilot experiments for the optimisation of the surgical workflow. In addition, wehave taken into consideration the sample size suggested by relevant international standards such as the ISO 10993-6, which specifies methods for the assessment of the local effects after implantation ofbiomaterials intended for use in medical devices.

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

We will strive to select the optimal experimental design to guarantee the scientific validity of our resultswhile using the minimum number of animals possible. We will also take steps to ensure that the experiments we perform are unbiased and adequately powered. For instance, the allocation of the animals receiving either the electrode controls or the LEs, as well as the order in which the surgeries will be performed will be randomised using a random number generator. Where possible, the processing of the samples retrieved for assessment will be blinded to reduce bias. In addition, data from the pilot experiments will be used to confirm the parameters for the design of the main study, suchas the effect size and the standard deviation for the primary outcomes. Lastly, we will use the NC3R's Experimental Design Assistant (EDA) to carry out and improve the design of our study, as well as the analysis of the data and the reporting of our findings.

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

Overall, we will aim to minimise error by ensuring adequate training of the staff and that all surgical equipment and infrastructure is in optimal conditions. Based on our experience, the initial pilot will provide valuable insight for the optimisation of every step of the experimental workflow and to identify any unforeseen contingencies prior to the main study. We will continue to work in parallel to develop more sophisticated in vitro models such as microfluidic organ-on-a-chip models, which are micro-scaledevices that reproduce some of the complex structures and functions of organs in vivo. We will also thoroughly characterise the engineered electrode constructs to ensure optimal performance prior to their assessment in vivo. Lastly, we will coordinate with other researchers to maximise the use of tissues and organs after the animals are humanely killed.

Refinement

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

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

Rats and mice are the most widely used models for intracortical electrode implantation since they share close resemblance to human biology. Adult rats are the preferred choice for this study becausetheir larger anatomical features will ease surgical procedures, which will minimise error and prevent unnecessary pain and suffering. Rats have also been selected due to their extensive use in the literature as models to assess tissue responses to NIs, which includes studies performed by our own group. This means that we not only have previous experience undertaking this type of work, but several peer-reviewed studies are available for benchmarking and refinement of our experimental approach. Following recovery, recording and stimulation regimes will be carried out on awake animalsvia the exposed head-mounted connector on the electrode array using either a tethered or a wireless connection. Tethering will be carried out using a cable attached to a swivel system that is anchored to the lid of the cage, with a length that allows free movement within the chamber. Alternatively, animals may be fitted with a custom rodent backpack containing a battery powered wireless module, which is connected to the array through a short cable. The selection of tethered or wireless set up will be madeto maximise animal welfare and comfort, and in consultation with the Named Persons at our establishment. Lastly, animals will be housed in stable pairs wherever possible. However, they may be temporarily housed individually during recording/stimulation in order to prevent any disruption that could compromise the welfare of the animals or the integrity of the implants.

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

Rodents are the simplest and most humane models that are compatible with our research aims. The use of less sentient or immature animals is not a viable option for this project due to the need for subjects with mammalian nervous and immune systems that are suitable for implantation studies overchronic timelines.

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

Overall, we will strive to follow the best possible practice for all experiments carried out in this project with a strong emphasis on those involving live animals. Prior to implantation, all animals will be habituated to experimenter handling, and those undergoing stimulation will be accustomed to the use of bespoke rodent backpacks containing a wireless module, if needed. Pain and discomfort will be minimised using proper aseptic technique, as well as post-operative monitoring and care involving an adequate analgesic regime. Animals will be routinely and carefully monitored after surgery and appropriate measures will be taken if any signs of pain or distress are observed. Moreover, to ensure the behavioural needs of the animals are met, rats will be housed in stable pairs where possible and institutional best practice will be followed with regards provision of environmental enrichment and other

husbandry needs. Our experimental approach will allow us to perform repeated measurements to maximise the amount of information extracted from each subject, while minimising the number of animals needed for the study. We will strive to select the most refined methodology to minimise animaldistress during the study. For instance, the choice of wireless vs tethered will be made by prioritising animal welfare and comfort. We will aim to minimise the footprint of the electronics used for tethered orwireless interfacing. We will also aim to optimise the duration and the number of sessions needed to achieve the desired biological response based on ongoing work from our group, the pilot surgeries from this study, and data from the literature. Animals may be singly housed temporarily during the sessions, if the presence of the cage mate leads to disruptions in animal welfare, and in consultation with the NVS and the NACWO.

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

We will follow all relevant guidelines available from the National Centre for the Replacement Refinement and Reduction of Animals in Research (NC3Rs), the Laboratory Animal Science Association (LASA), Planning Research and Experimental Procedures on Animals: Recommendationsfor Excellence (PREPARE), and Animal Research: Reporting of In Vivo Experiments (ARRIVE).

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

We will constantly revise our experimental strategy to reduce, refine, and replace the use of animals based on data produced by our own group and by staying up to date regarding current advancementsin the field. All staff involved in this license will attend relevant meetings held by our institution and other third parties related to recent advances in the 3Rs and their implementation. We will stay up to date with information and resources from external sources including the National Centre for Replacement Refinement and Reduction of Animals in Research resources (NC3Rs, https://www.nc3rs.org.uk/), as well as the 3Rs group at our establishment. We will also maintain closecommunication with all named persons to ensure that all possible measures are in place to preserve the welfare of our animals.

THE ROLE OF SEX CHROMOSOMES IN DEVELOPMENT AND DISEASE

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Genetics, Sex chromosomes, Germ cells, Developmental biology

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Men and women are genetically the same, with the exception of their sex chromosomes (men XY, women XX). Sex chromosomes have a special role in the formation of eggs and sperm, sex chromosomes are the most common known genetic cause of infertitity. Using mice, and the marsupialopossum, we aim to unravel how sex chromosome abnormalities arise, and how they cause infertility.Finally, we aim to design a system for creating litters that contain only males or females.

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

Why is it important to undertake this work?

Sex chromosome abnormalities are the most common known genetic cause of infertility in humans, buttreatments are lacking. By unravelling how sex chromosome abnormalities arise, and how they disturb the formation of germ cells, our discoveries could ultimately lead to reproductive therapies for these patients. Our findings could also identify specific genes on the sex chromosomes, and epigenetic processes affecting the sex

chromosomes, e.g. X-inactivation, that are responsible for diseases affecting men and women with different severity, e.g. heart disease and cancer. By understanding what pregnancy conditions are beneficial to the two sexes, we could improve the IVF culture conditions usedfor fertility treatments to maximize outcomes of male and female pregnancies. By understanding how male and female embryos differentially respond to suboptimal nutrient availability during pregnancy, wecould improve our understanding of sex-differences in pregnancy failure and optimize culture conditions for IVF that favour both sexes. Our efforts to design a system for generating single-sex litters could have a huge impact on research and agriculture. For examples, many research topics necessitate a specific sex, e.g. oogenesis, spermatogenesis, placental biology, sex-specific cancers, Y-chromosome biology and X-inactivation. In agriculture, the dairy industry requires females, and so each year 95,000 male calves are culled in the UK. In the egg-laying industry, which requires females, 3 - 4 billion male chicks are culled each year. By designing a system for generating single-sex litters, we could reduce the production and culling of animals of the unrequired sex. The ethical and economicbenefits of this technology could be considerable.

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

The main aims of our work are to understand how sex chromosome abnormalities arise, how they cause disease, and whether we can design systems for generating all-male and all-female litters. Themain project outputs will be new knowledge and peer-reviewed publications.

Expected outputs include:

understanding how errors in meiosis cause chromosome abnormalities, including those affecting thesex chromosomes (e.g., XO, XXY and XYY syndromes) how sex chromosome abnormalities cause infertility, and stem-cell based approaches to overcomethis infertility

how sex chromosome genes, and X-inactivation, influence sex differences in disease susceptibility

how sex chromosome composition of the embryo affects its predisposition to suboptimal maternalenvironment in early pregnancy, how this biases pregnancy outcomes and sex ratio at birth.

designing genetic systems for generation of all-male and all-female litters.

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

Our work on the origins and fertility-effects of sex chromosome abnormalities will benefit multiple stakeholders. They will be useful to researchers studying chromosome biology and the origins of aneuploidy (abnormal number of chromosomes in a cell), and those working on germline development and the genetic basis of infertility. Our stem cell-based approaches to overcome sex chromosome- related infertility will be of great interest to reproductive clinicians, and to patients with these conditions.A greater understanding of how male and female embryos respond to challenges, including suboptimalnutrient availability, will help to improve IVF embryo culture conditions. This could be of particular benefit to patients who are affected by sex-chromosome linked diseases (e.g., muscular dystrophy or haemophilia) who frequently rely on the disease-free female embryos only to achieve a pregnancy. In addition, since the sex chromosomes are the only chromosome pair that differs between men and women, their potential contribution to the abundant sex

differences in somatic diseases is considerable. Much focus has been placed on the Y chromosome as being a predisposing factor for male-biased diseases, and defects in X-inactivation a predisposing factors for female-biased diseases.By dissecting the functions of individual Y-genes, and the epigenetics of X-inactivation, we can providepotential mechanistic insight into such biases. Our genetic systems to generate single-sex mouse litters will in the short-term be useful to researchers who study topics that require a specific sex, because it will allow them to no longer produce and cull animals of the unwanted sex. More long term, our technologies could be more widely deployed to facilitate the generation of chickens, cows and pigsof a specific sex, and thus would benefit the international agricultural community.

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

We will disseminate unpublished work via preprint servers, to ensure immediate and free availability ofour findings internationally. Subsequently, our findings will be published in peer-review journals under Open Access. We will also regularly present our work at conferences and workshops, and at advocacygroups for patients with sex chromosome abnormalities. We will continue to regularly engage with media outlets on our work, thereby conveying our research findings to the public, as we did recently forour single-sex mouse litter study. We have a very strong focus on collaboration, and make all of our engineered stem cells and mouse lines available at our earliest convenience. This way, we will collaborate extensively nationally and internationally.

Species and numbers of animals expected to be used

- Mice: 32725
- : 1575

Predicted harms

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

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

For our experiments we use mice and a marsupial, the opossum. Mice are an excellent model system because their genetic make-up is similar to that of humans, and because egg and sperm formation in mice occurs in a manner similar to that in humans. In addition, they are useful because they can be genetically modified, which allows to identify precise mechanisms underlying the diseases we study. Opossums diverged from humans and mice about 160 million years ago in evolution. This makes thema really powerful model system, because mechanisms that are common, and thus have maintained over many years of evolution, are likely to be especially important for our understanding the diseases in which we are interested. For our experiments, we use tissue derived from both adults and embryos. We must use embryos because many of the processes we study initiate during embryonic development.

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

Most of our regulated procedures are focused on mice. We have a strong focus on germ cells and infertility. For this reason, we use genetic modifications that primarily affect developing eggs and sperm. In these instances, the only effect on the mice is infertility. We

typically harvest tissue, includingovaries and testes, post-mortem. Our genetically altered mice are maintained by breeding, of which wecarry out several hundred each year. In addition, we sometimes perform minor interventions such as injections, which we use to increase the number of eggs produced by female mice, or to induce a genetic modification in a specific tissue. The effects on the animal are usually minimal. In some cases, female animals will maintained on different dietary regimens for short periods of time (4-5 days a month) to establish how this affects the development of male and female embryos before they implant.Occasionally, we perform surgical procedures. This may involve implanting of newly-generated genetically modified mouse embryos into a recipient female's uterus, or removing one testis from a mouse, which allows us to assess sperm production without having to cull the animal. When surgery isrequired, we monitor animals closely, and pay close attention to anaesthetics, pain killers and other interventions to ensure minimal adverse effects. In all cases, animals will be humanely killed if there are signs of pain, distress or suffering above agreed limits. Sometimes, we may need to subject mice to a brief exposure of irradiation to remove their germ cells. We do this because we are interested in identifying the genes that control germ cell loss and infertility.

Making genetic modifications in opossums has not been widely performed. For this reason, the vast majority of our opossum experiments involve breeding opossums that are not genetically modified, andharvesting tissue and embryos postmortem. We are nevertheless attempting to set up genetic modification in the opossum, focusing on embryos. Although performed infrequently, this procedure involves surgery, for example to transplant genetically modified embryos into a recipient female's uterus. The effects on the opossum are usually minimal, but as described above for the mouse, we carry out close monitoring, provide ameliorating interventions where needed, and humanely kill theanimal if it exhibits signs of pain, distress or suffering above agreed limits.

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

Most of our experiments focus on germ cells, and therefore the effects we observe in genetically modified animals are largely restricted to the gonad. This may cause reduction in the number of eggs and sperm, and infertility, but other tissues are unaffected, so in the vast majority of experiments, no other adverse effects are observed. Rarely, a genetic modification that affects egg or sperm productionmay cause effects in other tissues, leading for example to increased cancer predisposition at older age. In most cases, we humanely kill and study tissues from these animals before they reach the age at which these other effects arise. Female animals fed for short periods of time (4-5 days/month) on modified diets may experience weight gain/loss, however the weight on similar regimens was shown toreturn to baseline shortly thereafter. In instances where we need to use surgery, we use appropriate anaesthesia and analgesia, and full recovery usually occurs within one or two weeks.

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

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

We expect a mild severity level of 10%, a moderate severity level of 10%, and the remaining 80% of animals to be classified as subthreshold severity (in the majority of cases, experiments will be performed on material obtained from animals post-mortem) Based on our experience, neonatal deathoccurs at a similar frequency (~2%) between GA

and wild type 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 haveconsidered and why they cannot be used for this purpose.

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

Most of our studies are focused on reproductive biology. We need to use mice and opossums becauseegg and sperm production in these organisms occurs in a similar manner to humans, making them excellent model systems. For mice, it is currently not possible to make sperm in a dish, and generating eggs in a dish is extremely complex and highly inefficient. This is probably because in order to form properly, germ cells require two-way interactions with other cell types in the gonad, as well as changinglevels of hormones provided via the bloodstream. For opossums, generation of sperm and eggs in a dish, or generation pluripotent stem cells is not possible. We therefore must study germ cell development within the context of a living model organism.

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

Tissue culture and organoid systems, which have been developed as models for other tissues, are notcurrently available for germ cells. Generation of testes, sperm and ovaries in a dish is not possible.

One group has reported successful generation of eggs from stem cells in a dish, but this is complex and highly inefficient, and requires co-culture with ovarian supporting cells, which themselves have to be harvested from female mice. Furthemore, other commonly used model organisms .e.g. fish or flies,cannot be used, because their sex chromosomes have a completey different gene content to the mammalian sex chromosomes. For this reason, there is no current alternative to animals available.

Why were they not suitable?

There are currently no in vitro systems for generating sperm in a dish, and production of eggs in a dishis very inefficient, and requires provision of ovarian supporting cells from female mice. Additionally, studying the impact of maternal environment on early development of embryos of either sex requires amodel where key gestational events occur under conditions similar to human development (early embryogenesis in the oviduct/uterus, followed by implantation and placenta development), and thus invertebrates or non-placental mammals cannot be used to address these questions.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 our mouse experiments, the numbers are based on how many strains we require to perform our experiments, and the minimum number of matings we need to generate GA animals, which derives from Mendelian frequencies. We also consider the breeding performance of particular GA lines, which can sometimes be lower than in strains without fertility-affecting mutations. We survey the literature forbest practice in terms of the numbers of GA animals required to reliably assess a given phenotype. Forour opossum work, our numbers are based on the minimum number required to maintain our modest colony size (n ~ 120), to provide embryos for analysis, and to allow us one attempt at genetic modification per month.

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

We implement a number of approaches to reduce animal numbers. This begins with experimentaldesign. Genes that we think are important for the processes we're studying are chosen based on published literature and data generated both by us and by other scientists. This vastly reduces thenumber of "false-leads". Genetically altered animals are only created if they are not available from existing sources. If this is the case, the genetically altered animals are created in-house by highly trained personnel, and are usually maintained as small colonies. We plan our experiments so that eachanimal provides the maximum amount of material for analysis, and that tissue harvested post-mortem from a single animal can be stored and repeatedly reused in different experiments. This approach, together with statistical approaches, means we use few animals to address a specific scientific question.

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

We are extremely efficient with our breeding program. Our lab has a designated lab member who has >20 years experience in mouse genetics and mouse maintenance who closely monitors breeding performance, and as such can reduce the numbers of many maintenance breeding cages to two per strain. As soon as lines are no longer needed, live animals of that genetic background are no longer kept and instead embryos are frozen down. This allows us to reconstitute via frozen embryo transfer ananimal line if needed at a further timepoint, without the need of breeding animals in the interim. We acquire commonly used strains from the institute's core animal facility rather than generating them ourselves. In instances where no harmful phenotypes are observed, we combine alleles in breeding lines so that we minimise the number of total animals required. We use expertise available within our institute and from collaborators to estimate the numbers of animals used per experiment. A particular achievement in our approach to reducing animals numbers relates to our opossum work. We have around 15 external collaborating groups that work on different organ systems and require opossum material, but our colony is only around 60 males and 60 females. We therefore carefully plan and coordinate to make sure that on culling, one opossum is used to provide material for all interested parties simultaneously.

Refinement

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

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

We use mice and opossums for our research. The opossum and mouse sex chromosome share similarity in sequence content to the human sex chromosomes, but other non-mammalian organisms do not, or they do not have sex chromosome at all, making them uninformative for our studies. The physiology of germ cell development and X-inactivation in mice and laboratory opossums is also similar to that in humans, making them a powerful comparative system. Mice are very useful because they are the most tractable mammal with respect to genetic manipulation. Many of our methods requiregenerating genetically altered animals, which are necessary to understand precisely what genes perform what functions in reproduction and disease susceptibility. To study these alterations in vivo, we must utilize protocols like: superovulation (injection of drugs similar to those used in IVF to obtain more embryos per mice, and hence use fewer animals for our experiments), embryo transfer (allowing an embryo produced from gametes of one female to develop in the body of a foster mother), or modification of the male germline, which in turn require surgery. Similarly, if we wish to study the impact of maternal diet on embryo survival, we must adjust the maternal diet accordingly. Most of our experiments are performed post-mortem, so there is no lasting harm or distress. For methods that are non-surgical, e.g., superovulation or irradiation, there is only transient discomfort and no long-lasting distress. In instances where surgery is required, we use anaesthesia with analgesia pre and post operatively to minimise suffering, killing promptly by an approved method in rare occasions when animals show signs of distress. We also monitor our animals daily to ensure that they are not suffering.We only proceed to in vivo work when preceding in silico and in vitro studies convince us of the validityof our approach.

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

Our projects aim to address how sex chromosome abnormalities contribute to infertility and suboptimalhealth. These questions can only be addressed in animals that show a similar sex chromosome biology and mechanism of sex chromosome dosage compensation between males and females.
Additionally, to study the impact of embryo’s sex on vulnerability to suboptimal conditions during pre-implantation development, we need to study placental mammals that show a similar pre-implantationdevelopmental trajectory.

Wherever possible, we use early life stages. For instance, in our irradiation experiments mice at post- natal day 5 are used. To assess the impact of maternal nutrition on embryonic development, we assessmale and female embryos before they implant, instead of waiting for the pups being born. Additionally, we complement our studies with tissue culture work and in vitro models of embryogenesis (e.g. gastruloids). We only proceed to in vivo work when preceding in silico and in vitro studies convince us of the validity of our approach.

We use mice and opossums for our research. The opossum and mouse sex chromosome share similarity in sequence content to the human sex chromosomes, and the physiology

of germ cell development and X-inactivation in mice and laboratory opossums is also like humans, making them a powerful comparative system. Mice are very useful because they are the most tractable mammal with respect to genetic manipulation. Most of our experiments are performed post-mortem, so there is no lasting harm or distress. For methods that are non-surgical, e.g., superovulation or irradiation, there is only transient discomfort and no long-lasting distress. In instances where surgery is required, we use anaesthesia with analgesia pre and post operatively to minimise suffering, killing promptly by an approved method in rare occasions when animals show signs of distress. We also monitor our animalsdaily to ensure that they are not suffering. We only proceed to in vivo work when preceding in silico and in vitro studies convince us of the validity of our approach.

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

We are acutely aware of minimising adverse effects. Where possible we use the most experienced personnel to perform procedures, and best practice to perform these procedures, gleaned from our own experience or that of others within or outside the institute. We use asepsis during surgery, we administer analgesia and anaesthesia carefully and we monitor animals during and after surgery for pain or distress. Any animal exhibiting unexpected harmful effects will be killed humanely. Only 10% ofour animals are expected to exhibit a moderate severity, 10% mild severity, and the remaining 80% subthreshold. In instances where it is not possible to fully predict the severity for all experiments, we will monitor animals closely and liaise with animal care staff, the Named Veterinary Surgeon, and the Home Office Inspector as required.

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

We will refer to the NC3Rs and to colleagues within and outside the Institute to ensure that we followbest practice.

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

We use the NC3Rs website to keep us up to date on these advances, as well as constant liaison withanimal facility and collaborators on best practice. Through our experiments on generating single-sex litters, we also communicate constantly with stakeholders in the research, agricultural and ethics arenas, and engage in public discourse on minimising animal numbers. We also keep up to date withthe research published in our field.

THE USE OF TISSUE ENGINEERING FOR RECONSTRUCTION AND REGENERATION IN CONGENITALHEART DEFECTS

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir 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 aimsmentioned in paragraph (b)

Key words

stem cells, tissue engineering, congenital heart defect, reconstructive surgery

Animal types	Life stages
Pigs	juvenile, neonate

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To assess the feasibility, safety and efficacy of novel tissue engineered constructs, such as valves, conduits, grafts and patches and to determine how they perform in response to the changes that occurduring growth.

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

Why is it important to undertake this work?

Around 1% of babies are born with congenital cardiovascular defects that require

corrective surgery involving the implantation of valves, stents or grafts. A high proportion of these patients will require further surgery during childhood and adolescence to replace the implants with larger ones to accommodate their growth and development. These repeat procedures are distressing for the child and their families, pose a significant risk to health and are highly costly to health care providers, such as the NHS. The development of replacement implants that grow with the patient's own body would offer huge benefits for the treatment of such defects by 1) the avoiding or reducing the need for patients to undergo repeat procedures, 2) reducing the overall health risk associated with treatment, 3)reducing the risk of complications such as tissue rejection, infection or graft failure, 4) reducing the costof treatment for health care providers.

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

This project aims to contribute to the development of surgical implants, for corrective cardiovascular surgery, that provide either a life-long or extended-life over those currently available. The study will generate data on the performance of novel implant being developed to grow with the patient, using arepresentative animal model, and thereby advance the progression of technological developments inthis field towards first in human clinical trials.

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

The primary beneficiary of the work undertaken will be scientists working either in academia or for companies specialising in soft tissue implants. In the medium term, the work is expected to lead to thedevelopment of life-long or longer lasting cardiovascular implants and to provide the data needed to facilitate their progression towards first in human clinical trials. In the long term, it is to be expected thatpatients will reap the benefit of the work as the need for repeat surgical procedures, together with the associated risks, will either be avoided or reduced, leading to an overall improvement in quality of life. In addition, health care providers, such as the NHS, will benefit from the overall reduction in the cost incurred in the treatment of congenital cardiovascular defects.

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

The work undertaken will be conducted in collaboration with scientists in both academia and industry inorder to achieve the long-term goal of developing vascular implant that fully integrate with the patient's tissue and grow in response to their development, as normal tissue would. The centre where the work will be undertaken is equipped with state of the art imaging facilities, that will ensure optimum data capture throughout the study in addition to the histological data obtained at the study end. The data generated will be presented at national and international meetings and form a fundamental part for MHRA application for the clinical upgrade of tissue- engineered products. The long-term benefits will be the translation of these models into the clinical setting for use in reconstructive congenital heart surgery.

Species and numbers of animals expected to be used

- Pigs: 240

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures,

includingduration of the experiment and number of procedures.

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

The outlined work will be conducted using pigs. Pigs have been selected for this work because they can be obtained at a size when their cardiovascular system is of a comparable size to that of human babies and therefore able to accept implants intended for use in babies. In addition, pigs grow to the size of an adult human within a relatively short period, thereby enabling the response of the implant to the animal's development to be assessed. The cardiovascular system of pigs shares a close anatomical and physiological similarity with that of humans and consequently this species has been used extensively for assessing cardiovascular interventions prior to first in human trials. In addition, models mimicking the surgical reconstruction of common cardiovascular defects are well established inthe pig.

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

Upon arrival in the unit, pigs will be group housed in environmentally enriched pens and allowed to acclimatise for a minimum of five days. During this period, they will be habituated to human contact byenticing them to take food treats from the hand. The pigs will be trained to voluntarily enter the weigh crate and transport trollies used when moving them to the surgical suite. Following the acclimatization period, pigs will undergo one or more procedures, all of which will be performed following the inductionand maintenance of general anaesthesia. The procedures undertaken may include 1) non-invasive imaging (e.g. ultrasound, angiogram, MRI), 2) the collection of small tissue samples from the heart, 3)the collection of a blood sample and 4) the surgical implantation of a tissue engineered construct into the cardiovascular system, including by open heart surgery. Following all procedures, pain controlling drugs will be given and maintained until no overt signs of pain are detectable. Pigs allowed to recover from the anaesthetic may be maintained for up to 9 months during which period they may undergo non-invasive imaging on a further five occasions to assess/monitored cardiovascular function. At the end of the experiment, the pigs will be killed by an overdose of anaesthesia and relevant tissues harvested for subsequent analysis.

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

The impact of the procedures undertaken on the wellbeing of the animals will vary in accordance with their severity. Animals undergoing non-invasive imaging, following the induction of anaesthesia, are expected to recover uneventfully and to resume normal behaviours shortly thereafter. Pigs undergoingtissue sampling are also expected to recover uneventfully and to resume normal behaviour within a couple of hours. Pigs undergoing graft implantation will require intensive care upon recovery and are expected to take several hours before they recover sufficiently to enable them to be returned to their home pen. Animals from which tissue samples have been collected or which have undergone surgery will be routinely provided with pain relief, which will be maintained until no overt signs of pain are detectable. Animals that have undergone surgery may lose a little weight over the first couple of days but are expected to regain this within the first week and thereafter to continue to thrive. Throughout thestudy all animals will be carefully monitored by experienced staff and prompt and appropriate action taken in the event that any adverse signs occur. At the end of the study, all animals will be humanely killed, following the induction of anaesthesia, to enable tissues to be collected for analysis.

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

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

The expected severity for the procedures undertaken is moderate, 90% of animals are expected toexperience this severity. The remaining animals will experience only mild transient distress during anaesthetic induction and will have a severity classification of mild.

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

- Killed

Replacement

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

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

Determining the safety and effectiveness of devices prior to clinical use requires testing in a relevant live animal model. The conduits being tested are ultimately intended for use in humans, consequentlythey can only be effectively tested in an animal model with a comparable cardiovascular anatomy, physiology and size to the human patient for which they are intended. There are no non-protected species that can meet these criteria.

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

Non-animal alternatives are not applicable to this study.

Why were they not suitable?

It is not possible to assess the integration, safety, efficacy and response to growth of tissue engineeredconstructs using either *in vitro or ex-vivo* systems or models based on non-protected animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 selected device performance as the primary outcome and marker of success, as this is the criteria against which it will be assessed in clinical practice. The experimental design will mirror that ofprevious studies which have shown that a group size of 12 animals is needed to achieve statistically significant result. Wherever possible, data obtained from

control group animals used in previous studies will be used to minimise animal usage. We expect to undertake studies on 4 groups of animals/year, during the course of the study, and therefore estimate the total animal usage as 240 animals.

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

Wherever possible, implants will be generated using autologous cells, thus avoiding the need for donoranimals, reducing the risk of graft rejection and thereby minimising the number of animals needed.
Wherever possible, data obtained from control group animals, used in previous studies, will be used tominimise animal usage.

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

Where the effect size is unclear, a stepped approach will be used involving pilot studies to establish thenecessary group size required to obtain statistical significance. Wherever possible, implants will be generated using autologous cells, thus avoiding the need for donor animals, reducing the risk of rejection, and minimising the number of animals 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

The pig has been chosen for this work because their anatomy and physiology closely replicate that of humans and they can be obtained at a size comparable to that of babies and infants, thus enabling thecardiovascular surgical procedures performed to correct congenital defects in babies and infants to be closely replicated. Furthermore, the rapid growth of the pig enables the response of the graft to the developmental changes that occur to be assessed within a relatively short time scale. Following surgery, the pigs will be given pain control, comparable to that given to babies and infants, which will be maintained until the animal shows no detectable signs of pain. Following surgery, the pigs are expected to resume normal behaviour within a day and thereafter to continue to thrive and grow normally.

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

It is not possible to conduct this study in less sentient species as it requires animals with a cardiovascular system comparable to human babies and infants in anatomical structure and size andthese criteria cannot be met.

How will you refine the procedures you're using to minimise the welfare costs

(harms) for theanimals?

The stress to animals will be minimised by habituating them to human handling prior to the procedure,the provision of an enriched environment and group housing. Throughout the procedure, the animals will receive appropriate levels of anaesthesia and analgesia, which will be delivered by a team of specialist veterinary anaesthetists and nurses who will carefully monitor the physiological status and vital signs of the animal. Our protocols for post-operative recovery have been optimised in line with techniques used routinely within the NHS. The experimental models used have been further optimisedunder our previous licence to cause the minimum injury consistent with achieving the scientific outcome. The team has significant experience in dealing with post-surgical recovery, and has full access to veterinary care and advice at all times. Advanced non-invasive imaging techniques (e.g. echocardiography, MRI) will be used to assess and monitor the performance of the conduit or patch invivo at predetermined timepoints, which ensures that in the event that an implant start to fail or be rejected that appropriate interventions can be enacted at an early time point.

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

All procedures will be conducted in accordance with LASA, NC3Rs Guidelines on best practice forsurgery and the administration of substance.

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

I attend the University's annual 3Rs events and receive regular updates from our NC3Rs representative and NIO.

EARLY HUMAN DEVELOPMENT AND THE GERMLINE

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Primordial germ cells, Totipotency, Pluripotency, Epigenetic reprogramming, Stem cells

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

Retrospective assessment

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

Objectives and benefits

Description 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 early human development and to determine how mouse and human cells develop into sperm or eggs. The interaction between these cells and cells of the developing ovariesand testis is critical for developing viable sperm and eggs.

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

Why is it important to undertake this work?

Much work on cells forming sperm or eggs has been done on mice. Using the knowledge from past work on animals allows us to attempt similar human studies, which is essential because we know that some crucial differences exist between mouse and human development. Our focus will be on cells thatappear in very early embryos that eventually develop into human eggs or sperm. For some studies, weuse human stem cells, which can potentially be used to make sperm or eggs in a dish, including fetal ovaries and testis, which might allow us eventually to make human eggs and sperm in a culture dish.

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

Sperm and eggs transmit information to fertilised eggs that direct their development to adulthood. Theprecise nature of the information, genetic and non-genetic, remains to be fully elucidated. The nature of the information has long-term consequences in subsequent generations for human health and disease. Since the initial events occur in very early human embryos, we might also acquire knowledgeof early human development. Advances in expertise from our research will also be informative for research on other areas of stem cell research.

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

The knowledge we acquire from our research will inform us of some causes of infertility, as well as about the cancers that develop from the cells that usually develop into sperm or eggs. We may also understand the causes of the current trend in the decline of human fertility and the potential development of approaches to arrest or reverse this trend. Environmental factors and pollutants also affect human fertility but how this occurs is unclear. The environmental factors can also induce non- genetic changes in cells that might be transmitted to subsequent generations with an impact on humanhealth, including mental health.

The information transmitted through sperm and eggs has also been implicated with a role in the evolution of organisms. The research might elucidate the nature of the information that may have beenimportant in, for example, the development of the human brain, which differs from that of chimpanzees,our closest non-human primate relatives.

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

As we complete each aspect of our research, we will aim to present it at scientific meetings and morewidely through various outreach programs. We will also publish high-quality research in leading journals to benefit advances in the field. We are also a part of the Human Developmental Biology Initiative (HDBI) established by the Wellcome Trust, a consortium of researchers working on all aspectsof early human development, which provides opportunities to present our work to a broader group of researchers. Some events organised by the HDBI also offer opportunities to inform a wider audience, including the lay public, about advances from our work and their implications for human health and policy decisions on sensitive research areas on human embryos and the germline.

Species and numbers of animals expected to be used

- Mice: 2000

Predicted harms

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

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

We have generated transgenic mice with fluorescent reporters specific for the mouse germline. We will isolate germ cells and early gonadal tissues from these mice to investigate their development. Inparticular, we have developed methods to generate germ cells from pluripotent stem cells with thesereporters and can conduct a significant amount

of our research using these authenticated organs outsde a living animals (in vitro) culture models. We do, however have to obtain embryos and germcells from mice for validation and to establish fresh pluripotent stem cell lines.

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

The animals are primarily used for breeding purposes to obtain authentic germ and gonads fromtransgenic and non-transgenic mice, and not for conducting research on animals directly in theirbodies/organs (in vivo).

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

Animals produced under this protocol are not expected to exhibit any harmful phenotype.

We have not observed detrimental phenotypes in transgenic mice over several years. If any animal displays an adverse phenotype (mostly due to natural causes), the incidence will be recorded and theanimal will be killed (Schedule 1).

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

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

We do not expect to see severe effects in our colony of transgenic mice.

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?

Human embryos are highly inaccessible for research. We use animals to test new experimental approaches, and the knowledge gained can be used to investigate similar events in precious humanfetuses and embryos.

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

We have developed several approaches where we start with the mouse or human pluripotent stem cells. For example, we have already developed experimental procedures with stem cells that allow us to generate large numbers of cells that resemble precursors of sperm and eggs. We use these cells tocarry out wide-ranging biochemical and molecular investigations leading to significant advances in knowledge. Consequently, we use far fewer animals for our research.

Why were they not suitable?

We have found many of our studies using stem cells are reliable for advances in knowledge.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 reduced significantly the number of mice we use from 4225 to 2000 for last 5 years and willcontinue to do so as we build new in vitro models.

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

As mentioned above, we can generate large numbers of early sperms/eggs from cultures cells. This has proved very important for research on mice and has enabled us to develop similarly in vitro modelsto study early human development and the germline. Thus, significantly reduced the usage of the animals for this project.

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

We use very few animals now because we have been able to develop in vitro methods using pluripotent stem cells. Importantly, the primary focus of our research is on early human developmentand the germline that has allowed us to use very few 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

We use GA mice (Oct4-GFP transgenic mice) in which we had introduced fluorescent tags associated with critical genes, which allow us to visualise and isolate pure precursors of sperm and eggs. Analysisof these cells will enable us to obtain information on authentic cells. We use the information to comparesimilar cells we make using stem cells in culture.

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

Developing precursors of human and mouse sperm and eggs have unique properties that do not occurin other vertebrates.

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

We have now reduced direct experimental work on animals. In the future, we would like permission touse Protocol 1 only for the Breading & Maintenance (B&M) procedure, which will be carefully monitored by the skillful technicians in the Institute animal house.

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

National Centre for the Replacement, Refinement and Reductions of Animal in Research (NC3Rs)

Breeding and colony management | NC3Rs

HOME OFFICE, Advice Note Animals Act 1986, Project Licence Standard Condition 18 notification

The ARRIVE guidelines: Home | ARRIVE Guidelines

The PREPARE guidelines: PREPARE (norecopa.no)

The NC3Rs is the best pratical guidance which has been followed through our project for last 5 yearsand will be a formal guidance for our next project B&M.

Another useful information is the 'User's Guide'. The booklet is informative for HO guidance; SC18 notification; study-plan; local rules for day to day practice etc, and also listed the useful links that weneed to carry out the project as Breading & Maintenance (B&M).

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

While our use of animals has declined very significantly, we will continue to be informed about 3R, anduse every available opportunity to reduce the use of animals.

EFFECTS OF ANTHROPOGENIC ENVIRONMENTAL CHANGE ON PHYSIOLOGY, REPRODUCTION AND SURVIVAL IN BIRDS

Project duration

5 years 0 months

Project purpose

- Basic research
- Research aimed at preserving the species of animal subjected to regulated procedures aspart of the programme of work

Key words

intergenerational effects, anthropogenic impacts, environmental change, wild animals, plasticcontamination

Animal types	Life stages
herring gull, Larus argentatus	embryo, neonate, juvenile, adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To understand the mechanisms by which environmental change caused by humans may impact on thecondition / state of the body / bodily functions (known as physiological state), reproduction and survivalof wild birds. Specifically, to examine how such environmental change may have impacts that transmit across generations, from parents to their offspring.

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

Why is it important to undertake this work?

Ecological impacts of environmental change caused by humans such as habitat and dietary alteration,or pollution often act at the population level, and can be measured in terms of changes in population sizes or how long a particular species stays in a habitat. However what actually causes these, for example, changes in population size, often

remains unclear. Populations of animals are made up of a collection of individuals, so it is important to understand how individual animals physiological state andbehaviour are affected by environmental change. If we can understand the impacts on individuals it may help to find ways to reduce or stop these impacts all together.

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

The main outputs from the project will be an unprecedented understanding of how man-made environmental stressors impact breeding animals and how these effects maybe passed from parents tooffspring. We will also gain an important, novel insight into how these environmental stressors effect the physiological state and reproductive capacity of parents, and the impacts on the development and survival of their offspring. Understanding these impacts at the level of the individuals will give us key insights to help to mitigate the effects of these stressors at the population level. Additional outputs of this project will be datasets, and knowledge-dissemination in the form of peer-reviewed articles published in international journals, and seminars delivered at international conferences. In addition, we will seek opportunities to engage with the media (e.g. newspapers, magazines, TV) and specific interest groups by giving talks and via internet blogs and web pages. Recent behavioural research on herring gulls by our team was covered by >190 media outlets worldwide, and has attracted the most attention of all papers published in Biology Letters. This shows there is huge public interest in the research outputs to be generated on our model system. Finally, we will use the research findings thatare generated during this project to apply for further grant income to extend the work.

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

The datasets and publications that arise from this project will primarily benefit other researchers, particularly those interested in animal ecophysiology, ecotoxicology, behavioural ecology, evolutionarybiology, conservation biology, and biomedical science.

There is considerable and growing interest in the impacts of anthropogenic disturbances in naturalenvironments, such as those through exposure to non-natural foods or exposure to xenobiotics.

However, to date most research has simply described such exposure (e.g. plastics in the stomachs of stranded marine mammals), or has reported associations between such exposure and impacts in terms of animal population size or persistence, without examination of the underlying physiological mechanisms. For example, sampling of seabird eggs for contaminants is considered to be an excellentmeans of monitoring ecosystem health. A recent American Association for the Advancement of Science(AAAS) conference presentation, in which the authors simply reported the presence of plastic additives(phthalate acid esters, PAEs) in seabird eggs in the Arctic, attracted much media coverage worldwide (e.g. https://www.theguardian.com/science/2019/feb/17/plastics-reach-remote-pristine-environments- scientists-say). PAEs have been declared “priority pollutants” by the United States Environmental Protection Agency (EPA), the European Union (EU), and the Chinese waters list. Remarkably, however, no previous study has asked: what are the intergenerational impacts of PAEs for the development and survival of offspring? Our research into mechanisms will bridge this gap in knowledge, and will provide crucial evidence to support the identification of cause-effect relationships between anthropogenic disturbances and ecological impacts. This will stimulate further research in other environments and animal systems.

Evolutionary biologists will benefit from new knowledge about how the nutritional state and

physiological condition of parents influences offspring development and survival. Existing life historytheory focuses only on the personal fitness consequences of ‘decisions’ such as how often to breedand how much reproductive investment to make. This ignores the potential for reproductive costs tohave impacts that transmit across generations. We have recently begun to develop new theoretical models that predict the intergenerational impacts of parental condition. The datasets that arise fromthis project will allow us to test the assumptions and predictions of this theory in a wild animal – the herring gull.

The research will also be of interest to conservation biologists interested in how resource availability and environmental conditions impact on reproductive capacity and the viability of future generations. For example, our supplementary feeding experiment could help to predict the impact of changes in resource availability for population productivity and group stability over time. Data on the physiologicalimpacts of anthropogenic activities could be directly relevant for our understanding of the causes of herring gull population declines in Europe. This species currently has a designation of 'Least Concern' on the IUCN's Red List; while the population trend is in decline, the total population is estimated tonumber 1,370,000-1,620,000 mature individuals. Thus our project will be timely. Because of this benefit, and despite the species' Red List status, we have successfully obtained a Natural Englandlicense to take eggs.

Our research will have relevance for biomedical scientists interested in the early life origins of health and the mechanisms that influence variation in reproduction. Our investigation of how maternal nutrition influences reproductive investment, oxidative damage and survival, and impacts on offspring development will help evaluate the generality of laboratory studies. Where appropriate, we will seek opportunities to promote the public understanding of science by publicising our findings via the media,and by engaging with specific interest groups (e.g. ornithological groups, schools, NGOs and other research organisations). A project collaborator currently holds a Royal Society Public Engagement grant to conduct an outreach program on herring gulls at local schools, and we have made strong connections with national and international media outlets already through our recent scientific publication on the model system.

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

We will look to maximise the outputs of the project by engaging with multiple end-users of the research, as detailed above, and by seeking new research collaborations that could unlock further,future funding streams to extend the work.

Species and numbers of animals expected to be used

- Other birds: No answer provided

Predicted harms

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

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

Gulls that inhabit urban and semi-urban landscapes are ideal models for studying impacts of anthropogenic environmental change on physiology and behaviour, for several reasons. Firstly, they are naturally exposed to a range of anthropogenic changes such as habitat

and dietary modifications,and exposure to xenobiotics that have the potential to cause deleterious impacts on breeding, health and lifespan. Secondly, wild gulls will readily take supplemental food placed at the nest, which will allow us to experimentally manipulate parental body condition and physiological state, and study the effects on breeding and lifespan. Thirdly, because bird eggs contain all the resources required for embryonic development in a self-contained package, it is possible to translocate eggs among nests in order to disentangle the impacts of environmental change on breeding success that are transmitted directly from mother-to-offspring through the egg, from those indirect effects that operate via parentalchick-rearing capacity.

A key objective is to study the impacts of anthropogenic environmental change on the breeding success of wild birds. In particular, we are interested in the mechanisms (direct and indirect) by whichsuch impacts are transmitted from parents to offspring. Breeding success is typically measured in terms of the numbers of offspring that are produced, and which survive to fledging. Therefore, we willstudy the behaviour and physiology of breeding adults of both sexes, and juveniles from hatching to fledging.

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

We will observe the behaviour and reproductive investment of adults throughout the breeding season, between April and July. As an option we may provide supplemental food to some individuals in order toalter their body condition and physiological state. As a further option we may translocate some eggs amongst nests. Both adults and young will be captured and a small blood sample will be collected up to 3 times during a breeding season. All birds will be released to the wild at the end of this procedure.

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

Some birds have small blood samples taken from a superficial vein. These will be taken by experienced researchers under aseptic conditions and are not expected to cause adverse effects. Onlyhealthy birds will be sampled.

One tail feather may be clipped from chicks. This will not cause lasting harm and does not influenceflight performance. Tail feathers re-grow.

Some adult animals may be offered non harmful dietary supplements, and for others clutch size maybe manipulated. This will be within the range naturally experienced by herring gulls. Parents readily accept cross-fostered eggs and resultant chicks; there is no risk of abandonment. We therefore anticipate no adverse effects of this experimental manipulation. We will ensure that the sequence oflaying is maintained in any manipulations, such that, for example a third laid egg would be used to increase a clutch size from 2 to 3.

Some animals will have a small GPS tracking device attached using a wing-loop harness. These havebeen used by the BTO to tag herring gulls in multiple locations. The harness thus does not cross or interfere with the flight muscles. These are designed to break at a specific point after a period of time and fall off without remaining partially attached to a bird. No adverse effects are anticipated.

Birds will be gently restrained by hand by experienced researchers for all procedures, and handlingtime is kept to a minimum. Individuals will be monitored during handling for any signs of pain or distress.

We anticipate that birds will resume natural behaviour within minutes of being released. Similarly,parents soon return to care for nestlings that have been handled: there is no risk of rejection of nestlings by parents as a result of handling. In the unlikely event that an individual shows signs ofsuffering or distress after the procedures it 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 animaltype)?

The expected severity limit is Mild in all cases.

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

- Kept alive
- 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?

There is no alternative to using live, sentient animals, if we are to gain an understanding of the mechanistic basis of ecological impacts of anthropogenic environmental change. Without research on animals, it is impossible to examine the fitness consequences (in terms of reproductive success and survival) of variation in physiological state, and, therefore, to determine the selection pressures drivinglife history evolution.

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

In parallel we are developing theoretical models that predict the conditions under which intergenerational impacts of maternal physiological state may occur.

Why were they not suitable?

Theoretical models can provide a valuable framework for examining intergenerational effects. However, for such models to lead to advance in understanding, it is essential that predictions aretested using empirical data.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 of the proposed methods have previously been used in studies of birds, including gulls, the majoritybeing well established methods that are known to successfully yield the types of data that are requiredto address this project's aims.

Sample sizes are based on previous studies using datasets from the established literature on physiological intergenerational effects of antioxidant manipulations and brood size manipulations inother bird species (e.g. wild jackdaws; captive zebra finches).

The applicant has extensive experience in use of advanced statistical models, and is in regular contactwith colleagues who are expert statistical modellers. Such analyses allow for data of various types of distributions, while controlling for repeated measurements of individuals or inclusion of other factors asrequired, ensuring the maximum statistical power for any given sample size.

The total number of individuals sampled will depend on the rate of population growth over the courseof the project, but numbers will be a maximum of 600 individuals (ca. 240 adults and ca. 360 chicks),and likely fewer (due to natural variation in nest numbers and breeding success), each year.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The research has been designed to make use of repeated (longitudinal) sampling of individuals wherepossible, rather than cross-sectional analyses of populations. Longitudinal sampling is extremely valuable for the study of reproduction and lifespan, due to the fact that selective mortality means that higher quality individuals tend to predominate in older age classes. Moreover, longitudinal sampling enables variation in an individual's physiological state to be followed contemporaneously with reproductive investment. By using longitudinal sampling it is possible to differentiate the variability in measurements taken during the study between the variability of an individual that has been sampled multiple times, and variability between different individual animals.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

In gulls the sexes look alike, so it will be essential to take small blood samples from all individuals inthe population for molecular sex determination. Blood and egg samples will be used for multiple assays, reducing the numbers of samples that are required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.
Our primary motivation for studying wild herring gulls is that this species readily inhabits

and breeds within human-modified landscapes, e.g. town centres and roof tops. This makes them an ideal model for studying the physiological and ecological impacts of anthropogenic environmental change. We alsoaim to study herring gulls at a nature reserve to quantify how gulls breeding at such more ‘natural’ sitesmight differ physiologically from the urban populations. Moreover, this species is extremely tolerant of human disturbance at the nest, including egg removal, and can be readily captured for blood and tissue sampling. Being a large bird species, relatively small blood samples are sufficient for all the analyses we will carry out. All the methods that we use are well established, and the applicant is very experienced in carrying them out in ways that minimise pain and suffering. None of these methods are expected to cause any lasting harm to the individual animals.

The timing of blood sampling has been chosen to achieve the project aims while avoiding disturbance during the most sensitive stages of reproduction (i.e. no sampling of adults during laying; chicks will besampled when they are sufficiently large to avoid adverse effects). The timing of longitudinal sampling will achieve the project aims while maximising the interval between sampling occasions. To minimise stress, blood sampling will be performed soon after capture. Blood samples will be small (<10% of estimated total blood volume on any one occasion), yielding no more blood than is necessary. Birds will be gently restrained and blood collected from the brachial vein; the area will be swabbed and then blood collected by venipuncture and heparinised capillary tubes. Bleeding will be controlled with gentlepressure, and once bleeding has completely stopped birds will be released from the hand, or placed back into the nest.

Why can’t you use animals that are less sentient?

By studying herring gulls in the wild at natural colonies we can examine real impacts of anthropogenicenvironmental change, and physiological trade-offs within the context that they have evolved. This willbe coupled with measures of ecological impacts in terms of fitness, i.e. reproductive success. There isno comparable, less sentient study system that would allow us to address our project's aims.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The only regulated procedure that we use is blood sampling, and the technique has been refined overmany decades of research on wild birds to ensure that the whole process from capture to release is minimally invasive, and unlikely to cause any significant pain or suffering, and no lasting harm.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow the Animals (Scientific Procedures) Act 1986 “Working with animals taken from the wild”Advice note 02/2016.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will check the Home Office website regularly, and will be informed by our Named Animal Care andWelfare Officer and Home Office Liaison Contact of any major advances in the 3Rs, which we will implement if practically feasible within the scope of our research program on wild herring gulls.

EVALUATING CANCER BASED THERAPIES

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Drug Delivery, Cancer, Targeted Therapy, Tumour models, Personalised therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Non-specific systemic side effects are a major problem with many anti-cancer interventions due to the non-selective nature of those therapies. This often limits the patients' tolerability and overall impacts the duration of treatment. Therefore, the aim of this project is to evaluate the therapeutic potential of novel site-specific cancer treatments such as nanomedicine-based formulations that aim to specificallydeliver anti-cancer therapy to cancer tissue taking advantage of enhanced blood vessel permeability incancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 significant progress in cancer treatments cancer continues to be a major cause of mortality in humans with the current estimated risk to develop cancer being approximately 1 in 2. Although currenttreatments such as radiotherapy, chemotherapy and hormone treatments can be effective against primary and secondary cancers, they can be

associated with significant toxic side effects, and therapeutic resistance often occurs. The spread of primary disease to other tissues (metastasis), tumour relapse and resistance to therapy remain the principal causes of death for patients with cancer.Metastasis is a particular problem and accounts for ~90% of cancer-related deaths. Therefore this project aims to improve cancer treatment by testing new anti-cancer treatments and / or reformulating cancer drugs as a nanomedicine formulation that should be able to specifically reach the cancer area while saving the rest of the body from side effects that often limit the effectiveness of therapy and the duration of the treatment.

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 National and International Conferences.

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 andinternationally recognised work that will be published in high impact journals.

Output from this project may be utilised by others including preclinical academics, industrial partnersand 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), the clinical impact may take a longerperiod to be achieved. This may have the potential to be a therapeutic tool to be used for patients.

The time taken to fully evaluate a given therapeutic is likely to go beyond the five-year duration of theprogramme of work due to the complexities involved in the research.

How will you look to maximise the outputs of this work?

It is anticipated that the findings from these outputs may be disseminated through peer-reviewedjournals and conferences.

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.

All of our experimental procedures will be performed in either wild-type or genetically

altered mice. Allthe studies in this project will use adult mice from 6 weeks of age.

Mouse models have many similarities to humans in terms of anatomy, physiology and genetics. The animals used in our research will help us understand the mechanisms that underpin cancer, such as the growth and spread of tumours, and to develop new ways of diagnosing, treating and preventing thedisease.

Mouse models are essential in cancer research. They are used to understand the genetic basis of tumour development and cancer progression. They can also be used to test the efficacies of differentanti-cancer agents. Moreover, in translational cancer research, they represent a powerful tool in assessing the potential validity of targeted therapy.

Typically, what will be done to an animal used in your project?

- Implantation of tumour cells.
- Administration of substances
- In vivo imaging
- Blood sampling

Animals will be humanely killed at the end of the experiment

The animals will be implanted with tumour cell lines (e.g. KMS-12-BM or MM1S) to establish the appropriate tumour growth rate. Blood samples may be taken from a superficial vessel to monitor theblood circulation half-life for the therapy and correlate that with the effect of treatment. Repeated imaging may be performed up to 5 times in which case the animals may be injected with substrate prior to imaging.

The animals in this project may be administered compounds by one or more of the following routes -subcutaneous, intravenous, intraperitoneal, intradermal, intramuscular, intranasal, gavage, topical, drinking water, diet or infusion via a pump or pre-implanted cannula(e) attached to a subcutaneouslyimplanted mini-pump) once or repeatedly to monitor the effectiveness of cancer-based therapy over time.

The typical duration of these types of studies will vary from between 4 weeks to 12 weeks and beyonddepending on the design of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

It is anticipated that the animal may experience some transient discomfort after the injection of cancercells or administration of substances. Animals will be clinically observed daily, however, the frequencyof observations may increase should a deterioration be observed in the animal's clinical condition.

All animals will be weighed at least twice a week to establish body weight development. However, thefrequency of body weight measurements may increase should a deterioration be observed in the animal's condition.

The animal may undergo up to 3 imaging sessions during which they will experience mild transient discomfort as a result of the administration of imaging agents. They will then undergo general anaesthesia followed by the acquisition of the image, typically each imaging session, from the injectionof imaging substrate, anaesthetic induction, and

acquisition of image will take around 15-45 minutes.
The animal will be allowed to fully recover between each imaging session.

Animals may be placed into a warming box to dilate superficial veins prior to an intravenous injection orblood sampling. In addition to this, a terminal blood sample may be taken. A typical animal on a study may have up to 3 in life blood samples taken in addition to the terminal blood sample at the end of the study.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

It is anticipated that up to 70% of the animals may experience moderate severity and up to 30% mildseverity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 this research employs an extensive portfolio of in vitro laboratory techniques, these cannot adequately model the complete array of biological and immunological events that are involved in tumour growth and the changes in blood vessels permeability that is essential for testing the selective accumulation of cancer-based therapy in the tumour tissues and the generation of protective anti- tumour effect. Hence, in vivo studies in mice are therefore essential to understanding the effectivenessof new cancer treatment candidates compared to conventional treatment.

Which non-animal alternatives did you consider for use in this project?

In vitro studies with 2D and 3D cell cultures will be implemented whenever possible to assess the effect of a given treatment on a tumour cell line or primary cells from patient samples. However, theycannot completely replace the information gained from animal experiments as the entire tumour microenvironment is difficult to replicate in a laboratory setting.

Why were they not suitable?

Although in vitro models are very helpful for early evaluation of the therapeutic effect of cancer treatment candidates, they cannot fully replicate the in vivo conditions such as tumour microenvironment and vascularisation that play a key role in the selective infiltration of anti-cancerdrugs to the tumour area.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers were estimated following discussions with research collaborators using similar models toestimate the expected usage.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We will ensure that we use the minimum number of animals required to answer the scientific questionby performing power calculation studies. We will also apply the NC3Rs experimental design assistanttool 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 ofanimals you plan to use in your project?

Sample sizes will be estimated from pilot studies and previous data using power analysis in addition topublished manuscripts by others in the field. Animals will be randomly allocated to the experimental conditions.

Pilot tumour growth studies will be performed where appropriate to establish tumour growth rates andhumane endpoints prior to starting therapeutic studies. Where no data is available in the first instancepilot studies will be performed to assess the tolerability of therapeutic agents before proceeding to themain 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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Prior to implantation, all cell lines will be checked for mycoplasma and prepared in a sterile environment and assessed for sterility prior to administration.

Substances will be prepared and administered using sterile techniques. The route of administration is via the least invasive method appropriate to the model. The volume of substances to be used will be inaccordance with the Laboratory Animal Science Association (LASA) good practice guidelines.

Pilot studies will inform on tumour development rate and end-points prior to moving into a therapeutic setting and these will be used to determine the most appropriate method of assessment. Tumour development will be monitored via imaging which will require transient anaesthesia to immobilise themwhile an image is acquired. Once completed, the animals are expected to make a full recovery within 30 minutes. Each method will allow us to identify development rates and end-points.

Blood sampling will be performed using sterile techniques and volumes collected will be in accordancewith the LASA / NC3Rs guidelines. We will aim to take the smallest volume which will allow for adequate analysis.

All animals will be humanely killed by a Schedule 1 method or as part of terminal (AC) procedures.

Why can't you use animals that are less sentient?

The studies proposed in this project could not be undertaken in animals with a lower form of the immune system (e.g. Drosophila, C.elegans) because those models do not show comparable responses that are seen in humans. Furthermore, to achieve the maximum relevance and utility fromthis project, it is essential that we use genetically altered animals e.g., NSG™ mouse model variants that are the most highly immunodeficient model that supports the development of several cancer xenografts and PDX modelling.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The procedures that are in place to administer substances and monitor the animals are 'fluid' wherebyany opportunity to refine a technique or ensure additional monitoring is performed where necessary, which is carried out by the licensed scientists. Any relevant refinements made are discussed and disseminated to the other users by the animal care scientists.

The veterinary surgeon also observes the work undertaken under this project and will offer suggestionsfor refinements where necessary.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow the guidelines published by the Workman Group on the welfare and use of animals incancer research published in 2010. The ARRIVE guidelines Animal Research: Reporting of In Vivo Experiments) and LASA Good Practice Guidelines.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will maintain close interactions with the relevant welfare, training and information officers as theyoversee 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 arecirculated monthly.

Moreover, the PPL holder will attend appropriate seminars, symposiums and conferences deemedsuitable.

GENE FUNCTION IN CANCER DEVELOPMENT AND TREATMENT

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Cancer, Tumour formation, Tumour progression, Cancer therapy, Genetically modified models

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall goal of our studies is to understand the cellular and molecular processes that contribute totumour formation, progression to aggressive disease and response to treatment with an aim to provideinformation that will help improve cancer patient 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?

Although great strides have been made in improving treatment for cancer patients, there is still much that needs to be done, with some tumour types having few treatment options and successful treatments sometimes only working in a subset of patients. Understanding the processes that contribute to tumour formation, progression and response to treatment

is essential for the developmentof novel and better cancer therapies.
What outputs do you think you will see at the end of this project?

We expect to generate genetically modified models of cancer to understand disease progression and response to therapies. Our studies of these models will provide information that will help improve cancer patient therapy, which is part of our aims and objectives. The data generated in this project willbe communicated to the research community as part of peer-reviewed publications and talks at international scientific conferences.

Who or what will benefit from these outputs, and how?

In the short term, the outputs of the work will benefit other clinical and research scientists in the cancerfield. The availability and analysis of genetically modified tumour models will help advance the discovery of novel cancer treatments. In the long term, we expect our data to inform the design of clinical trials in cancer patients by our clinical colleagues.

How will you look to maximise the outputs of this work?

Our close collaboration with clinicians ensures that our work contributes to the impact on patient benefit. The data generated in this project will be communicated to the research community as part of peer-reviewed publications and talks at international scientific conferences. The models generated willbe available to the scientific community and may be part of collaborative projects.

Species and numbers of animals expected to be used

- Mice: 12500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Animal models are required to fully replicate the properties of the three dimensional tumour tissues growing within specific organs in cancer patients. These properties cannot be adequately recapitulatedin vitro. Similarly, the effects of drugs need to be tested in vivo so that the effects of the natural microenvironment where the tumour resides and how the drugs access the tumour and how specific isthe drug to its target can be assessed. Mice are the most effective choice of species for these experiments and the availability of genetically modified strains make it ideal for studies involving the complex genetic make up found in tumours.

Typically, what will be done to an animal used in your project?

Mice will be housed in cages with sterile bedding, food, and water. Trained competent personal withexperience of using animals in research will perform all procedures. The welfare of mice entering a study is closely monitored throughout each procedure.

Mice with the appropriate genetic background will be generated either by breeding relevant

strains, inducing gene editing and expression through addition of compounds or DNA or by injecting geneticallymodified cell lines or 3D organoids into organs such as the prostate and salivary gland or through injection of tail vein or sciatic nerve to mimic metastatic spread.

Tumour development will be assessed through phenotypic studies, including non-invasive imaging,and, in some cases, mice will be treated with therapies to investigate drug sensitivities. Mice will receive optimal drug dosing that has been previously shown to be effective.

What are the expected impacts and/or adverse effects for the animals during your project?

The mice used in this project may exhibit clinical signs of cancer similar to those seen in humans suchas weight loss, lethargy and pain. Where we are testing the effects of anti-cancer drugs, mice may have established tumours and will therefore inevitably show some or all of these clinical signs. At all times humane endpoints will be established to ensure that the mice do not suffer any more than is absolutely necessary.

During surgical procedures aseptic techniques will be used to avoid and minimise the likelihood ofwound infection, general anaesthesia coupled with peri- and post-operative analgesia will be administered to limit the transient pain/discomfort from surgical procedures

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The severity for the majority of animals used for breeding (around 30% of animals on this licence) is expected to be mild. Procedures covered in other protocols in this project are expected to have effectsof a moderate severity as mice will have spontaneous tumours or grown from cells implanted using well refined techniques, including minor surgery for internal organs and may have anti-cancer therapies with known doses and frequencies.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 strive to develop in vitro systems, the study of the complex interactions of tissues that arerequired for tumour development and progression, including metastatic spread, can only satisfactorily be done using in vivo systems. Similarly, the effects of drugs need to be tested in vivo so that the effects of the natural microenvironment where the tumour resides can be assessed.

Which non-animal alternatives did you consider for use in this project?
We are continually developing genetically modified in vitro cell culture and three dimensional organoid assays and comparing them to the in vivo models in an effort to establish animal replacements. The advent of protocols to grow mini organs such as the prostate and salivary gland in 3D has allowed us to generate more relevant in vitro data that informs our choice of the most appropriate models to use invivo.

Why were they not suitable?

Although in vitro models allow us to test novel hypothesis and treatment regimes, they are not suitableto address the contribution of the tumour microenvironment to tumour growth and response to treatment. In addition, they are not able to reproduce the process of tumour spread to different organs.For these reasons, studies on in vivo tumour models need to be performed, in which the benefits are weighted against the likely adverse effects, and humane endpoints utilised.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of mice have been estimated based on our experience from previous research combinedwith the use of statistical tools to calculate optimal and minimum number of animals for each experiment. We are estimating that we will use 4000 animals over 5 years in our breeding protocol, which includes complex inter breeding programmes, and 1000 in the phenotypic analysis of geneticallymodified animals with tumours. For organ specific protocols, we are expecting to use 4000 animals over the 5 years for cell implantations into the salivary and prostate glands and 1500 animals for subcutaneous and kidney capsule implantations, which are alternative sites that promote tumour growth. 2000 animals in the 5 years will be used for metastatic models, either in the lung or sciatic nerve.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We do the following to ensure that we are using optimum number of animals for each experimentalprotocol:

We consult colleagues with statistical expertise, including our clinical colleagues, to ensure that the optimum and minimum number of animals are used to obtain significant data. These will be most relevant for our therapeutic studies and will depend on the therapy to be studied and tumour diversity.

We incorporate methods to avoid bias such as using automated software or when manual, the operatoris blinded for data analysis; animals in drug studies will be allocated to experimental arms according to tumour size and matched across arms.

We use the NC3R experimental design tool to help design experiments that generate robust data.

We base our work on data from previous studies using similar tumour models. Pilot studies are usedwhen outcomes are unpredictable.

We are continually developing methods to introduce genetic changes in vitro either in cells, 3D organoids or in tissues that we then grow as grafts to minimize experiments which require complexgenetic breeding and therefore reducing animal number.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We take a number of measures to ensure that the minimum number of animals is used including:

Using non-invasive imaging to follow the development of the tumour in time and so that we do not haveto kill mice at different time points during the course of experiments.

We ensure that the maximum amount of information is obtained and analysed per experiment toreduce the need for repeats.

We refine our breeding protocols such that a minimum number of animals is used to generate micewith the appropriate genotype as well as consulting the NC3Rs breeding and colony management.

Using optimum procedures to reduce the number of mice and to reduce experimental variability. Forexample, where possible, we use ultrasound guided tumour cell inoculation into organs to achieve higher precision.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use the mouse as a model system because of the available techniques that allow genetic manipulation and the vast background knowledge we have of its organ formation, cell biology and pathogenesis. In addition, mice have been shown to be the most appropriate animal model to study thecontribution of genes to tumour development and to serve as preclinical models of cancer. The animals are maintained in ventilated cages using sterile food and bedding and all procedures are carried out in laminar flow cabinets to avoid infections. Animal suffering is minimised by keeping tumour burdens within acceptable limits. Therapeutic drugs will have been assessed for toxicity and therefore we expect high tolerability of the regimes. Where possible, imaging will be used for earlier

study end points.

Why can’t you use animals that are less sentient?
The similarities in genetic make up between human and mouse makes them appropriate in vivo models to study the process of carcinogenesis. This is particularly the case for the tumour microenvironment and organ physiology, which cannot be fully reproduced in less sentient animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Mice will be housed in cages with sterile bedding, food, and water. Competent personal trained in non-aversive mouse handing methods with experience of using mice in cancer research and who are familiar with the effects of anti-cancer drugs on rodents will perform all procedures. Studies will be designed to use the minimum number of mice. The welfare of mice entering a study is closely monitored throughout each procedure using welfare assessment protocols to determine when humaneendpoints have been reached. Where possible, non clinical humane endpoints are used. Anaesthesia and analgesia will be used to minimise stress and suffering during surgical procedures. We structure our breeding protocols to generate mice with the appropriate genotype using the minimal number of animals. Where possible, we will use ultrasound to guide tumour inoculation at specific sites as a refinement. The procedures chosen are always considered to be the least severe ones that would produce satisfactory results.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?
We maintain the highest levels of care and welfare. All our activities are covered by standard operatingprocedures. Our BSU produces quarterly newsletter that will keep us informed of any new information relevant to animal research, including 3Rs. We attend our establishment’s BSU user meetings which includes minutes from the Named Persons meetings and Technician Discussion Forums. Every team member also receives NC3Rs newsletter and publications which will inform them of any new information. All team members attend national or international conferences to stay informed of advances in the field.

Published guidelines that are used:

Workman et al, 2010. Guidelines for the welfare and use of animals in cancer research, British Journalof Cancer 102, 1555-1577.

LASA 2010 Guiding principles for preparing for and undertaking aseptic surgery. A report by the LASAEducation, Training and Ethics section. (M. Jennings and M. Berdoy eds).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We follow NC3Rs resources for practical guidelines, such as use of the NC3R experimental design toolto help design experiments and the NC3R breeding and colony management tool for our breeding protocols, and ARRIVE guidelines to report our animal research to ensure that enough detail is reported.

GENE REGULATORY NETWORKS IN DEVELOPMENT ANDDISEASE

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Embryonic development, Genetic interactions, Phenotypic robustness, Regeneration, Zebrafish

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 notrequired.

Objectives and benefits

Description 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 advance our understanding of the gene-regulatory mechanisms underlying embryonicdevelopment and associated genetic 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?

Embryonic development is a complex process that requires precise temporal and spatial control of gene expression. A lot of the same gene-regulatory interactions are also important at later stages inlife, for example during regeneration, and can be erroneously reactivated in carcinogenesis.
Understanding the mechanisms that ensure robustness of developmental pathways against environmental or genetic disruptions will provide insight into developmental disorders and otherdisease etiologies.

An important mechanism of robustness is the ability of some genes to compensate for

each other's loss of function. These genes with compensation ability often stem from genome duplication events, where one gene gives rise to two copies, called paralogues. It is currently poorly understood why someparalogues can compensate for each other while others can't. Studies in human cancer cell lines have tested paralog interactions, but effects are mostly cell-line specific. Identifying the rules underlying this genetic interaction in a vertebrate and in vivo context will provide insights into a fundamental process of gene regulation and genetic diseases caused by paralogous genes. It will also potentially open up gene-therapeutic avenues.

What outputs do you think you will see at the end of this project?

Expected outputs include:

- Identification of the gene features that make genetic compensation between paralogous genesduring embryonic development more likely
- Establishment of the gene-regulatory events that underlie genetic interactions of selected paralogpairs
- Advanced understanding of the role of the gene kdm2aa, and its functionally related genes indevelopment and regeneration
- 5 or more publicationsin internationally recognised and peer-reviewed scientific journals to furtherthe understanding of gene regulation

Who or what will benefit from these outputs, and how?

Long term benefits:

Academics, human geneticists and wider population:

The identification of gene features that make compensation between paralogs more likely will shed light on a long-standing question of evolutionary and developmental biology, i.e. why are some paralogs maintained with the ability to fulfil each other's function. This knowledge may open up therapeutic avenues for genetic diseases caused by paralogous genes as it will help to identify genepairs which might be amenable to reciprocal compensation through targeted upregulation.

Kdm2a is a gene that is difficult to study in mouse or cell culture since knockouts are incompatible withlife. Studying the partially sub-functionalised paralogs in zebrafish will advance our understanding of the role of DNA packaging in the nucleus during development and regeneration. Especially its role in regeneration may point towards therapeutic approaches in regenerative medicine.

Short term benefits:

We use zebrafish, specifically the larval form, as our model species. This contributes to refinement inanimal use as it reduces the use of mammalian models. We will publish our findings in peer-reviewedjournals and present them at scientific conferences.

How will you look to maximise the outputs of this work?

To maximise reach and impact we will publish in renowned journals, disseminate findings via social media, the university's public engagement office and at national and international conferences. We willcontinue our existing collaborations and forge new ones, within our

field and in cross-disciplinary approaches.
Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): Adult wild-type or genetically modified fish: 6,000, wild-type orgenetically modified larvae: 30,000

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.

This project takes advantage of the teleost-specific whole genome duplication to investigate paralogueinteraction in vertebrate development and disease. Zebrafish have been used extensively as a geneticmodel to study development and protocols for phenotypic characterisation, such as labelling of specificproteins or mRNA in the animal, or gene editing techniques, are well established. The vast majority of experiments will be conducted on larval forms before 5 days of development since our main focus is embryonic development. Some experiments on tissue regeneration will use adult individuals. Studies in yeast and C. elegans have uncovered compensatory interactions between genes, but how well these findings translate to a vertebrate context has not been explored. There is no other model systemin which this process can be studied in vivo and in a vertebrate organism.

Typically, what will be done to an animal used in your project?

The vast majority of adult fish, wild-type or genetically modified (i.e. fish carrying non-harmful reporter constructs or genetic mutations showing no clinical signs of harm), will only be used for natural matingsto produce offspring. Some may be used for egg or sperm collection for cryopreservation or in vitro fertilisation. Some of the eggs resulting from natural matings or IVF will be injected with compounds, ortreated with drugs, to generate transgenic lines or modulate gene function. For the characterisation of regeneration some fish will undergo a fin-clip procedure. We will also weigh some adult fish or take other body measurements (e.g. length). No other surgical procedures will be performed on adult fish.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are expected from natural matings, gamete collection or the phenotypic characterisations. Rarely fish do not recover from anaesthesia (<5%). Where a new transgenic or mutant line is generated injected constructs may cause developmental abnormality (<25%). No animalshowing abnormal development will be reared.

Where fish are genotyped using finclip, fish may experience brief <24 hr post-operative pain. Swabbing(https://www.liebertpub.com/doi/10.1089/zeb.2016.1348) will be used as an alternative method for collection of DNA for genotyping wherever possible.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category

(per animaltype)?

> 95% of animals (larval or adult) are expected to be used in protocols of mild severity. <5% ofanimals are expected to be used in protocols of 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 research focusses on the interaction of paralogues during vertebrate embryonic development, sousing animals is unavoidable. If we find tissue-specific effects that could be studied in cells or organoids we will consider these experimental models.

Which non-animal alternatives did you consider for use in this project?

We will collaborate with a computational geneticist and take advantage of existing data from humanand mouse to identify gene features that are potentially predictive of paralogue compensation usingmachine learning.

Why were they not suitable?

The experimental verification of the prediction model is not possible using computational approachesonly.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 studies in our lab and the lab of others in the field.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We use research experimental tools like the NC3Rs Experimental Design Assistant and power calculations based on previous work to ensure appropriate study design and to help reduce thenumber of animals used.

For the generation of GA models where possible we take advantage of mutant archives in the form offrozen sperm samples. The archive contains disruptive mutations for over 60% of zebrafish protein- coding genes. This avoids the need for producing new mutant lines.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

To minimise number of animals used we will optimise genotyping protocols so as to allow genotypingat early life stages to increase breeding efficiency. Pilot studies will also be undertaken for all new studies to help minimise animal usage. Wherever possible animals that are to be killed following analysis will be used to provide tissue for molecular studies and tissue will be shared. We will use fishof optimal breeding age to maximise clutch size and thus minimise the number of adults use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use zebrafish as our model system. Zebrafish are non-mammalian vertebrates with high genomic and functional conservation with humans. Zebrafish are fertilised and develop ex vivo and aretransparent. They are therefore ideal for the study of developmental processes. Moreover, this project requires high-throughput combinatorial knockout studies which is facilitated by the large clutch sizes female zebrafish can produce. Such experiments are not possible in mouse or rat. The vast majority ofour work will involve embryonic and larval forms up to 5 days post fertilisation, therefore this project uses the least sentient vertebrate model system possible. We will use a small number of adults to study fin regeneration. This is an established regeneration assay that causes the least pain when compared to other regeneration models such as heart or spinal cord regeneration, or experiments using rodents.

Why can’t you use animals that are less sentient?

This project seeks to uncover the rules of paralog interaction in vertebrate development and disease, specifically taking advantage of the teleost-specific whole genome duplication. Zebrafish embryos andlarvae are therefore the least sentient animals that can be used in this work.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Refinements to minimise suffering include increased monitoring for all experimental animals, particularly following any invasive procedure (skin swabbing/anaethesia/finclip) or drug exposure. Weregularly review protocols and the use of analgesics, for example post

finclip, as discussed with the NACWO and NVS.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We follow FELASA and LASA best practice guidelines (e.g. see Alestrom et al 2020), the PREPARE(https://norecopa.no/prepare) and the ARRIVE guidelines (https://arriveguidelines.org/arrive- guidelines).

Aleström P, D'Angelo L, Midtlyng PJ, et al. Zebrafish: Housing and husbandry recommendations. LabAnim. 2020;54(3):213-224. doi:10.1177/0023677219869037

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We are kept up to date with advances in the 3RS through the NC3Rs newsletter direct to personal email addresses and via Institutional notification from the NACWO, the Named Information Officer (NIO) and the NVS, and through communications from the AWERB. We also receive from the NIO details of specific scientific meetings and courses related to our research activities, including NC3Rsinitiatives, and those from the RSPCA and FRAME and other welfare organisations.

GENETIC CONTROL OF CARDIOVASCULAR DEVELOPMENT

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Heart development, Congenital heart defects, Genetics, Cardiovascular

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Genes play a major role in the development of the heart and the great blood vessels that carry blood away from the heart. Defects that are present at birth affecting the heart and the great blood vessels are a major cause of illness and death in childhood but the genetic and developmental mechanisms underlying most of these defects remain unknown. Using genetically modified mouse models this project aims to identify genes that work together in a network to control normal heart and blood vessel development. By identifying the genes responsible for normal heart and the blood vessel development,and how these affect development when mutated, could help treat or support patients in the future.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Heart and blood vessel defects that are found at birth affect approximately 1% of all births worldwideand are a major burden for the patient as well as the health care system. Data obtained from this project will help us to understand the interaction of the genes that

contribute to the development of these heart and blood vessel defects. Also, by furthering our understanding of the genetic pathways that control heart and blood vessel development we may be able to devise screening strategies for prospective parents that can highlight any potential risk to the child.

What outputs do you think you will see at the end of this project?

The most likely output from this project will be in the form of scientific publications. These will be peer- reviewed articles that will be published as open access to allow anybody to read about our research. These publications will contain new information about how genes that are important for heart and bloodvessel development interact with each other which will enhance our understanding as to how genes work and what could go wrong if these genes have mutations.

Who or what will benefit from these outputs, and how?

The scientific community will be the main beneficiary of our publications, as this is the standard way to communicate research data. Immediately after publication the general public will also have access to our data so will be able to gather information about the genetics underlying normal heart and blood vessel development. Whilst not in the scope of this project, identifying the genes involved could lead tonew treatments and/or better care for patients. For example, prospective parents could be screened formutations in key genes that could adversely affect the children they have in the future, allowing them to make decisions about their family building plans.

How will you look to maximise the outputs of this work?

We aim to collaborate with national and international colleagues during our research and to publish alldata collected during the project. New knowledge will be disseminated at national and international scientific meetings and conferences, which will increase opportunities to form new collaborations with other scientists. Publications will be highlighted using social media (e.g. Twitter).

Species and numbers of animals expected to be used

- Mice: 6500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are being used in this project because this is the simplest organism that has similar heart and blood vessel structure to people, and that can be used to investigate the roles of different genes in mammalian heart and blood vessel development. We will examine the heart and major blood vessels, as well as the tissues in which these organs and blood vessels form, as they develop from mid-way through mouse embryo development. These stages are chosen because we want to identify the genesthat are expressed in these tissues and the role they play in heart and blood vessel formation.

Typically, what will be done to an animal used in your project?

As we are investigating the effect of genetic mutations on the developing embryo and fetus, the main procedure we carry out is by inter-breeding different genetically altered strains so that embryos or fetalforms can be collected from the pregnant mother who will be killed. In some experiments the pregnantmother may be injected with substances that do not cause harm but can later be detected in the developing embryo.

What are the expected impacts and/or adverse effects for the animals during your project?

The genetically altered adult mice used in the project are normal and healthy, with no obvious defects caused by the mutation. The adult mice are used for mating to create embryo and fetal forms for analysis. The adult mice, therefore do not suffer any adverse effects, particularly as mating is considered a natural act. The embryo and fetal forms obtained may present with developmental abnormalities but the majority will be humanely killed prior to birth, and will therefore not suffer any adverse effects. For the majority of experiments, embryos less than two-thirds through gestation, whichare not regulated, will be collected. Adverse effects, therefore, are related to the adult 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 animaltype)?

The expected severity for the majority of animals used as breeding stock is subthreshold as they will experience nothing beyond standard breeding practices. For some experiments, a small proportion (upto 15% of adult mice used on this project licence) may receive an injection which is classified as mild as the injection will only cause mild and temporary discomfort.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Due to the complexity of the processes involved in heart and blood vessel development, and the manydifferent cell types and tissues that need to interact with each other during this process we need to useembryo and fetal forms to analyse the development of the heart and great arteries from genetically altered mice. Due to this complex interaction within the embryo this work cannot be replicated outside of the experimental animal.

Which non-animal alternatives did you consider for use in this project?

We considered using stem cells and computer modelling in this project, but found that

these were notappropriate due to the complex interaction of cells and tissues within the developing embryo.

Why were they not suitable?

Whilst these are useful tools, there are limitations of cell culture systems, these include;

Cells grown in petri-dishes sit on plastic, which is much stiffer than where they reside in the body. The increased stiffness can change their behaviour and they become "super activated" or fail to do thejob they would in the body. These abnormal behaviours could lead to the identification of non-relevant pathways or fail to predict drugs which are likely to be ineffective in the disease.

Organ disease development and resolution is regulated by many different types of cells communicating with each other within the damaged organ as well as through communication with white blood cells and receiving signals from other organs (delivered by circulating blood). Recreatingall of these internal and external organ damage signals is extremely difficult to model in culture.

Maintaining cultures alive and sterile for weeks, months or years is very challenging and will not mimic all the natural events cells or organs deal with and so will lack context that is very important inmost diseases or normal processes.

Using genetically engineered mice will help advance our understanding of both natural processesand those in of disease to help identify new targets for drug discovery.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals estimated is based on our experience of the minimum number required for each experiment to produce statistically robust outcomes. Our work involves analysing heart and bloodvessel defects caused by genetic mutation so a suitable number of mutant embryos need to be collected for analysis to determine the penetrance of these defects. The embryos are taken before theyare protected at two-thirds of the way through gestation. We will also collect control and mutant embryos for tissue to use in laboratory techniques and experiments where 2 to 6 samples are required to achieve sufficient depth of testing and to enable us to distinguish differences between groups not caused by underlying biological variability.

We have consulted with the Animal Welfare & Ethical Review Board (AWERB) statistician and theColony Management Team in estimating these numbers.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Group sizes are constantly being re-evaluated and updated when necessary. When using new strains,or combinations of currently used strains, small numbers of animals will initially be analysed to check for the predicted defects. This will either ensure that large numbers of mice where the genetic mutationdoes not produce a heart or blood vessel defect in a robust and reproducible manner will not be used, or will inform the outcome of subsequent experiments. The development of animal alternatives, such as cell culture models, will be used when appropriate and may lead to the reduction of animal numbersin future experiments by being able to address specific scientific questions that do not rely on whole animals. For example, we will use cell-based assays to test for gene expression and interactions.
Genes of interest identified from our experiments can be introduced into cells purchased from a company to see where they are expressed inside the cell. Genes can also be introduced with othergenes to see if they interact with each other.

Where appropriate we will use tools such as the Experimental Design Assistant to support ourprogramme of work.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We will only use animals up to their optimal breeding age, and not beyond, and we will not breed animals that display unexpected background specific phenotypes that are harmful. We will conserve tissues by storing as frozen samples, from mutants and extra control embryos, so they can be preserved and used over long periods without needing to produce new animals for experiments. Thiswill maximise outputs from animal procedures and minimise numbers of animals used.

In conjunction with the Colony Management Team, breeding colonies will be monitored carefully to avoid over-production of animals. Breeding colonies that are not required in the medium/long term willbe stored as frozen embryos or frozen sperm, to minimise continued production of genetically altered animals.

Where specific genotypes are available from academic or commercial sources, mice will be acquiredfor each study, to avoid maintaining a breeding colony.

We will share tissue with multiple groups for example the University participates in a number of projects for archiving and sharing mouse lines and phenotyping information (e.g. the European MouseMutant Archive (EMMA), the Medical Research Council Mouse Network) as well as maintaining informal arrangements for sharing lines such as tissue-specific Cre lines.

We will also take advantage of tissue culture models if they are relevant and can address ourhypothesis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will typically use mouse models with genetic modification of genes of interest in heart and great blood vessel development. We will breed these mice together to generate embryos, fetal forms and neonates for tissue collection for downstream analysis outside of the body. Animals will be humanelyeuthanised by a method which is minor in terms of pain, suffering or distress.

The genetically modified mice that we use generally carry mutations for one copy of the gene (the normal animal has two copies of each gene) and this usually has no adverse effect on the mouse. These apparently normal mice are bred together to produce embryo and fetal forms with the geneticmutation on both copies, and these are collected from the humanely euthanised pregnant female.
Other strains carry ‘hidden’ mutations, such that the mice are completely healthy until they are crossedwith another mouse strain expressing, for example Cre recombinase, an enzyme that causes a geneticchange in the cells of the body, to activate the mutation. The majority of mice used for investigation in this project will be collected before two-thirds of the way through gestation and a minority as fetal formsor neonates for analysis, thereby reducing any clinical effects to the absolute minimum necessary for the project. Any adverse effects therefore relate to the adult animal.

Why can’t you use animals that are less sentient?

Due to the complexity of the processes involved in mammalian heart and blood vessel development,the use of other animal models such as the chick, zebrafish or insects (e.g. flies) are not appropriate. Moreover, there are no suitable cell culture systems that can be used to replace a mammalian model.Mice are being used in this project because this is the simplest organism that has a similar heart and blood vessels to human, and that can be used to investigate the roles of different genes in heart and blood vessel development.

We will mostly be collecting animals as embryos and fetal forms, so these are already at a moreimmature life stage.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

All animals, regardless of the line used, will be checked regularly and supportive care will be provided to minimise distress or suffering and to improve welfare, for example low stress handling techniques will be used and habituation periods allowed when new mouse strains are brought into the animal unit.Regular meetings with the NACWO will be held to discuss animal welfare issues. Most of our experiments will involve the collection of embryos and fetal forms which will minimise the welfare coststo the animals.

The majority of the mouse lines we use are apparently normal, healthy and viable. For the breeding protocols for colony maintenance, mice with only one copy of the gene mutated will be mated with wild-type mice to avoid producing mice with two copies of the mutated gene that will die on the day of birth from heart and blood vessel defects.

For any other lines that may present with welfare issues, health monitoring will be carried out regularlywith the aid of a local establishment score sheet, and data recorded, for example, weight, activity, appearance, and body condition. If a welfare risk is identified, the

impact will be minimised by workingwith the Veterinary team to administer appropriate treatment.

To demonstrate proactive management and care for animal welfare, we will limit genetic drift in ourstrains and therefore increase reproducibility by:

- Cryo-preserving new and non-commercial strains as soon as practicably possible after arrival
- Look for unexpected gene mutatons by performing single nucleotide polymorphism (SNP) analysis for new non-commercial strains at arrival, and before each backcross
- Backcross each strain after 5 generations (for colonies of 1-6 breeding pairs) or 10 generations(for colonies with 6-10 breeding pairs)

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Alongside the guidelines listed below, I will also adhere to local AWERB standards for research animals, and where appropriate, support the development of new local standards for refinementsdiscovered during the project licence.

Code of Practice for Housing and Care of Animals Bred, Supplied or used for scientific purposes

LASA Guidelines

RSPCA Animals in Science guidelines

UFAW Guidelines and Publications

NC3R's and Procedures with Care

I will consult with the Colony Manager to review genetic health, breeding practices and overallcolony health and management at regular intervals.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

The local Animal Welfare & Ethical Review Board (AWERB), Named Animal Care and Welfare Officer (NACWO), Named Training and Competency Officer (NTCO) and Veterinary team regularly inform anddisseminate improvements related to animal work, including recent studies involving reduction, refinement and replacement. This also includes information from external resources including (but not limited to): collaborators, peers, conferences, lab animal and animal welfare bodies.

For the 1st, 3rd and 5th year review of the project licence, updates will be provided on implementationor considerations for reduction, refinement and replacement that has occurred during the previous period, alongside a review of the linked training plan. This will be in collaboration with the NACWO, NTCO and Veterinary team, with a particular focus on refinements.

IDENTIFICATION OF DETERMINANTS OF PATHOLOGY ANDPROTECTION IN INFECTIONS OF BARRIER ORGANS

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Lung infection, Gut Infection, Immune response, Tissue damage and repair, Immunopathology

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required,and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures
- Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to identify determinants of severity in the lung and gut that wereinjured as a result of infection, with two separate but closely linked aims:

First, to identify immune-mediated mechanisms of pathology and protection in viral infections such asinfluenza or COVID-19, and bacterial infections caused by Streptococcus pneumoniae, Yersinia pseudotuberculosis or Yersinia enterocolitica.

And **second**, to study requirements of epithelial differentiation and repair in health and disease, insteady state and following infections, immune responses or pollutant exposure.

A retrospective assessment of these aims will be due by 16 August 2028

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Epidemics and pandemics of both respiratory and enteric infections pose a major global health burdenand can bring societies to a worldwide standstill. It is unknown why some people suffer severe symptoms but others only mild or no symptoms when infected with the same pathogen. Understandingthis better is the first step to make predictions about disease outcome and to develop host-directed therapies that may even have a protective effect across several types of infection (known as pandemicpreparedness). The immune response to infection is complex and involves many cell types residing in and moving between several organs. Only experiments in live animals can reproduce this complexity and allow us to understand infection better.

What outputs do you think you will see at the end of this project?

We aim to push the boundaries of our understanding of host determinants of severity in lung and gut infections. The new information will be made widely available, in scientific publications, press releases,at scientific conferences and through outreach activities to a wider lay audience. We aim to identify interindividual differences that explain different disease outcomes, and environmental factors that change course of disease. We will thereby contribute to deeper knowledge of how infectious pathogens and the host response to them influence severity of infectious disease. Building on this newknowledge, we also expect to identify novel targets for immunotherapy of infectious disease to be tested in preclinical models and, eventually, in clinical trials.

Who or what will benefit from these outputs, and how?

While our work is basic research, it has strong translational potential, due to the fact that all infectious settings we work on closely mirror relevant clinical situations of high public health impact. This applies to influenza, COVID-19, other respiratory viral and bacterial infections, and influenza-bacterial or influenza-viral coinfections, known to be among the most severe complications of influenza. In particular, the coincidence of influenza and SARS-Coronavirus-2 in the winter season is cause of greatconcern. We also seek to understand mechanisms underlying susceptibility to gut infections, and complications arising from resulting enteric pathology in different patient groups. Our study of zoonotic foodborne Yersinia enteropathogens in mice reflects natural human infection where the pathogen disseminates from the gut to deeper tissues such as spleen and liver, and provides a model very similar to typhoid-like syndrome observed after salmonella infection. Thus, studying these infectious agents will facilitate understanding on intestinal barriers, gut tissue immunity, inflammatory consequences of bacterial dissemination from the gut and sepsis, and immunity to common enteropathogens.

The outputs of the present project will be deeper knowledge of the mechanisms and

agents driving increased severity in these infections: For instance, we know that interferons (IFNs), a family of hormone-like messenger substances with antiviral effects leading to protection, can sometimes promote severe disease. However, we don't know how and when the damaging effect prevails over thebeneficial effect, and we plan to study the mechanisms leading to either tissue damage or to antiviral protection. As in vivo testing often involves blockade of the relevant pathogenic pathway to show improvement, we will produce data that directly paves the way towards human treatment. No IFN inhibitors are known, but through our mechanistic studies, upstream or downstream modules in the IFNpathway may be revealed as promising drug targets. Understanding how different subclasses of IFNs work will also allow us to identify more specific pathogenic drug targets to inhibit. In addition, our studies will show which dynamic pattern of IFN induction is linked to protection and which one to pathology, findings that might lead in the future to blockade of the pathway specifically early or late during infection.
Also, we know that genetic differences between inbred mouse strains change IFN levels from protective to excessive, but we don't know which is the causative genetic difference. As oneof our efforts is to identify the upstream IFN regulator that differs between these mouse strains, we will be able to identify genes that may define at-risk groups in the human population, due to their tendency towards excessive IFN responses. We are already studying a mouse model of a human genetic syndrome that may fall into this category. Therefore, all outcomes of this project represent direct and indirect benefits for alleviating respiratory and gut disease in humans. Similarly, for COVID-19, blockingthe excessive inflammatory processes that characterise the late phases of severe disease would have a dramatic and immediate beneficial impact in the clinic. All results generated in our studies will be made available to the scientific, clinical and lay communities, by publications with open access, presentations at scientific and clinical conferences, and outreach to the lay public which is a priority in our organisation. Reagents will be made freely available to the scientific community upon request.

Interactions with clinical groups will be sought to confirm or refute our findings in existing humandatasets or to set up trials to test this. These interactions will be developed as soon as we have certainty of our findings in our in vivo models, so depending on the individual programme this will be between the coming months and several years from now. Where applicable, IP support will be sought within the institute to determine whether patent applications are possible. Should our work lead to the identification of novel targets, then the medium- and long-term beneficiaries would be the general public (having access to novel immunotherapies against severe infections), and industrial partners whodevelop the products that we have tested or who develop products against the novel targets that we have identified. As far as research with pollutants is concerned, showing clear molecular links betweencomponents present in pollutants and changes in immune responses or in epithelial differentiation will inform and support policies towards improving air quality, the use of diesel exhausts and the reduction in smoking.

How will you look to maximise the outputs of this work?

Maximal dissemination of our results on all levels, including scientists, clinicians, health policy makers,patient groups, the general public will be sought using all available channels, e.g. scientific publications, seminars, conferences, press releases, radio and TV interviews, blogs, social media, outreach activities such as school visits or the pint of science, and others. Wherever collaborations allow us to improve or accelerate outputs, we will use them, we will share reagents and make data relevant for ongoing epidemics and pandemics available as pre-prints prior to peer review.

Unsuccessful experiments will be published as far as possible, to minimise unnecessary

repetition by colleagues. The translation team in our institute will be involved as early as promising targets are foundto expedite bench – to bedside development.

Species and numbers of animals expected to be used

- Mice: 50,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To understand the complex, multi-organ processes that characterise immune responses to infection, invivo models are indispensable, as no in vitro system is able to reproduce the complexity of the immuneresponse. Among the species available, mice are the species of choice, given the unparalleled range of tools for analysis and manipulation. Many immunological therapies in use today were first discovered and studied in mice. As the physiology and pathology of the immune system is similar between humans and mice, mice are very good predictors of mechanisms in action in human disease. Data obtained in our mouse experiments are complementary to other studies in our lab using representative in vitro systems and activities to compare our data to available patient data. As respiratory infections hit humans at all stages, and at-risk groups are the very young, the very old and obese individuals, we may use mouse stages covering the whole life span and conditions such as obesity. For enteric infections we will focus on adult mice initially, to understand disease processes.

Typically, what will be done to an animal used in your project?

Typically, mock-treated versus animals with immune-modulating treatments such as blocking antibodies or inhibitors (mostly administered intraperitoneally) or genetically wild-type and gene- modified mice will be compared. Animals will be infected intranasally or by oral gavage with a defined dose of a known pathogen strain, and either killed at a pre-determined time point to assess lung or gutdamage, as well as specific immune parameters that we consider relevant for severity, or mice will be monitored over the whole course of infection for clinical signs and weight loss to determine severity of the infection. All infections we study are self-limited and are resolved within maximally three weeks, unless animals reach humane end points before. Where pre-determined time points are chosen, we always aim to maintain duration of experiments to the minimum required to address the scientific needand obtain meaningful scientific data. In some protocols, two infections will be applied in short succession or with longer delays, to mimic better the human condition of sequential or overlapping infections.

What are the expected impacts and/or adverse effects for the animals during your project?

As the disease models we study are respiratory and gut infections, mice suffer flu-like and enterocolitissymptoms respectively, including weight loss, hypothermia, laboured breathing, diarrhoea, hunched position, lack of movement, all to varying degrees. For all models, we have detailed knowledge of the days of highest severity (which, depending on the pathogen and the mouse strain, may last between one and four days) and will monitor

mice daily on these days, using a detailed clinical score sheet and/or weight loss as the main parameters. Once these critical days are overcome, mice recover rapidly and are undistinguishable from uninfected mice within a few days, similar to human influenza or Yersiniosis. Only a minority of mice will undergo a full-time course of infection to link interventions or genetic ablation to changes in severity, while the vast majority of mice will be killed at predetermined time points to study lung damage and immune parameters.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

We utilise a severe or a moderate protocol depending on the scientific questions we are addressing. Where severe influenza or COVID are modelled, we may need to use a severe protocol, but as outlined above, only a few mice will undergo the whole course of infection, while most will be killed at predetermined time points prior to the days of highest clinical scores. As the same immune parameter may be protective in mild to moderate disease but disease-enhancing in severe disease (similar to e.g.interferon or IL-6 in COVID-19), we often cannot study severe disease in moderate infection protocols.Initial comparisons will tell us whether or not this is the case, and the mildest possible protocol to model human disease will always be applied. This is further exemplified in moderate, self-limiting Yersinia infection models where the main study outcome is to investigate memory immunity, and as such the lowest infectious dose possible will be used that is still able to generate these memory immune cells while avoiding excessive pathology during primary infection. Also, when recovery from lung damage, either infection-induced or in other lung damage models, is studied, we need to have certainty of the lung damage occurring in the first place, which is more reliable and reproducible in severe protocols. More moderate protocols show a higher interindividual variability, which make interpretation of results difficult and require higher mouse numbers per group. Again, we will weigh upin each individual experimental setting which is the earliest time point possible to assess crucial immune parameters.

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 16 August 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animalswere affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

As the host response to infection involves multiple immune and non-immune cell types, several organsand extensive cell recruitment, it cannot be fully modelled in vitro. For

instance, the immune response to a respiratory infection normally involves the bone marrow, blood vessels, the lung, lung-draining lymph nodes and the spleen; while Yersinia infections are characterised by dissemination from the gutto distal organs like the spleen and liver. Furthermore, resident immune populations (important targets of this work) are shaped by the complex interplay of tissue-specific signals and stromal cell types within different organs, that cannot be effectively recapitulated in vitro. However, whenever complementary in vitro experiments can be performed in organotypic models or on specific immune cells we will do this. We have an established primary lung epithelial cell culture system that we heavilyrely on to test a multitude of conditions, thus reducing mouse numbers drastically. Additionally, a recently funded grant in the group aims to model intestinal human memory immune cells in vitro by developing an organ-on-chip system for gut tissue, with great potential to reduce mouse numbers needed to study these populations in future.

Which non-animal alternatives did you consider for use in this project?

We use cell culture systems in vitro that reproduce, at least in part, the complex composition of lung tissue. For these cultures, we use primary cells taken from lungs, instead of tumour cell lines as do most in vitro studies, to be able to study the behaviour of normal lung cells rather than cells that can grow indefinitely in cell culture. We closely follow the development of even more complex cell culture systems (called “lung-on-a-chip), but so far they are not able to mirror all the interactions between many different cell types in the living lung. Our existing models of primary lung epithelial cell or cultures of specific immune cells can be used to address specific questions in isolation, which allows us to replace some animal experiments by experiments in these cell culture systems. We are collaboratingwith clinicians and screen cell availability in biobanks to use infected patient samples for lung infections, to validate that our findings in vivo reflect clinical data, and to reduce the usage of mice.

Why were they not suitable?

Culture systems cannot reproduce the high complexity of immune responses that are dependent on cell movement from the bone marrow through the blood into infected organs, under the influence of systemic factors such as hormones, microbiota or the central nerve system. It is also impossible to fullyreproduce lung or gut tissue, composed of a great variety of different cells on a complex extracellular matrix. The possibility to replace animal experiments by in vitro experiments is so far limited to specific questions, and we actively pursue further development of in vitro systems in the lab to extend their potential. For example, we add immune cells to our lung tissue cultures with the aim to gain a more complete picture in cell cultures of lung immune responses.

A retrospective assessment of replacement will be due by 16 August 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design

studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 on the volume of experiments we have performed in the last 13 years doing similar research, and on the current lab size and range of projects we are pursuing or plan to start,factoring in that we will be able to perform in vitro part of the experiments addressing our biologicalquestions.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

For all infection experiments, we have long-standing experience for experimental design and optimal handling to reduce operator-dependent variance. For the design of mouse experiments, we follow therecommendations as outlined in the PREPARE guidelines (https://norecopa.no/PREPARE0) and the NC3R's Experimental Design Assistant. Some of the basic principles we apply are as follows:

We try to include several conditions (e.g. depletion or blockade of several cell types or cytokines)in separate treatment groups in the same experiment, so save on untreated controls and make full useof multifactorial design to enhance statistical power. Using the above online tools, power calculations will be performed to determine minimal treatment group size for maximal statistical information. In- house advice by statisticians in the bioinformatics facility is routinely sought.

We routinely perform experiments on both males and females, to make full use of the mousecolony and to avoid sex-bias in our results or discover sex-specific effects.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We participate in institute-wide animal tissue sharing from experiments to enable multiple studies-exvivo.

We store many organs from experimental mice in case that novel findings or future research directionsrequire analysis of these organs.

We constantly improve education and training for those working under this project license to ensure and improve animal welfare and minimise operator-dependent variability in results. This happens forinstance through the 3R newletters circulating in the institute, through conference attendance of lab members reporting back on new improved methods having an impact on the 3Rs, and through updating among colleagues using similar protocols in the institute.

Different treatment groups (e.g. mice treated with control or depleting antibodies) will be co-housed inthe same cage, to avoid artefacts driven by differences in microbiota composition. Where genetically modified mice are tested, litter mates will be used for similar microbiota and to avoid artefacts due to minor differences in the genetic background.

Where use of littermates is not possible, co-housing prior to experiments or exchange of

cage beddingwill be ensured. This is easier for females but can be achieved for males if they are co-housed immediately after weaning, thus requiring early genotyping or homozygous lines. Co-housing will be particularly challenging for ageing experiments with males, given that constant in-cage fighting often requires separation of males. We use guidance from the NC3Rs on monitoring aggression among males and when to take action when aggression is evident (https://www.nc3rs.org.uk/minimising- aggression-group-housed-male-mice). We also use enhanced environments in our mouse cages help reduce stress and in-cage fighting.

We mostly breed the genetically altered animals that we use ourselves to be able to respond to theexperimental needs by prompt and often transitory colony size adaption. This reduces waste from overbreeding.

To decide to archive lines by cryopreservation when not required over a longer period of time, we takelead from HO efficient breeding of GA animals https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773 553/GAA_Framework_Oct_18.pdf).

Obtaining wildtype mice from in house facility-shared breeding allows better efficiency for largercolonies.

We often use small pilot studies to estimate the effect size and the directions for future experimentalsettings.

A retrospective assessment of reduction will be due by 16 August 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use wild type and genetically altered mice of different inbred and potentially outbred genetic backgrounds in in vivo infection models, using human pathogens or the mouse equivalents of them, to identify determinants of disease severity und the mechanisms underlying severity. We are actively seeking to refine severity parameters to go beyond the present clinical scores and weight measurements. This includes the employment of lung function measurements where mice undergo using non-invasive techniques, such as unrestrained whole-body plethysmography that is used to analyse breathing behaviour in mice. This physiological technique allows the analysis of lung and airway function and has several advantages: data obtained can be directly compared to patient data asclinical

plethysmography measures similar parameters, we measure directly lung function rather than indirect disease measures, and we may be able to detect severe lung injury earlier by these measurements than by the clinical scores we presently use. Similarly, labelled GFP- or luciferase expressing or -encoding bacteria and viruses can be potentially used for non-invasive imaging of pathogen spread during infection. We are already using fluorescent bacteria for ex-vivo measurementsof bacterial load, but the ultimate refinement will be to measure this in vivo in a non-invasive manner, atechnology that we are actively developing. This would not only represent reduction (as fewer mice areneeded in repetitive measurements) but also refinement (as mice are not killed for measuring bacterial load but monitored with a non-invasive method). Such newer and more sophisticated parameters needto be linked firmly to the above-mentioned morbidity measures which still represent the gold-standard indicators of disease severity, before we can replace the latter to achieve refinement.

Why can’t you use animals that are less sentient?

Less sentient animal species do not provide the physiological similarity to humans and the wide availability of tools for analysis and manipulation. More immature mice are not an option as embryos and newborns have only an immature if any immune system. We need a physiology in our in vivo models which is as close as possible to humans. Only mammalian organisms show the same degree of complexity as humans in their immune response, lung or gut structure, and other basic physiologicalparameters like metabolism.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We always aim to refine our procedures. For instance, we have started to replace intraperitoneal tamoxifen administration by oral gavage, and for serial blood collection we currently explore the use ofsaphenous vein rather than tail vein puncture. As mentioned above, we are attempting to pioneer imaging and lung function analysis techniques to follow pathogen spread during infection by non- invasive means, to reduce harm to the animals. Furthermore, tunnel handling will be rolled out across the institute within the next years, which causes less anxiety than traditional tail handling. We are guided by and seek advice from local NVS policy on non-invasive procedures, as well as improved pain management, to minimise harm.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Unless otherwise specified, the work in this project will be designed using the principle outlined in PREPARE guidelines for planning animal research and testing (2017) and in the LASA Guiding Principles on good practice for Animal Welfare and Ethical Review Bodies. We will also follow LASAGuidelines administration of substances.

With regards to infection models we take advice from the "Considerations for Infectious Disease Research Studies Using Animal " (Lesley A Colby, Lauriane E Quenee, Lois A Zitzow. Comp Med.2017 Jun; 67(3): 222–231; PMID: 28662751) as well as from “Refining procedures for the Administration of substances” (https://doi.org/10.1258/0023677011911345) and from the “guiding principles aseptic surgery” (https://www.lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf).

How will you stay informed about advances in the 3Rs, and implement these

advanceseffectively, during the project?

We are regularly updated within the institute on advances in the 3Rs from NC3Rs (https://www.nc3rs.org.uk/nc3rs-newsletters) and NORECOPA, and we actively seek information onpossible improvements in discussions with colleagues and collaborators at in-house meetings and external conferences. Whenever we are able to refine techniques without impacting the scientific validity of our work, we aim to implement advances, for example enriched environments for mice to reduce stress levels and avoid overgrooming.

A retrospective assessment of refinement will be due by 16 August 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved forfuture work of this kind? During the project, how did you minimise harm to the animals?

IMMUNE REGULATION OF HEALTH AND DISEASE IN MUCOSAL BARRIER TISSUES

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Immunology, Inflammation, Diet, Microbiota, Infectious Disease

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description 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 the immune system in "mucosal barrier tissues", such as the intestine and lungs, acts to maintain health and prevent disease by mounting different and appropriate responses to infections, "good bacteria" that live in our guts, (commensal bacteria) and thediet.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Immune responses in tissues such as the intestinal tract and lung continually act to keep

us healthy.The immune system is continually being challenged by infections, as well as otherwise harmless stimulus from the diet and the "good bacteria" that reside in our guts. Disturbance of this normal immune function in humans and other animals results in a wide range of chronic illnesses.

A better understanding of how the immune system functions in health and disease has the potential tolead to new therapeutic approaches for a number of chronic diseases and is of clinical and veterinary importance.

What outputs do you think you will see at the end of this project?

Advances in our knowledge of how the immune system functions in health and disease withinmucosal barrier tissues.

Ultimately, we hope to generate information which will inform new drug discoveries and therapies forhuman and animal disease.

Communication by peer-reviewed publications, research conferences and seminars, and wherepossible through engagement with public

Benefit across the scientific community in terms of advancement of research methods and generationof genetically altered animals.

Who or what will benefit from these outputs, and how?

This project aims to generate new insights into fundamental biological processes that help to maintainhealth and prevent disease in organs such as the intestine and lungs. As such the work has the potential to be relevant to a broad range of both human and animal conditions.

In the **short term** the research community will benefit from this work. In the **medium term** the dissemination of knowledge will benefit the public, funding bodies and disease focussed charities andinterest groups. And in the **long term**, we hope this work will allow drug developers will develop new therapies for patients.

How will you look to maximise the outputs of this work?

Dissemination of knowledge: We aim to communicate our findings to the largest possible audience, where appropriate. Primarily this will be via the publication of peer-reviewed findings in internationally recognised, open-access journals with a broad audience. We will use open access repository sites, funder-backed open access journals and pre-print servers, where appropriate, to expedite sharing of our research and to share findings that may not fit the scope or depth of typical publisher-led journals. This will ensure even negative findings or observations generated through this project can be shared toreduce unnecessary duplication of studies.

Communication: Publication will be complemented by presentation of research findings at nationaland international seminars and conferences, as well as incorporation into outreach and public engagement forums such as “Pint of Science”, or engagement with patient groups. We routinely engage with social media (Twitter) and our institute’s press office to share our research as widely aspossible.

Collaboration: We additionally collaborate extensively both nationally and internationally

with otherbasic researchers, as well as clinicians and have pre-existing links with several pharmaceutical companies. Where possible we will share our findings at the earliest opportunity.

Translation: The institute has extensive infrastructure for the translation of basic research findings andfor technology transfer, as well as for harnessing intellectual property and engaging with the pharmaceutical industry to expedite knowledge transfer, drug development and impact upon clinical practice.

Species and numbers of animals expected to be used

- Mice: 15000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice, including genentically altered animals (GAA), are the most appropriate species for these studies as they resemble humans, as well as other animals, and biological insights have a strong track record of translation to clinical advances.

Typically, what will be done to an animal used in your project?

In this project there are several common procedures that will be performed on mice:

Mice will receive molecules or drugs that activate or suppress the immune response, throughinjection or administration via oral routes.

Mice will be subjected to infection with bacterial, fungal or parasitic organisms that model humaninfectious diseases, through injection or oral administration.

Mice will be subjected to models of human inflammatory or allergic diseases, through administrationof chemicals or allergens via injection, oral administration or administration into the airways.

Mice will receive antibiotics to study the role of the non-infectious, beneficial intestinal bacteria (“themicrobiota”) or receive beneficial microbes from defined cultures or transferred from other animals, to study their effects on immune health and disease. Mice will receive diets with altered nutritional content, or defined nutrients, to study the effect of dietand dietary metabolites on immune cell function and tissue health.

Typical routes of intervention include administration via the oral route (ad lib in food or drinking water,gavage), intranasally or via intraperitoneal, intravenous or subcutaneous injection. Blood may be sampled via the tail vein, or fecal matter sampled passively. In some cases, animals may undergo transient anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals used on this project may experience adverse effects, including transient stress or pain and discomfort associated with i) sample extraction for purposes of genotyping or sample collection, ii) theexperimental induction of human-relevant inflammatory and infectious diseases, or iii) associated withthe delivery method, restraint or anaesthesia required for the experimental procedures. Specific expected adverse effects will vary and are outlined within individual protocols.

In many cases the stress and discomfort will be transient and range from a few minutes to up to 12 hours. However, in protocols associated with i) administration of substances to alter the immune system, ii) administration of infectious microbes, iii) experimental induction of inflammatory disease, iv)administration of antibiotics to alter intestinal bacteria and v) alterations in diet and nutrient administration, it is expected some prolonged adverse effects of mild to moderate severity will be experienced, with duration exceeding 12 hours.

A common expected adverse effect across multiple experimental protocols is weight loss, which will typically be less than 10% and resolve within 48 hours or treatment/experimental intervention, althoughin some protocols weight loss associated with inflammation may exceed this value. In all cases steps will be taken to refine experimental approaches to reduce weight loss as much as possible.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The studies outlined in this project will results in the following proportions of animals actuallyexperiencing the following severity ratings:

Sub-threshold – approximately 30%Mild – approximately 45% Moderate – approximately 25%

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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are currently no good in vitro (i.e. cell lines) or in silico (i.e. computational) models that can fully and faithfully replicate the complex biological scenarios we are studying. In contrast, a mouse is a powerful and flexible model to experimentally investigate these questions using well-defined methodologies and experimental systems in a controlled environment. The mice used will also be inbred, reducing the genetic variation inherent in trying to study complex biology in human populations.Only through defining the full complexity of the immune system can we truly understand these important biological

processes and make new therapeutic breakthroughs for the treatment of disease.

Which non-animal alternatives did you consider for use in this project?

- cell culture with in vitro derived primary cells, or cell lines.
- *in silico* (e.g. computational) analyses or meta-analyses of publicly available.
- parallel studies utilizing human materials.

Why were they not suitable?

The complex tissue environment in which immune cells are found in humans and mice cannot be fullyreproduced in common non-animal approaches or accurately modelled through in silico studies. We specifically study the function of rare immune cell populations within the context of complex tissues, e.g. the gut and lungs. While we aim to use alternative approaches – such as public data sets, cell lines, organoids, organ-on-a-chip – to expand our investigations, they do not reflect or fully capture thecomplexity of organs.

Furthermore, while we aim to complement our research with human samples, access to cells derived from organs such as the intestine and lung is limited and/or often not feasible in healthy humans due tothe invasive nature of obtaining these samples, while tissue-associated immune functions are not accurately represented in easily obtainable human tissue, such as the blood. Moreover, humans exhibit a vast degree of genetic and life-style associated variability which can only be controlled for via the use of inbred mice within a controlled and defined environment, and it is not ethical and/or feasible to knowingly infect or induce disease in healthy human beings.

Finally, animal models also offer invaluable opportunities to genetically manipulate immune cells to better understand their functions in health and disease that are not possible, or currently technicallychallenging with human samples alone.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers were estimated through consultation with professional statisticians, the NACWO(s) and other named persons, and senior license holders in my department with significant experience inrunning a license.

The majority of animals utilized on this license will be generated through breeding of genetically alteredanimals (GAAs) (Protocol 1) and supplemented in subsequent protocols with wild type animals purchased from commercial vendors. As such the numbers required takes into account mice required for breeding and experimental work as well as the crossing and maintenance of new GAA lines.

Animal numbers in individual experimental protocols were calculated based upon prior

knowledge of typical experimental design and experimental units associated with these procedures. Where possible,numbers were also based on typical prior and current usage of GAA lines and commercially purchasedanimals across each procedure and based upon typical usage over the last 5 years, where the procedures were routinely performed.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Experimental design was informed through consultation with an approved statistical agency. We will utilize power calculations, alongside the National Centre for Replacement, Refinement and Reduction (NC3Rs) experimental design assistant, throughout the duration of the project to ensure each experiment is designed optimally for the central experimental readouts. In the majority of cases we willbe able to utilize previously generated data from our laboratory, colleagues or publicly available data sets to inform these calculations. However, in some cases where experiments with a given procedure or combination of procedures, new GAA models etc have not previously been performed we will beginby performing a small-scale pilot experiment – often with smaller group sizes (n=3-4) if animal availability is limited - to determine whether any effect is present and to inform experimental design of future replicate or related experiments.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

The use of GAAs, bred in-house, is critical for the success of this project. Thus, efficient breeding strategies and management of breeding colonies will be essential to minimise animal usage throughthe course of this project. To address this we will undertake several complimentary approaches;

For the generation of new GAAs via crossing of existing lines we will limit breeding to the minimumrequired to propagate the colony towards the final experimental genotype.

To the best of our abilities we will strive to continually estimate the requirements for each individualGAA line.

To reduce animal usage, experimental procedures performed for the first time without prior experimental data will be run as small scale "pilot studies" to determine the presence or absence of aneffect. This pilot data will be further incorporated via sequential analysis or pooling of data when performing additional validation experiments to maximise statistical power.

In all situations we strive to share tissues from experimental animals both within the laboratory groupand with colleagues and collaborators within the university. Where possible tissue samples may additionally be taken and frozen for subsequent use to maximise experimental readouts from an individual animal and reduce the need for additional animals to generate separate readouts.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime

of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will utilize mouse models of infection and intestinal and pulmonary inflammation to study interactions between the immune system, diet and beneficial microbes that reside in the intestine.These models are the most appropriate because they have been extensively studied over severaldecades and their relationship to clinical human diseases and therapeutic potential have been extensively demonstrated. Moreover, mice enable application of cutting-edge scientific tools and techniques, genetic alterations and ease of breeding and handling that make mice the most informativeand ethical model for the studies proposed.

Our models of infection and inflammatory, allergic and metabolic disease will lead to weight loss or gain, gastrointestinal symptoms (e.g. diarrhoea) or short term breathing difficulties - which were chosento mimic clinically relevant human diseases. The experimental models are designed to induce the minimum clinical signs and symptoms necessary to model human disease and the associated immune response, and all animals will be humanely euthanised prior to the onset of symptoms approaching theseverity limits indicated. Throughout these studies we aim to determine how the immune system can be boosted or blocked therapeutically in order to prevent disease symptoms and suffering.

Why can’t you use animals that are less sentient?

We are studying complex interactions between multiple biological systems (immune system, intestinal resident beneficial bacteria, diet) which can only be fully reproduced in adult mammalian species, suchas mice, that have a fully developed immune system, consume solid foods - equivalent to the adult human diet, and which are colonized by commensal microbes.

The long-term nature of many of the experiments, need to study responses at multiple time points andneed for sequential experimental steps prevent the use of terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Following the establishment's established policies, we will adopt the latest techniques in animal handling and husbandry to significantly reduce the stress associated with experimental procedures as much as possible. Furthermore, where possible, the least invasive methods of dosing or sampling will be applied (e.g. repeat intraperitoneal injections will alternate sides to reduce any adverse effects), andanalgesics or anaesthetic use considered to manage pain and post-operative care, e.g. humane restraint during a procedure, close management of pain, temperature and conditions where necessary).

Experiments in which animals receive an infectious or inflammatory agent will always be performedwith the lowest dose possible and with end points as early as possible, to prevent the animals fromexperiencing unnecessary or severe harm.

While the lab has significant experience in all experimental procedures herein, we are also

continually refining our procedures and will consult with the NC3Rs and institute staff to take advantage of changes in best practice or new opportunities to refine these methodologies. Best practice is discussedwith lab members at regular group or one-on-one meetings and we maintain close contacts with other labs locally, nationally and internationally who run the same experimental models and discuss new advances that may help to refine experimental procedures and reduce harm.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

The lab consults the latest literature on recommended standard practice and refinements (e.g. Mortonet al Lab Anim 2011, PMID [11201285]), the recommended resources from the NC3Rs at https://www.nc3rs.org.uk/3rs-resources regularly to inform ourselves of new advances, and videos of best practice techniques, and regularly receives updates and suggestions from the institutes NTCOs.

For the specific experimental models employed here we read published research papers from laboratories worldwide utilizing the same approaches, and consult with local, national and internationalcolleagues to discuss methods to refine these procedures.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We are routinely informed about advances in the 3Rs via internal newsletter emails. In addition, we willaim to stay up to date with advances via the NC3Rs website, publications of methodology and handlingrefinements, and attendance at seminars offered by the NC3Rs and similar organisations - both online and in person. As an example, we are in the process of adopting the improved rodent handling methods that reduce animal stress (detailed by Hurst et al. Nat Methods 2010) and now provide environment enrichment as standard

In addition, we will maintain regular contact with the Named Veterinary Surgeon (NVS), Named AnimalCare and Welfare Officer (NACWO) and other named staff to inform ourselves of opportunities for refinement with the animal facility, and where possible discuss suggestions for refinement arising fromour experimental work.

IMMUNE RESPONSES TO INFECTIOUS AND INFLAMMATORY AGENTS

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Inflammasomes, Macrophages, Inflammatory disease, Innate immunity, Infection

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 notrequired.

Objectives and benefits

Description 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 we respond to infection and other chronic diseases such as diabetes, obesity and atherosclerosis. We will particularly focus on a set of molecules that come together to form a complex known as the inflammasome. We will study how the inflammasome complex is regulated in a particular kind of cell, called a macrophage, to decipher novel strategies thatwill help ameliorate infectious and inflammatory diseases.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Activation of the inflammasome complex results in the secretion of potent molecules called cytokineswhich contribute to the clearance of the disease-causing agent during infection.

In other instances, inflammasome assembly is important in wound healing. However, during dysregulation, our immune system goes into overdrive causing unnecessary activation of the inflammasome complex which thenresults in tissue damage and contributes to the progression of inflammatory and metabolic diseases like atherosclerosis and obesity. We will identify whether the targets and pathways we have identifiedthrough experiments with isolated cells in the lab are important from the point of a whole animal. We will investigate how targeting these pathways using a range of approaches will alter the formation of the inflammasome complex, clearance of the pathogens, and tissue damage in mice.

W**hat outputs do you think you will see at the end of this project?**

We will gain a further understanding of how our immune system, which consists of pathways that fightinfection, contributes to microbial clearance and the pathogenesis of inflammatory diseases. We will compile these results for publication in open access and high visibility journals and share our results widely with the scientific community at national and international conferences.

Who or what will benefit from these outputs, and how?

The experiments proposed here focus particularly on understanding the regulation of inflammasomes. We are particularly interested in understanding how the synthesis and degradation of fat-like moleculeslipids (lipid metabolism) affects inflammasome activation. Thus, first and foremost, this research will beof interest to a broad spectrum of scientists working in the area of inflammation, particularly those studying obesity and cardiovascular disease. In the mid-term, this research has the potential to attract pharmaceutical research by those companies who are interested in developing compounds to dampen inflammation or promote pathogen clearance. Although the first focus may be on diseases with defective lipid metabolism, such therapies can potentially be used for a range of inflammatory and autoimmune diseases.

In the long term, advances in understanding inflammatory pathways could result in potential economicbenefits as a result of reduced healthcare support required. Moreover, as several lipids are obtained from dietary sources, our research will further inform how food and nutrition can affect health and impact disease risk, and how dietary metabolites affect processes that influence health. This might eventually translate into economic benefits for food manufacturers.

Since potential therapies from these studies may be useful for a range of inflammatory and autoimmune diseases, these would significantly enhance the quality of life for these patients. Thepolicymakers including the World Health Organization are also likely to benefit from this research.Additionally, medical charities may be influenced by our research, and this may inform funding priorities.

How will you look to maximise the outputs of this work?

We will maximise the output by collaborating with other researchers working in similar areas. We willshare the results of these experiments with our collaborators. The findings from this research will bepublished in open access journals, presented at scientific meetings, and collaborations with pharmaceutical companies will be initiated which may help the development of novel therapeutics.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Since this study proposes to investigate immunological parameters involved with the inflammatory response, mice are the most appropriate host to use, based both on the availability of reagents for immunological investigation and the number of animals needed. Most of the innate immune response genes in humans are represented in mice, and mice lacking critical molecules are available for many ofthose. As the mouse model has been extensively evaluated and validated for use in studies evaluating host immunity to inflammation and infection, studies in mice will thus provide an abundant resource for comparison of the experimental data.

Our research largely relies on understanding a normal immune response to bacterial infection or an appropriate agent. Therefore, it is important to model these responses in a mammalian system that hasan immune system like humans. Other animals lack the necessary immune system and the repertoire of immune receptors and pathways that are present in humans. Furthermore, it is important to use a model system that breathes air through a nasopharynx similar to humans and produces immune responses in mucous membranes and skin similar to humans. This can only be modelled in mammals, and the mouse therefore represents the most conservative and appropriate system to use avoiding use of higher mammals or mammals from the wild. Mice are also easy to deal with in terms of genetics, thus it is possible to use mice that have been modified to better represent humans, or that are deficientin a specific immune checkpoint that is under investigation. Adult mice are required since immune responses are fully developed and stable by then, meaning that the outcomes of in vivo experiments are more reproducible between individuals. However, we also know that our immune system respondsdifferently when we age. Therefore, we will use aged mice, in order to identify pathways that may be more amenable to therapeutic targeting in aged individuals.

Typically, what will be done to an animal used in your project?

This project is focused on understanding immune responses to appropriate agents and bacterial infection, and in many cases, we will evaluate the role of lipid pathways in regulating immune functions. Therefore, in most cases, we will treat the animal with an agent to block a certain lipid pathway before inoculating them with an immune agent or bacterial infection. We may need to test an animal’s baseline level of immunity to the agent, and also check what bacteria the animal is already carrying; we can do this by taking a small volume of blood from a superficial vein. A blood sample mayagain be taken at the end of the experiment after 2-3 weeks. The mice will be monitored and weighed daily or every few days depending on the model. Most of the experiments would typically end in one week after infection or inoculation of an agent but may sometimes continue for up to 4 weeks at whichstage animals will be humanely killed and different organs, again depending on the model, will be collected for evaluating the progression of infection or to perform other assays in the lab.
In some cases, mice will be made available a modified diet for up to six months. This is particularlyimportant in cases where the subsequent aim is to block a certain lipid pathway

or to induce a particular chronic condition. The subsequent experiments investigating how mice respond to an infection or an agent which is injected intraperitoneally or intravenously are usually shorter and cancontinue for up to 4 weeks.

In some cases, mice will be injected intraperitoneally by an agent before infecting them either intravenously or intranasally to model either a systemic infection or to model a respiratory tract infection. The animals will be monitored and weighed daily to find out their susceptibility to infection. These infections can continue for 3 weeks, but in general, are limited to 7 days as most mouse strains fail to carry bacteria beyond that time point. In general, mice do not get sick from this infection providedthat the volume administered is tiny.

Because human infections can sometimes enter the bloodstream via the skin some models might include inoculation of bacteria into or just under the skin, or application of bacteria onto the skin that has been gently shaved, to replicate a superficial graze. Some mice might be infected intramuscularly(into the muscle), as a model of more invasive infection. These mice will usually only be infected for amaximum of 72 hours.

It is harder to monitor the bacteria during infections that are ‘internal’ to the body (so-called invasive infections), so we may take small blood samples during infection to check for bacteria in the blood. Wewill weigh animals regularly as this provides an early indication of the infection progression. At the endof any infection model, the mice are humanely killed, and bacteria are counted from the site of the original infection and also from as many organs as possible, including the blood.

To understand the effect of novel treatments, or of specific components of the immune response, wewill sometimes administer substances to the mice, either before, or during the infection. These substances may include inhibitors because it is important to understand how novel treatments work together with routine treatments that patients receive. Most substances are administered intraperitoneally (into the abdomen) or intravenously (into a vein) for the easiest rapid spread into thebloodstream. Mice that are infected, are then monitored for progression of infection.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice that experience intraperitoneal or intranasal inoculation are not expected to undergo any adverseevents, although some may experience moderate systemic effects such as reduced appetite/eating less for 24-48h, however, most will recover from these symptoms. Inoculation using larger volumes may lead to peritonitis, or lung infection and early pneumonia in case of intranasal infection leading to gradual onset of rapid breathing. Mice with abnormal breathing lasting more than 6 hours will be humanely killed.

Mice that experience intramuscular infection will demonstrate a limp after a few hours and may experience some pain; such experiments do not normally last more than 24h and pain killers will beused.

Any mouse that is undergoing inoculation with an agent administered into the skin, muscle, intraperitoneal or intravenous may show signs of systemic illness, such as weight loss or ruffled fur forup to a maximum of 24h. Mice that will show abnormal breathing lasting more than 6 hours 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 animaltype)?

Overall, for this project, it is estimated that for mice, 40% will undergo mild severity and 60% moderateseverity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project aims to understand how an animal immune system responds to infectious and sterile stimuli, and to assess this by investigating the levels of secreted cytokines and chemokines and the level of tissue damage that occurs to different tissues and organs; this can only be modelled in livinganimals or humans. Some of the bacteria and the agents used in our project are considered too dangerous for use in humans. These experiments would not be possible with fish or insects.

Which non-animal alternatives did you consider for use in this project?

To study the immune response to infections, we collaborate with clinicians and use blood samples frompatients and tissue biobanks. We also continually use human blood cells as a model system although itis necessarily a static system and limited to the cells in a tube- this cannot mimic the dynamic movement of immune cells to the site of infection or tissue damage.

Why were they not suitable?

These samples provide very useful information but do not allow us to undertake experiments or testnew interventions.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We use statistics to help us decide the lowest possible number of mice needed to get a meaningful answer to a specific question. The projected number of animals reflects our best estimate of the number necessary to achieve the proposed scientific objectives. We

also considered the number of mice funded in our new grants, as well as an estimate of transgenic breeding required to deliver the required numbers. We estimate that a maximum of 600 per year might be used if our in vivo projects are active however this is an upper estimate and the actual number used is likely to be very much less.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Our experiments incorporate longitudinal monitoring of mice inoculated with bacteria or other agents over a period of 1-3 weeks as required. These experiments are designed only after we have carefullycollected data from our in vitro experiments with both immortalized cell lines and human peripheral blood mononuclear cells, thereby greatly reducing the number of animals that will be used.

For most of our experiments, we try to identify a suitable fixed single time point to end the experiment;this ensures that we obtain as much quantitative (numeric) data as possible from that final, planned time point. This has largely replaced the practice of waiting for a particular symptom to develop, as thepractice is too unpredictable and would require continuous monitoring of mice overnight.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We will undertake pilot experiments using a very small number of mice on each occasion where there is a new variable, for example, a new pharmacological agent, as the effect of this will be unknown. Thisinformation will be used to undertake statistical tests to ensure the optimum number of mice are used per group, wherever 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will inoculate animals with a pathogen or a sterile agent depending on the natural route of infectionor the model most appropriate to study. Some mice which will be infected may additionally receive treatments by injection. The models used have been scientifically validated and shown to provide valuable information while emulating the disease features of human infections. The duration of infection, and bacterial dosing regimens in these models has been adjusted and improved upon over several years to avoid severe outcomes and to cause least distress.

Why can’t you use animals that are less sentient?

In order to gather data from a mature mammalian immune system in response to infection or sterile stimuli, it is necessary to use mature (adult) mice. We cannot use species that

are less sentient, suchas insects or fish, because these do not provide the context required to model infection in humans i.e.being able to breathe air, and with a mature adaptive and innate immune response.

For a large majority of our experiments, we will use bone-marrow-derived cells which will be differentiated into macrophages or other cell types. In these cases, we will humanely kill mice by aschedule 1 method.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The animals will be group housed and environmental enrichment will be provided to improve animalwelfare.

For any model where there is a risk of systemic illness, the frequency of monitoring will be increased.Where there is a risk of pain, analgesia will be provided in advance, as advised by our Named Veterinary Surgeon.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will adhere to guidelines issued by LASA and NC3Rs. Furthermore, we will endeavour to report ourfindings accurately using ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Institutional seminars and workshops will provide continuing professional development in the 3Rs,while the NC3Rs website provides a readily available resource at all times.

IMPLANTATION OF SEEDED SCAFFOLDS FOR TISSUE/ORGAN 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, ortheir effects, in man, animals or plants

Key words

Stem cells, scaffold, organ/tissue, therapy, transplantation

Animal types	Life stages
Sheep	adult, juvenile
Pigs	juvenile, adult
Minipigs	Juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description 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 create functional tissues/organs using a combination of cells and biomaterials to either repair,replace or regenerate diseased or damaged tissues and/or organs.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 meet the shortage of organs/tissues needed for organ transplantation or tissue repair, both of whichare on the increase globally.

What outputs do you think you will see at the end of this project?

The outputs from this study will be publishable data which will be disseminated through peer reviewjournals and meetings (we intend to do this after each set of experiments if appropriate).

Additionally, the output may be patentable products (identified once a a detailed analysis of the competing market has been undertaken) which can be commercialised and offered clinically to patients(once regulated studies have been completed). If a successful product is identified, it is likely to take more than five years (outside the duration of this licence) before being offered clinically.

Who or what will benefit from these outputs, and how?

The ability to supply organs or tissues for transplantation, possibly from a xenogeneic source (i.e. non,human), which will not be rejected by the host, will transform the prospects of patients who currently suffer considerable morbidity due to failure of tissue function. This will not only transform the lives andaspirations of the recipients but will also considerably reduce the financial burden on the NHS and Health Care systems.

There are currently 6,079 people waiting for a transplant in the UK and many more who are notон the waiting list would benefit from having a diseased or damaged tissue replaced or repaired.

However, the benefits from our platform technology (cell and biomaterial combination) to create functional organ/tissues are not restricted just to patients and health care providers; there are substantial benefits to the broader scientific and pharmaceutical community. The data generated is vital for the scientific arena. Both in terms of the bioengineering concepts and in providing basic informationon how cells interact with their environment and how their growth and development might be beneficially modified using existing molecular cues present on the extracellular matrix of biologically derived scaffolds.

Additionally, complex perfusion models, whereby the organ's/or tissue's vascular circulatory system areused to repopulate some organ scaffolds prior to implantation, could also be used as miniature validation models. Whilst the 3D culture systems needed to acquire sufficient numbers of different cell populations within a single system may also be used as drug screening platforms.

How will you look to maximise the outputs of this work?

Output (both positive and negative) will be maximised by:

- presenting at clinical review meetings to obtain clinically relevant feedback in order to ensure thefinal approach can be clinically adopted (e.g. is the surgical implantation approach feasible, relevant and appropriate).

- explore collaborative opportunities to better understand how to combine cells and scaffolds exvivo prior to implantation.

- Present at relevant focused meetings/ international conferences

- Publish in peer reviewed journals appropriate for the topic e.g. F1000Research

Species and numbers of animals expected to be used

- Sheep: 225
- Pigs: 375
- Minipigs: 375

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The pig's anatomy, size, diet, and physiology are similar to humans and is the most appropriatetranslational transplantation model.

Additionally, as there is potential for xenogeneic implantation for **some** of the tissues/organs clinically in the future, the donor species needs to be of similar size to humans. Hence pigs and sheep are proposed for most of our studies. Additionally, this is reflected in our de-cellularisation / re- cellularisation technology, which has been directed towards using pig (porcine) tissue/organs to createthe initial implantable biologically derived scaffolds.

De-cellularisation is the removal of cells from a tissue or organ, whilst re-cellularisation is the additionof cells to a tissue or organ in which all cells have previously been removed.

Typically, what will be done to an animal used in your project?

Animals will fall into two groups; donor or recipient

Donor animals will be used to provide the tissues or organs harvested under **terminal general anaesthesia (GA)** to create the scaffold. Organs (e.g. liver, small bowel/colon, pancreas, trachea/larynx, oesophagus, thymus, or kidneys) will be harvested with their associated vascular supply intact and patent, as this will be used to re-establish a connection to an active blood supply when the tissue scaffold is subsequently re-implanted. Donor animals will also be used to harvest multiple tissue specific cells and tissue biopsies. The harvested organs or tissue will be taken to the laband undergo a process known as de-cellularisation to create a biological acellular scaffold (ie all the cells will be removed leaving begin a collagen based extra cellular matrix).

Recipient animals under general anaesthesia (GA) will receive either
a seeded matrix/scaffold - Biological matrices will be developed using the tissue or organs harvested from the donor animals. Each matrix will be seeded with tissue specific cells or progenitors. Cells used to seed the scaffold may be autologous (from the same animal) or allogenic (different animal but same species). If Autologous, animals will undergo GA one monthprior to receiving a scaffold at which point the appropriate cells/ tissue biopsies will be harvested.These seeded matrices will be implanted (up to 6 months) without comprising the tissue or organand will provide information on how the matrices integrates with the surrounding tissue and cellular fate on a smaller scale.

a seeded organ/tissue scaffold (i.e. intact organ scaffolds) which will be implanted in situ (i.e. tissue and site specific with connected vasculature). Cells used to seed the scaffold may be autologous or allogenic. If Autologous, animals will undergo GA one month prior to receiving a scaffold at which point the appropriate cells/ tissue biopsies will be harvested.

Implanted scaffolds will be left in situ for up to 2 years and assessed for tissue/organ regeneration and integration. During this period they will be monitored in terms of potential functionality using bothimaging (e.g. endoscopy, bronchoscopy, MRI/CT/PET) and non imaging techniques (e.g. blood tests and functional assays).

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects will be different for each organ or tissue under investigation.

For **donor animals,** we expect no adverse effects as tissues/ organs will be harvested under a non-recovery terminal GA.

For **recipient animals receiving a seeded matrices,** we expect no clinical adverse effects other thanthe surgical procedure for which they will be given analgesia. Animal suffering will be kept to a minimum by regular monitoring by experienced husbandry staff.

For **recipient animals receiving a seeded scaffold,** we expect the adverse effects to be influencedby the organ/tissue being replaced. For example, animals receiving a tracheal tissue scaffold would require greater monitoring and post-operative care than animals receiving a bowel segment in a two-step procedure. In each case, and for each organ, we will have a clinical post-operative care plan based on human clinical care criteria (e.g. blood analysis) and parameters to monitor and maintain overall animal health.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Pig Moderate 100%

Sheep Moderate 100%

Mini pig 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 haveconsidered 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 this research is geared toward using animal tissues or organs to create scaffolds that areidentical to the tissue/organ that needs to be replaced in human patients. By using animal tissue/organs to produce the scaffolds, we will not compete with human tissue destined for transplantation which is already in short supply.

Which non-animal alternatives did you consider for use in this project?

A non-animal alternative animal was not considered as we require a large animal model for directclinical translation.

Substantial initial work using rodents for biocompatibility, degradation and immune response to the biological scaffolds has already been undertaken. In vitro cell culture work to assess cell-scaffold interaction has been completed. Additionally, in some cases, complex ex vivo perfusion systems andbioreactors have been used to determine how best to deliver cells to scaffold using its existing associated vasculature before in life implantation studies.

Why were they not suitable?

We require a large animal model for direct clinical translation, especially as the experiments have nowdeveloped to the point where we need to show function and efficacy in a full physiological model, and for that, there is no substitute for 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?

As our in vivo (in life) studies will aim to establish function of the seeded scaffolds and tissues, they areobservational rather than statistically driven which means we will be able to use very few animals to prove function (for example, typically no more than 6 per tissue/organ per experiment- based on previously published and peer reviewed studies and following discussion with the Regulatory Bodies (e.g. MHRA) before progressing to regulated studies.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

By assessing cell-scaffold interaction in vitro, using perfusion and bioreactor systems and conductingas much of the pre-implantation logistics on the bench by conducting detailed pilot studies, we have reduced the number of animals used.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Post-mortem tissue sharing between different projects and collaborators will also ensure maximum usage of each animal. Additionally, by conducting initial studies with seeded matrices before progressing to seeded organs, we can make iterative changes to ensure the successful outcome of theimplanted seeded organs. Thus helping to limit the number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The pig is the most appropriate animal model with regards to anatomy and physiology when comparedto humans for organ transplantation and tissue repair.

Sheep may, under certain circumstances be a better anatomical model for tracheal/laryngealimplantation.

Minipigs provide an opportunity for implanting seeded organs when assessing for long term paediatricdevelopment.

Why can't you use animals that are less sentient?

These experiments have now developed to the point where we need to show function and efficacy in aphysiological translational model.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

By acclimatising the animals to single housing prior to the initial surgery (including increased handling and interaction, accompanied by enriched environment), any adverse effects or injury to the wound sitefrom pen mates can be minimised. Post surgery, if necessary animals will continue to be single housedbut in sight of litter mates. Animals will be monitored regularly, pain relief administered (if appropriate).Detailed post-operative clinical care plans will be developed to ensure the highest standard of animal welfare.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

The use of:

- best surgical and human clinical care practice associated with organ/tissue repair and transplantation and subsequent monitoring practices
- adherence to the principles set out in the LASA (Laboratory Animal Science Association) guidingprinciples document combined with good pre- and intra- operative care and monitoring will minimise unnecessary suffering.
- The Norecopa, NC3Rs, PREPARE guidelines, and LASA (and similar animal research andwelfare) websites

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will review:

- the current literature (encompassing changes in veterinary research and human surgery, (e.g.transplantation surgery) and
- Any revisions to the regulatory guidelines along with input from thelocal Named Information Officer (NIO), Named Animal Care Welfare Officer (NACWO),
- Named Veterinary Surgeon (NVS) and other local animal care staff

As well as checking the Norecopa, NC3Rs and LASA (and similar animal research and welfare)websites and implement any changes where appropriate.

LARN webinars.

INTESTINAL IMMUNE RESPONSES

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, ortheir effects, in man, animals or plants

Key words

Intestinal immunity, infection, inflammatory bowel diseases, T lymphocytes, nutritional immunity

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We are investigating how the intestinal immune system contributes to protection against infection, yet itcan also drive autoimmune inflammatory bowel diseases. The aims of this project are to explore the crosstalk between immune cells in the gut, the intestinal lining, diet, and the normal micro-organisms that live in the gut, in health and disease, including genetic predisposition to intestinal inflammation.
We aim to discover the basic processes involved as well as identify potential new therapeutic targetsfor treating disease.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 a million people in the UK live with a serious bowel inflammatory condition, for which

treatment options are limited. The gut is also the site of nutrient absorption, thus playing a major role in metabolicdisease and obesity. Moreover, many life-threatening viruses, parasites, and bacteria cause infections in the gut. Our research aims to provide fundamental insights into how the immune system of the gut is linked with how the body responds to infection, to diet and to stress. These findings can then be leveraged to identify ways to treat infectious diseases, improve vaccination strategies, and treat or prevent inflammatory bowel diseases.

What outputs do you think you will see at the end of this project?

- The outputs will take the form of measurable outcomes, such as: High quality scientific publications in peer-reviewed journalsPresentations at international and national meetings.
- Press releases and public talks to communicate the outcomes of our research and its potentialimpact on public health.
- Specific tools and reagents for studying the gastrointestinal immune system, for example,genetically altered (GA) animal models specifically targeting the gut immune system

Longer-term outputs based on the knowledge accrued over the course of the programme and beyond include:

- targets for the design of mucosal vaccines
- targets for the treatment of autoimmune and inflammatory bowel diseases
- targets for modulating immune responses to infection
- intellectual property rights (IPR) that can lead to the development of diagnostic and therapeutictools for gastrointestinal diseases.

Who or what will benefit from these outputs, and how?

All of the above are expected to benefit the scientific community as well as the public at large. We aimto identify the roles of molecules that are genetically associated with intestinal diseases; we aim to elucidate the molecular mechanisms allowing the intestinal immune system to respond to harmful but not innocuous bugs in the gut; we aim to understand how dietary nutrients can modulate immune responses and thus make us more or less susceptible to disease. We will evaluate findings in mice fortheir applicability to human guts, thus increasing the translational potential of our research. In the longterm, this work will provide a basis for therapeutic strategies targeting inflammatory diseases of the gut.

How will you look to maximise the outputs of this work?

The outputs of this work will be discussed at international and national conferences. We will maximise the reach of our work by publishing our research on open-access archives such as BioRxiv. In addition,all peer-reviewed research published in journals will also be made open-access.

We will also publish any new methods and resources generated by the lab so that they can be used byother labs. For example, we have generated a new mouse model, which

will be very useful for studyingthe roles of a number of different immune cells. We plan to share this mouse model widely with other researchers. We also collaborate with researchers in the UK and in Europe with whom we discuss and are informed of ongoing research.

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.

For these studies, we need access to primary cells and tissues, as there are no culture systems or celllines available to study the cross-talk of the many different cell types present in the gut. Mouse models(adults) are most appropriate because their immune systems have similar complexity as the human immune system and it is very well characterized. The immune responses of mice to several oral pathogenic infections, diets, and tumour models have already been extensively characterized. The immuno-metabolic changes that occur during T cell activation have been studied in great detail in many different T cell subsets in mice, allowing me to make predictions based on the information already available. The many genetically modified strains of mice that are necessary for the execution ofthis project are also readily available, many already established in-house. On the other hand, human intestinal immune cells can only be obtained in small quantities from tissue biopsies, and are mostly taken from patients with inflammatory symptoms, thus precluding the analyses of resting ‘healthy’ cells.Moreover, experiments such as infection studies would not be possible in humans. Overall, the reagents and tools that I need to investigate immune and metabolic processes in the gut are all available for mouse studies, making this system the most useful.

Typically, what will be done to an animal used in your project?

Procedures to be carried out on the mice include tube feeding of infectious microbes, injections of immune-modulating or immune-boosting, or cell-tracing agents, irradiation and transfer of immune cellsby injection, and optical imaging; all of which are not intrinsically expected to cause harm. All the infection models utilised will be mild to moderate severity, with some diarrhoea, and will run for a maximum of 5-10 days, after which the mice will be killed humanely. Colitis and intestinal inflammation models can either be acute or chronic, and may reach moderate severity. The acute models run for a maximum of 10 days, the chronic models may run for between 8-12 weeks, and experimental drugs may be administered to try to treat the disease. Mice may also be fed diets with different nutritional content, which may affect the energy balance in the mice. Bolus injections of nutrients may also be provided. These diet modulation studies will normally run for 4 weeks, but may run for a maximum of 8 weeks. Immune-modulating agents or drugs may be injected or given orally to alter the course of dietary interventions or intestinal inflammation.

What are the expected impacts and/or adverse effects for the animals during your project?

In most studies, including infections and intestinal inflammation, mice may show signs of weight loss, ruffled fur, listlessness, and diarrhoea. Mice will be monitored closely, assessed using a local clinical scoring system, and significant deviation from normal behaviour (normally involving more than one clinical sign) will result in the mouse being immediately and humanely killed. In mice which develop chronic intestinal inflammation, the mice will be kept for the minimum time required for the scientific endpoint to be achieved. Most dietary interventions planned, where nutrients will be modulated, are not expected to cause significant deviation from normal, other than high-fat diets, where weight gain, and impaired glucose metabolism can be expected. However, these studies will normally only run for 4 weeks, long before such adverse effects would be 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 animaltype)?

Based on previous experience, infection of mice is likely to reach mild severity in >70% of mice, with only about 25-30% proceeding to moderate severity. Similarly, for injection of immunomodulatory agents and dietary interventions mild severity is expected in >90% of animals (based on our previous studies). Animals in which genetic modifications are present that lead to intestinal inflammation or induction of intestinal inflammation using chemical or biological agents will most likely experience mild severity for 30% of the animals, with the rest reaching symptoms of moderate severity. All physicalprocedures are not intrinsically expected to cause more than transient discomfort for the animals.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

The project involves studying the responses of a number of different cell types, nutrients, and microorganisms working in concert to define intestinal immune responses. No tissue culture system isyet available that can integrate all the different cell types and allow measurement of tissue responses.Moreover, many of the cell types are not yet culturable outside the gut. Therefore, in vivo studies in animals are the most appropriate here.

Which non-animal alternatives did you consider for use in this project?

We have established in vitro co-culture systems involving intestinal epithelial cells and immune cells, and are using these systems wherever possible. We use 3D primary intestinal organoids systems as well, especially for imaging studies. We also occasionally use intestinal epithelial cell lines, however nocell lines exist for the other cell types we are working with. We have also considered isolating cells from human intestinal biopsies for some measurements. However, the numbers of cells obtainable from human intestinal biopsies are too low for many analyses. In addition, these biopsies are normally taken from

patients with intestinal complaints, therefore are not really representative of healthy tissue.

Why were they not suitable?

While simplified culture systems for intestinal tissue exist, these are by definition, simplified, and therefore do not have all the cell types present in the gut. Moreover, no cell culture systems exist for some of the cell types we have considered, and they do not exist in lower vertebrates. Another important consideration is that the interactions between the gut and the micro-organisms that normallylive in it, and the effects of dietary nutrients cannot be modelled in non-animal systems. Therefore, these systems can only replace animal studies in a small part of our research. For human cells, the numbers of cells obtainable from human intestinal biopsies are too low for many analyses. In addition,these biopsies are normally taken from patients with intestinal complaints, therefore are not representative of healthy tissue.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on our previous usage and experience, our current funding levels, and anticipating an increasein the number of people working under my supervision on this project licence, I have estimated the number of mice.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The numbers of animals to be used on this project are based on refined estimates from our prior experience in these models, and on optimised data collections that allow maximum information to be obtained from the minimum number of animals. We have consulted with the NVS and other local experts on best design of experiments. The experimental design follows best practice guides includingthe ARRIVE guidelines and the NC3Rs Experimental Design Assistant. Where pilot data does not exist, numbers have been estimated based on published studies.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Efficient breeding practices will be utilised to reduce animal numbers, taking into account the recommendations on colony management from the NC3Rs. The minimum number of mice required to show scientifically and biologically significant data will be used. As experiments may be started over a period of days, a randomised block design will be implemented, whereby animals are randomised to treatment type and order of dosing on any given day. This will reduce experimental variability, and thusanimal numbers. Longitudinal studies on the same mouse will be performed using non-invasive imaging studies and faecal analyses to follow infection or development of intestinal inflammation in theanimal, reducing numbers. We will also use specialised techniques for metabolic

measurements in mice to study the animals while conscious and conduct longitudinal analyses wherever possible to maximise the data acquired from each animal. In all experiments, once the animal has been killed, extra tissue pieces, faeces and intestinal lumenal contents will be collected when possible, and archived for subsequent analyses, such as RNA/DNA sequencing, imaging, proteomics, etc, to maximise the information that can be obtained from each experiment, minimising the need to repeat experiments for different analyses.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

For these studies, mouse models are most appropriate because their immune system has similar complexity as the human immune system and it is very well characterized. The immune responses of mice to several oral pathogenic infections and intestinal inflammation models have already been extensively characterized, particularly in terms of the pain and suffering experienced, allowing me to make predictions based on the information already available. Oral gavage of pathogens mimics the natural route of infection, and will only be performed by well-trained staff, minimizing pain and distress. Similarly, injection of immunomodulatory agents are all well-established and will be through the optimised route. Wherever possible, immunomodulatory or labelling agents or dietary nutrients will be introduced into the diet or drinking water, causing the least distress to the animals. We will use previous work to determine appropriate clinical scoring systems that will allow us to identify the earliestpossible humane endpoints.

Why can’t you use animals that are less sentient?

Mice have a similar physiological response to infections, dietary alterations, and inflammatory boweldiseases as humans. Less sentient animals do not share these similarities and lack the immune celltypes that we are investigating.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

For each experiment, a specific study plan will be drawn up detailing the rationale for the experiment and detailing the monitoring and specific endpoints to be used. These will be agreed in conjunction with the named vet (NVS) and will use the minimum severity possible in order to address the scientifichypothesis to be tested. The choice of dose and route of infection will be made to mimic the natural progression of infection, but with the lowest degree of clinical signs. Pilot studies will be performed as appropriate to help to minimise the risk of unexpected responses in lines of genetically altered animalsthat have not been used before so that the severity of further studies can be minimised. Monitoring during intestinal infection is performed at least daily and then at least twice per day as the infection progresses. Weight changes are also monitored closely and will be compared

with established weight charts for animals of similar age, gender and genetic background, as well as being compared to changes seen in other similar studies, so that we can detect animals that are responding badly to infection as soon as possible.

We will also use the services of the animal facilities, that have highly competent members to either perform the techniques themselves or carry out training of staff in regulated procedures, minimising welfare costs. We also rely on the skilled personnel in the animal facilities for routine breeding and maintenance of genetically altered (GA) animals, and for assistance in some procedures. In addition, we will have access to dedicated staff, shared with other investigators to assist with animal husbandry,genotyping and strict adherence to 3R principles.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Best practice guidance for experiments will be acquired from discussions with local and national experts, the Research Animal Training online programme, the NC3Rs and other appropriate websites.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We have regular discussions with other local users and authorities where the latest best practices arehighlighted and discussed. Our local animal users' group is kept updated with recent advances. I will also attend webinars and workshops organised by NC3Rs or other sources to keep myself up to date.

INVESTIGATING CELL COMMUNICATION IN SALIVARY GLAND REGENERATION

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Salivary glands, Regeneration, Stem cells, Cancer

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to understand how the different cells of the salivary gland communicate and co-operate during normal cell turnover (to replace older cells) and following injury (inorder to replace damaged or dead cells). The majority of patients who are diagnosed with head and neck cancer are offered radiotherapy to treat the cancer. While largely effective at targeting the tumour,patients are often left with the side-effects radiotherapy has on other organs in the vicinity, including thesalivary glands. In addition, many patients undergo surgical resections of the salivary glands during treatment. There is currently no treatment for this collateral damage. By better understanding cellular communication and co-operation in the salivary gland we can find which cells to target to promote regeneration.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Head and neck cancer is the 8th most common cancer in the UK and the 7th most common cancer worldwide, with 900,000 cases worldwide every year. The primary treatment for head and neck canceris radiotherapy and more than 75% of patients undergo radiotherapy during their treatment. While thisis a largely effective treatment at targeting the cancer other organs within the region are also inadvertently irradiated, leading to injury and dysfunction. The salivary glands are one of the most adversely affected organs, and injury leads to a loss of saliva production or a change in saliva composition in the majority of patients. This impacts on speaking, eating and sleeping, and leads for poor mouth health, tooth decay and infections. Collectively, this severely impacts patient quality of life.There is currently no cure for irradiation-induced salivary gland injury and patients rely on short-term treatments, such as saliva substitutes or constantly sipping water. However, these short-term treatments do not recapitulate the protective features of saliva and are inadequate. Furthermore, the cost of these palliative treatments exceeds £1 billion/year in the UK. To date no permanent therapy exists, signifying the need for alternative approaches. Regeneration of the damaged organ offers a way that salivary gland function may be restored in head and neck cancer patients.

We, and others, have previously demonstrated that progenitor cells (immature cells that can make newfunctional and mature cells) exist within the salivary glands and these cells can make new cells when provided with the right cues. These cues come from nerves and blood vessels that surround the gland and other cells within the gland. When these cues are disrupted, in the case of injury after radiotherapy, the progenitor cells can no longer replace the damaged tissue and this leads to a dysfunctional organ. In particular, we have demonstrated that signals from the nerves are vital in maintaining progenitor cells and that replacing these signals can reactivate progenitor cells to replace damaged cells.

Furthermore, we have shown that macrophages, a type of immune cell, are the predominant immune cell in the salivary gland, and are vitally important in removing damaged and dead cells after radiotherapy. Macrophages are known to play a positive role in the regeneration of other organs and macrophage therapy is now a viable treatment option for liver disease. We know that macrophages communicate with progenitor cells in other organs and that without macrophages these organs cannotregenerate. However, we are yet to find out if macrophages also communicate with progenitor cells inthe salivary gland and if this is important for cell replacement and regeneration.

Collectively, understanding how the different cells of the salivary gland co-operate will lead to long-term, permanent treatments that can vastly improve health and quality of life.

What outputs do you think you will see at the end of this project?

The main outputs that I expect to have at the end of this project are:
New datasets detailing specific cell types that are important in the salivary gland. We will makethese datasets freely available to the research community once the data has been published.

New information on how cells communicate and co-operate in the salivary gland and what happenswhen a particular cell is missing or damaged.

Identification of a novel drug, gene or cell therapy that has a significant impact on salivary glandregeneration in a pre-clinical model

Peer-reviewed publications detailing these findings

Further research funding arising from these findings

Who or what will benefit from these outputs, and how?

In the short term, datasets will be made publicly available for the use of other researchers, in both the UK and worldwide. This would therefore benefit not only those working in salivary gland research, bothalso those working in immunology and regenerative medicine as a whole. Protocols and published knowledge from this project will be of benefit to a range of other researchers working in all these fields,and will overall inform future work. The results of this project will also have broad implications for both fundamental and clinical research in other organs, such as the intestine, skin and lungs, where cellular communication is essential in regeneration. In many of these areas regenerative medicine potentially offers an important way forward, and as such data from this project will be of considerable relevance.

In the longer term, this project will generate data that will lead to important future studies investigating whether targeting different cells in the salivary gland can promote regeneration. If this were to succeedit could eliminate the need for salivary replacements for a lifetime for all patients suffering from salivarygland dysfunction. In addition to the 900,000 head and neck cancer patients diagnosed every year, such a therapy could also impact others who suffer from salivary gland dysfunction (including the 2 million people living worldwide with the autoimmune disease Sjogren’s Syndrome). This has the potential to save over £1 billion/year as well as achieving for every patient a vast improvement in quality of life.

How will you look to maximise the outputs of this work?

In order to maximise the outputs of this research we will:

Publish our findings in a timely manner in Open Access journals

Present our findings at scientific conferences and meetings

Present findings via social media accounts (personal, group, institution) to ensure patients andthe public hear about progress

Disseminate findings through partner patient groups

Seek to press release important results to the media

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To better understand how regeneration occurs requires studying adult organisms, since earlier life stages have a different regenerative capacity. In addition, in order to fully understand the cells and thegenes that are involved in these processes requires the use of genetically modified mice. Since organ damage and regeneration both involve the interaction between a multitude of different cell types (nerves, epithelial tissue, blood vessels, inflammatory cells) recapitulating this in vitro does not providea faithful representation of the effects. Thus, alternatives to in vivo experiments are not possible.

Ex vivo experiments are used in place of some in vivo experiments. My research group members and Ihave developed a number of ex vivo culture assays where tissue explants or slices are irradiated and manipulated in culture, and I have demonstrated how closely such assays reflect the in vivo situation. Tissue for ex vivo experiments is collected from mice sacrificed using a humane killing method having not previously undergone any regulated procedure. No established in vitro cell culture assays exist thatfaithfully recapitulate epithelial repair/regeneration in primary tissue obtained from mice, largely due to the complexity and numerous cell types involved.

Human tissue (collected from surgical procedures and surplus, to be discarded) replaces some mouseex vivo experiments.

The models used in this project will be reviewed at regular intervals and the possibilities of incorporating alternatives will be addressed.

Typically, what will be done to an animal used in your project?

Mice will be used for breeding and colony expansion.

Mice will sometimes be injected with drugs. In some cases, these drugs will delete a gene or celltype. In some circumstances the deletion of a gene or cell may result in changes in the function of the salivary gland.

Some mice will also undergo salivary flow measurements as a measurement of function.

In order to study regeneration following injury some mice will undergo irradiation of the head andneck region, which will mimic radiation treatment that patients have for head and neck cancer.
The rest of the body will be lead shielded as protection. Other mice will undergo surgical injury ofthe salivary gland or cell transplants into the gland.

In order to study the role of nerves in regeneration some mice will undergo surgical or chemicalimpairment of the nerve supply to the salivary glands.

Animals will be humanely killed at the end of the 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 effects are expected from breeding any genetically modified mice and/or the techniques used for general husbandry. However, if the deletion of a cell or gene by breedinga new combination of genetically modified mice results in a change to the salivary glands mice may exhibit changes to saliva production. Mice will be monitored for ill health and will be humanely killed if a humane endpoint is reached.

The injection of drugs will result in minimal and transient discomfort and we do not expect anyadverse effects.

When mice have saliva flow measured, we first administer a drug which stimulates saliva production, due to the small volume of saliva produced normally. This drug also activates the gutand can lead to transient diarrhoea. Mice will be monitored for ill health and will be humanely killed if a humane endpoint is reached.

Dry mouth/dry eye may occur following glandular or nerve damage (radiation, surgery) and willbe alleviated with wet food/eye drops. Mild irritation and/or inflammation of the surgical wound site may occur. Mice will be monitored for ill health and will be humanely killed if a humane endpoint is reached.

Mice will take a little time to come round from anaesthesia. They will be kept warm in order torecover quickly and will be monitored for signs of distress or ill health. Mice will be humanely killed if a humane endpoint is reached.

Mice may experience some pain and discomfort after surgical procedures. In order to preventpain mice with be given pain relief at regular intervals

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

All animals used under breeding and maintenance and assessment of genetic alteration would beexpected to be of a mild severity.

All animals that undergo irradiation, surgery or cell transplant would be expected to be of a moderateseverity.

It is estimated that overall in this project 80% of animals will experience mild severity, and 20% willexperience 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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Since organ damage and regeneration both involve the interaction between a multitude of different celltypes (nerves, epithelial tissue, blood vessels, inflammatory cells) recapitulating this in vitro does not provide a faithful representation of the effects. Thus, alternatives to in vivo experiments are not possible.

Which non-animal alternatives did you consider for use in this project?

Some molecular pathways and cellular response to stimuli can be tested in in vitro assays. However, no established in vitro cell culture assays exist that faithfully recapitulate epithelial repair/regeneration.

Human tissue biopsies are used whenever possible in order to test the significance of our findings forhuman relevance.

Why were they not suitable?

In vitro cell culture assays do not recapitulate epithelial repair/regeneration in vivo, largely due to thecomplexity and numerous cell types involved. Human tissue biopsies, while informative, can only model the complex organ environment for a shortperiod out of the body.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 progress on the previous PPL and are based upon theplanned experiments for the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We will utilise experimental design tools, such as the NC3Rs Experimental Design Assistant, inorder to design robust and meaningful experiments and to reduce the number of animals required for the project.

Mice will be randomly assigned into groups and samples will be labelled such that staff assessingthe effects of the treatment and analysing the data will be unaware of treatment groups.

Decoding will be provided only after experimental endpoints have been measured. This ensuresthat we are performing high quality, non-biased research.

All members of the team will make use of an in-house Research Optimisation Course which focuses on rigorous design, conduct, analysis and reporting of research using animals and TheCollaborative Approach to Meta Analysis and Review of Animal Experimental Studies (CAMARADES) team for training and advice.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

The project will optimise colony management in order to reduce surplus breeding.

We will undertake pilot studies first when investigating a new intervention or model, in order todetermine the most appropriate and meaningful parameters (e.g. dose of drug, timepoint of analysis).

We will share or archive tissues from our animals whenever possible, in turn reducing the needfor multiple experiments for other tissues of interest. The project will also make use of collaborative colonies to share tissue as a resource. We actively use historical tissue/samples which allows new avenues to be pursued without theuse of new animals.

The project will use both male and female mice and powerful statistical analysis methods in orderto maximise the amount and quality of information obtained from each animal.

We will freeze down sperm when the line is no longer being used

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used in this project. Mouse models provide essential tools to specifically and temporally target specific cells of interest and the molecules that may be involved in the bidirectional crosstalk between these cells (e.g. Cre and loxP model systems).

In some studies we will simply look at what happens in the salivary gland when a cell or gene is labelled or deleted, using genetically-modified mice. No adverse or harmful effects are expectedin the genetically-modified mice. The use of tissue-specific (i.e. salivary gland epithelia, macrophage, nerve) deletions will avoid whole-body genetic alterations which may have harmfuleffects (for example, on the brain or heart). Furthermore, data obtained from such models has been shown to be relevant to human salivary gland injury.

For drug/substance administration we will use the most appropriate route which causes the leastanimal distress/suffering.

In some studies we will induce salivary gland radiation injury in mice, similar to what patients experience. This is the only way to study this type of injury in genetically-modified mice. However,we have refined all our studies to ensure that the injury is the most minor it can be while still mirroring what human patients experience.

Similarly, when undertaking surgical procedures, we ensure that the operation is as minor as possible while achieving the purpose of the procedure (i.e. cutting a nerve, removing a piece ofsalivary gland).

Why can’t you use animals that are less sentient?

To better understand how regeneration occurs requires studying adult organisms, since

earlierlife stages have a different regenerative capacity. Less sentient species of animals (such as zebrafish or fruitflies) either do not have salivaryglands or have salivary glands that are very different in structure and function to those in mammals, meaning they are not a good model.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Wherever possible we will use tissue-specific (i.e. salivary gland epithelia, macrophage, nerve)gene/cell deletions to avoid whole-body genetic alterations which may have harmful effects.

My research group members and I have developed a number of ex vivo culture assays where tissue explants or slices are irradiated and manipulated in culture, and I have demonstrated howclosely such assays reflect the in vivo situation. Tissue for ex vivo experiments is collected from mice sacrificed using a humane killing method having not previously undergone any regulated procedure or having undergone injections to induce gene/cell labelling or deletion only.

When irradiating the salivary glands (i.e. the neck) the rest of the body and head of the mouse will be lead-shielded to prevent whole-body effects and injury elsewhere. Furthermore, we havedemonstrated that a single dose of gamma irradiation to the salivary glands in mice elicits the same tissue injury as fractionated low dose irradiation (as experienced by patients during radiotherapy) and as such we have adopted this as a refinement in our mouse studies.

Following surgical procedures mice will be given appropriate painkillers/anti-inflammatory medication.

Wet food will be provided following irradiation or surgery to alleviate symptoms of dry mouth andsalivary dysfunction.

Mice will be checked routinely post-operatively and throughout the study for symptoms of pain/discomfort. Mice will be humanely killed if any health-related issues arise that cannot beimmediately treated.

For certain procedures (e.g. saliva measurements) we will train the animals beforehand so they get used to the method, before taking measurements. This allows the animals to get used to thehandling and apparatus, and reduces distress during measurement-taking.

All mice will be provided with appropriate housing that allows expression of normal behaviour,including but not limited to, places to hide and climb and nesting material. Mice will be housedtogether unless circumstances such as aggressive behaviour and fighting do not allow it.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

When possible, we use published best practice guidelines in our experiments. We use Standard Operating Procedures (SOPs) for all procedures and ensure that all members of the team follow theseduring training and experiments to ensure best practice.

How will you stay informed about advances in the 3Rs, and implement these

advanceseffectively, during the project?

We will stay informed about advances in the 3Rs by receiving the NC3Rs newsletter, and by attendingour establishment's annual 3Rs day. All members of the team will attend the PIL refresher course annually, which updates on important new methods, such as handling and needle use.

INVESTIGATING MECHANISMS INVOLVED IN PAEDIATRIC AND ADULT ACUTE LEUKAEMIA

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Acute leukaemia, blood cancer, therapy, leukaemia stem cells, tumour microenvironment

Animal types	Life stages
Mice	adult, pregnant, neonate, embryo, juvenile, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description 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 deeper knowledge of the mechanisms that regulate blood cancer inchildren and adults. In this way we will identify weaknesses in blood cancer cells that can be used to generate new treatments for disease appropriate for both children and adults. This knowledge will enable us to enhance current treatments options of patients with the development of new drugs and/ornew drug combinations.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Acute leukaemia of the myeloid and lymphoid types (AML and ALL) are aggressive types

of blood cancer. Whilst ALL is predominantly a childhood disease, AML occurs in both children and adults. Theincidence of AML is highest in the aged, and prognosis is worse (~5-15% overall survival) with increasing age. In children, the incidence is highest in infants (<1year) and this age group have the lowest survivorship. One in three children diagnosed with AML will die. Clinical trials in children are difficult due to low numbers and are normally designed based on adult data. The disease in children and adults has significant differences which need to be considered for novel treatments and improve outcomes in both children and adults. New treatments are urgently sought that are appropriate for theage of the patient.

What outputs do you think you will see at the end of this project?

This project aims to find out what makes acute leukaemia in children different from acute leukaemia inadults and figure out how best to treat the disease in the different age groups. These results will be made available to doctors and scientists by publishing in high-quality cancer and haematology (blood cell) journals, and sharing unpublished data at conferences in the form of abstracts and poster/oral presentations.

Who or what will benefit from these outputs, and how?

The results of this project will benefit children and adult patients with blood cancer by aiding in the development of kinder and more effective treatments, and discovering new markers (biomarkers) to help clinicians identify those patients that will respond to specific therapies. Understanding the diseasebiology specifically in children and adults will teach us similarities and differences in the disease and help to focus targeted therapies towards appropriate patient populations, establishing a personalized care pathway, and deliver quality adjusted life years for patients, through the reduction of disease burden. Identifying new drug targets that are age-relevant will permit the delivery of clinical trials topatients.

How will you look to maximise the outputs of this work?

The results of this work will be discussed at scientific meetings with other experts in the field. Any scientific data generated from the experiments will be made available to other scientists after they havebeen published. We will collaborate with other researchers and will share our knowledge and expertiseas much as possible.

Species and numbers of animals expected to be used

- Mice: 7500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our previous work has shown that if we give mice leukaemia, either by transplanting mice with mouse cells engineered to replicate acute leukaemia, or patient cells isolated from leukaemia patients, these cells grow and spread around the mouse body in a very similar way to the human disease. Using thesemice with leukaemia, we have already discovered important markers on the surface of leukaemia cells that can help separate normal cells

from leukaemic cells, thus providing an improved approach to determine disease levels in patients. Furthermore we have used these mice to identify important genesthat might be responsible for keeping the leukaemia cells alive, and further demonstrated that these genes are also important in patients with leukaemia. We have used mice at different ages to show that the biology of the disease is different depending on age. This means that mice are very good "model systems" for studying leukaemia and how it will respond to treatment in different age groups. We typically use prenatal fetus, neonate mice (1-2 days old), young mice (usually 6-8 weeks old) and adultmice (usually 20 weeks old) in these experiments. As acute leukaemia is more generally a disease of the elderly, we sometimes transplant these mice with blood cells isolated from older (up to 24 months old) mice. In addition, in some experiments we will use older (up to 15 months old) mice to allow us to look at the impact of aging on leukaemia development. As AML in children is most commonly seen in infants, we sometimes use neonates to look at the impact of leukaemia at very early age.
Understanding these differences will allow us to develop more personalised treatments for patients.

Typically, what will be done to an animal used in your project?

The project covers a broad range of techniques, with around 60% of mice used for breeding and maintenance purposes, generating the mice required for experimental purposes. This includes genetically altered (GA) mice that develop leukaemia due to the genetic mutations they carry. Of the remaining 40% of mice, 5% will be used to generate tissue, 5% will be aged or placed on special diets,and the remaining 30% will be transplanted with leukaemia cells.

For transplants, 30% of the time we will use mice with a defective immune system that are injected with human leukaemia cells and 70% of the time we will use mice with a functioning immune system(WT and GA) that are injected with mouse leukaemia cells (called xenograft experiments).

For xenograft experiments, the preferred method for conditioning the mice for receiving the cells is treatment with an agent called busulfan (1,4-Butanediol dimethanesulfonate, a myeloablative alkylatingdrug) instead of irradiation. We have optimised the protocol for using busulfan injections in the strains and ages of mice in this project. For busulfan conditioning, the mice may receive busulfan prior to being injected with human or murine leukaemia cells in a vein. In weaned mice this will be a tail vein, inneonates this will be superficial vein - this is relatively painless and quick. In some cases we may needto use a non-lethal dose of irradiation to condition the mice (50% of a lethal dose) prior to being injected with mouse or human leukaemia cells. This is done on weaned animals only as neonates do not withstand irradiation well. Pregnant females are treated with busulfan for neonate conditioning. Theconditioning treatment makes room in the bone marrow of the mice for the transplanted cells, improving engraftment of the cells similar to the conditioning that human patients receive prior to a bone marrow transplant in the clinic. In some cases (10% of xenografts) the mice will be put under anaesthetic to allow us to inject the leukaemia cells directly into the bone marrow using a fine needle inserted into the leg bone - they will be given painkillers to relieve any discomfort which usually lasts less than a day. In both cases, the leukaemia cells grow in the mice and spread around the body. The mice will have regular health checks (at least twice a week) and may undergo blood tests and possibly total body imaging under sedation to look at how fast the leukaemia is growing. These tests are usuallydone once a week. Depending on the leukaemia progression, the mice will start to appear unwell between 2-40 weeks. At this point we will terminate the experiment to prevent suffering.

For 60% of all transplanted mice, we will try out new treatments to see if they can get rid of the leukaemia or slow its growth. Treatments can be given by injection, by mouth or added to drinkingwater or food and will typically last up to 4 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Myeloablative busulfan treatment of pregnant mothers causes:

- Increased risk that include early delivery of pups and rejection of newborn pups.
- Increased risk of infection
- Irradiation causes:
- Weight loss, diarrhoea at the beginning of week 2, which resolves by around the end of week 3 postirradiation.
- Increased risk of Infection

Tooth growth in specific GA mouse strains Leukaemia causes:

- Low blood counts (anaemia) - causing reduced activity and tiredness - this lasts 1-2 weeks onaverage
- Increased risk of infection
- Weight loss - this is seen in the last 1-2 weeks of the illness
- Small tumour formation can occur throughout the body (e.g. in lymph nodes, liver and spleen) in thelater stages of the disease.
- Treatments may also cause side-effects such as loss of appetite, diarrhoea or fur loss
- Injection of leukaemia cells directly into the bone marrow can cause discomfort which usually lasts lessthan a day and is helped by painkillers.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

- Mild severity (causing no more than momentary discomfort) - 50%
- Moderate severity (discomfort lasting no longer than 72 hours on any occasion) - 50%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is difficult clinically to study and compare the disease in children and adults. We therefore rely on pre-clinical models in the laboratory to give us the information. Mouse

models have provided enormousvalue and progress in designing better treatments. To develop new drugs that are age-relevant we need to understand how leukaemia cells behave in different environments (age-relevant) within the body. We are currently unable to recreate these complex biological environments (comprising multiple different cell types, nutrients and blood supply) in test-tubes or tissue culture flasks. By using mouse models, we can not only identify molecules responsible for leukaemia maintenance or therapy resistance but also modify the leukaemia cells using genetic approaches and/or test new drugs to see if this can be overcome. These are essential to develop new clinic-ready therapies for patients withleukaemia and other blood cancers.

Which non-animal alternatives did you consider for use in this project?

All preliminary work (such as testing whether drugs kill leukaemia cells and what dose to use) will be done in the laboratory with cells grown in culture, enabling us to rationalise which experiments to carryforward into mice. Since we need to study cells taken directly from different sites around the body we have two alternatives - animal models or patient samples taken from the bone marrow and peripheral blood. We have permission to use patient samples and use these when they can address our researchquestion.

Why were they not suitable?

Cell culture: Our central aim is to understand how leukaemic cells at different ages behave in differentenvironments within the body and how treatments are affected by these different ages and environments. We are currently unable to recreate these complex biological environments (comprisingmultiple different cell types, supporting matrix, nutrients and blood supply) accurately in test-tubes or tissue culture flasks.

Patient samples: While we do use patient samples, patient material is often limited and generally doesnot survive or grow once outside the body. Importantly, we cannot test experimental therapies directlyin humans and cannot genetically alter leukaemia cells and inject them into patients.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 active researchers we are routinely exposed to new experimental approaches and will incorporate them into our future research programme if they prove to be as reliable and robust as the proposed animal experiments. In this way we will endeavour to reduce the number of mice that are proposed foruse in this project licence.

I have used statistical methods to determine that I will require around 5-7 mice per experimental groupand typically experiments will compare 3 or 4 groups. Based on our current funded work and planned future work we will require 1,500 mice per year to perform our research.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

I have used both expertise from statisticians and the NC3Rs' Experimental Design Assistant. We haveconsiderable experience in carrying out the protocols described and will gain the maximal amount of scientific information from an appropriate number of mice, using serial non-invasive imaging and/or blood sampling of cells from the mouse during disease development and/or treatment to gain a detailed picture of the blood cancer developing in the mouse. Therefore data will not just be generatedat the endpoint, but throughout the course of the experiment.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

I will employ the most efficient breeding strategy at all times. Ongoing monitoring of a given cohort (mouse group) allows experiments to be stopped as soon as there is enough data (information), thusminimising suffering whilst obtaining meaningful and publishable results. For any new treatments wewill always treat a small pilot cohort to estimate the effect size before performing the full experiment. When possible control mouse cohorts can be shared across studies, to reduce mouse numbers required for individual studies. We always maximise all the information we get from each mouse.

During the experiment blood sampling and non-invasive imaging techniques will be carried out to obtain as much data from a smaller number of animals. At the end of the experiment we will analyse asmany tissues as possible per mouse by harvesting spleen, bone marrow, lymph nodes, and blood and sharing this tissue between different members of our research group working on different projects.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use a variety of mouse models in this project.

We have all but switched from using irradiation to using the agent busulfan for myeloablative conditioning of the mice for transplantation. This method is a refinement to the transplantation experiments, to minimise animal suffering and reduce severity risks, in order to address the impact of age in leukaemia disease.

The use of busulfan in pregnant females as a method of preconditioning neonate animals prompted this refinement, as younger animals do not withstand irradiation as well as older adult mice. Irradiation will only be used in models where we have tried, tested and deemed busulfan to not be appropriate, or instances where scientific replications are necessary. Busulfan is nottoxic at the dose and scheduling we are using, as the host bone marrow

microenvironment is not/minimally affected, and there remains host blood cells to combat the risk of infection associated with all myeloablative treatments.

Some of our models have been developed to be more susceptible to developing leukaemia and therefore we can study them without having to expose the mice to any myeloablation and will do thiswhen possible.

Other models have markers that are identifiable by luminescent/fluorescent imaging of the mice, thusallowing more straightforward investigation of processes within the organ(s) of interest without the need for additional invasive procedures. All these models were developed to cause the least pain, suffering or distress possible while providing us with valuable data on the disease of interest.

Furthermore, we have introduced detailed methods of recording the health status of animals undergoing procedures which enables changes in their well being to be detected earlier in the diseaseprocess.

Why can’t you use animals that are less sentient?

Mice are still sufficiently closely related to human beings to adequately represent the human condition, whereas other model organisms more distantly related would not replicate the disease sufficiently (on agenetic and disease characteristics level).

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Animal monitoring, post-operative care, and pain management are an important part of all the procedures within the project. The need to minimise suffering is always considered when planning experiments. None of our experiments exceed a moderate severity level. All mice on experimental procedure will be frequently monitored (a minimum of 2 x weekly) and humanely culled when exhibitingsigns of altered health status and/or tumour burden or another specified endpoint is reached. We have developed a stringent distress scoring system that allows an immediate identification of mice with adverse effects. All researchers working on this project will undergo specific hands-on training in monitoring leukaemia development and health status in our models using our monitoring forms and health status check forms. We will refer to the literature to identify any likely adverse effects of a new agent and when a genetic cohort is given a treatment for the first time, pilot studies will be carried out and closely monitored before extending to a larger cohort. All animals are housed in a dedicated facilityproactive with environmental enrichment and the use of anaesthesia and analgesia under guidance from the named vet is routine practice.

To minimise infections, immunocompromised mice will be housed in barrier caging under sterile conditions and handled in sterile cabinet that protects the mousefrom infections. Post-mortems are carried out to investigate any unexpected deaths.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

For all studies we will refer to the Guidelines for the welfare and use of animals in cancer research(Workman et al, 2010) and ensure best working practice. We consult the NC3Rs guidelines and monitor refinement when practice advances are published (https://www.nc3rs.org.uk/).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

All our work observes government guidelines and we adopt new guidance, from the Home Office or ourlocal AWERB, as soon as these are recommended. We are also actively involved in our establishments 3Rs Day, and there are a number of Culture of Care events organised throughout the year to ensure everyone stays up to date with current guidelines. I also routinely check the following website:

https://www.nc3rs.org.uk/ for updates and will address any updates at our weekly lab meeting and disseminate this information to all personal licence holders working on the project.

INVESTIGATING THE NICHE OF MUCOSAL PARASITIC NEMATODES AND THEIR INTERACTIONS WITH THEIR HOSTS

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Whipworms, Infection, Host-parasite interactions, Immunology, Gut
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 notrequired.

Objectives and benefits

Description 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 mucosal parasitic nematodes, specifically whipworms,invade, colonise and persist in their hosts gut.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Whipworms are parasites that live inside the gut lining of 500 million people worldwide causing the disease Trichuriasis (also known as whipworm Infection). Whipworms remain in their host for years by interacting with the gut lining and surrounding cells to manipulate gut structure and immune responses.How the parasite mediates these interactions is not understood. There are no vaccines available and current drugs are ineffective to cure the

disease. Knowledge generated from this work will open new avenues to eradicate whipworm infections and control gut inflammatory diseases.

What outputs do you think you will see at the end of this project?

By the end of this project, we will have new information on the host-whipworm relationship, including:

(1) how the parasite efficiently invades, colonises and persists in the gut,
(2) how the host responds tothis infection,
(3) how the gut lining adapts and repairs over chronic infections.

These findings will be published in peer-reviewed scientific journals following the ARRIVE 2.0 guidelines (a checklist of recommendations to improve the reporting of research involving animals).Moreover, these results will be presented at local, national and international scientific meetings.

Finally, the data obtained in this project will support future funding applications.

Who or what will benefit from these outputs, and how?

A better understanding of how whipworms invade, colonise and persist in the gut, and how the gut repairs during chronic infection will be of considerable value to the wider helminthology and intestinalimmunology research community within the time-frame of this five year project.

Findings on how whipworm invade and colonise the gut will guide future studies on how the parasitesenses the gut lining cells and on the pathways it uses to invade them. In the long term, this knowledge will help to develop vaccines and to discover drugs that are desperately needed to effectively and efficiently control whipworm infections at endemic areas in the tropics and subtropics.Whipworms infect 500 million people worldwide, particularly affecting children 5-15 years of age.
Whipworm infections contribute to the vicious cycle of poverty and neglected tropical infectious diseases that prevent the economical development of endemic areas. Eradicating parasitic worm infections will help to break this cycle and positively impact the health and economy of those regions.

Data on how the gut lining adapts and repairs over chronic infections will lead to investigations on howthese adaptations affect: 1) the establishment of subsequent whipworm infections; and 2) predispose to gut cancer. Moreover, these findings will yield broader insights into the mechanisms of gut lining repair upon damage that could be used to better understand other gut inflammatory diseases and provide new avenues towards controlling them. Thus, in the long term, this work could result in the development of new treatments for diseases such as Inflammatory Bowel Disease, which has an incidence of 6.8 million cases globally.

How will you look to maximise the outputs of this work?

To maximise the outputs of this work, I will:

- Collaborate with researchers in national and international institutions, who will provide materials andknow-how that will complement this research project.

Disseminate openly and promptly the data, methods and results (including both successful and unsuccessful approaches) of this project to the scientific community via:

1) sharing data in open access online databases;
2) publications in leading international peer-reviewed scientific journals;
3) presentations in local, national and international scientific conferences.

Participation on conferences will lead to the development of new collaborations in which the know-how and knowledge generated inthis project will be shared with others to tackle important questions focused on the interactions other parasitic worms with their hosts and their impact on tissue repair and immunomodulation.

Species and numbers of animals expected to be used

- Mice: 5830

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Currently, there is no laboratory model to study the human whipworm (*Trichuris trichiura*). Therefore, for our studies we use a mouse model of infection with the natural rodent-infecting species *T. muris.* This is a good and widely accepted model for this research as it closely mirrors infections in humans.

We infect adult mice (6-8 weeks old), both wild type and genetically altered animals, where the immunesystem has completely developed.

Typically, what will be done to an animal used in your project?

Genetically altered mice will be bred in order to study the function of intestinal epithelial, immune and stromal (connective tissue) cells and to label and visualise these populations in the tissue. These micemay require a fresh tissue sample taken for confirmation of their genetic make-up (genotype) by ear punching (commonly 2 mm in diameter). This is not expected to cause lasting harm.

Wild type and genetically altered mice will be infected via ingestion (oral gavage, OG) of mouse whipworm (*T. muris)* eggs or larvae. A high dose (200-3000 eggs/larvae) recreates an acute infection,where mice expel the worms after 2 weeks, and a low dose (20 eggs) recreates a chronic infection, where mice remain infected for months. Mice will be infected for different times (up to 10 weeks) depending on the experimental question.

In some experiments mice will be administered orally (OG) or injected into the abdomen, muscle, veinor under the skin (intraperitoneally (IP), intramuscular (IM), intravenously (IV) or subcutaneously (SC))with molecules, drugs or cells that activate or suppress the immune response, to evaluate the role of specific mediators during infection.

In some experiments, mice will be administered in diet or drinking water, orally (OG), or injected into the abdomen or under the skin (intraperitoneally (IP) or subcutaneously (SC))

with substances to induce or delete genes in genetically altered mice, to label and visualise cells in the tissue and analysethe fate of their daughter cells.

In some experiments mice will be given drugs to kill the whipworms via ingestion (OG).

In other experiments, mice will be exposed to radiation (irradiated) to deplete their immune system andreplace it with immune cells from another mouse strain, to understand the importance on genes in different cellular populations during infection.

In some cases blood and/or faeces will be sampled to measure immune response in the blood over thecourse of the infection and changes in the faecal microbiota, respectively. Faeces will be collected by scruffing the mouse or placing it on an empty or shallow container for sufficient time (maximum 5 minutes) to allow the mouse to defecate.

Mice will be subjected to non-recovery anaesthesia, under which the mice remain deeply unconscious,in experiments where blood will be collected from the heart (via cardial puncture). Mice will be humanely killed immediately following the cardiac puncture procedure.

Mouse gut lining, immune, stromal cells and whipworm responses during infection will be assessed bydoing an extensive study of mouse organs after the animals are killed at the end of each experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

Expected adverse effects for the mice during this project would include:

Wasting disease with hunched body posture, ruffled fur, weight loss and diarrhoea that occurs after19 days of infection. However, these symptoms (phenotypes) are rare and in cases where this is observed, mice will be closely monitored to ensure that the animal shows no signs of pain or distress and does not become dehydrated. In case the condition of the mice does not improve, and if signs reach predetermined humane endpoints such as weight loss greater than 15% plus clinical signs of disease, the animals will be humanely killed.
Upon injections, one could observe bleeding at the site of injection or inflammation at the injectionsite. Animals that display any of these signs will immediately be humanely killed.

Toxicity and/or allergic reaction to anti-parasitic (helminth) drugs. However, these drugs have beenextensively employed in mouse (murine) models of trichuriasis, and rarely induce toxicity and/or allergic reactions at the doses to be employed in the current project.

Suppression or over-activation of immune pathways due to administration of immunomodulatory substances that could lead to signs of sickness behaviour or transient weight loss. We expect in themajority of cases adverse effects will resolve within 48 hours of the treatment being administered, ortreatment being ceased.

Administration of substances to induce or delete genes could lead to transient weight loss. Weexpect in the majority of cases that this will resolve within 2-7 days of the treatment being administered.

Irradiation of mice could result in infection. However, the implantation of donor cells prevents adverse effects of irradiation, and therefore, when used in this way, irradiation is

not expected to result in any adverse effects. In the result of an infection, antibiotics may be supplied in the drinking water. However, in rare occasions irradiated animals can develop acute radiation toxicity and clinically present with one of the following signs: weight loss, lethargy, poor coat condition, goosebumps (piloerection), hunched body posture and diarrhoea. In the event of at least one sign of acute radiation toxicity, mice will be immediately culled. Moreover, all mice will be closely monitored and daily weighed, following all aspects of the procedure as well as at days 7-10 post procedure for unexpected graft rejection causing signs of pain and distress displayed by adverse clinical signs such as hunching, reduced movement and piloerection. In the event of an animal showing up to three of these clinical signs, i.e. from 1 to 3 signs, it will be killed immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Mouse Sub-threshold: approximately 30%.Mouse: Mild 60%.
Mouse: Moderate 10%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

Currently, there is no model to study human whipworms *(T. trichiura)* and the disease they cause,Trichuriasis. The use of mice is essential to study the overall gut lining and immune responses towhipworm infections. This is because the gut lining and immune response to whipworms is very complex and cannot be replicated in cells in a laboratory *(in vitro)*.

Which non-animal alternatives did you consider for use in this project?

In this project, I will use an *in vitro* model of whipworm infection that uses mini-guts (organoids), which Irecently developed. Currently, this model uses mouse organoids and the mouse whipworm *(T. muris)*, but I am actively working to translate this system to the human whipworm *(T. trichiura)* using human organoids.

Why were they not suitable?

Unfortunately, this *in vitro* model does not fully recapitulate the complexity of the gut or the life cycle of the parasite. Thus, to study complex whipworm interactions with the gut lining and immune cells duringinfection and to validate the *in vitro* observations, infections of live animals are essential.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

I have extensive previous experience of infecting mice with whipworms, which enables me to makegood estimates of how many animals will be required for each experiment.

To estimate the numbers of mice I will use in this project I have first identified the experimental unit andcalculated the group size by using statistic methods (power calculations) based on previous experiments. Then, for each experiment, I have calculated the numbers of groups required and considered each experiment needs to be performed independently up to three times.

Moreover, I have estimated the numbers of animals I need to breed from the different strains I will usein this project, in order to obtain the mice required for experiments.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Extensive review of the scientific literature has been conducted to enable predictions for likelyoutcomes of experiments planned, so the minimum number of mice can be used.

Then, for designing my experiments, I run power calculations based on pilot or previous experiments and use the NC3Rs Experimental Design Assistant (EDA) and the PREPARE guidelines. I randomise mice on experimental groups, and when possible, I blind the experiments. Control animals, either uninfected or wild type (depending on the experiment) are always included. I use age and sex matchedcohorts to reduce variability. These measurements ensure animal numbers are at a minimum and welfare is maximised.

I will conduct my experiments in such a way that I will be able to publish according to the ARRIVEguidelines.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

To optimise the number of animals I plan to use in this project, I will:

Optimise genetically altered mice colony breeding and management, and track it using a software tomanage the mouse colonies. This will ensure there is as little over-breeding as possible. This project will aim to keep “surplus” animals to a minimum. In order to reduce the numbers of breeding pairs the mice will be kept as purebred lines (when appropriate), provided that they do not show any signs of disease.

Perform pilot studies for experiments I will run for the first time. Data from these experiments andfrom previous projects will feed into power calculations.

Participate in animal sharing schemes when possible to make others aware of available tissue ofuninfected mice used as controls of my experiments.

Some of the samples collected from mice as part of experiments planned will be stored long term at -20C or fixed for histology. This will make the samples available for future analysis by scientistsworking on this project, and also for any collaborators.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

During this project, I will use the mouse model of whipworm infection with the natural rodent-infecting species *T. muris.* This model has been selected for this project as it is the best way to study human gutlining and immune responses to whipworms. The mouse also benefits from well-established and robusttechnologies for modifying their genes. Where possible we will use experiments of the shortest duration so long as to do so will yield satisfactory data.

In this project mice will be infected with whipworms, then their gut lining and immune responses will bestudied by collection and analysis of different organs after death. In order to understand the function ofhost genes on the response to whipworms and visualise different cells, we will need to: 1) infect with whipworms genetically altered mice lacking those genes or where cells are labelled ; 2) treat mice withsubstances (including, but not restricted to, antibodies, inhibitors, drugs) or 3) replace their immune system with the one from other mice. These procedures can cause clinical signs, but it is essential thatwe follow the outcome of infection to fully investigate how worms invade, colonise and persist in the gut. Previous experience has shown that weight loss can correlate closely with the disease caused by whipworms. Monitoring weight loss is therefore an effective way of determining infection, and is a widely used and accepted measure. However, weight loss is not the only measure of infection and we will use a comprehensive monitoring and scoring system to assess the animals throughout experiments and ensure they do not undergo pain and suffering.

Procedures will be performed by competent personal licensees and we will endeavour to minimise animal suffering. We have access to a state-of-the-art animal facility, staffed by highly trained and dedicated animal technicians and managers that have access to a sophisticated database to track thehealth status of every animal and alert when there is a loss of condition.

Infection is achieved by ingestion of eggs via feeding (oral gavage). This route of infection replicatesinfection in nature.

In some experiments, during the infection, mice will be injected with substances in the

abdomen, veins,muscle or under the skin using the minimal volumes required and adequate needle gauge to cause theleast pain.

In other experiments, mice will be given substances in their diet or drinking water.

In some experiments, mice will be given with drugs via feeding (oral gavage) to ensure the animalsare given the correct dose.

In other experiments, mice will be irradiated to deplete their immune system and injected with immunecells from another mouse strain (intravenously) using the minimal volumes required and adequate needle gauge to cause the least pain. After these procedures, mice will be closely monitored to early identify any animals showing signs of rejection of the replacement immune cells so that appropriate intervention can be taken promptly to avoid unnecessary suffering. In the event of infection, irradiatedmice may be treated with antibiotics in their drinking water.

Mice will be subject to non-recovery anaesthesia in experiments where blood will be collected from theheart. Finally, this project aims to use non-surgical embryo transfer for breeding genetically modified mice.This method is a refinement to the existing surgical method of embryo transfer.

Why can’t you use animals that are less sentient?

Mice are the least sentient animals available that share the anatomy and functions of this disease ashumans. Most of the mice in this license will display mild symptoms upon infection with whipworms.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

In this project, to minimise the harms for the animals the following husbandry/care measures will betaken:

Monitoring will be specifically tailored for the procedure in question and take into consideration thestrain of mice, the stage or phase of the disease development and the rate of change of the animal’scondition. Thus, mice will be checked at least daily and weighed at least weekly from the point of infection to carefully monitor for adverse clinical signs and general welfare of the animal. A record of weights and animal condition will be kept on an electronic database.

Refinement of housing and care will be assured, e.g. use of soft, non-tangling nesting material,provide effortless access to easy-to-eat food and water.

Before all procedures, mice will be acclimatised to handling.

For any procedures requiring anaesthesia, mice will be monitored closely for the duration of theirrecovery.

Carefully consider needle gauge, and keeping volumes and doses to the minimum necessary, forinjection of substances.

The duration of experiments will be reduced, provided this is compatible with the study aims, i.e. allthe necessary data can be obtained within the study time.

The risk of aggression will be reduced by establishing groups early, using littermates, ensuring thatanimals are not subsequently mixed where possible, and selecting appropriately designed refuges inthe cage.

Assure that all those responsible for assessing animals should receive adequate training in recognising indicators of suffering associated with murine trichuriasis such as diahrrea, dehydratation,piloerection and huntching.

Mice will be monitored in the next hour after they undergo any regulated procedure.

Ensure that appropriate welfare assessment protocols are defined and regularly reviewed.

Keep up with development in animal welfare monitoring technology.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

In this project the following guidance will be followed to ensure experiments are conducted in the mostrefined way:

Laboratory Animal Science Association (LASA) guidelines to make sure all experiments are conducted appropriately. In particular we will follow the 'Guiding principles on good practice for AnimalWelfare and Ethical Review Bodies'.

PREPARE guidelines for planning experiments, and ARRIVE guidelines for thorough, responsiblereporting of results.

NC3Rs recommendations on non-aversive mouse handling, genetically altered mice, welfareassessments, euthanasia and anaesthesia, and administration of substances to mice.

LASA and NC3Rs guidance on dose level selection for regulatory general toxicology studies forpharmaceuticals.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

To stay informed on advances in the 3Rs, the duration of this project the following websites will beregularly checked:

NC3Rs website: http://www.nc3rs.org.uk.

RSPCA website: http://science.rspca.org.uk/sciencegroup/researchanimals

Moreover, we will register for the regular NC3Rs e-mails and newsletter updates. Regular reference toguidance documents provided by Laboratory Animal Science Association (LASA) and the RSPCA will be made.

Researchers working under this project will also engage regularly with the organisational teams in the facilities in which our mouse work is conducted, including NVS, NACWO and Named Information Officer, to discuss on how to implement 3Rs advances. Any new recommendations will be incorporatedinto our experimental plan wherever possible.

LYMPH NODE STROMA DRIVEN PROTECTIVE IMMUNITY AGAINST INTESTINAL HELMINTH PARASITES

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, ortheir effects, in man, animals or plants
    - Improvement of the welfare of animals or of the production conditions for animalsreared 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 aimsmentioned in paragraph (b)

Key words

Stromal cells, Type-2 immunity, Lymph node, Intestinal helminth infection, B cells, T cells, Dendriticcells

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 notrequired.

Objectives and benefits

Description 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 current project addresses how lymph nodes (LNs) stromal cells influence humoral and cellular immunity during homeostasis and inflammation. I will be focusing on defining the immunological pathways and their subsequent impact on humoral and cellular immunity using intestinal helminths asmodel organism.

Potential benefits likely to derive from the project, for example how science might

be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 gastrointestinal tract (GIT) has the challenging role of digestion, absorption, and tolerating foreign nutrients and the microbiome while eliminating pathogens; a failure in these processes leads to a range of intestinal diseases such as food allergies, Celiac Disease, Inflammatory Bowel Diseases, andGIT infections. The intestinal helminth infection is a global health problem that also impacts susceptibility to other diseases such as HIV/tuberculosis and malaria. In the past decade, the field of immunology has grown through an increase in our understanding of tissue-draining lymph nodes (LNs)using various imaging and quantitative approaches. LNs are important sites for the induction of adaptive immunity where various immune cell priming, activation, and expansion plays an important role in driving protective immunity against helminth. It has been well established that CD45 negative cells (commonly referred to as stromal cells) within the LNs underpin protection against diverse infections and autoimmunity by undergoing structural remodelling, proliferation, and expansion. Over decades, many fundamental studies have addressed the involvement of secondary lymphoid organs such as lymph nodes and spleen in successful vaccination, sequelae of autoimmunity and effective immune responses to pathogens defining the outcomes in acute and chronic infections as well as vaccination. It is now generally accepted that stromal cells play overwhelmingly important roles in T- and B- cells activation and antibody production contributing to productive immunity. These abundant non-hematopoietic stromal cells govern the B cell and T cell's survival, and activation, as well as program their various fundamental functions in host defence. In recent years it has become evident that hematopoietic cells have the ability to shift their metabolism to maintain immunity, thus in this project, we will try to understand the metabolism of LNs stromal cells at various stages of development as well as during helminth infection. As it is hard to get LNs from the helminth-infected pregnant motheror children, our in vivo mice experiments combined with deep tissue imaging will allow us to understand LNs biology from such perspective to address global health problems like helminth. As our understanding of the mechanism about disease increases, the understanding of LNs stromal cells will likely help us to design multiple key interventions targeted to improve the immune system which will likely synergize with the vaccine and other therapeutics for GIT disorders including helminth infection.

What outputs do you think you will see at the end of this project?

My research relies upon a multidisciplinary approach (immunology, imaging, genetic mice models, metabolomics, modelling, etc.) to understand a disease common in countries where hygiene, poverty, child deaths, malnutrition, and hunger are common attributes. Considering the fact that lymph nodes are important sites for immunological outcomes ranging from infection to cancer progression, the proposed research should find a direct application in various diseases. Helminth infections are also common in livestock and often associated with multiple co-infections leading to antimicrobial resistanceand socioeconomic burden. Therefore, the outputs generated through this project will generate new information related to host-pathogen interaction, and vaccine biology and will yield high-impact publications. This information will be published and shared through public engagement as well as through scientific meetings and conferences for maximum dissemination.

Who or what will benefit from these outputs, and how?

Scientific research is an important component in the enlargement of what we call the ‘knowledge- based society’ where interaction between research and society drives an innovation system. The proposed work has direct relevance to developing countries where the lack of sufficient resources for research may inhibit this important aspect of innovation to improve human health. Present estimates indicate that approximately 2 billion people worldwide, mainly children, currently suffer from intestinal helminth infection which impacts human health through effects on nutrition leading to growth retardation, vitamin deficiencies, and poor cognitive function. Despite their prevalence and high impacton morbidity, there are currently no vaccines available against human intestinal helminths. Before this can be achieved, we need to improve our understanding of the parameters governing the induction, specificity, and function of vaccines by studying the lymphoid compartment where responses are initiated. The proposed work strategically addresses the role of lymphoid organs stromal cells and the niches they generate for B cells/T cells which have become an attractive new target for therapeutic intervention. Therefore, the expected findings should also help researchers in the development of better drugs/vaccines/nutrient regimes to improve immune responses when beneficial (vaccines, infections) or to ablate them when deleterious (autoimmunity, allergy).

The dissemination and implementation of results obtained in this project will also find direct relevanceto livestock infections and antimicrobial resistance. I strongly believe in sharing the data with the outside scientific community and therefore all confirmatory data will be presented to international meetings and conferences (considering no patent application filed). All participants in the project will publish the results of their work. Any reuse and other related concerns will be dealt with according to university guidelines and with pre-consent. Data sharing with other researchers upon reasonable request and within a reasonable time will be taken into consideration and the sensitivity of data will be decided based on mutual consent. All significant findings will be evaluated and published in peer-reviewed international journals.

Most importantly, this data-driven research program aims to define the energy usage within the stromalcells (for ex. Fibroblastic reticular cells or commonly referred to as; FRCs) and gain new insights and answers at both cellular and system levels. The proposed discovery research will help us to understand the host-pathogen interaction with direct relevance to human diseases. The proposed research also aligns with the Medical Research Council (MRC) health focus themes that aim to promote research in stromal biology, global and mental health that is strongly linked to the both host’s nutrition state and parasitic infections, therefore holding immense translational opportunities for the long-term future. As such, the metabolism aspects of the proposed research are truly aligned with the MRCs and UK priorities on nutrition research. Furthermore, an understanding of stromal cells, during homeostasis and inflammation, will foster multidisciplinary collaboration across the various field which is central to UK research.

Interestingly, angiotensin-converting enzyme 2 (ACE2), a key receptor for SARS-CoV-2 (COVID-19) ishighly expressed on LNs stromal cells (FRCs) and many human pathogens like HIV, Ebola, Marburg, and Lassa viruses can target FRCs. Therefore, FRCs (stromal) research will not only bridge the fundamental immunological concepts underpinning homeostasis, immunological memory, and immunometabolism but may also find relevance for a direct application in developing novel vaccinationstrategies- benefiting global society at large. Collectively, the findings of this research will benefit researchers with a long-standing interest in host-pathogen interaction, cellular immunity, metabolism, lymphoid biology, and vaccine research.

How will you look to maximise the outputs of this work?

We will share the data between lab members without any limitations. We will make datasets openly available on appropriate digital data repositories at the latest at the time of publication. We may makedata available before publication upon demand by potential new collaborators.

Data obtained during this project will be shared (though not limited to) through lab meetings, departmental seminars, other University meetings, Joint meetings, Annual courses/Seminar series,annual immunological/stroma/imaging/metabolism meetings, public meetings and social outreach programs (data will be scaled for a general audience), international conferences (poster and oral presentation)

Species and numbers of animals expected to be used

- Mice: 3600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Access to helminth-infected individuals is a difficult task and much of our knowledge regarding host immunity has been necessarily derived from experimental murine models. Of these, our laboratory hasworked extensively to study the protective immunity in mice against model murine Heligmosomoides polygyrus (Hp) which is placed in the same suborder as ruminant parasites Haemonchus contortus and Teladorsagia circumcincta as well as the human hookworm (Ancylostoma duodenale and Necatoramericanus) infection. The immune response occurring within the intestine against soil-transmitted helminths can only be studied in live animals at the present time as these organs represent complicated tissues exhibiting microenvironments which cannot be replicated in vitro. Moreover, the parasitic life cycle of these infectious agents cannot be replicated in vitro as we do not know the host cues that prompt parasite migration or development through the distinct life cycle stages (seen in humans). We have recently completed many independent studies showing that protective immune responses against helminth are raised in the mesenteric lymph node, whilst the intestinal lymphoid tissues raise regulatory responses against the worm. Altogether these studies have contributed greatlyto our knowledge of immunity against helminths, the knowledge that we hope will ultimately lead to thedesign of effective vaccines or food/metabolite-based therapeutic strategies. We now hope to expand on this knowledge to investigate host factors that regulate the immune response against these helminths within the mesenteric lymph node by using laboratory mice to develop tractability of these findings especially when mice and humans share approximately 95% of their genome.

Typically, what will be done to an animal used in your project?

We will use a natural murine model organism to study the host-pathogen interaction. A typical experiment involves an oral gavage of helminth followed by analysis at different time points. The experimental procedures we propose are all considered mild to moderate

and infections are usually asymptomatic. In brief, animals will be infected with natural murine helminth which, at the doses used,do not have any side effects, and they will be humanely sacrificed at 3, 6, 12 or 21- or 28-day post- infection (dpi) before parasites or various cells can be retrieved and analysed using standard methodsestablished in our lab.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals subjected to helminth infection are typically asymptomatic. For mice used in the bone marrow chimera experiments animals may have increased susceptibility to infection or pain in the first few weeks after irradiation and bone marrow reconstitution. To counter this all-irradiated mice will be closely monitored to support their well-being, with enriched food and antibiotic and analgesia treatmentif required.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The treatments (50% of mice) in a given experiment will be of mild severity such as general discomfortfollowing infection established by a single oral gavage or transiently moderate (50%) transient irritation during an injection or immunisation models for studying vaccination. In most of the cases the animalsresume normal activity almost immediately.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered 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 like mice, serve as a great tool for biomedical research because they have many similarities to humans in terms of anatomy and physiology. In addition, the use of mice has economic advantages as they require little space or resources to maintain, have short gestation times but relatively large numbers of offspring, and have fairly rapid development to adulthood and relatively short life spans. Access to helminth-infected individuals is a difficult task and much of our knowledge regarding host immunity has been necessarily derived from experimental murine models. Animal models have been used extensively to study the protective immunity development against helminths. Chronic helminth infections remain a huge global health burden, causing extensive morbidity in both humans and livestock and currently, there are no vaccines available to tackle these infections. Many ofthe most prevalent helminth parasites are difficult to study in the laboratory, as they have co-evolved with, and are closely adapted to, their definitive host species. However, model organisms such as Heligmosomoides polygyrus, a natural mouse parasite, offer tractable and informative systems to explore the mechanisms of immunity and immune evasion in helminth infections. Therefore, the use of mice will allow us to obtain results which can be harnessed to understand immunology as well as in

developing novel vaccines against the helminths. In line with this, we have recently completed many independent studies showing that protective immune responses against helminth are raised in the mesenteric lymph node, whilst the intestinal lymphoid tissues raise regulatory responses against the worm. Altogether these studies have contributed greatly to our knowledge of immunity against helminths, and we hope will ultimately lead to the design of effective vaccines or food/metabolite-basedtherapeutic strategies against these evolutionarily conserved pathogens.

Which non-animal alternatives did you consider for use in this project?

The immune response occurring within the intestine against soil-transmitted helminths can only be studied in live animals at present as these organs represent complicated tissues exhibiting microenvironments which cannot be replicated in vitro. Moreover, the parasitic life cycle of these infectious agents cannot be replicated in vitro as we do not know the host cues that prompt parasite migration or development through the distinct life cycle stages (seen in humans). However, we constantly use the in vitro cell lines (derived from both mice and humans) to study the hypothesis andvalidate the relationship between the parasite antigen and immune responses towards protective immunity. These in vitro experiments allow us to mimic the physiological single-cell population response to parasitic antigens which can only be generated using mature worms (after completing thelife cycle in live animals). Unfortunately, these systems are still at their primitive stage to reveal the global complex host-parasite relationship and these in vitro models will, however, not be able to replace our animal work. Whenever possible, we will use cell culture models (cell lines of stroma, B cells and T cells) to stimulate and evaluate the impact of helminth-derived antigen (generated using adult worms) on pathways of cellular inflammation. These models can also be used to provide a primary essay or recall response for the first evaluation of the efficacy of a molecule against a specificsignalling target to study proof of concept.

Why were they not suitable?

The immune response occurring within the intestine against soil-transmitted helminths can only be studied in live animals. At present these organs (small intestine, draining lymph nodes, spleen etc.)represent complicated tissues exhibiting microenvironments which cannot be replicated *in vitro*.
Moreover, the parasitic life cycle of these infectious agents cannot be replicated *in vitro* as we do not know the host cues that prompt parasite migration or development through the distinct life cycle stages(seen in humans).

Furthermore, the stromal cells lose their distinct cytokine and chemokine expression profile post-*invitro* culture which makes the *in vitro* system non-suitable for some (if not all) of the major goals proposed during this project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The minimum number of animals will be used in all experimental groups, as determined by comparablepublished literature and statistical calculation of numbers required for statistical significance testing (power calculations). There will be careful planning of experimental timing to minimise the breeding of excess animals and the use of control animals. Harvest of tissue/samples will be pre-planned to try to ensure that all tissues are subjected to an analysis pipeline, preventing/minimising the need for repetition of procedures/models detailed in the licence. For all experiments at least 5 animals per treatment, the group must be used to gain information about the statistical variability of the result. In addition, each experiment will be repeated at least 3 times in order to validate the results. These numbers are based on our previous experimental design, power calculations and statistical calculation and are expected by any external reviewer within the field. We will use both male and female mice in all our experiments.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Pilot studies will be performed to assess variability and time courses of effects, to optimise and minimise the final group sizes for assessments. We will refer to developed Experimental Design Assistant tool (EDA https://eda.nc3rs.org.uk/) and InVivoStat (http://invivostat.co.uk/) where relevant toassist in design and analysis of data from animal experiments.

Furthermore, various cellular response will be tested using our in vitro culture system to further reducethe animal usage.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Mice will be bred at the lowest numbers to maintain the genetically modified colonies. Harvested tissues will be shared between different group members where possible. Whenever possible, we will obtain specific animal strains (e.g., genetically-modified strains) in small numbers from collaborators. Itis not our intention to breed a large number of animals and setting up large colonies for this project. If abreeding approach is chosen for a specific experimental cohort, we will take a very targeted approach to ensure a close match between breeding and experimental requirements with minimal waste in line with the guidelines relating to 'Colony management best practice' provided by the N3CR. As suggestedby the NC3R, we will follow the advice outlined in 'Breeding strategies for maintaining colonies of laboratory mice' from the Jackson Laboratory. We will generate maximal data output from each experiment by capturing organ function through imaging and blood samples. Furthermore, over the years we have developed a robust, scalable and translatable analysis pipeline for tissues harvested from control and infected animals. This pipeline allows us to maximize the data output and help us to gain insight into the pathophysiology and/or mechanism of action of any intervention used as well as reduce the number of experiments and in turn use 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority of animals we will use will be mice. We use inbred laboratory mice for the vast majority ofour experimental work because of the enormous range of available reagents. We use a natural murinehelminth model where the natural host is a mouse, the mouse is used to keep parasites alive (none can be maintained in culture). These can be used to assess in detail the mechanisms by which the cells and proteins of type 2 immune response cause wound repair, changes in metabolism and killing of parasites along with the cellular and humoral response.

The mouse genome is phylogenetically closer to the human, compared to any other model organisms. The availability of complete genomic sequence, and extensive chromosomal synteny, makes the identification of homologous human genesconsiderably easier. Mice are the most appropriate small animal model for the study of tissue biology as the biological, molecular and genomic information available for these animals is unparalleled when compared to other small animal models in biomedical research.

Why can’t you use animals that are less sentient?

For homeostatic analysis, the more immature life stages will be used. We will also use terminally anaesthetised animals to address some of the questions. However, for the infection experiments, werequire adult animals as the parasite requires the living host to complete the life stages. Our studies rely on looking at the immune response to infection or other conditions in the context of the whole body. The immune response is a highly complex process involving multiple different cell types and molecules that work together. The function of these cells or molecules depends on where they are inthe body, and often they move around the body. We cannot replicate these processes outside the body. No alternatives exist for parasite migration through the body, and it is not possible to mimic helminth infection in vitro. Whenever possible, we use cell culture systems to address specific questions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Procedures will be minimised in frequency and adapted if more refined techniques should become available. All procedures that are likely to cause stress and pain will be done under appropriate anaesthesia and analgesia. The most refined surgical procedures and routes of drug administration willbe employed. Animals are closely monitored for any ill effects, typically by visual assessment and weighing. Weight loss can predict ill effects before they are seen visually. When performing experimental manipulations, we will use the least invasive and distressing procedures available, and use the least number of manipulations, to reduce pain, suffering, distress, and lasting harm to animals.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will refer to the PREPARE (Planning Research and Experimental Procedures on

Animals: Recommendations for Excellence) guidelines as well as **ARRIVE guidelines** to improve our standardsof reporting and making sure that the data from animal experiments can be fully evaluated and utilised.

We will also follow the published literature in the field and adapt some (if not all) reporting guidelines toimprove the clarity and data presentation to ensure experimental reproducibility.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Firstly, we will subscribe to the NC3Rs e-newsletter to get regular updates and will discuss these updates during our lab meetings. By attending NC3Rs events and international workshops/conferences (with in vivo work discussion). By having regular discussions with the named persons and animal technicians at our institutions, we will review our current approaches and whetherthere are any new 3Rs opportunities available to refine or replace current practices.

MECHANISMS AND TARGETS FOR 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, ortheir effects, in man, animals or plants

Key words

neuropathic pain, immune system, neurones, targets, inflammatory pain

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 required,and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Pain accompanies a variety of clinical conditions and the management of pain remains a difficult task. Currently chronic pain is poorly treated by opiates and is resistant to alleviation from the use of non- steroidal anti-inflammatory drugs (NSAIDs). This lack of appropriate and effective treatments is due inlarge part to the incomplete understanding of basic neurology. More work needs to be undertaken to better understand the 'mechanisms' of chronic pain, so that we can develop new treatments that directly target the pain itself and provide pain relief in the absence of overt side-effects.

A retrospective assessment of these aims will be due by 07 August 2028
The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Given this shortage of suitable therapies for both neuropathic and inflammatory pain our research aimsto identify new mechanisms underlying chronic pain in order to find new targets for analgesic therapies(pain relief). Neuropathic pain is associated with trauma to a nerve or chemotherapy for cancer treatment. Inflammatory pain is associated with conditions such as rheumatoid arthritis.

What outputs do you think you will see at the end of this project?

This project will result in publications and data that will enable applications for more funding and generate new knowledge that can guide the identification of new targets for pain relief. Our pre-clinicalresearch aims at developing a mechanism based approach to the identification of new therapies whichreduce chronic pain in the absence of overt side-effects.

Who or what will benefit from these outputs, and how?

This project will identify new mechanisms which are responsible for chronic pain in animals and humans. The identification of key players in such mechanisms will provide new therapeutic targets for the relief of chronic pain in diseases like arthritis. The usefulness of our models lies in them replicatingboth the symptoms and the disease progression seen in patients. Thus, the findings of our pre-clinicalresearch will have direct relevance to the clinical problem and will provide important evidence for the therapeutic potential of new targets for the treatment of chronic pain. In the short-term we will identify new targets for drugs which reduce chronic pain. In the long-term, our findings will result in the development of new analgesics for chronic pain patients and the availability of new analgesics will improve their quality of life.

How will you look to maximise the outputs of this work?

We will present our data at national and international meetings, and publish in scientific journals. We will keep active collaborations with industry so that we remain in contact with translational approach toscience.

Species and numbers of animals expected to be used

- Mice: 25,500
- Rats: 5,700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 adult rodent models is crucial to our understanding of pain pathophysiology and the development of novel analgesics. Rodents will be employed in these studies as they are the lowest vertebrate group on which these types of experiment can be conducted to provide unique systems responding to drugs used in the clinic. In addition, the extensive use of rodents in biological researchhas already provided much information on pain processes.

Typically, what will be done to an animal used in your project?

We will induce conditions that resemble chronic pain in humans by surgical procedure or administrationof specific agents. We expect that our animals will walk less and lose some of their explorative behaviour. The duration of the experiments will be kept to the minimum time required to address specific aims: 1. To elucidate new mechanisms and mediators involved in chronic pain with a special focus on neuro-immune interactions in the spinal cord and in peripheral nerves including the dorsal root ganglia. 2. To determine the effectiveness of compounds as analgesic and anti-hyperalgesic agents in models of inflammatory and neuropathic pain with acute (hours) or chronic duration (several weeks).

We are interested in acute and chronic pain mechanisms. Some studies of sensory phenomena are possible in humans. However, some mechanistic questions require more invasive techniques so we undertake our work in rodents for this. We are interested in using animal models of human disease orpathology. Some of these models have short onset and duration (hours) and can be studied acutely. For example, the injection of a chemical agent like zymosan in the pad of hind paw is associated withpain-like behaviour that is observed and recorded for up to 24 hours.

The hind paw pad is innervated by the sciatic nerve and the cell bodies of these sciatic afferents are located in the lumbar dorsal rootganglia and can be studied as representing the treated afferents. Other models develop and change over time and experiments may last weeks. For example, the injection of collagen as a model of arthritis is associated with pain-like behaviour that is observed and recorded for several weeks. The prolonged time course of some experiments and the assessment of animal behaviour require the useof recovery protocols. Furthermore, the treatment of chronic disorders usually requires persistent or chronic treatments. Pain associated with the procedures in this licence will be controlled by analgesiaduring procedures (for example surgery), except where the principal aim is to study chronic pain mechanisms and in these models alternative pain relief will be used as directed by the NVS.

At the start, in models of pain (neuropathy, arthritis) animals will either receive administration of vehiclecontrol (no treatment), neuroactive substances and/or undergo alteration of gene expression and/or depletion of immune cells (Treatment).

In behavioural tests, animals will be trained and then assessed before and after treatment (Assessment). Functional assessment may be associated with pathway tracing and neuroimaging. Atthe end of assessment animals will be killed by schedule 1 and tissue may be collected for analysis (End).

Start:
We start with normal or genetically altered rodents, initially purchased from licensed breeders, or transferred from other academic institutions. Some of the genetically altered animals will be bred at ourestablishment and may be used where appropriate; these are

particularly useful for defining the role ofa particular target in the pain process, or for providing the means for examining the activity of a particular compound at a human target protein. We don't expected our genetically altered animals to show a harmful phenotype. Safety and efficacy of neuroactive agents (commercially available compounds or compounds generated by discovery programmes with satisfactory biological activity in vitro and/or known pharmacokinetic profile) will be determined in normal or genetically modified animals before use in disease models.

Model:

In a typical study, after having spent at least five days in the animal facility, animals are used in one ofthe pain models. Animals may undergo induction of a neuropathy or arthritis. The models are used to mimic some aspects of the clinical pathologies and understand pain mechanisms. In these models wetest the effect of compounds acting at specific targets and assess the role of a specific mechanism in the induction and maintenance of pain.

The models are as follows:

Peripheral neuropathies: Chronic pain associated with nerve trauma is poorly treated with current drugs. We use models of peripheral nerve injury with the final aim to elucidate new targets for innovative pain therapy. The nerve injury models consist of surgical lesions created under anaesthesia,such as cutting or crushing a particular peripheral nerve in one of the back legs of the animal. The cell bodies of sciatic nerves are located in the lumbar dorsal root ganglia and account for some 70% of the cells that can be studied as representing the treated nerves. Animals recover well from surgery and show increased sensitivity to painful or unpleasant stimuli in the hind-paw of the leg that the surgery was for several weeks and mild impairment of locomotor function for up to 1 week. Systemic drug- induced neuropathies closely model some neuropathic lesions frequently precipitating clinical problemssuch as chronic pain during cancer therapy in patients. In cancer treatments a dose-limiting side-effect of chemotherapeutic agents is the development of neuropathic pain, which is poorly managed by available drugs. We mimic clinical protocols with the aim of finding new mechanisms and targets for pain. For example, 5 day cycles of daily injections of vincristine is associated with significant mechanical hypersensitivity and a moderate cold hypersensitivity.

Induction of inflammation:. Inflammatory (nociceptive) pain is partially controlled by nonsteroidal anti-inflammatory drugs (NSAIDs) and the development of new analgesics would improve pain treatment.The study of the mechanisms of clinically relevant, inflammatory pain requires the use of animal models that induce some tissue injury. We will use models of inflammation that are restricted to one body part but also models of systemic inflammation. We will also use models that are relatively shortlasting (measured in hours to days) or longer lasting (several weeks). Control animals will be either untreated animals or sham treated. The several models listed are necessary as they underlie diverse mechanisms of activation of the pain pathways. However, most of them share comparable severity which will be addressed by keeping their duration short.
The models of inflammatory (nociceptive) pain facilitate the study of pain transmission and the characterization of novel analgesic compounds. In these models acute (seconds to hours) nociceptivepain is measured by spontaneous and evoked behaviour. Longer lasting pain is measured by evoked stimulation.
Induction of collagen-induced arthritis (CIA) and inflammatory arthritis. Chronic diseases such as rheumatoid arthritis display ongoing inflammatory disease associated with pain that is poorly managedclinically, especially when pain is present despite joint swelling

being controlled by disease-modifying drugs.

To mimic this type of disease, we need recourse to chronic models of active or passive immunization.These models are potentially more distressing for the animals and we therefore use them only for crucial studies and then only maintain them for the minimum time required to answer the experimentalquestion.

All compounds will not be tested in all animal models but rather they will be initially examined in the model and species most appropriate for that class of compound in which activity could be anticipated.

Thorough experimental procedures requires that the effects of a surgical procedure, whether on biochemical, morphological or behavioural end-points, be compared to the same endpoint in animalswhich have undergone a sham operation. This ensures that the effects observed are a result of the intended injury rather than a non-specific effect of surgery. This would require an extra group of animals in every experiment. However, the majority of the surgical techniques with recovery in this project are well established and we know from extensive experience that there is no sham effect, particularly with relatively simple procedures such as sciatic nerve injury. Therefore, in these cases we will not include a sham group but rather refer to historical control data to minimise the animals used under this project. For all techniques the effect of sham surgery will be reviewed periodically, and whena new experimenter is using the technique. This will ensure that their data are consistent with previouscontrol data before they can proceed with routine testing.

Treatment:

Our proposed experiments involve the treatment of animals with neuroactive compounds. The range ofcompounds to be tested requires the use of a variety of delivery techniques (including slow delivery directly to the CNS), sites and frequencies which will vary according to the behavioural test to be used.We may also investigate the neuromodulation potential of altering gene expression. The cellular inactivation of depletion of immune cells will be used to investigate the role of these cells and derived mediators in the development and maintenance of chronic pain states.

The animal may then be administered with a test substance either before or immediately after assessment of behavioural responses. In some behavioural tests there may be a period of trainingprior to entering the animal into a study model.

Assessment:

One way of achieving our objective of assessing the performance of the somatosensory system is to measure how manipulations affect the ability of the animal to detect and respond to its environment and to applied stimuli. Some of this information can be obtained simply by observing the animals in their cage or for instance in an open field. For assessment of nociceptive capacity, it is necessary to challenge the animal with painful or unpleasant stimuli that can be thermal, mechanical or chemical. The standard response of the animal to such stimuli is a co-ordinated withdrawal response or a periodof licking of the skin area that was affected by the stimuli. Most of the stimuli we apply are threshold stimuli: stimulus intensity is increased until the animal detects a minimal (threshold) amount of pain, atwhich point it is free to generate a withdrawal reflex. The time taken to achieve this, or the level of the stimulus at which this occurs, is a measure of the sensitivity of the nociceptive system, and varies withanalgesic drugs. Such tests do not damage (or

produce only minimal damage) to tissue (and are terminated by cut-off times in any case). We may therefore apply them repeatedly. Typically, experiments are either short term, when 3 or 4 testing sessions will take place in one day and the experiment then terminated; or long-term - perhaps lasting 3 weeks, when animals might be tested on alternate days.

For many of these tests, the animals need to be habituated and pre-trained on the relevant apparatus.This can sometimes require a few weeks of exposure to the tasks before a treatment or intervention isstarted. On many occasions results from these tests then can be used as a baseline to compare backto once the treatment or intervention has started.

For tests of chemical sensitivity, it is not usually possible to use threshold testing. Here it is more common to provide a dose of chemical (from which the animal cannot escape) and measure the amount of pain-related behaviour induced. A robust stimulus we use is the capsaicin test that featuresa brief period of paw flinching and guarding of the treated paw, which subsides after 30 minutes. We quantify the degree of nocifensive behaviour produced by the stimulus, and then kill the animal. We willnot combine more than two tests of chemical sensitivity in any given animal.

Another way in which we assess changes in sensory systems is an analysis of anatomical connections. To do so, we use anterograde and retrograde labelling techniques. The essence is that atracer compound is injected or applied to the neurons of interest that internalise and transport the tracer to other parts of the neuron. One example is the injection of tracers into a joint, where they are transported to the cell bodies of sensory neurons in the dorsal root ganglia and reveal for instance thesize of the cells that innervate the joint. The transport of tracers throughout the treated neurons typically takes a few days, and for some tracers can take up to 2 weeks. It is therefore necessary to apply the tracers in one operation (which might be before or after putative therapeutic treatments), allow the animal to recover for one or more days before taking tissue for post-mortem analysis, under terminal anaesthesia.

Functional imaging of brain structures is performed to evaluate whether microglia and neurones are activated in supra spinal areas. When testing the effect of a neuroactive substance on brain imaging, itis necessary to perform prolonged imaging sessions within a 24-hour period. This is in order to performanalysis before and after acute treatments. We may need to use neuromuscular blockers to reduce animal movements that would interfere with imaging measurements. Animals will be anaesthetised throughout the period of neuromuscular blockade.

A final method of assessment is to examine the biological properties of tissues taken from the animalsstudied under this licence. In some cases, isolated tissues will be studied in tissue culture. In others, tissues preserved with fixatives for (immuno)histochemical studies.

End:

At the end of the assessment animals are killed by methods deemed ethical by the law and tissue istaken for further experiments in the laboratory.

What are the expected impacts and/or adverse effects for the animals during your project?

During most of the protocols in this project we expect that our animals will feel pain as this

is what we are trying to assess in the experiments however from our experience the animals normally do not showany pain behaviour in their natural environment unless evoked during one of our pain assessment behaviour tests. As well as this it may also have an impact on the animals walking ability and they maylose some of their explorative behaviour. The duration of these impacts can vary between the pain models used, for example animals that underwent nerve injury surgery may experience less mobility for the first few days after surgery and then gain it back and animals that have inflammatory induced may experience this impact for the duration of the swelling of the paw. Because of these impacts animals may gain less weight than than controls and in the case of the collagen-induced arthritis model, rats may lose weight. To help prevent weight loss animals are given diet on the floor for easy access and sometimes wet diet which is more appealing to help them keep weight. We make sure thatthe severity of our model is "moderate" whenever possible by limiting the time for which the animals are kept following the induction of the pain model. Furthermore, the use of neuromuscular blockers willbe limited to specific cases when we need to image activity of neurons and animal movements may cause interference. Animals will be anaesthetised throughout the period that neuromuscular blockers are used. The KBxN model of arthritis causes all paws to transiently swell (peak swelling at approximately day 5 followed by a gradual recovery until complete recovery by approximately day 17). Pain persists beyond 17 days but this can only be clearly obnserved when pain responses are evoked.This model causes a moderate level of suffering in our experience. The paw swelling associated with the collagen-induced arthritis model starts at approximately 11 days and peaks at approximately 18 days after induction of the model and the animals experience swelling of all paws. Again, pain persists persists beyond the swelling. In our experience, the degree of suffering lasts for longer than the KBxN model and the animals have a slow recovery. Animals with collagen-induced arthritis are killed up to 28days (rats) or 42 days (mice) after model induction.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

We have included eight protocols. Severity will be moderate in 6 protocols for about 60% of all animalsin each protocol and the remaining 40% control animals will experience mild severity. Severity will be mild in one protocol for few animals. Severity is considered to be severe for one protocol which includes the KBxN arthritis model in mice and the collagen-induced arthritis model in mice and rats. In this model, 100% of mice and rats that receive collagen to induce arthritis will experience severe severity (approximately 20% of all animals used in this protcol). However, control animals that do not receive collagen will experience moderate severity, as will animals that are part of the KBxN model (approximately 80% of animals in this protocol in total).

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 07 August 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animalswere affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of animal models is crucial to our understanding of pain pathophysiology and the developmentof novel pain killers. The translational animal models which are going to be used in this project provide unique systems responding to drugs used in the clinic. We are principally interested in pain mechanisms. Some studies of sensory phenomena are possible in humans. However, some mechanistic questions require more invasive techniques that are not possible or feasible at present in humans. In vitro techniques are also not sufficiently advanced so they can model the integrated actionsof the nervous system. Thus, we will need to undertake some of our work in animals. Mostly we are interested in animal models of human disease or pathology, and so some of our experiments will makeuse of such models. Some of these models are short onset and short duration (hours) and can therefore be studied acutely in animals. For example we inject a chemical agent in the hindpaw and then measure the amount of pain-related behaviour that is induced. A given chemical agent produces abrief but strong period of pain, which subsides over a few minutes. In animals one sees a brief but intense period of flinching of the treated paw and guarding behaviour which are quantified. Flinches a defined as the number of times the animal raise hind paw; the spontaneous guarding behaviour is measured as the time of animal held the hind paw while stationary.

Which non-animal alternatives did you consider for use in this project?

As well as the animal models, we use in vitro techniques to dissect mechanisms and look for alternatives, such as translatable markers. We also use cell lines when appropriate and primary humancells to look at mechanisms however these may not be suitable or all models and mechanisms we lookfor. For instance, we use sensory neurons in culture and immune cells in culture. We routinely search for non-animal alternatives outside of our lab, for example in the literature or through collaborations.
These techniques are always used prior to/alongside our work in animals.

Why were they not suitable?

The non-animal alternatives are not sufficiently advanced so they can model the integrated actions ofthe nervous system so we cannot asses the aims in these methods alone.

A retrospective assessment of replacement will be due by 07 August 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We routinely seek to reduce the number of animals studied by careful experimental design, the adoption of sensitive outcome measures with small variation and the study of only the most relevant time points. Where possible each animal is used as its own control. Where this is not possible groups of animals will be utilised. In these cases the numbers in each group will be the minimum required to allow valid statistical analysis. In all in vivo tests the number of animals in each group will be the minimum required to allow valid statistical analysis. The number of animals used will vary from procedure to procedure depending on the degree of variability in the experimental measures, but our extensive experience with these models has shown that group sizes of 6 – 8 animals are generally appropriate. For those procedures involving surgery it is scientifically more rigorous to include sham operated control animals in an experiment. However, for techniques which are well established and forwhich we know from experience that there is no sham effect we will not include such animals in every experiment, but refer to historical control data. The effect of sham surgery will be reviewed periodicallyand when a new experimenter is using the technique. It is possible in some instances to use in vitro cell systems to analyse, for example, the effect of inflammatory mediators on receptor and we plan to use such studies extensively. However, it is not yet possible to make stable cell lines of sensory neurones, our main cell type of interest, or to mimic the long term effects of neuronal damage or inflammation that occur in vivo. In these instances the use of animals is unavoidable. Also, by cryopreserving embryos we will reduce the number of animals culled in breeding programmes. Indeed,we have recently cryopreserved embryos from a number of our colonies, which are not going to be used until more research funding is available.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

One of the methods used to help reduce the number of animals used was using the NC3Rs design assistant online tool to help calculate the most efficient number of animals for the experiments. As wellas this we have optimised some of our protocols so that we can use previous data from experiments rather than repeating and when possible each animal can be used as its own control. For some protocols this is not possible so groups of animals will be used and calculations will be made so we only use the minimum required to allow valid statistical analysis. We also utilise multiple behaviour testing when possible for the protocols to get more robust results leading to less repeating of the experiments and have optimised the behaviour tests used.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We have several years of experience doing these protocols and most techniques are well established in our laboratory. Therefore, we often can reduce the number of control animals to the very minimum and use historical data. For examples, to reduce numbers of

animals may be retested with more than one compounds and we always make sure that retesting won't cause more harm. As well as this we have had many experience in breeding animals and planning experiments so we can produce efficientbreeding to only get the animals we need for the experiments and animals that can't be used for initial experiments will be used to tissue and in vitro work. We will use the NC3Rs design assistant where possible to help optimise the number of animals required and also perform pilot studies for any new compound/substance or experiment we use.

A retrospective assessment of reduction will be due by 07 August 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rodents will be employed in these studies as they are the lowest vertebrate group on which these types of experiment can be conducted to get reasonable results and their extensive use in biologicalresearch has already provided much information on pain processes. This is why we will continue touse mice and rats for our pain models. We use more mice than rats as mice can be genetically modified. We use rats in models for which they are the species of choice such as collagen-induced arthritis.

The models we use are designed to study pain and at this time these are the only models available to accurately study this. The severity of most models is moderate but severity is mild for control groups ofanimals in each model. Because we cannot change the models we have refined them to cause the least amount of extra pain and suffering than what we need to explore. This means that in some of ourmodels the pain we use is normally evoked pain so the animals should not feel pain during times where we are not testing them or at least they do not show pain or distress in their behaviour during times of not testing. As well as this we have maximised the methods of these tests and procedures used to create and study this model. We clarify the type of model we are using such as neuropathic pain or inflammatory pain and have limited the methods needed to study each as well as the limiting the time animals are needed to be kept following the induction of each pain model. An example is that one model induces swelling of the paw so to prevent any unnecessary distress and suffering we keep this model for a short time and monitor the animals daily and measure swelling.

We also have refined our procedures so with invasive procedures like surgery all animals will receive post-operative intensive care to ensure high standards of welfare are maintained as well as analgesicsto reduce any extra pain from the procedures. This care

will include cages remaining on heated mats, administration of saline, provision of soft, easily digestible food. On some occasions we may not provide analgesia as for instance, neuro-active agents under examination are likely to exert analgesic effects themselves.

Why can't you use animals that are less sentient?

Behavioural tests require the animal to be vigilant and less sentient animals would not be able toperform our tests.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

To minimise welfare costs to the animals we adopt several measures, all animals will receive environmental enrichment and when possible we always adopt group housing. As well as this we minimise stress in the procedures we do, such as giving animals training prior to behavioural testing toreduce stress from the test and giving post-operative care after surgeries or invasive procedures. All animals will receive post-operative intensive care to ensure high standards of welfare are maintained. This will include cages remaining on heated mats, administration of saline, provision of soft, easily digestible food.

We will provide analgesia where possible and the most appropriate method will be selected to minimise any welfare costs to the animals (this could also include the route and dose of administration or choice of pain model/assay) and that the duration of any suffering will be minimised. When analgesia may not be provided as it would interfere with our studies of pain mechanisms, for animals inpain because of pain induction we make the animal comfortable and provide group housing and intensive care.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We follow the International Association of Pain (IASP) guidance on the use of animals in pain researchand the NC3Rs ARRIVE guidelines which provide recommendations to improve the reporting of animalexperiments and for improving the reliability of published research.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We are regularly updated and informed by our animal facility colleagues, who organise meeting andsessions that I regularly attend.The NC3Rs regional program manager communicates any new initiatives and incentives.

A retrospective assessment of refinement will be due by 07 August 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved forfuture work of this kind? During the project, how did you minimise harm to the animals?

MECHANISMS OF FUNGAL INFECTION AND DRUG RESISTANCE

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Infectious disease, Aspergillosis, Drug targets, Resistance

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Aspergillus fumigatus is a fungal pathogen that causes significant morbidity and mortality in humansand animals. This project aims to further our understanding of how and why *A. fumigatus* is pathogenic, how resistance to our current antifungals emerges and how infection can be overcome.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
Infection by Aspergillus causes more deaths globally than any other fungal disease. Aspergillus fumigatus alone is responsible for over 400,000 deaths every year, placing it among the leading causes of infection related mortality globally behind TB and alongside malaria. Voriconazole is the firstline therapeutic for the treatment of all forms of

aspergillosis. However, since we first demonstrated theemergence of azole resistance in A. fumigatus in 1991, we have documented a worrying increase in itsfrequency, now posing an imminent global public health crisis that requires immediate action. We only have a limited understanding of how and why A. fumigatus is such a successful pathogen, while other Aspergillus species are not. Gaining a greater understanding of the nature of pathogenicity in this organism will allow us to develop novel methods of treatment that can overcome the emergence of resistance. By addressing this challenge, we have a potential to have an immediate impact on the lives of millions of people afflicted with these diseases.

What outputs do you think you will see at the end of this project?

This project will yield a number of critical outputs:

We will generate an understanding of how and why A. fumigatus is such a successful pathogen. Thisknowledge can be used to develop novel antifungal drugs.

We will explore if strains that are resistant to antifungals are still pathogenic. This will help us understand which drugs or combinations of drugs may work best to suppress resistance, and how todevelop tests to develop resistance rapidly in the clinic.

Improved methods to reduce the use of animals in infection studies will be developed.

Our work will be published in peer reviewed journals, and at the earliest opportunity will be presented at scientific conferences. We also undertake outreach activities with key stakeholders to inform them ofthe critical role infection models play in improving patient outcome.

Who or what will benefit from these outputs, and how?

Beneficiaries will include:

Immediate: Scientists, clinicians and industries pursuing novel anti-fungal therapies and looking to understand the pathogenic impacts of drug resistance mechanisms. We are already working with drugdiscovery companies who have compounds in different clinical phases of development, clinicians whowish to optimise treatment regimes, and groups involved in diagnostic development who are keen to understand what mechanisms of resistance may appear clinically.

Medium-term: Pharmaceutical companies seeking novel antifungal agents will be able to exploit ouroutputs to progress novel drugs through pre-clinical trials. I have significant experience in this area of translational science and there is currently a significant interest to fund ongoing preclinical antifungal studies.

Long-term: Individuals suffering, or at risk from, Aspergillus-related disease: The health burden of diseases caused by fungi is considerable. Over 300,000 people are thought to get invasive aspergillosis every year. Around 150,000 of these patients will die. In addition, around 3 million sufferfrom chronic pulmonary aspergillosis, mortality rates are around 15% per year. Improvement in treatments will reduce the burden of this disease.

How will you look to maximise the outputs of this work?

We have a long standing track record of rapidly publishing our scientific outputs in peer

reviewedjournals. Prior to publication, we will seek to present our work at both national and international conferences.

Many of our research programs are collaborative and we frequently exchange staff between labs. Ourbest practice methods, which will seek to implement the 3Rs at every stage, will be shared with our collaborators.

Species and numbers of animals expected to be used

- Mice: 2400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To establish an infection in a human lung Aspergillus fumigatus must bypass a patient's immune system. To appropriately study the mechanisms of disease a similar environment is required. Rodent models and specifically mouse models of invasive aspergillosis have been established for many years.

Over 700 independent studies using mouse models have been published and these works have beencritical in identifying high quality drug targets, novel antifungals, and host and pathogen factors that contribute to immune recognition. While there have been significant advances in the development of non-mammalian models over the last 20 years, the vast majority of the aforementioned discoveries would not have been possible using the alternative models.

We will use adult mice with completely developed respiratory systems, to model the disease setting ofthe adult human lung. Similarly aged mice will be used throughout the lifetime of the licence to ensureour data are reproducible and informative.

Typically, what will be done to an animal used in your project?

In most cases, infections cannot be established in a mouse without first suppressing the immune system. Licenced drugs are therefore used to suppress the immune system of the mice, administeredmost frequently via subcutaneous or intraperitoneal injection. Infections are established via intranasaladministration of a set dose of fungal spores. To achieve this in a way to reduce stress to the animals,the procedure is performed under gaseous anaesthetic. For studies measuring fungal burden and fungal strain fitness, animals will be humanely culled at a set time point, usually 2 to 5 days after infection. For studies assessing fungal virulence, animals will be humanely culled prior to exceeding 20% body weight loss, relative to start of experiment, or as they begin to exhibit physical symptoms ofinfection, defined in our health monitoring procedures, that indicate mice are unlikely to recover. All animals will be culled 14 days after the day of infection.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals that are infected with A. fumigatus would normally exhibit signs of lung infection. Initial signs ofdisease include body weight loss and decline in general body condition associated with a lack of grooming. Animals may become less responsive to external stimuli and exhibit laboured or rapid breathing at which point the animal will be humanely culled. Rarely (<1%) Aspergillus has caused signsof neurological damage when it has spread to the CNS e.g. ataxia, muscle tremors or seizures.
Immunosuppression might lead to anaemia, blood in urine or diarrhoea. Some animals might develop renal impairment, which leads to yellow staining around the anal region and/or blood in urine. A changein body posture with the head held to one side or staggering might be observed in up to 5% of mice.
Animals exhibiting neurological or renal involvement will be immediately culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

All mice included in these studies would be expected to experience at least mild severity procedureslinked to the administration of immunosupression, anaesthesia and A. fumigatus spores.

For studies measuring fungal burden and fungal fitness of strains exhibiting typical levels of pathogenicity, severity would be expected to be mild for most mice (60%). These mice would experience weight loss of no more than 10% and would not exhibit other symptoms. For the remaining mice (40%) a moderate severity would be expected; these mice would experience weight loss ofbetween 10% and 20%, coupled with one or more of the other symptoms described above.

For virulence studies 90% of mice infected with strains exhibiting typical pathogenicity would be expected to experience moderate severity symptoms as defined above. The remaining 10% would experience mild severity symptoms limited to reduced weight loss of no more than 10%. For strains with reduced virulence, severity would be reduced. It is exceptionally rare that strains are identified with increased virulence. In these cases it would be expected that the severity of symptoms would beno greater than that for strains with typical levels of pathogenicity however the onset of symptoms would be faster.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Aspergillus infections are typically localised to the lungs and have to overcome challenges posed by the host immune system. Models that mimic the complexity of the host cannot yet be captured in in vitro or non-mammalian systems. This is because we have little understanding of how immune cells combine with each other and the lung environment

(levels of oxygen, nutrient levels, fever) to eliminatean infection. These stressors and other as yet undefined host factors that contribute to the clearance offungal infections need to be assessed in combination in order to validate specific fungal targets and therapeutic strategies. The only way to do this currently is to perform experiments in mammalian hosts.

Which non-animal alternatives did you consider for use in this project?

We have worked extensively in recent years with in vitro and tissue culture models and wherever possible, and scientifically justified, we replace in vivo studies with in vitro alternatives. In addition we have made extensive use, and pioneered the use of insect (*Galleria mellonella*) models of infection. In some circumstances, these studies can be used to effectively replace the number of experimental procedures that ultimately require the use of animals. Unfortunately these models are unable to replicate the complexity of a murine system in its entirety. We have work ongoing in the wider group that seeks to provide alternative models to replace the use of animals (e.g. the use of human-induced pluripotent stem cell derived lung tissue organoids; single-cell models to explore immune recognition offungal cells) and when validated they are likely to replace some of the animal experiments described inthis project.

Why were they not suitable?

The Galleria mellonella model of infection can be useful in screening strains for pathogenicity studies however there are many significant differences between insect models and mammalian models that are not adequately replicated. The immune system and the nutrient composition of larvae is distinct from that of the murine lung. Our prior data evaluating the fitness of multiple strains of *A. fumigatus* in parallel has revealed that the insect model is a poor alternative for murine infection. In vitro models canbe exceptionally useful in defining specific aspects of pathogenic behaviour but cannot replicate the complex nature of the host environment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

To ensure that rigorous experimental design is employed in the conduct of all experiments expert biostatistical advice has been sought and the suggestions implemented. Continued biostatistical support will be accessible for the lifetime of the project. Required sample sizes have been estimated based on understanding of treatment effect size and variability in response measures. Where there is limited or no previous relevant information for a specific experimental design or endpoint a pilot study will be used to generate information for subsequent statistically powered studies. Data analysis will be conducted according to a pre-specified statistical analysis plan drawn up in conjunction with bio- statisticians. Important experimental results will be repeated and validated via an independent follow-up experiment to minimise the likelihood of spurious non-replicable results. Overall numbers of animalsrequired are based on initial sample size estimates. These numbers

will be updated as more recent and relevant data becomes available.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We have worked for many years to develop models which use the fewest animals to achieve accurateand statistically significant outcomes.

For studies that involve the identification of possible drug targets, prior to performing any comparativevirulence model (which requires the use of more mice than any of our other models) significant evidence must exist that virulence or antifungal drug sensitivity could be affected. This would involve first screening strains through our suite of phenotypic assays, host cell assays, fungal burden modelsor our recently developed competitive fitness models of infection.

The competitive fitness model is a highly sensitive measure of fungal disease progression and permitsus to test (in a single animal) many different fungal mutants simultaneously, thereby massively reducing the numbers of animals required for infection studies. For example, a study of 25 strains in parallel would require the use of 561 mice. In our competitive study all strains are pooled in a single infection and a strong indication of virulence defects for most strains in the pool can be obtained from only 5 mice. Unfortunately it is not possible to assess the virulence of all strains using this model as the defect in virulence from some strains can be overcome by cross feeding by other strains within the pool.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

As previously mentioned, our studies will be staged so only strains that are likely to have a virulence defect, or have been shown to have altered drug resistance will be used in our animal models. Wherepossible we will perform our experiments in parallel as wild-type isolate controls can serve as comparators across several 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our practice in performing virulence assays over past 8 years has been informed by our collaborators.With them, we have used species that have the least advanced nervous system whilst retaining the ability to closely resemble human disease. Advantages for studying fungal diseases in mice include ease of use, reproducibility and availability of immunosuppression regimes which mimic host factors ofhuman disease. Additionally murine disease models permit the use of well-established human clinical biomarkers of disease, making them highly amenable to evaluation of novel therapies and diagnostics,

which will become a major focus of our work in years to come. We routinely adopt the minimum experimental duration and infectious inoculum that provides meaningful data.

In all models, intranasal inoculations are performed under anaesthesia to reduce stress to animals. Wehave 3 well established models in which to study invasive aspergillosis in leukopenic or corticosteroid- treated mice. Herein we describe these as our fungal burden, competitive fitness and comparative virulence models. Fungal burden and competitive fitness analysis requires a lower fungal inoculum, results in a milder disease progression and earlier end-points than comparative virulence models, therefore we will utilise the former models whenever possible.

Specifically the fungal burden and competitive fitness models use a standardised time-limited end- point (such as day 4 post-infection at which point all mice are sacrificed). For comparative virulence studies where infections progress beyond 4 days, suffering will be minimised by ensuring mice do notexceed 20% weight loss from the start of the experiment (which has been previously established as a surrogate marker for moribundity). Close monitoring of mice will be undertaken to ensure to ensure they are humanly culled before exhibiting signs of distress that exceed our stated severity endpoint.

It is estimated that 75%-90% of mice used for comparative virulence experiments with strains with wild-type virulence characteristics will reach a moderate endpoint.

Why can’t you use animals that are less sentient?

We have historically employed a larval model, using the larvae of the greater wax moth (*Galleria mellonella*), of virulence as a proxy for virulence in mice. Recently we have been able to interrogate thevalidity of this model with greater accuracy using our competitive fitness technology. Although there is significant overlap between strains which show decreases in virulence in mouse models of infection, this is only the case for around 60% of strains. One of our objectives over the next 5 years will be to assess why this discrepancy exists and how it can be predicted. This will enable more precise and scientifically accurate use of the Galleria model.

There are several reasons why these models may be inadequate including significant differences in theimmunological and abiotic environment of the hosts, the significant dynamic changes in a murine lung that are not replicated in larval hosts.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Where possible, we will choose to conduct fungal burden experiments as opposed to virulence studies.The set endpoint for fungal burden experiments is usually before any animals would be expected to reach the humane endpoints we observe in virulence studies, thereby greatly reducing suffering.

Throughout all experiments we will adhere to a robust monitoring schedule, scoring each animalaccounting for a number of visualised factors, once signs of infection are present.

Through our previous work, we transitioned from using cortisone acetate to the steroid triamcinolone inan immunocompromised mouse model, either alone (steroid model) or in combination with cyclophosphamide (leukopenic model). Triamcinolone will be administered as a single dose 1 day priorto infection, replacing cortisone acetate, which required up to 5 doses, reducing the number of subcutaneous injections required. An

additional advantage to this drug is the solubility, improving the accuracy of dosing and subsequent immunosuppression over cortisone acetate.

Non-aversive handling techniques will be used to minimise the level and duration of stress, and animals will be handled as infrequently as possible. Animals will be placed in cages with sufficientenvironmental enrichment to minimise stress.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will adhere to PREPARE guidelines (https://norecopa.no/PREPARE) to ensure our experiments areas robust as possible, and contribute to the scientific community in the clearest possible way, removingthe need for similar experiments to be conducted by other researchers in the field. We will follow guidelines published by the Joint Working Group on refinement with regards to dosing routes and volumes, and take guidance from the wealth of NC3Rs resources available for conductingall regulated procedures, ensuring animals experience the lowest level of suffering possible.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

To maintain a broad knowledge of advancements in the 3Rs, our infection technician and other PIL holders will continue to regularly attend events organised by appropriate organisations, ensuring wehave an up to date knowledge on animal research as a whole, which can then be applied to our ownresearch.

We will monitor the literature on aspergillosis research for advances in our field of research, and attendconferences, providing the opportunity to discuss best practices with other fungal researchers. Over the lifetime of the project, we will incorporate any progression in the field into our experiments.

MECHANISMS UNDERLYING ABNORMAL HEART RHYTHM AND FUNCTION

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Heart disease, Arrhythmia, Cardiomyopathy

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo
Rats	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required,and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it’s addressing.

What's the aim of this project?

Disorders of the rhythm and function of the heart are an important cause of death in clinical medicine.Our proposal focuses on how disturbances in heart cells lead to abnormal cardiac rhythm and contraction and how the nervous system, infections or hormones in the body might regulate this pathology in genetically modified rodents.

A retrospective assessment of these aims will be due by 13 July 2028

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 hope our studies will lead to increased understanding of the pathobiology of heart rhythm disorders, animal models of human arrhythmic syndromes and cardiomyopathies and potentially newtherapies for those diseases.

What outputs do you think you will see at the end of this project?

We will communicate our advances through presentation at international meetings and publication inwell recognised peer review journals. Our work may also result in new research tools or medicines.

Who or what will benefit from these outputs, and how?

In the short term new ideas will be circulated in the cardiovascular research community. In the longterm we hope this feeds into the development of new therapeutic strategies for patients.

How will you look to maximise the outputs of this work?

We have national and international collaborations where we have made our mice available to researchgroups in the UK, Europe and USA. We will continue these collaborations but also forge new ones as new ideas emerge. We are also regularly approached for collaboration and training as we have a unique set of technologies we implement in the study of the cardiac electrophysiology of rodents.

Species and numbers of animals expected to be used

- Mice: 2000 mice per year

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We typically use young adult mice in most of our protocols. We use mice as there are a range of wellcharacterised genetic tools and resources. Furthermore, our technical interventions can be implemented reliably with scientific credibility in this species and in these age groups.

Typically, what will be done to an animal used in your project?

A stepwise approach will be used so that initial studies will be carried out using tissues from animals bred under the licence or will be done under a general anaesthetic from which the animals will not recover. As the lines of inquiry progress studies may involve surgery to, for example, implant devices that allow blood pressure and other measurements to be made. Some of these transmit radio waves to allow recordings to be taken from the animal following its recovery from the surgery without the animal being aware that the measurements are being made. In some studies the animals use wheels or treadmills so that the effects of exercise can be examined. Finally we use surgical procedures to mimic common cardiovascular pathologies. The protocols which cause the most deleterious effect for the animals involve an operation to place a thread around a coronary artery so that the blood supply topart of the heart is restricted to mimic a heart attack or to apply controlled pressure around a blood vessel in the abdomen to mimic high blood pressure. All surgery is conducted using general anaesthetics and the same types of aseptic measures to prevent infection as are used in human operating theatres. The animals will receive pain killers following the surgery. The animals are also monitored very closely and will be euthanised to prevent unnecessary suffering if they develop signs set out in the licence.

What are the expected impacts and/or adverse effects for the animals during your project?

The most likely impact is expected to be pain from the surgical procedures. All surgery is conducted using general anaesthetics and the same types of aseptic measures to prevent infection as are used inhuman operating theatres. The animals will receive pain killers following the surgery. The animals are also monitored very closely and will be euthanised to prevent unnecessary suffering if they develop signs set out in the licence. A number of the protocols may lead to impaired cardiac function and heart failure. For example, after ligation of a coronary artery and recovery as a model of myocardial infarction and heart failure the animal may suffer pain and distress from the surgery. We have optimised the surgery so that the vast number of animals now recover (>95%) an do not suffer from sudden cardiac cardiac rupture. These animals may develop heart failure indicated by reduced activity,piloerection, increased respiratory rate, weight loss and pedal oedema. We monitor these animals carefully for these side-effects and keep the animals no longer than is necessary to achieve the scientific objectives of the study (typically less than a month in this example).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The majority of procedures are mild in severity (70% often related to breeding mice for ex-vivo studiesafter schedule 1 killing) with only 25% being moderate in severity. A remaining 5% of mice are subjected to a severe protocol.

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 13 July 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animalswere affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The analysis of cardiac rhythm and function and associated pathophysiological situations often requires the use of intact animals. Complex physiological processes involving the function of a numberof interacting body systems are being examined. The normal working of the heart requires the function of several distinct organ systems including a functioning nervous system, vascular and renal function and respiratory function. As such these cannot be reconstituted fully using in vitro experiments. Thus such analyses require an in vivo analysis of the integration of the function of different organs and for the results to be extrapolated to human physiology these analyses need to be performed in intact animals.

Which non-animal alternatives did you consider for use in this project?

We use various cell based approaches including cell lines and human induced pluripotent stem cellsdifferentiated into heart and other cells to investigate cardiac arrhythmia and function. We have extensive links with clinicians looking after patients with these diseases. We are also performing genome wide association studies in am for ECG traits and these yield new molecules for study.

Why were they not suitable?

It is impossible to fully understand the interaction of different organ systems without the use of animalsand the function key molecules needs to be observed in their cognate tissue. There are limitations to the kind of interventional studies and tissues you can obtain from patients.

A retrospective assessment of replacement will be due by 13 July 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are based on the number required to deliver our scientific aims in our current fundedprojects. I anticipate maintaining this level of activity over the five years of the licence.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The numbers of animals used will be minimised by maximising tissue use from each animal and by designing experiments according to good statistical and scientific principles. Important experimental design features will ensure that the correct physiological conclusions are reached. Useful online tools and policy documents are available (e.g. ARRIVE guidelines and NC3R's Experimental Design Assistant). The structure of our study protocols also allows repetitive imaging and prolonged telemetrythat reduces the number of animals that to be used to address the scientific questions.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We have optimised the breeding stategies for many of our lines. We have ongoing collaborationswhere tissues are shared.

A retrospective assessment of reduction will be due by 13 July 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We mainly study genetically manipulated rodents particularly mice. Using new genetic technologies wecan delete genes in only some organs and\or at only some times during the lifetime of the animal. In general this allows us to reduce the severity of overall impact to the animals' health. The most severe aspects of the proposal are the models of heart attack achieved by tying off the coronary artery and increased blood pressure achieved by constricting the main blood vessel leaving the heart. The ligationof the coronary artery mimics the process of thrombotic vascular occlusion in the large arteries of man during myocardial infarction and constricting the aorta that of hypertension and\or aortic stenosis.

There are no real alternatives to mimicking these important pathological processes in a

controlledfashion. It should be borne in mind that cardiac arrhythmia is a significant health problem in thesesettings and results in a significant numbers of patients feeling unwell or even dying.

Why can't you use animals that are less sentient?

The use of more immature life stages does not mimic the behaviour of organ systems in the adult animal. Other less sentient animals have a role but they do not reproduce the physiological responsesin mammals. For example, fish such as zebrafish don't have lungs and the development of the heart isdifferent. In many of experiments we need to observe the behaviour of key physiological process suchas heart rhythm, blood pressure in the awake animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We are developing and using cutting edge technologies that remove the need for surgery during datacollection. For example the "ECGenie" which avoids the need to implant telemetry devices and sequential imaging techniques reduce animal numbers as it is possible to watch the progression ofpathology in a single animal.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

The NC3Rs website has a very useful library of resources and guidelines (https://www.nc3rs.org.uk/3rs-resources). Important advances are reported in the scientific literature.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Our local NVS and animal staff continuously provide helpful information. I receive regular notificationsfrom the NC3Rs and attend conferences showcasing advances in the 3Rs. I have been a committee member and reviewer for the NC3R Crack-IT initiatives and I am currently on the MRC Genetically Engineered Mouse Models committee. All these channels allow me to stay up-to-date with best practice and care.

A retrospective assessment of refinement will be due by 13 July 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved forfuture work of this kind?

- During the project, how did you minimise harm to the animals?

Home Office

MODULATION OF WOUND HEALING IN SMALL ANIMALS

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, ortheir 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 aimsmentioned in paragraph (b)

Key words

Wound Healing, Acute, Chronic, Therapies, Efficacy

Animal types	Life stages
Mice	adult, aged
Rats	adult, aged
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description 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 assist in the development of new therapies to: - minimise blood loss and disfigurement after traumatic injury, and promote or otherwise improve the healing of acute and chronicwounds.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
Acute traumatic injury to the skin and underlying tissues (due to accident or elective surgery) is extremely common, effecting millions of people each year. The control of blood loss immediately afterinjury and the subsequent development of disfiguring scars are key

issues following such acute trauma. The NHS spends ~£8.3billion annually on treating wounds, ~£5.6billion of which is spent on chronic wounds (venous leg ulcers, pressure areas and diabetic foot ulcers). These debilitating and slow healing wounds preferentially afflict (and severely impact) the elderly. Reflecting the progressivelyaging population their prevalence is rapidly increasing, year on year. Slow-healing (or otherwise defective) wounds are a significant burden to patients and their Quality of Life, their families, the NHS, and the economy. Existing treatments are largely ineffective – new, effective therapies are urgently required.

What outputs do you think you will see at the end of this project?

Based on past experience (gained from work undertaken under the authority of previous project licences), it is anticipated that work undertaken on this project will assist in the development and clinical uptake of new, more effective, therapies able to: - prevent excessive blood loss and scarringafter acute traumatic injury, and accelerate or otherwise improve the healing of acute and chronic wounds.

While our work is invariably commercially sensitive and undertaken under confidentiality agreements,we have and will publish our findings in respected peer-reviewed journals - wherever possible.

Who or what will benefit from these outputs, and how?

The process of new product development, testing and receipt of approval for clinical use is very timeconsuming - and as such, it is unlikely that any of the therapies tested in this project will achieve approval for clinical use within the lifetime of this licence.

That being said, three developmental therapies tested over the past 5 years (under our previousProject Licence) are now nearing approval for clinical use.

In the long-term, it is hoped and expected that patients with problem wounds or individuals that displayabnormal responses to injury will be the principal beneficiaries of our work under this licence.

The development of new more effective wound healing therapies, that accelerate or otherwise improvewound healing, would also be expected to reduce costs incurred by the NHS, and increase the profitability of those companies engaged in the development of these therapies.

How will you look to maximise the outputs of this work?

We will maximise the value of our outputs by dissemination through a variety of means. Subject to approval from study sponsors, we will endeavour to present our findings at scientific wound healingmeetings and publish as much information as possible from studies conducted under this Project License.

Species and numbers of animals expected to be used

- Mice: 2500
- Rabbits: 150
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult (and aged) mice, and adult rats and rabbits will be used in this project as they have repeatedlybeen shown to be effective screens to assess the impact of a wide range of therapies on the woundhealing process.

As it would be unreasonable and unacceptable to fully recreate the extreme conditions and complex pathologies of human chronic wounds in an animal, models have been established that exhibit delayedhealing due to one or other of the pathologies associated with chronic wounds in man. Studies involving the use of adult and aged diabetic animals, specifically the spontaneously diabetic 'db/db' mouse (which display characteristics similar to those of Type II diabetics - including delayed wound healing) are particularly widespread and considered clinically relevant. Similarly, the surgically-inducedrabbit ischaemic ear wound healing model is widely accepted as a clinically-relevant impaired healing model.

Typically, what will be done to an animal used in your project?

Prior to any procedures, animals will be acclimatised to their new environment for a period of between7 and 10 days - during which they will be largely left undisturbed other than to replenish their food andwater provisions and to refresh their bedding materials.

Under general anaesthesia, the fur at the planned wound/implantation site will then be removed byclipping and/or depilatory cream and the skin cleaned and disinfected.

The subsequent 'typical experience' of animals will depend on the species of animal and the protocolbeing followed.

For mice and rats undergoing wound healing protocols

Standardised (incisional or excisional) wounds will be created under general anaesthesia and asepticconditions. Animals will be given appropriate pain-relieving drugs and antibiotics to minimise post- surgical discomfort prior to recovery. Wounds will usually be dressed to prevent contamination with soiled bedding. Animals will then be allowed to recover from anaesthesia under warmed conditions.
Animals will be re-anaesthetised at various time points after injury (typically every 2 to 3 days for non- diabetic mice and rats, and every 3 to 7 days for diabetic mice) to permit photography, wound site assessments and re-application of topical treatments. Pain-relieving drugs and antibiotics will usually be given by injection under the skin on each occasion. These studies will typically run for up to 14 days(20 days in diabetic mice as they heal slower) after injury - though longer-term studies examining the effect of treatments on long-term parameters (such as scarring) may be performed. On conclusion of the study, animals will be humanely killed and tissues harvested for histological investigation (or other analysis).

Test or control treatments may be applied topically to the surface of wounds or to the animal as a whole (systemically). Topical treatments will usually be applied immediately after injury and subsequently re-applied at each dressing change (i.e., every 2 to 3 days

for non-diabetic mice and rats, and every 3 to 7 days for diabetic mice). Systemic treatments will usually be applied to restrainedfully-conscious animals by oral dosing or injection under the skin on a daily (or less frequent) basis forthe duration of the study.

For rabbits undergoing wound healing protocols

Under anaesthesia and aseptic conditions, the vessels supplying blood to one of the rabbits ears will be tied- off to reduce blood flow and thereby delay wound healing. Biopsy wounds will then be createdin both ears. Test or control treatments will then be applied to the biopsy wounds, and dressings applied to prevent contamination with soiled bedding. Animals will be given appropriate pain-relieving drugs and antibiotics by injection under the skin prior to recovery. Animals will then be allowed to recover from anaesthesia under warmed conditions. Animals will be re-anaesthetised at various time points after injury (typically every 3 to 5 days) to permit photography, wound site assessments and re-application of treatments and protective dressings. Pain-relieving drugs and antibiotics will typically begiven on each occasion. These studies will typically run for 15 to 20 days after injury. Test or control treatments will usually be applied topically, immediately after injury and subsequently re-applied at each dressing change (i.e. every 3 to 5 days). On conclusion of the study, animals will be humanely killed and tissues harvested for histological investigation (or other analysis).

For mice and rats undergoing the sub-cutaneous (under the skin) implantation protocol

Depending on how test products present (liquid, gel or solid), they will be inserted under the skin byhypodermic injection (liquid or gel) or surgical insertion (gel or solid) under general anaesthetic.

Surgical insertion will typically involve the creation of small scalpel wounds (typically 1cm in length) followed by the creation of pockets under the skin by blunt dissection (separation of the skin from underlying muscle involving minimal damage). Test substances will then be inserted into the pockets. The insertion (scalpel) wounds will be sutured closed and animals allowed to recover under warmed conditions. Animals will be given appropriate pain-relieving drugs and antibiotics by injection under theskin prior to recovery from anaesthesia. Sutures will be removed under general anaesthesia 5 to 7 days later. Animals will be killed and implantation sites harvested (for histological, or other analyses) atvarying time points up to 12 months.

All animals following these wound healing and subcutaneous (under the skin) implantation protocolswill typically receive a marker of cellular proliferation (or other tracer) by injection into the abdomen whilst restrained and fully conscious - one hour prior culling.

All animals following the wound healing protocols will be singularly housed for the duration of the study - as when housed in groups they have a tendency to groom one another which invariably results indressing damage. This can result in loss of test materials and contamination of wounds with soiledbedding - which can impact on the progression of wound healing and thereby invalidate the study being undertaken.

All animals following the 'under the skin' surgical implantation protocol will usually be singularly housedfor 7 days after implantation (to allow incisional wounds to heal) and group-housed thereafter; whereas, animals following the 'under the skin' hypodermic injection implantation protocol will be singularly housed for 2 days after implantation and group-housed thereafter.

What are the expected impacts and/or adverse effects for the animals during your project?

Based on over 20 years of conducting studies using similar protocols, we believe the protocols to befollowed in this project to be very well tolerated.

The adverse effects we expect to observe in animals following protocols in this project are: localisedwound pain, wound infection 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 bedisplaying signs of distress or discomfort, that does not respond to remedial actions (as advised by theNVS), will be killed by a schedule 1 method.

Wound infection

The loss of the skin barrier as a result of experimental wounding in rodents and rabbits can occasionally result in localised wound infection (occurrence under previous Project Licences <0.5%approx.). In order to minimise the likelihood of infection, surgical procedures will be carried out in accordance with Laboratory Science Animals Association Guiding Principles for Preparing for and Undertaking Aseptic Surgery. All animals will also be given appropriate antibiotics (as advised by theNVS) prophylactically (unless there is a potential for interference with scientific data).

Wound site infection is associated with increased (cloudy) exudation and wound malodour together with elevated peri-wound inflammation (heat, pain, redness & oedema). These indicators will be monitored, and if evident appropriate systemic antibiotics will be given. Wounds on rodents and rabbitswill be monitored for infection on a daily basis for the first week after injury and subsequently on a twice weekly basis. Where systemic antibiotics are ineffective, and the infection is considered to be compromising well-being, animals will be killed by a schedule 1 method. With frequent monitoring for wound infection, the duration of this adverse effect will be limited to 1-3 days, which we believe is insufficient time for significant clinical manifestation of adverse signs.

Weight loss

Repeated anaesthesia, initially for the purpose of wounding and subsequently for follow-up assessments and re-application of substances, and the repetition of other 'mild' activities (such as dosing) 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 obese animals. Animals will be monitored regularlyfor weight loss, and where greater than 5% loss is observed in wild-type (normal) animals, or greater than 10% in observed in obese 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 weightstabilisation and often in gain. Any animals that display greater than 20% loss in body weight (25% for diabetic 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 animaltype)?

Mice (including diabetic mice) - wound healing protocols- expected severity **MODERATE** - proportionof animals 100%

Rats - wound healing protocol - expected severity **MODERATE** - proportion of animals 100% Rabbits - ear wound healing protocol - expected severity **MODERATE** - proportion of animals 100%Mice - sub-cutaneous implantation protocol - expected severity **MILD** - proportion of animals 40% Mice - sub-cutaneous implantation protocol - expected severity **MODERATE** - proportion of animals60%

Rats - sub-cutaneous implantation protocol - expected severity **MILD** - proportion of animals 40%

Rats - sub-cutaneous implantation protocol - expected severity **MODERATE** - proportion of animals60%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The mammalian response to injury is multifaceted and complex - involving actions, interactions and responses of numerous cell types present at the site of injury and delivered to the site from other partsof the body via the blood stream.

Wound healing research in animals is necessary as, while laboratory investigations on cells or pieces of skin (from animals and humans) can generate important preliminary data (such as toxicity data, andsome indication of 'likely' efficacy); they are unable to fully replicate the multiplicity of physical and biochemical reactions, cell types and cell interactions, that occur in and around wounds as they heal.

In vivo wound healing studies in animals also offer the possibility of investigating mechanisms of actionof, and/or, the development of unexpected adverse interactions to wound healing substances/therapiesat the tissue/cell/molecular level, which, as this invariably requires wound tissue excision, would be largely ethically unacceptable in the clinical setting.

Which non-animal alternatives did you consider for use in this project?

We considered, and use (together with academic collaborators), a variety of non-animal approaches toachieve our research aims.

These include undertaking wounding assays (called scratch-assays) on sheets of cultured skin cells,and studies of the healing of wounds created on pieces of cultured (live) human skin (taken from patients with excess abdominal skin or following surgical amputation).

Why were they not suitable?

While these non-animal alternatives can provide useful information, that can assist and guide the design of animal studies, such alternatives cannot replace them. The complexity of the wound healingresponse, particularly the involvement and interaction of numerous cell types from different parts of thebody means that wound healing and the effect of developmental therapies on wound healing, can onlybe studied in intact animals.

Reduction

Explain how the numbers of animals for this project were determined.

Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientificobjectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissueand reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals needed for each protocol based on our experience of usingthese approaches in previous projects and anticipated future requirements.

This includes both our experience of the minimal number of animals required, for each treatment groupto provide valid and useful data, alongside our experience in, and understanding of, the wound healingsector, and the likely demand for our research models. We have made assumptions on future requirements based on our best assessment of the science and our previous experience.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Group sizes for all of our protocols are based on 20 years previous experience with these animals andprotocols, the relevant scientific literature and power calculations. We utilised available online resources such as the NC3Rs experimental design assistant to plan experiments and perform power calculations to determine sample size. These calculations were based on knowledge of the mean values and variability of the primary outputs for each protocol - taken from our prior experience and that of others.

Our group sizes are the lowest possible to allow for the infrequent “in study” loss of animals (due to death or complications); whilst maintaining a high probability that a study will be sufficiently powered for statistical analysis on completion. This reduces the likelihood that animals will undergo unnecessaryprocedures in statistically underpowered studies.

We design experiments so that multiple experimental readouts can be derived from a single animal. We use imaging when possible so that wound healing can be tracked non-

invasively and confirmed bytissue approaches after humane killing.

The creation of multiple wounds sites in individual animals allows for “within animal” controls to beused (where appropriate) which reduces the total number of animals required for a given study.

We standardise our experimental variables in order to minimize variation, such as using animals thatare closely matched in terms of age and of a single strain. This also reduces the total number of animals required for a given study.

As we have managed to reduce variation, we have established very reproducible protocols. Thismeans that it is not always necessary to repeat certain positive control groups and that historicalcontrol data can be used – thus reducing the number of animals required in a given study.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We work closely with an academic group that have extensive experience in the development and useof non-animal alternatives – including wounding assays on sheets of cultured skin cells (scratch- assays) and assays of the healing of wounds on cultured (live) human skin.

Where appropriate, these non-animal alternatives will be used to screen-out inappropriate investigational agents and determine the most effective dosing regimens for subsequent animal studies - thereby reducing the numbers of animals required for effective *in vivo* investigation.

In instances where the pre-existing data (in relation to potential efficacy) is considered insufficient to undertake a fully powered study, or where a large number of formulation variants exist, preliminary pilotstudies, involving small numbers of animals/wounds, will be undertaken to determine the need for more extensive investigation or to screen-out less effective variants – and thereby reduce the numbersof animals used.

We regularly share animal tissue with other research groups and have a good communication networkwithin the University to alert other groups to available 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mouse, rat and rabbit models of wound healing, mouse and rat models of *in vivo* implantation, and a rat model of haemostasis.

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.

The surgical methods used to create wounds or implant materials, the routine use of pain killing drugsand warmed recovery, the size and number of wounds or implants/implant sites per animal, and the frequency, form and duration of follow-up assessments and dosing procedures/dosing regimens that we use, have been progressively refined to minimise harms - during the course of our previous licences.

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 assessmentsand substance administrations by the most refined route, possible.

Wherever possible, we avoid single housing of animals unless it is essential for scientific reasons or animal welfare. Group-housed animals have a tendency to groom one another, which for our protocolsinvariably results in removal of sutures or damage to dressings - which in turn can lead to rupture or contamination of wounds - and ultimately invalidation of the study. That being the case, all animals arehoused-individually for at least 7 days after wounding, and where necessary for the duration of the study.

Wherever possible, environmental enrichments (e.g., forage food, nesting materials and wooden chew-sticks) will be provided to animals following our protocols.

Why can’t you use animals that are less sentient?

The mammalian response to injury is multifaceted and complex – and as yet, these complexities haven’t been fully recapitulated in less sentient species. Recent research undertaken on Zebrafish suggests that whilst their response to injury is similar to mammals in some ways – it is clearly differentin others; and it is well known that not all data derived from Zebrafish studies and other less sentient models is relevant to humans.

Because wounds take days or weeks to heal, it is not possible to study the entire wound healing process under terminal anaesthesia. That being said, one of our protocols, in which we determine theimpact of agents on the process of haemostasis (halting blood flow after trauma), is undertaken entirely under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

As noted earlier, the surgical methods used to create wounds or implant materials, the routine use of pain killing drugs and warmed recovery, the size and number of wounds or implants/implant sites per animal, and the frequency, form and duration of follow-up assessments and dosing procedures/dosingregimens that we use, have been progressively refined to minimise harms - during the course of our previous 3 project licences.

Opportunities for further refinement are are continually sought.

The following refinements were made during our most recent project licence:
We moved from oral (gavage) dosing with steel gavage 'needles' to dosing with less rigid andthereby less damaging flexible plastic 'needles'.

We routinely use 'black-out' felt head covers (like falcon hoods) when dosing conscious animals.This has an immediate calming effect and appears to make dosing procedures less stressful.

While we do not expect any significant physical, behavioural or physiological deviation from normality in animals following our protocols, we undertake regular monitoring of key well-being parameters whichfacilitate the early detection of unexpected adverse effects (that may impact on animal pain, discomfortor distress) and thereby allow the rapid deployment of remedial action.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will use the following resources in planning and conducting experiments:ARRIVE Guidelines 2.0. https://arriveguidelines.org/arrive-guidelines PREPARE Guidelines. https://norecopa.no/prepare
NC3Rs Experimental Design Assistant. https://eda.nc3rs.org.uk/

NC3Rs guidance on blood sampling in mice. https://www.nc3rs.org.uk/3rs-resources/bloodsampling/blood-sampling-mouse

NC3Rs guidance on blood sampling in rats. https://www.nc3rs.org.uk/3rs-resources/blood-sampling/blood-sampling-rat

NC3Rs guidance on blood sampling in rabbits. https://www.nc3rs.org.uk/3rs-resources/blood-sampling/blood-sampling-rabbit

NC3Rs guidance on microsampling, including the microsampling decision aid. https://www.nc3rs.org.uk/3rs-resources/microsampling

NC3Rs Mouse Grimace Scale. https://www.nc3rs.org.uk/3rs-resources/grimace-scales/grimace-scalemouse

NC3Rs Rat Grimace Scale. https://www.nc3rs.org.uk › 3rs-resources › grimace-scales › grimace-scale-rat

NC3Rs Rabbit Grimace Scale. https://nc3rs.org.uk › 3rs-resources › grimace-scales › grimace-scale-rabbit.

NC3Rs guidance on anaesthesia. https://www.nc3rs.org.uk/3rs-resources/anaesthesia
NC3Rs Guidance on analgesia. https://www.nc3rs.org.uk/3rs-resources/analgesia
NC3Rs Guidance on handling and restraint. https://nc3rs.org.uk/3rs-resources/handling-and-restraint

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery. https://www.lasa.co.uk/wpcontent/

uploads/2018/05/Aseptic-Surgery.pdf

EFPIA/ECVAM good practice guide to the administration of substances and removal of blood, includingroutes and volumes.
https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/abs/10.1002/jat.727

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Our group will stay informed through the NC3Rs website. Relevant information, including the NC3Rsnewsletter, is circulated within our institution by email to all personal and project licence holders. Wewill attend local events organised by our Animal Welfare and Ethical Review Committee and information sessions on NC3Rs funding streams organised by our institution's Research & InnovationService. We will share best practice within our institution and have well developed interdisciplinary networks to facilitate this. We attend regular local user-group meetings for project licence holders at which the group will receive updates on any changes to best practice or requirements.

MOLECULAR CONTROL OF NEUTROPHILS AND ENDOTHELIAL CELLS IN INFLAMMATION AND REPAIR

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

endothelial cell, neutrophil, 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 required,and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it’s addressing.

What's the aim of this project?

This project investigates molecular processes that underpin the generation, propagation and resolutionof inflammation by analysing (i) the molecular control of neutrophils, abundant circulating immune cellsand endothelial cells, the building blocks of blood vessels as well as (ii) cross-talk between cell types, in particular the key players mentioned above, in inflammation.

A retrospective assessment of these aims will be due by 07 August 2028

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Inflammation is an important part of any immune defence against infections. It is tightly regulated to ensure that infectious agents are killed while the body's own tissues are preserved, and any bystander damage is promptly repaired once the threat has been removed. As part of the 'healthy' inflammatory response, vessel walls become a little leaky, allowing plasma fluid and proteins to enter the surrounding tissue. Moreover, immune cells, notably neutrophils, breach vessel walls to reach inflamedsites and fight infections. However, breakdown of the tight regulation of immune cells and their interplay with blood vessels can result in important tissue damage. This is the case in chronic inflammation, where the immune system is in overdrive and harms the body's own tissues. Similarly, inacute situations, an overblown inflammatory response is not helpful. This is illustrated for example by COVID-19 or 'flu, where severe disease, e.g. in the elderly, is the result of an excessive inflammatory response rather than the viral infection per se. The interplay of immune cells with blood vessels is particularly obvious here, and can result in accumulation of plasma fluid in the lung, which in turn can interfere with breathing.

What outputs do you think you will see at the end of this project?

This work will result in an improved understanding of molecular events in both neutrophils and endothelial cells that occur during inflammation and its resolution. We aim to identify why things go awry, and how neutrophils contribute not only to generating inflammation but also to healing of bystander damage in healthy organisms. By making use of disease models in conjunction with analysing patient samples, we aim to understand how these mechanisms go awry in disease with a view to identifying better treatment for patients in the long run. In the short term, this work will result innew knowledge which will be published in specialist journals.

Who or what will benefit from these outputs, and how?

In its different guises inflammation is one of the biggest killers, but unfortunately at present, we only have access to a small number of blunt treatments, that often target the symptoms but not the cause ofinflammation. In the long term our work will contribute to improved therapies for those suffering from excessive inflammation. Our contribution will be an indirect one, because we aim to understand how inflammation works in so-called pre-clinical work. In other words we prepare the foundations for future clinical work that will use the knowledge we and others generate to identify the best drugs for particular inflammatory processes.

How will you look to maximise the outputs of this work?

Prior to embarking on the study, pilot experiments will be used for calculations called 'power analyses' that work out how many repeats of any experiments will be required to

reach statistically sound conclusions. Once completed, this work will be published in specialist journals and discussed with specialist audiences at conferences. To ensure that the work will reach its full potential, publications willinclude detailed study protocols and information on statistical analyses that were performed.
Furthermore, publications will not only report those approaches that were successful, but any pitfallsencountered along the way will also be written up. The aim of this is that ultimately unsuccessful experimental approaches will not be repeated by others.

Species and numbers of animals expected to be used

- Mice: 4600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Inflammation is very complicated and cannot easily be modelled. Mice will be used to interrogate neutrophil and endothelial cell function and neutrophil-endothelial interactions in inflammation. Using genetically altered mice can allow us to interrogate the function of a particular gene in both endothelialcells and neutrophils. Mice are the model of choice since their immune system is very well understood
and serves as a model of the human immune system. The models we use are well established modelsfor human disease and they are performed using adult mice .

Typically, what will be done to an animal used in your project?

Most of our models involve the induction of inflammation. These models involve administration of substances that cause inflammation in a specific place, e.g. the lung. We then analyse signs of inflammation, e.g. by weighing the animals and analysing their tissue (e.g. blood, lungs). In some cases we administer drugs to test whether doing this will reduce the inflammation. The actual experiments last from several hours to a couple of weeks to analyse early events during the inductionof inflammation or induction and resolution of inflammation.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of our experimental models are of very short duration and do not result in very noticeable adversesymptoms. An exception is the viral infection model, where mice will display weight loss and have a reduced activity level prior to making their recovery following infection with influenza virus. In most models used here, preparatory steps are likely to be associated with more adverse effects than the inflammatory models themselves. In some cases mice are reconstituted with stem or progenitor cells after having received a dose of irradiation that ablates the stem cells which form the blood. Unless newblood forming stem cells are administered (i.e. the mice are being 'reconstituted'), this is lethal. The lethal dose of irradiation is required here to avoid rejection of the graft by the original immune cells (which would make the mice very unwell). If, however, reconstitution with haematopoietic stem cells is successful, the irradiated mice will recover and be absolutely fine. This is a well established tool in the

laboratory (and the wider field); in many instances it can avoid the use of a much larger number of mice. In our experience deaths following irradiation and reconstitution is extremely rare (far less than 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 animaltype)?

Of the total of 4600 mice projected to be used over the course of 5 years, it is expected that the severity experienced by 41.5% (1900) will be sub-threshold, by 15% (680) mild, and by 40.5% (1870)moderate, while the severity of 3% (150 mice) is expected to be severe.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

A retrospective assessment of these predicted harms will be due by 07 August 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animalswere affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Using genetically altered mice allows us to interrogate the function of a particular gene in both endothelial cell and neutrophil. Moreover, to analyse inflammation we need the entire body. For thisreason we perform experiments in mice.

Which non-animal alternatives did you consider for use in this project?

Isolated (human) neutrophils and cultured endothelial cells alone and in combination.

Why were they not suitable?

Human neutrophils are not amenable to genetic manipulation, and specific inhibitors do not exist for allprocesses of interest. Co-culture models allow us to study an interaction between isolated endothelial cells and neutrophils. While instructive this is unfortunately not adequate to fully model inflammation that occurs in an organism, which is much more complex and includes involvement of additional factors in the body.

A retrospective assessment of replacement will be due by 07 August 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Publications and previously conducted studies offer an insight into mouse numbers typically requiredfor studies. The estimation provided above is based on such estimates.

Since neutrophils are extremely short lived they cannot be cultured and genetically modified. Thereforelarge numbers of animals (hundreds per year per project) are used as a source of genetically modified neutrophils for analysis in vitro. Moreover, the generation of a new genetically modified mouse line requires a lot of breeding, in particular where more than one genetic modification is required, e.g. for a conditional knock-out, or even just for re-derivation into a unit followed by breeding to homozygosity or where back-crossing to a particular genetic background is required. This results in the use of a large number of mice (>100 per line even with modern approaches)

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

For those experiments that analyse inflammation in vivo, we perform pilot studies prior to designing definitive experiments involving larger cohorts; experiments will be planned, conducted and reportedaccording to NC3R and ARRIVE guidelines.

One of our projects analyses conditionally HoxB8-transformed haematopoietic progenitor cells from mice. We and others already showed that these can be used to derive HoxB8 neutrophils, which behave like neutrophils in many ways. We currently have a project underway in which we aim to genetically manipulate HoxB8 neutrophils to avoid the generation of new mouse lines as reservoirs ofneutrophils for analysis in vitro. We also trial whether HoxB8-transformed progenitors can be administered to mice for in vivo experimentation, potentially further reducing the requirement for breeding genetically altered animals for experimental purposes with a view to solely analysing neutrophil behaviour.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

The majority of mice we use are bred in-house. Wherever possible we plan experiments very carefully so that we can answer several questions at once by using more than one read-out for the study, e.g. byanalysing more than one tissue. Breeding all animals in-house means careful planning to ensure that not too few nor too many mice are born.

However at times there will be some mice that we cannot use in experimental cohorts. We use tissue from such mice to optimise new in vitro experiments, and for pilot studies.

A retrospective assessment of reduction will be due by 07 August 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Inflammation is a highly complex process that cannot be modelled based on cells in culture alone. We use mice and have carefully chosen experimental models, employing models of very short duration foranalysis of early events in inflammation. This reduces the time in which suffering can occur. Where weanalyse also the resolution of inflammation, we carefully titrate the stimulus again with a view to induceminimal suffering required while achieving our scientific objectives.

Why can’t you use animals that are less sentient?

Although different to humans, mice represent at present the best available animal model that is representative of inflammation in human beings. Not only do mice have comparable immune system and inflammatory responses to humans, there is also a wealth of genetic mutants that allow us to decipher the importance of individual regulators. This is important since some of these may representgood drug targets, which is what we hope to find.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Most of our inflammatory models are very short with a duration often only of hours. These models werecarefully selected because they will reduce the length of any suffering. In our previous experience with these models we have not observed clinical signs in mice on these models.

The pulmonary infection model (protocol 7) is the exception to this. This model involves administrationof virus (e.g. a mouse adapted influenza A) to cause lung inflammation. Dose finding experiments areperformed where viruses are titrated carefully to ensure that the lowest titre commensurate with the aim of the experiment is being used. While animals typically make a full recovery from this model, the protocol has been classed severe in case some of our GA animals may be more susceptible to the lung inflammation caused by the viral infection.

Animals on this model are weighed daily and are carefully monitored and scored for clinical signs (posture, activity level, responsiveness, temperature, breathing, piloerection), with weighing and scoring performed twice daily while the mice are acutely unwell. Clearly defined endpoints are applied for any animals exceeding severity levels (no spontaneous activity, no response to touch or a clinical score >4 for more than 24 hours; loss of >25% body weight compared to original body weight, or >20%for >5 days).

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We stay up to date on best practice guidelines and updates by making use of the NC3Rs website(www.nc3rs.org.uk)

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We make use of the online resources provided by NC3R and follow developments in the literature. We moreover keep informed on new developments with the help of informational seminars and events heldlocally, and where applicable change our practice accordingly.

A retrospective assessment of refinement will be due by 07 August 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved forfuture work of this kind?
- During the project, how did you minimise harm to the animals?

MOLECULAR ECOLOGY OF BATS

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Ageing, conservation biology, bats, epigenetics, genetics

Animal types	Life stages
Bats (Chiroptera)	juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the causes and consequences of genetic diversity in bats.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 understand how genetic variation affects reproductive success and survival in the longest-runningstudy in mammal ecology worldwide. We now have a genetic dataset spanning 30 years. Long-term studies are important for understanding how populations respond and adapt to climate change for example. The genetic dataset has allowed establishment of a 30-year pedigree which allows insightsinto topics such as the heritability of traits and biomarkers associated with ageing, now a major focusof research under this licence.

To understand the genetic mechanisms underpinning extreme longevity (relative to body size) in bats.Bats live remarkably long given their small size and provide key insights into ageing. Determining biological characteristics associated with ageing within and across taxa assists in the identification of factors associated with extreme longevity, and studies

on animals with exceptionally long lifespans areespecially relevant. Insights into the mechanisms underpinning exceptional longevity in bats may havevalue for extending healthy lifespans in humans and could therefore be of value to medical scientists and researchers working on the biology of ageing.

To inform conservation and management strategies of rare and protected bat species. For example,populations with low genetic diversity can be identified and mitigation for improving gene flow can beimplemented. We can identify important sites for mating and inform conservation measures such as fitting grills to reduce disturbance by humans.

Our research involves educational activities, the training of PhD students and postdoctoral scientists,many of whom are likely to continue careers in academic situations or in conservation biology.

What outputs do you think you will see at the end of this project?

Publications in high impact, open access journals.

New information on genetic and epigenetic mechanisms promoting extended lifespans that may confera better understanding of ageing.

Training of PhD students and postdoctoral researchers.Outreach activities to promote bat conservation. Evidence to assist conservation organisations in making management decisions, for exampleconcerning impacts of habitat fragmentation and climate change on genetic variation.

Who or what will benefit from these outputs, and how?

Scientists working on conservation, life histories and ageing will be provided with new data via openaccess publications.

Young scientific researchers will be trained in methods in both the field and laboratory through PhDprojects and postdoctoral experience.

Evidence will be made available (by sending publications) to Statutory Nature Conservation Organisations (e.g. Natural England) and Non-Governmental Organisations (e.g. Bat ConservationTrust) to inform management decisions needed to sustain or enhance the viability of populations ofendangered bats.

Our work will benefit researchers working on ageing and may have implications for better understanding human ageing and healthspan. For example, we have previously identified genes associated with telomere length maintenance (telomeres typically shorten with age resulting in breakdown of cellular mechanisms in most animals), and epigenetic changes associated with cancer resistance in bats. Information will be provided via publications and through presentations at scientificconferences.

How will you look to maximise the outputs of this work?

The work is interdisciplinary and highly collaborative, involving collaborations with internationally leading researchers in the USA and Ireland. We strive to publish findings in the highest quality scientificjournals, and by making regular presentations at international conferences. We will publicise key findings via outreach activities including on information boards and through videos available to visitors at our main study site and will use social

media to announce new publications.

Species and numbers of animals expected to be used

- 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This study will use bats, as it is the ecology of these animals that is the basis of the work being undertaken. Bats are remarkable in having some of the longest lifespans in relation to their body size of any mammal. Of 19 mammal species with exceptional longevity quotients (lifespans longer than expected for their body size), 18 are bats with one 6g species living for at least 42 years. They have been targeted as organisms of special interest by scientists working on ageing to better understand themechanisms underpinning long life spans.

We have been working on a long-term study that allows unique insights into life histories over decades.The work builds on an extensive database of genetic information already in place, allowing us to track longitudinal changes in genetic parameters within individuals.

Our methods are of mild severity and we work on infants and adults with no adverse effectsdocumented to date.

Typically, what will be done to an animal used in your project?

Bats are captured in nets and approved capture devices at field and roosting sites. These methods arelicensed by the Statutory Nature Conservation Organisations, are used widely by scientists and ecological consultants, and cause no major harm to the bats. They are held individually in cloth bags and then measured and weighed. We take small (3mm) biopsy punches from the wing and tail membranes and place these in tubes of preservative. We also take small blood samples from blood vessels in the wing or tail by pricking a superficial blood vessel and collecting blood samples in capillary tubes. Animals are put back into their holding bags, then released at the site of capture typically within 3 hours of capture (all bats are typically released in one batch so that processing of the whole sample is uninterrupted, complete and efficient, and so that bats are reunited with all colony members soon after release).

What are the expected impacts and/or adverse effects for the animals during your project?

Bats experience mild stress during capture and during procedures. All procedures are conducted on animals caught in the wild and released at the site of capture, so no transportation of animals for procedures to be undertaken is needed. We aim to minimise stress by minimising the amount of timethe animal is held for. Bats are recaptured regularly over the year so we can detect any adverse effects. We have experienced no obvious adverse effects of conducting these procedures during 30 years of doing them. During capture we follow guidelines issued by the Joint Nature Conservation Committee

that are considered best practice in terms of animal welfare.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Both tissue and blood sampling are of 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 haveconsidered 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 our studies is to explore the causes and consequences of genetic variation within andamong bat populations. There is no realistic alternative to using living animals for these studies. Although some aspects of the study could be attempted outside of the ASPA, e.g. some success has been achieved with genotyping bats via their faeces, this approach is not appropriate for the proposed work for the following reasons (as would probably be the case for urine):
1) The amounts of DNA obtained using this method are very small and often below the minimum required for reliable detection.
2) The quality of DNA extracted from faeces is unpredictable and often of poor quality, making analysisdifficult.
3) The times needed to obtain a sufficient quantities of faecal material (often several hours) is much longer than that required when using a regulated procedure (can be as brief as several minutes) and consequently imposes a significant stress on the animal.
4) we have been using standard protocolsfor quantifying genetic profiles over 30 years and need to adhere to these for consistency. We need reasonable quantities of tissue material so we can extract DNA to use in several analyses (paternity, telomeres,DNA methylation), and we need to sample blood to obtain RNA for blood transcriptomes, asthese are considered representative of a wide range of bodily functions.

Which non-animal alternatives did you consider for use in this project?

There are no alternatives, especially as we need to track genetic profiles over many years in a wildmammal population.

Why were they not suitable?

Our main study population is unique and has been studied over decades. We work on wild populationsand need to understand how life history and environmental factors shape genetic variation in nature.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 will be kept to the minimum need to achieve the aim of each study. In studies of relatedness and mating associations at the intensively studied population of greater horseshoe bats, it will be necessary to sample as many individuals in the population as possible to obtain as accurate estimations of paternity as can be achieved. We already have DNA samples from many bats in our study population but need to sample all individuals born into the colony, all immigrants, and occasionally bats that were sampled many years ago under previous licenses whose DNA may have degraded, though whose genotype it may be necessary to determine at new markers through continued use. At present we are sampling about newborn and immigrant 100 bats/year at our focal study site, and this may rise if the colony grows. We will need to take biopsy samples from the same bats across different years to assess within-individual changes in telomere length.

We will sample each individual in our main study colony once annually for DNA used in telomere length measurements (ca. 150 individuals annually). We use the same DNA samples for telomere length measurements and for DNA methylation methods used in epigenetic clock analyses. We use smallernumbers of blood samples in our analyses. We also sample at feeding sites for population genetics studies, may have new studies that develop, and 600 bats sampled annually should cover all eventualities. My Home Office PPL returns have under 500/year during my current PPL.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

All animals need to be sampled annually for longitudinal telomere length and DNA methylation change measurements. All newborns need to be genotyped for parentage studies. Regarding transcriptomics, my collaborators have extensive experience in gene expression studies on other bat species, and we will use their findings to guide sample sizes for blood sampling. For example, we have submitted a proposal to investigate how age and reproductive effort affects immune function quantified by gene expression. We will need an estimated 120 samples (60 females and 60 males in 3 age classes) basedon similar studies in a bat and baboon populations where 2000-6000 differentially expressed genes were identified in a similar number of age groups.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We have used power analysis in grant proposals for example to estimate the precision of age acceleration in different sample sizes from DNA methylation profiles, and will continue to pursue this approach. We perform these analyses with advice and input from biostatisticians and epidemiologists.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The methods we use are established as best practice throughout the world and enable use to obtainreliable sources of DNA using the least severe methods needed to provide high-quality data. We willmanage the capture, sampling and release of the bats carefully based on over extensive field experience. Capture methods such as hand netting and mist netting, together with holding methods,are all licensed by Statutory Nature Conservation Organisations such as Natural England, with methods described in The Bat Workers' Handbook available at http://jncc.defra.gov.uk/page-2861.

The licence holder has over 30 years experience in handling bats and is competent at recognising potential signs of injury or disease prior to animals being used in regulated procedures. No such issueshave arisen to date in our research. We have a high recapture rate of individuals within and across years, so any long-term welfare consequences can be monitored. Our capture and holding methods result in all animals being released together, and permit efficient processing by licensees when procedures are being conducted.

Why can't you use animals that are less sentient?

Our methods are mild in severity, and we have detected no adverse effects. For genotyping studies(where age is not of importance in analyses) we sample infants because the holes made by biopsypunching typically heal within days or a few weeks.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Bats are captured repeatedly throughout their lives in our long-term study so any long-term impacts resulting from procedures can be detected. I read the specialist literature and have considered findingson healing times of biopsy punching in relation to age and season, and will factor findings that can minimise any potential harms into the sampling design.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Capture methods such as hand netting and mist netting are licensed by Statutory Nature ConservationOrganisations such as Natural England, with methods described in The Bat Workers' Handbook available at http://jncc.defra.gov.uk/page-2861

Published work on blood and biopsy sampling is published in textbooks such as 'Ecological and Behavioral Methods for the Study of Bats', and papers on methods appear in journals that I subscribeto including the Journal of mammalogy, where ethical guidelines

for studies on mammals are also provided.

Best practice for blood sampling is provided at the NC3Rs in vivo methods website, and will befollowed.

https://www.nc3rs.org.uk/3rs-resources/blood-sampling/blood-sampling-general-principles

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Regular updates on courses and resources are provided by the institution's Named Information Officerand accessed as appropriate.

NEURAL MECHANISMS OF MEMORY, FROM CELLS AND CIRCUITS TO BEHAVIOUR

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

memory, neurons, hippocampus, brain dynamics, circuits

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to establish how cells and circuits in the brain work together to gate andcontrol selective memory recall.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Memories are not typically overwritten by new learning. Instead, we continue to acquire memories throughout our lifetime, which we can recall in a highly selective manner. However, we don't understand the specialized biological mechanisms within the brain that control selective memory recall.This has important implications for our understanding of diseases such as Alzheimer's disease and schizophrenia where memories may remain inaccessible or become inappropriately activated. What prevents us from establishing the biological mechanisms that control selective memory recall within thebrain? Perhaps the greatest challenge to contemporary neuroscience is that it is simply not possible to record cell- and circuit-level activity in the living human brain, except in rare circumstances.

Instead, non-invasive methods are coarse, providing an aggregate readout of the diverse responses of thousands of neurons over space and time. To gain access to neural activity at the cell- and circuit- level we instead rely on invasive procedures in animals. To relate findings in animals to measures in humans the work proposed in this project will contribute to a broader programme of work that promotescross-species research.

What outputs do you think you will see at the end of this project?

Publications

Open source software, for common and parallel analysis of data across animals and humans

Open source data of relevance to pharmaceutical companies developing new drugs for treatment ofmemory disorders, including psychiatric disorders such as schizophrenia

New technological developments

Who or what will benefit from these outputs, and how?

Short-term (1-5 years): new discoveries made as part of this research program will allow us to furtherunderstand the role of neurons in the brain. These findings will provide insight into the brain mechanisms that underpin memory.

Long-term (5 years +): By conducting parallel experiments in humans, findings that derive from the proposed research in mice will be related to an understanding of memory in humans. The long term benefit of this approach is that we can use our research output to inform our understanding of memorydeficits that occur in disease states and thereby facilitate drug discovery.

How will you look to maximise the outputs of this work?

Through collaboration, sharing of data and code, dissemination of new knowledge via conferences andseminars

Species and numbers of animals expected to be used

- Mice: 8000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In this project we will use mice. Mice are the most widely used animals for research involving behavioural paradigms. The structure of the mouse brain is well described together with activity of cellsin the mouse brain. The technology that we propose to use has been specifically designed for mice. We will use adult mice that are 3-6 months old to allow investigation of memory mechanisms that canbe related to those in the adult human brain.

Typically, what will be done to an animal used in your project?

Typical procedures implemented in this project will include intracranial injection and implant of devicesin the brain to allow recording of brain cells during behaviour. Typically each mouse will undergo 1 or 2surgical procedures. Following recovery, a typical recording experiment includes active waking behaviour with intermittent rest/sleep sessions which include long rest periods in a familiar home environment. Recording typically takes place for 1-2 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Following brain surgery, all animals are expected to make a rapid and unremarkable recovery from theanaesthetic within two hours. Administration of analgesics for control pain will be performed alongside food enrichment to promote good and fast recovery. Full recovery is expected within 7-10 days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Mild: 20%

Moderate: 80%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The objective of this project is to identify cell and circuit mechanisms within the brain that are relevant to higher-order cognition and behaviour in health and disease. To address this objective there are at present no possible replacements for small rodent-related experimental work. In the proposed work wewill use mice which show clear structural and functional equivalents to the human brain, despite differences in brain size. In addition, there is good availability of mouse transgenic strains for the proposed anatomical work and for use of causal manipulations.

Which non-animal alternatives did you consider for use in this project?

Recordings in humans, in-vitro methods and computational models.

Why were they not suitable?

Recordings in humans: Recording brain activity in humans is currently restricted to the use of coarsenon-invasive tools which measure the average response from thousands of cells. Therefore, activity atthe level of single cells and synapses cannot be readily inferred from non-invasive measures in humans.

In vitro recordings: In vitro recordings provide limited insight as brain networks do not necessarilyremain intact and behaviour is not preserved.

Computational models: Computational models are not a viable alternative as they do not fullycapture behaviour-related brain dynamics.

Conclusion: Findings reported from studies using all of the above methods have been used to informthe predictions and design of this project. However, to address the objectives of this project there is noalternative model for the recording of brain activity associated with higher-order cognition and behaviour. It is therefore necessary to use animals to meet the aims of the project. The project will generate a valuable data set that can in turn be used to inform future studies performed using the above alternative methods.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 a small number of animals in this project primarily because we are using a multi-neuron recording technique, an approach that leads to the collection of large datasets from each individual. Therefore, more data are collected from each individual animal (from ~40Gb to ~120Gb of data/individual/day) compared to more traditional electrophysiological preparations. The estimated number of animals is based on more than 7 years of previous experience in conducting multi-neuronrecording techniques.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The behavioural tasks implemented in this project will be developed from previous tasks that we havedesigned, developed and replicated in the lab.

To reduce the number of animals used in this project the following steps will be taken:

The project will use mice: Mice are the lowest vertebrate for which experimental tasks designed toinvestigate memory and higher-order cognition are well-characterised, thus reducing the need for extensive task development and further use of animals.

Within-subject design: The design of our experiments is such that each mouse is used as its own control (within-subject analyses), thus reducing variance and, in turn, reducing the number of animalsused.

Randomisation: to reduce experimental bias, randomization will be implemented using computer- generated sequences. This includes randomizing allocation of animals to different experimental conditions (e.g. causal manipulation vs control group); randomizing the assignment of stimuli to eachmouse; randomizing the order in which stimuli are presented in the test sessions.

Keeping variables constant: To further reduce bias, other variables will be held constant, including animal housing and handling protocols which will be logged across all experiments. Testing will be conducted at approximately the same time each day. Variables that are difficult to control, such as foodintake relative to body weight, will be logged and accounted for in experimental analyses using statistical methods such as multiple regression. Where possible, recordings and analyses will be conducted blind to the condition allocation.

Multiple recordings and rich data analysis: The numbers are also kept small because multiple recordings are made from each animal. In addition, in each animal we collect multivariate physiologicaldata to allow application of rich, multifaceted data analysis techniques.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Efficient 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 breedinganimals 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.

Pilot studies: We will typically analyse data rapidly after data collection. This approach effectively allows the initial experimental cohort to be used as pilot data for subsequent experiments. This reducesthe number of animals used by minimising technical errors and ensuring that the project is fully optimised to address the aims of the 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use small rodents (mice) because: (a) they are the most widely used animals for research involving both sophisticated behavioural paradigms and research methods that rely on specific genetically altered strains; (b) the anatomy and physiology of the mouse brain is well described; and
(c) the proposed procedures have been specifically designed for and successfully used in this species.

In our project we will record brain activity using microdrives: small, compact devices that includes recording electrodes/fibers and/or lenses. We have been recording using microdrives since 2015 and this product has undergone a series of refinements. As a result, the device and implantation techniques are optimised to minimise impairment to the animals natural behaviour. Mice implanted witha microdrive can easily voluntarily eat, drink at the spout of the water bottle, groom, nest, and express normal exploratory behaviour such as rearing.

For intracranial injection we will use sterotaxic guidance which uses a set of three coordinates that, when the head is in a fixed position, allow for the precise administration of substances to the relevantarea of the brain. This method means the risk of damage to surrounding tissues is minimal. The substances we will inject are tracers that tag or label cells in the target brain region and are unable toreplicate. Therefore, the injected substances do not integrate into the host genome and our approachis the least harmful. Indeed, these traces have been used widely by the neuroscience community andno adverse effect has been detected

Behavior will be recorded using video tracking while animals explore an arena or maze, or while animals sleep in an environment similar to their home cage. In most of our experiments we are seeking for the animal to use its natural exploratory behaviour to learn about an environment. This approach minimises any potential harm to the animal and provides the most refined approach to recording brain activity. Animals are therefore given the opportunity to explore arenas and mazes which often include sensory cues such as auditory and visual cues. We will also use appetitive cues such as sucrose to incentivize animals to learn about their environment. Appetitive cues such as sucrose will sometimes be used while animals have controlled access to food or water. Controlled access to food or water involves reducing the normal baseline weight by up to 10-15%. This approach motivates mice to learn rapidly and minimises the overall duration of an experiment. In addition, it is recognised that schedulesof food or water withdrawal are not harmful. During controlled access to food or water, the body weightand condition of the animal are regularly monitored and the amount of controlled food/water given is recorded. Overall, our approach to animal behaviour is refined to minimise any distress or harm to the animal.

Why can’t you use animals that are less sentient?

Non-mammalian animal models that are considered less sentient, such as fish or invertebrates orthose at a more immature life stage, do not share the same brain circuits as adult mammals. For example, the hippocampus and related circuits, which are the focus of this application, are not preserved in non-mammalian models and not fully developed in ore immature life stages.

To address the objectives of this project, which aim to characterise brain activity during behaviour, it isalso not possible to use animals that have been terminally anesthetised.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Animals will receive training in their behavioural tasks, to familiarise the animal with the recordingenvironment and promote learning.

Our surgical procedures will continue to undergo refinement by liaising with our veterinary surgeon. Analgesia is given prior to surgery and continues until the animal is fully recovered. Warmth and easilyaccessible food and fluids are also provided. Implanted

animals will be housed alone as cage-mates can potentially damage the recording electrodes. However, animals will be placed in sight of other animals and, if possible, placed next to old cage-mates during rest periods. After animals have recovered from surgery and prior to recording, animals will be handled frequently. This will help minimise stress during behavioural tasks and recording, and help accustomize the animal to the experimenter. During a typical recording day we will include intermittent rest and sleep sessions, including in a familiar home environment. During controlled access to food or water, the body weight and condition of the animal will be monitored daily. Periods of fasting will occur during the day and controlled food/water delivered in the evening. Where possible, systemic administration of substances/drugs will occur via the animal's diet or drinking water.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

LASA, NC3R's, ARRIVE and PREPARE guidelines

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

I will stay informed about advances in the 3Rs via the *Named Information Officer* and *NC3R's regionalmanager* who sends out 3Rs News Letter and organises the annual 3Rs Research Day for the establishment. I also attend the termly meetings within the establishment that focus on 3Rs.

Incremental advances will be communicated to all researchers working on the project and implemented after consultation with advisory staff at the establishment.

NEUROPROTECTION IN 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, ortheir 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 aimsmentioned in paragraph (b)

Key words

Neuroprotection, neurorepair, traumatic brain injury, focal stroke, neuroplasticity

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this programme of work is to focus on the discovery of new compounds that could be beneficial in patients with acquired brain injury, such as traumatic brain injury (TBI) or focal stroke. At present, there are no treatments that protect the brain in the acute and subacute phase of an injury, ortreatments that support neurorepair and promote neuroplasticity.

The way we choose the compounds we are going to test will be based on i) the on-going research in other related neurology fields, where neuroprotective agents are identified, which may target pathological cascades which are relevant to brain injury, and ii) on-going research worldwide on new mechanisms underlying damage in brain injury. Both these strategic approaches will feed our pipelineof compounds. Furthermore, iii) we will explore the interaction of treatments with rehabilitation approaches, so that we reproduce realistically the clinical setting of such injuries, and the patient experience.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In the last 50 years, the adoption of new standards of care and advances in routine medical management, have led to improved life expectancy and better health for people living with the consequences of a brain injury. Some progress in specialised rehabilitation has also led to some incremental improvements in function post-injury - although the provision for neurorehabilitation is veryvariable worldwide.

However, overall, despite much progress in neuroscience research over recent decades, there is still no available treatment for protecting the brain against the major loss of tissue that is the consequence of the spread of the injury beyond the zone of the initial impact (in both TBI and stroke) and the loss of further function that results from it; so far, no consensus has emerged as to the best strategy to use forbrain protection and repair in individual patients, and the degree of long-term recovery of patients remains rather unpredictable. These remain among the largest unmet needs in neurology and trauma medicine, and therefore research efforts in this area are still needed.

What outputs do you think you will see at the end of this project?

We aim to develop a variety of innovative treatments for use at different phases following injury: acutely, subacutely, and in the chronic phase, and thus provide neuroprotection against protracted lossof tissue and also support regenerative processes. The main expected outputs are:

- the identification of key novel compounds for neuroprotection and repair of the brain after acquired brain injury; neuroprotective approaches could subsequently also be used prophylactically (inthe military context, before exposure to increased brain injury risk) or for prevention of cumulative injury load in repeated concussion in sports
- the identification of combinatorial treatment modalities (including, for example, combined use ofpro-repair compounds and rehabilitation procedures) for protecting against the long-term changes seen in the brain after injury and for the support of regeneration;
- the identification of strategies that would be effective in newly-injured patients or those alreadyliving with a chronic injury, from brain injury in young adults to injury in late middle-age;
- a better understanding of the mechanisms of action of novel therapies and also identification ofnew targets for treatment

All these outputs would be reflected in original publications and they may lead to pilot exploratoryclinical trials.

Who or what will benefit from these outputs, and how?

In the short-term, our research will inform the scientific community working on TBI and stroke of new potential approaches for neuroprotection and repair. But the main aim of our approach is its translationfor the benefit of the patients. TBI and stroke affect a significant number of patients worldwide and areassociated not only with dramatic consequences for

the concerned individual and their family and carers, but also with major health and social care costs.

The acute management of TBI is mainly focused on medical interventions to stabilise patients and reduce the risk of high intracranial pressure. At present, there is no specific pharmacological treatmentto limit the consequences of the secondary injury that continues to propagate within the central nervous system tissue in the aftermath of injury, expanding the injured zone. There is thus a large unmet clinical need for agents that would protect the injured brain from the acute phase and into the chronic phase of the injury.

A preparation that could be administered by the emergency team in the first hour after trauma, before or during transfer to the hospital, would represent a major medical innovation and would lead to much-improved prospects for patients. This could be followed by interventions aimed at supporting long-termprotection of the brain and support of its repair, through enhancement of neuroplasticity. This integrative and multifaceted approach which follows the clinical care path, would aim to improve the autonomy of the patients and their quality of life, with the long-term goal of reducing the size of the population of individuals living with a disabling TBI. It is realistic to envisage that the clinical translationof some of the approaches we propose to test in animal models could start within the duration of this project. This has already happened in the context of our previous brain injury licence: a specialised medical multinutrient which improved significantly the outcome in mice with a brain injury, is tested at present in a pilot study in patients with TBI. Such dietary interventions could be also investigated as a pre-emptive protection against increased risk of TBI (e.g. military cohorts), or in the civilian context, asa protection against the impact of repeated concussions during a sports season in young athletes.

How will you look to maximise the outputs of this work?

We have ongoing national and international collaborations with experts in neurotrauma research, lipidsand lipidomics, and medicinal chemistry, and we have established programmes of work on neuroprotection and repair with various pharmaceutical companies involved in the development of innovative approaches to acute injury and neurodegeneration. Some of our partners have been involved in the development of new chemical forms of fatty acids, for improving their stability and delivery to the central nervous system (CNS), and this will allow us to expand for example our work onthe potential of fatty acids as neuroprotectants, by producing new therapeutic formulations. Other collaborators have developed new peptides with neuroprotective and pro-regenerative properties, that we have been testing in our laboratory. Therefore, we have a network that will help us disseminate widely the knowledge generated by our research. The network also includes clinical collaborators, andthis will facilitate clinical translation of our experimental research.

Species and numbers of animals expected to be used

- Mice: 2500
- Rats: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 rats and mice in the experimental models of TBI and focal stroke described in this project proposal. Although there are some differences in certain aspects of the pathology of brain injury in these species as compared to human pathology, differences which are inherent to evolutionary aspects, rodent models are the most widely used to study brain injury – most of the literature publishedso far in neurotrauma research has been generated in these species. The anatomy of their nervous system is well understood and has similarities with the human nervous system, well-established functional analysis techniques are available to study their recovery following injury, and many therapeutic interventions have progressed towards the first phase testing in clinical trials in TBI and stroke, based on rodent studies. Mice have the added advantage that they can be modified genetically,and there are various genetically altered mice which target pathways that are relevant for the pathophysiology of injury in the central nervous system; genetically modified rats have also become available. This availability of genetically modified animals offers the potential of providing information on key molecular determinants of the injury mechanisms and the resulting pathogenesis, thus leading to new possible drug targets. For translational purposes, and to avoid the risk of possible biases of effect seen in a specific species, in translational medicine the use of mice and rats gives the opportunity of confirming the efficacy of tested strategies in two species, which is a key factor for increasing the probability of successful clinical translation. Because TBI disproportionately affects the younger population and results in a life expectancy that isstill below that of the general population, young adult and middle-aged rats and mice will be mostly used in this project for TBI models. However, as stroke affects older patients, the project will also use aged animals, mostly for the focal stroke studies.

Typically, what will be done to an animal used in your project?

In a typical experiment, the animals' baseline neurological status would be assessed up to four weeks before undergoing an experimentally induced TBI or focal stroke, which is also the period during which the animals would be trained on more complex behavioural tasks. In some cases, animals may receive a treatment, such as a modified but palatable diet, that will correspond to variations of aphysiological diet, with altered levels of certain components - to study how these modify the response to injury. They would then undergo a controlled moderate the injury. The injury may be a head injury (with a closed skull approach or open skull approach), or a focal stroke. Following this, animals might be injected with an active compound in the tail vein or through other parenteral routes – single bolus or repeated injections, or start another form of treatment (e.g., a dietary intervention). Treatment could be started either immediately after injury (first 1-2 h), in the subacute phase (i.e., over the first 1-3 weeks after injury), or once a state of chronic injury has been reached (e.g. after the first month). Post-injury, the animals' neurological status and behavioural recovery would be tested regularly under a specific schedule (daily, every 2-3 days, weekly, monthly) that would depend on the task and duration of the experiment. The tests chosen for assessing neurological outcome will address project-specific questions, and animals will not be exposed unnecessarily to a comprehensive set of tests testing all neurological domains. We will use a graded approach, i.e. for the treatment strategies tested, we will evolve from simple global neurological assessment, to specific domains of neurological function. This will avoid cumulative suffering aspects. The duration of the experiment would vary depending on the question being investigated – e.g. from 1 month to investigate the new compounds' efficacy for neuroprotection, up to 6-8 months to investigate the efficacy for repair in the chronic phase and the support of neuroplasticity. In some animals we willalso carry out tract tracing, which is a method of analysis that allows us to look at individual neural projections in the brain and assess the

integrity of brain circuits, after injection of a molecule that acts as a tracer, to assess the degree of plasticity of the brain circuits. Some animals will also undergo imaging, using translatable imaging markers. The analysis of the brain circuits using tract tracing and using imaging, which will add to mechanistic understanding, will only be carried out after we obtain some initial proof-of-concept evidence that newtreatments that we are investigating have significant potential to improve neurological outcome. This will lead to minimising cumulative suffering aspects.

What are the expected impacts and/or adverse effects for the animals during your project?

Our team has extensive expertise in inducing well-controlled experimental interventions, and in the supporting care and welfare of the animals during the studies. Animals may be at risk of developing discomfort or pain during the acute phase (24-48 h) that follows surgery (post-operative recovery period), which will be managed by providing appropriate perioperative analgesic relief. Despite the local CNS injury, animals recover well, requiring very limited special care; yet all animals will be closelymonitored during the studies, to regularly assess their cognitive and physical behaviour, along with clinical assessments. **Animals will also be administered substances, will be anaesthetised and blood samples collected, and the behavioural responses of the animals will be explored withspecific tests. All these procedures could induce a mild, transient discomfort for 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 animaltype)?

All interventional procedures are kept under a mild to moderate severity range, inducing focal,controlled injuries.

Mice: mild, 50%; moderate, 50%

Rats: 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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Trauma in the central nervous system triggers a complex cascade of events, both in the key affected organ and in other organs around the body (e.g. a systemic inflammatory reaction). There is no alternative that would entirely replace the use of living animals for studies of the complex response of the injured nervous system. Clinical studies can only be carried out when new investigational entities have been shown to have efficacy. This

element of prediction of efficacy in humans needs to be tested first in experimental species, to have some indication whether efficacy occurs in vivo**.** In vitro modelling can be used to explore certain cellular mechanisms which are characteristic of the response of various brain cells, but this approach fails to mimic the systemic injuryresponse. Therefore, animal studies remain a necessary strategy to improve our understanding of traumatic injury and to support the development of new treatments.

We are working closely with clinicians to fully integrate clinical information and knowledge in our studies, and implement experimental outcomes such as MRI imaging or blood biomarker analysis, thatare clinically relevant. We are also using various in silico (e.g. Machine Learning) and in vitro models (e.g. 2D cultures and organotypic brain slice cultures), that allow us to undertake a lot of early discovery and compound screening testing. More recently, we have started developing work on induced pluripotent stem cells, to be able to study human cells and thus reduce and replace the use ofanimals. We can also use cell lines to undertake early testing studies, prior to translating potential treatments into whole animal studies. Our Centre has various collaborations with other national and international teams, with the objective to promote sharing of resources, including clinical and animal data /tissues, to maximise the impact and efficiency of our studies and enable the replacement of earlydiscovery phase animal studies.

Which non-animal alternatives did you consider for use in this project?

Because of the nature of the research, addressing the issue of protection of a complex organ such as the brain against the consequences of injury, there are at present no non-animal alternatives that we could use in this project, as it is essential to understand the injury response at both organ level and systemic level. However, the in vitro analysis of the response of cultured cells of human origin (for example, human induced pluripotent stem cell-derived neurons) is a complementary approach that weare currently developing in the laboratory. This approach will enrich the field of brain injury and will provide very relevant human cell-specific information which will replace, in time, some of the animal use in this field. However, the response to injury involves not only the central level response but also aperipheral response, and to reproduce this complexity, it is necessary to carry out whole animal studies.

Why were they not suitable?

The only non-animal alternatives that could be used at present are in vitro systems based on human-derived cellular material, but these cannot reproduce the systemic response to injury, which is an important contributor to the outcome.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 carried out following good laboratory practice and will be designed after carefulexamination of all the relevant literature (e.g. PREPARE guidelines), which will

inform us as to the appropriate group sizes. All the animal studies will be designed so they are based on solid mechanistic evidence which is already available from in vitro experiments, and they address clinically relevant questions, based on clinical observations, thus having immediate utility for translation.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Sample size calculations will be done before each experiment, so that studies are adequately poweredstatistically. We are using for reference the information about group size that we have accumulated over decades of our own experience in neurotrauma research. We are also using the guidanceprovided by the NC3R's Experimental Design Assistant.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

For the exploration of new concepts in pilot studies, we will use a minimum group size, just as a pilot. In the design of the experiments, we can also optimize the number of animals by sharing controls, when we test multiples substances. We will also maximize the amount of information obtained from every animal, by correlating the effects of treatments on the functional outcome, with theeffects seen in terms of protection of the brain tissue. This will be achieved by carrying out the tissue analysis at the end of the behavioural assessment, thus obtaining tissue information in the same animals where we have the behavioural data. Furthermore, in some instances we will carry out studiesusing approaches such as imaging, which will allow us to follow the same animal for a period of time, therefore reducing the number of animals used in chronic assessments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice and rats, as these are the major species used in the research carried out so far by specialist groups working on brain injury worldwide. Most literature on acquired brain injury (trauma or stroke) has been produced in models of injury in rats and mice, as they have a nervous system which is similar to the human nervous system. Both rodent species reproduce many of the relevant sensory- motor and cognitive deficits seen in the brain injury affecting human patients. Furthermore, these smallrodents can be modified genetically. This could help establish which genes could confer increased resistance to injury, and would also help understand why certain compounds are neuroprotective, by elucidating mechanisms – and even why they may have different efficacy in different individuals, through elucidation of resilience factors.
The models used for the moderate traumatic injury (controlled cortical impact) and mild concussive injury (single or repetitive) and the model of focal stroke, are commonly used in

the field and their useis now well-established in our group. These experimental interventions are well refined, supported by perioperative analgesia, good supportive care and appropriate housing and handling to ensure their natural behaviour (e.g. animals being group-housed during the behaviour assesments). The fact thatthey are used by the leading researchers in the field means that there is an abundant literature whichallows us to compare our findings to what other progress is taking place in the field. These models of brain injury used in our studies induce little distress (mild to moderate severity) and the animals display abnormalities which can only be detected using specific neurological tests. The differences do not affect the overall welfare of the animals. Furthermore, humane endpoints are all well established inour studies.

Why can't you use animals that are less sentient?

The injury to the adult brain affects neural circuits which have undergone stabilisation and refinement after the developmental period is completed. Therefore, the use of immature animals would not be appropriate. Similary, as brain evolution has reached a high degree of complexity in higher species, theuse of species that are less sentient and whose brain has a lower level of complexity would lead to results which would not help understand the disruption induced by injury in more complex circuits.
Finally, the use of animals terminally anaesthetised is not relevant for therapeutic studies, becausedeficits incurred after a brain injury need to have full expression as motor, sensory and cognitive capabilities, and these cannot be expressed under anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Any animal which shows adverse clinical signs which deviate from the effects expected after injury willbe killed humanely and immediately. The neurological abnormalities associated with our models of brain injury will be mild to moderate and will not be allowed to progress beyond the minimum required to achieve the scientific objectives of the project (moderate severity). Our group has developed good expertise on working with TBI models in laboratory rodents, and has improved the clinical and behavioural assessment in these models, particularly using non-harmful behavioural assessments in grouped housed animals. We have also carried out studies based on 24/7 constant monitoring and video recording in the housing cages.

All the animals will receive appropriate analgesia after the procedures and be appropriately nursed with food/drink support and recovery housing (quiet, warm environment and back to their original group). Our previous work has allowed us to develop specifically designed humane endpoints to address the severity of the procedures and ensure the well-being of the animals. Critical neurological impairments are not expected in our studies, and are well-described in our humane endpoints to avoidany suffering in our animals. We will also be implementing the use of imaging and biomarker analysis to assess the disease progression in living animals, to continue promoting the use of minimally invasive approaches.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

For all our research on injury in the central nervous system, researchers will follow the guidelines produced by the NC3Rs. "The Responsibility in the use of animals in bioscience research" general guidance document will be used as a reference document, as it sets out the expectations of the funding bodies for the use of animals in research. The updated

ARRIVE guidelines (2.0), designed fortransparent reporting of animal research methods and findings, will be consulted for the planning and design of new experiments to ensure reliability and reproducibility of findings. In this respect, the “Guidelines for planning and conducting high-quality research and testing on animals” (2020, ref. 1) and PREPARE (2018, ref. 2), offer comprehensive guidance and a 15-point checklist for planninganimal studies.

We will follow the guidelines of the UK Research Integrity Office (UKRIO), including the use of both sexanimals to avoid any sex bias, and will aim to register our study protocols in an international online register of protocols for preclinical animal studies.

Smith AJ (2020) Guidelines for planning and conducting high-quality research and testing onanimals Lab. Anim. Res. 36:21. doi: 10.1186/s42826-020-00054-0. eCollection 2020.

Smith AJ et al. (2018) PREPARE: guidelines for planning animal research and testing. Lab. Anim.52(2):135-141. doi: 10.1177/0023677217724823.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We are regularly updating our knowledge using the NC3R website, so that we are aware of the latestadvances in the 3Rs. Our Named Veterinary Surgeon and Named Information Officer, are also a source of information and he keeps us updated. We regularly attend the events focused on animal welfare and best animal research practice organised by our institution.

NOVEL GENETIC REGULATORS OF CARDIOVASCULAR SYSTEMS BIOLOGY

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

RNA metabolism, cardiovascular system, vascular biology, atherosclerosis, ageing

Animal types	Life stages
Mice	embryo, neonate, juvenile, pregnant, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description 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 evaluate how the regulation of our gene affect the blood vessels in health,atherosclerotic vascular diseases (when fat buildup in our vessel) and ageing. This work may help us to develop novel pharmacological interventions to improve our cardiovascular health.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Atherosclerotic vascular diseases is a serious condition where arteries become blocked with fatty substances called plaques. The most important behavioural risk factors of

atherosclerotic vascular diseases are unhealthy diet, physical inactivity, tobacco use, harmful use of alcohol and ageing. In theworld, atherosclerotic vascular diseases is the number one cause of mortality and morbidity in the world. According to the British Heart Foundation factsheet, 7.4 million people are living with heart andcirculatory diseases in the UK meaning that every 8 minutes someone dies from coronary artery disease. There is an urgent need of studying the molecular mechanisms involved in atherosclerotic disease to improve diagnosis and treatment options.

On a biological level, our bodies are formed of many networks that are integrated and communicatingwith each other. From the DNA to the cells composing the organs of the body, the human species is fundamentally a network of networks. Cardiovascular systems biology focuses on these networks based on the understanding that the whole systems is greater than the sum of the parts. It is not enough to understand only one part of a system when studying the complexity of biology.

The foundation of all of these network's rests on this statement "DNA make RNA and RNA make protein". The genetic architecture of the human body comprises more than 40,000 genes with big or small effect contributing to the genetic diversity of the population. However, each individual has morethan a million proteins. How is this possible?

The answer lies on the biology of the RNA. RNA is an important biological macromolecule that functions to convert the genetic information of DNA into proteins. These RNAs can be subjected to modifications and this how a single gene can give rise to multiple protein products. The RNA modification studied in our work are called RNA editing. These novel genetic regulators (or RNA editors) can change the code and the behaviour of RNAs and are carried by molecules named ADAR1,ADAR2 and WTAP.

There is now increasing evidence that cardiovascular diseases are influenced by changes in RNA. Thisproject will therefore investigate the role of RNA modification in health, atherosclerotic heart disease and ageing using genetically modified mice having parts of the RNA modification pathway altered (ADAR1, ADAR2 and WTAP). The information these mice will provide will increase our understanding of cardiovascular biology and may lead to the discovery of novel potential therapeutic targets and improved diagnostic tools.

What outputs do you think you will see at the end of this project?

We are hoping, with the present multidisciplinary project, to achieve the following outputs:

New information:

- Discovery of novel genetic regulators regulating circulatory system health.
- Discovery of novel genetic regulators regulating circulatory system in atherosclerotic heartdiseases.
- Discovery of novel genetic regulators regulating circulatory system in ageing.

Publications:

Scientific publications to disseminate our knowledge helping to drive new research forward. Newsletters for the general public to understand how our research translates into public benefits..
Products:

Developing patents based on our discoveries.

Discovering new therapies to modulate the cardiovascular system in health and disease withcollaborative work with a health company.

Who or what will benefit from these outputs, and how?

Identifying RNA modifications as a signature of cardiovascular diseases will be of great importance for developing innovative interventions. It will have significant consequences for researchers studying cardiovascular dysfunction in many age-related diseases. This project will impact different stakeholdersnationally and internationally by providing a wealth of novel insights in cardiovascular pathophysiology:

The research community:

- by developing new knowledge about how vessels and heart work.by providing new pathways of research.
- by providing a model to prevent cardiovascular disease

The healthcare and clinical community:

by discovering new genes that may cause a disease similar to those observed in humans.
by studying the role of genes already known to be involved in human diseases.

The patients:

- by being able to understand the fundamental mechanisms of their disease.
- by providing the therapeutic potential of targeting RNA modifications metabolism to prevent ortreat cardiovascular diseases.

The general public:

- by providing new knowledge on the genetic components of the circulatory system.

How will you look to maximise the outputs of this work?

Our laboratory is a multidisciplinary network of scientists, clinical academics, clinicians and healthcareprofessionals aiming at reducing the cardiovascular disease burden. This will help us to maximise theoutputs of this work by:

- sharing the positive and negative outcomes of our *in vitro*, *in vivo* and clinical work.
- designing each experiment to maximise the amount of information gathered. For example, ourintention is to keep blood and tissue samples from mice for potential use to answer additional questions posed by our group or in collaboration with other research groups.

The dissemination of the new knowledge emerging from this project will be made available:

To the scientific community:

through presentations, posters and talks at local, national and international meetings. our findings from this project will be published in peer-reviewed multidisciplinary journals. through professional web platforms (Research Gate, Linkedin)

To the general public:

- through a comprehensive outreach program in order to communicate the most interesting andbroadly relevant elements by working with different centres for public outreach in the UK:
- to present our work at science festivals
- to participate in public talks and workshops organized in collaboration with our Establishment.
- through patient seminars
- through social networking platform (our website and twitter account, our University's website)
- through open access to our scientific publications.

Species and numbers of animals expected to be used

- Mice: 10850

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In this project, mice will be used due to:
- Their similar cardiovascular structure to humans.
- The ease to investigate target genes by genetic manipulation.
- In this project, mice at different life stages will be used:
- Embryo, to measure the impact of the genes on development. Neonatal, to measure the impact of the genes on vascular growth.Adults, to measure the impact of the genes on organ functions.

Typically, what will be done to an animal used in your project?

Wild type or mice produced under breeding protocols with standard Home Office practice will be used.Mice are bred using standard breeding practices (Protocol 1, 6600 animals and Protocol 2, 1250 animals). Depending on the strain, the animals will have one the following three procedures to assesstheir cardiovascular function (Protocol 3, 3000 animals; see flow diagram):

Gene activation:

Mice will be subjected to tamoxifen injections up to once a day for a maximum of 9 days to repress a specific gene.

- Mice may have administration of single or a combination of two different biological substances(optional step). The least invasive method of delivery appropriate for each

substance would beused.
- Mice may have blood sampling of the tail vein (optional step) OR animals may be imaged in suitable equipment (e.g. MRI, PET or CT scan, echocardiography, ECG, Laser Doppler) for amaximum of 5 times under general anaesthesia (AB) (optional step).

Adult animals will have terminal procedure from one of the following procedures (mandatorystep):

Terminal anesthesia to assess the ventricular function (AC).Exsanguination (AC) Perfusion fixation (AC)Schedule 1 killing

Atherosclerosis:

- Animals will be be fed by Western rich diet for a maximum of 20 weeks.
- Mice may be subjected to tamoxifen injections subcutaneously or intraperitoneally up to once aday for a maximum of 9 days to repress a specific gene.
- Mice may have administration of single or a combination of two different bioactive substances (optional step). The least invasive method of delivery appropriate for each substance would beused.
- Mice may have blood sampling by venepuncture or venesection of the tail vein or saphenousvein (optional step) OR animals may be imaged in suitable equipment (e.g. MRI, PET or CT scan, echocardiography, ECG, Laser Doppler) for a maximum of 5 times under general anaesthesia (AB) (optional step).

Adult animals will have terminal procedure from one of the following procedures (mandatorystep):

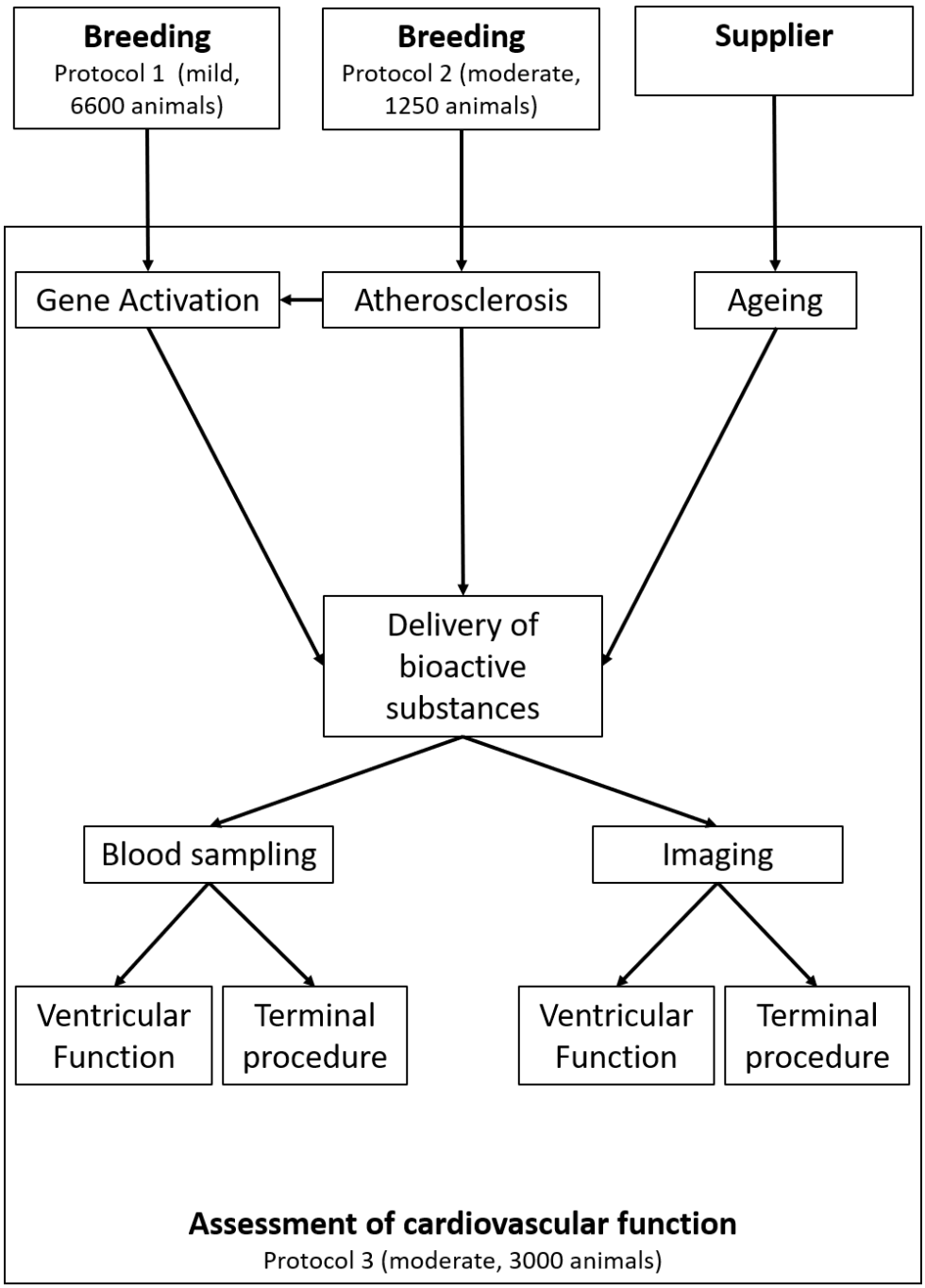
Terminal anesthesia to assess the ventricular function (AC).Exsanguination (AC) Perfusion fixation (AC)Schedule 1 killing

Ageing:

- Animals will be allowed to age up to 24 months.
- Mice may have administration of single or a combination of two different bioactive substances (optional step). The least invasive method of delivery appropriate for each substance would beused.
- Mice may have blood sampling by venepuncture or venesection of the tail vein or saphenousvein (optional step) OR animals may be imaged in suitable equipment (e.g. MRI, PET or CT scan, echocardiography, ECG, Laser Doppler) for a maximum of 5 times under general anaesthesia (AB) (optional step).

Adult animals will have terminal procedure from one of the following procedures (mandatorystep):

Terminal anesthesia to assess the ventricular function (AC).Exsanguination (AC) Perfusion fixation (AC)Schedule 1 killing



What are the expected impacts and/or adverse effects for the animals during your project?

Breeding and maintenance of genetically altered mice (protocol 1, mild):

- We expect the observable characteristic(s) to be sub-threshold and genotyping will generally beundertaken using surplus material from ear notching for identification.
- Breeding and maintenance of genetically altered mice (protocol 2, moderate):
- We will be using transgenic mice carrying mutation to remove the RNA editor WTAP in vascularendothelial cells (cells lining our blood vessels). Mice carrying this mutation may be embryonically lethal.
- We will be using transgenic mice to mimic the atherosclerosis vascular disease. These mice willhave a marked increase in fat in the blood vessels. These mild characteristics will lead to very little (if any) adverse effects.

Assessment of cardiovascular function (protocol 3, moderate):

- Administration of tamoxifen for gene deletion: mice may develop adverse effects due to gene deletion (e.g. inactivity, rough hair coat, closed eyes, failure to eat or drink or laboured breathing),although they are not expected to exhibit any harmful phenotype.
- Atherosclerosis model: Atherosclerotic mice show a marked increase in total plasma cholesterolthat is unaffected by gender or age. Fatty streaks are found in the proximal aorta at 3 months ofage. The lesions increase with age and progress to pre-atherosclerotic lesions. Rarely there aresigns of ill health such as poor feeding, poor coat condition, loss of weight (>20%), and reducedmovement (Incidence <1%).
- Ageing model: Aged mice often display a 'scruffy' fur, with the presence of piloerection, hunchedposture and reluctance to move which are part of the normal ageing process. In addition, aged mice are often larger and may be overweight.
- Delivery of bioactive substances: Delivery of bioactive substances may result in unanticipated problems, such as poor feeding, poor coat condition, loss of weight (>20%), or reduced movement. Adverse effects may occur depending on the delivery process, the vectors used orthe bioactive substances themselves:
- Altered food or water: Unpalatability of the diet/drinking water treatments.Gavage: lung injury, weight loss and stress.
- Minipump: There may be poor healing or infection of the skin where the minipump has been inserted. Wounds breakdown or infection may occur. Occurrence of these signs is:bleeding (<5%), swelling or redness (<5%), wound not healing (<1%).
- The vector injections may provoke non-specific symptoms of subdued behaviour (e.g. piloerection, hunched posture; 2-3 day duration) but are not associated with long-term adverse effects on the animals. The aim of this delivery is to improve animal health.
- Blood sampling: Potential problems that may arise as a consequence of poor sampling techniqueinclude stress, haemorrhage, bruising, thrombosis, infection at the site of needle entry, phlebitis, scarring and nerve damage.

Non-invasive imaging in adult animals:

- Anaesthesia: Anaesthetic overdose could occur, the likely incidence of this is less than <1%. Adverse effects will be recognised by the fact that the animal would show altered respiratory patterns with gasping respiration. Repeated anaesthesia (5 times maximum) forimaging could result in a reduction in body weight, the likely incidence of this is < 20% of animals. Animals might fail to make good recovery from isoflurane anaesthesia. Adverse effect will be recognised by the fact that the animal does not return to normal mobility and activity within a few minutes of being taken off the facemask (incidence less than 1%).
- Hypothermia: Mice are cold to touch, or have low body temperature (<36.5°C). After anesthesia, if the hypothermia is severe, the animal will become sluggish or possibly nonresponsive. Hypothermia of anaesthetised animals while imaging is rare (<1%).
- Dehydration: If severe then eyes that appear recessed and the facial fur will be raised (piloerection), or if the skin is pinched over their shoulder blades, and will not return quicklyto its original shape, but instead will remain bunched. Dehydration is rare (<0.1%) for shortperiods of anaesthesia, but becomes much more likely (>50%) for animals given more than2 hours anaesthesia. However, if subcutaneous fluids are given in advance, the likely incidence is rare (<0.1%).
- Stress due to transfer: Animals are less active, food and water consumption may be reduced, and animal may lose weight.

Expected severity categories and the proportion of animals in each category, per

species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The breeding and maintenance of genetically altered mice (protocol 1) is mild. However, we expect thephenotype(s) to be sub-threshold and genotyping will generally be undertaken using surplus material from ear notching for identification (around 95%).

The breeding and maintenance of genetically altered mice (protocol 2) is moderate. However, we expect that 50% of animals (1 out of 2 lines) are likely to experience moderate levels of severity, and50% will be mild.

Assessment of cardiovascular function (protocol 3) is moderate.

Severity expected	Animals (number; percentage)
Sub-threshold	6270; 58%
Mild	955; 9%
Moderate	3625; 33%

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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice are being used in this project because:

- Mice are the best characterised and most widely used experimental model.
- Mice represent the simplest organism with a comparable heart and blood vessels (circulatory)system to humans.
- Mice allow genetic manipulation enabling investigation of target genes, and there are a numberof genetically modified mice available which are required for our research.
- Less sentient animals, such as the Zebrafish, are not suitable for this work due to their evolutionary distance to humans and research results are not translatable.
- Mouse models replicate vascular growth and health in humans, allowing valuable insights intohuman physiology.

Which non-animal alternatives did you consider for use in this project?

- Non animal alternatives will be used to complement mice work and contribute to reducing the totalnumber of animals in this project:
- *in vitro* specific cell culture models available on the market to investigate the effects of specificsignalling pathway.
- *ex vivo* engineering of living tissues with adult stem cells.

- *In silico* computer analysis will help establish potential interactions between signalling pathways .Clinical tissue banks from patients will be used to establish relevance of findings.

Why were they not suitable?

While the main non-animal alternative to these studies is to use cells in culture, unfortunately no cell culture, tissue engineering or computer modelling can currently mimic human cardiovascular functionsuccessfully due to the following limitations:

One dimensional system of single-cell population whereas cardiovascular system is multidimensional, consisting of many different cell types.

Cardiovascular system has a number of complex interactions, notably with the immune system,which cannot be modelled by non-animal models.

These processes occur over long time periods which make it difficult to perform similar studies innon-animal models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 design was created by the PPL holder (with more than 10 years of experience in running mouse colony), the University colony manager and a veterinarian (with mouse expertise andis involved in colony maintenance and preservation University-wide) working together:

We used good experimental design (e.g. group sizes, gender, strain and age are matched for control and experimental groups) to assure reproducible outcomes and maximise the informationobtained from the minimum resource.

Pilot experiments were conducted by local collaborators (aged mice, delivery of bioactive substances) or external collaborators (Germany licence for atherosclerosis model) using smallgroup sizes (up to n=3-5) to help predict expected outcomes and minimum number of mice needed to provide statistically relevant results.

All our protocols are already published in the literature by our group and others. Therefore, an extensive literature of baseline studies using our different animal models is available allowing usto predict the number of experimental animals needed.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Experimental design was planned with advice from experienced investigators, the University animalunit staff, and using the NC3Rs Experimental Design Assistant.

Experimental design:

Experiments carefully designed and performed, including randomisation of treatment or controlgroups, allocation concealment, blinded assessment of outcome, and explicit inclusion and exclusion criteria (e.g. group sizes, gender, strain and age are matched for control and experimental groups).

Mice are of a specific genotype so randomisation will not be performed, but littermate controls willbe used whenever possible. For each of the readouts, tissue processing and staining will be carried out by an operator who will be blinded to the mouse genotype and treatment.

We use online tools for power analyses (eg https://eda.nc3rs.org.uk/ as discussed recently in Nature. 2016; 531(7592):128) to predict group sizes needed to detect differences with statisticalsignificance based on pilot data. This key feature of good experimental design makes analyses more efficient and minimises the risks of overpowered or underpowered (and therefore wasteful)experiments.

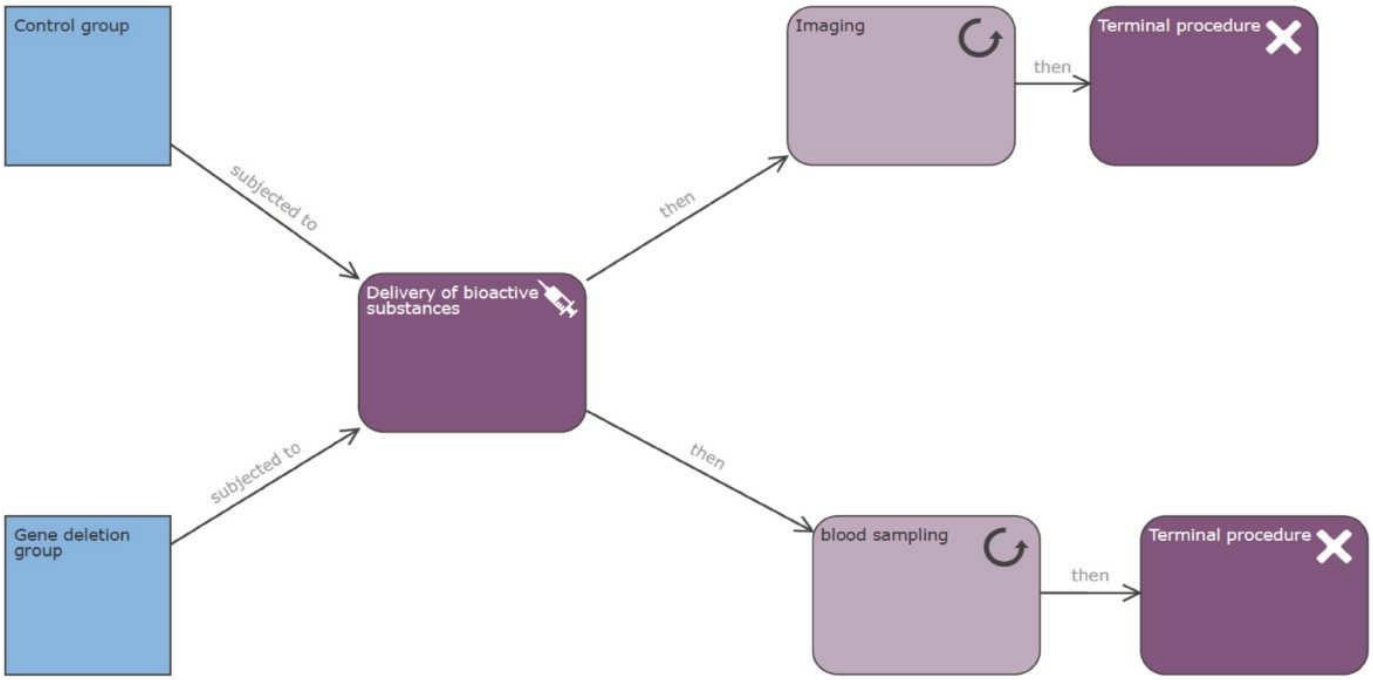
Normality of distribution will be determined using D'Agostino-Pearson omnibus normality tests. Statistical significance will be determined by one-way analysis of variance (ANOVA) followed by Bonferroni post-test when more than two groups were compared and a two tailed Student's t-testto compare two groups of data using Prism 6.0 (Graph Pad). Differences will be considered statistically significant with a P value<0.05.

Justification of the proposed sample size:

Experimental group numbers have been calculated from previous similar studies to provide the minimum numbers required to detect differences in multiple parameters. For most quantitative experiments, sample sizes may be set using power analysis. Generally, the significance level willbe 5%, and the power 80%. For example, in a two-group experiment, if the least practicable difference between groups is chosen to be 25% and if the coefficient of variation is estimated to be 15%, then about 7 animals/group would be required.

The exact numbers of animals required will vary with the particular experimental design and theestimate of the coefficient of variation. For example, the use of atherosclerotic or aged animals introduces further variation into the experiments (e.g. plaque size, vascular stiffness, cardiac function).

This will be repeated as more data accumulates to ensure the minimum number of animals isused throughout the project.



EDA flow diagram of the main experimental procedure described in this application.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

To optimise the number of animals used in this project, we will follow these steps: Breeding:

Use animals with the same genetic background to ensure proper reproducibility of our research by other research teams in different Establishments. We will use the following steps to avoid anygenetic drift and refresh the genetic background:

Cryopreservation of the breeding colonies as frozen as soon as they are reliably established thus minimising continued production of animals and ensuring greater surety inmaintaining their genetic integrity.

Mice will be genetically screened by SNP analysis at arrival, before backcrossing and/or at cryopreservation.

Mice will be backcrossed after 5-10 generations as appropriate for colony size (1-6 breeding pair: 5 generations; 6-10 breeding pair: 10 generations).

Where specific genotypes are available from academic or commercial sources, mice will be acquired for each study, to avoid maintaining a breeding colony (e.g. aged mice or atherosclerotic mice purchased from appropriate supplier).

Efficient breeding colonies will be monitored carefully to avoid over-production of animals incollaboration with the NACWO and according to the published guidelines.

Experimental procedure:

Every new experiment will include pilot studies using small numbers of animals (3-5) to determine the onset and progress of adverse effects and to identify criteria for humane

endpoint.

Studying mice at different ages through longitudinal approaches (e.g. imaging) over the life course of the same individual in pilot study reduce the number of mice necessary to determinewhether an intervention acts in an age-dependent manner or an age-independent manner.

Routinely monitor experimental results allowing proactive adjustment of numbers to use theminimum animals required to achieve robust statistical analyses.

Sources of variability will be identified and minimised wherever possible (e.g. variability in vascular phenotypes following gene activation with tamoxifen is minimised by ensuring the optimised tamoxifen dose is used).

Tissues from the same animal will be used in as many analyses as possible to minimise thenumber of animals required.

We will use cells isolated from transgenic and wildtype mice to better understand the biology of individual cell populations using ex-vivo primary cell cultures (e.g. cardiovascular cells will be isolated from young and old mice for short term culture to allow the comparisons of their gene expression profiles in order to understand how these are influenced during the ageing process).

Participation in collaborative projects for archiving and sharing mouse lines and phenotyping information (e.g. EMMA, MRC Mouse Maintaining formal arrangements for sharing mice with other researchers locally such as control aged mice and atherosclerotic mice to allow a reductionin the number of animals utilised for research 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this project, we will use the following animal model:

Genetically modified mice carrying “silent” mutations (e.g. floxed alleles) that generate no phenotype. These animals are completely healthy until administration of tamoxifen to inactivate the gene of interest, reducing any clinical effects to the absolute minimum necessary for theproject.

Genetically modified mice that have had certain genes constitutively inactivated (e.g. knockout)or activated (e.g. knock-in). These animals will be maintained as heterozygous animals, and homozygous animals will only be produced for experimental procedure.

In this project, we will manage a breeding colony:

Active monitoring of defects in breeding colonies will occur with support from the colony management team and technicians. This will identify early reoccurring health defects that mayindicate genetic drift. Likewise, we will remove non-standard animals from breeding programs.

We will adhere to the local AWERB standards: Rodent 12 Month Breeding Age Limit and RodentBreeding Defect Management.

In this project, we will use models of cardiovascular dysfunction:

Model of atherosclerosis to mimic atherosclerotic heart disease. This model is characterised extensively in the literature.These mice are normal until fed with Western diet. By limiting the dietto 20 weeks, we can control the extent of atherosclerotic lesion.

Mice may be kept until 24 months of age to mimic vascular ageing. Aged mice may demonstratenatural aged related conditions including arthritis or tumour formation. In addition with increasingage, mice demonstrate a progressive increase in morbidity. Before an experimental procedure, abody condition scoring will be performed. Only mice that are in suitable condition before the interventions will be used in the protocol. The adverse cardiac remodelling and decrease in cardiac function are well tolerated, and we do not anticipate any adverse effects above that of mild due to ageing alone.

In this project, we will use experimental procedures:

Delivery of bioactive substances: Where substances are used to reduce clinical symptoms wewill use the oral route wherever possible (e.g. in drinking water). Initial work with any new treatment with a bioactive substance will use small pilot groups of 3-5 animals that will be carefully checked to detect whether there are unanticipated harmful effects before treatment begins in larger groups.

Blood sampling: The saphenous vein and tail vein are more refined and appropriate routes ofsampling for most studies and strains of mice.

Non-invasive Imaging techniques: Non-invasive longitudinal imaging will lead to a reduction inanimal numbers because mice do not need to be harvested at different time points to monitor gene repression or activation. By using appropriate anaesthesia and analgesics in the procedures to alleviate pain and discomfort, the protocols cause the minimum possible discomfort to the animals.

Why can’t you use animals that are less sentient?

Less sentient animals, such as the Zebrafish, are not suitable for this work due to their evolutionarydistance from humans and research results are not translatable. (i.e. cold-blooded, two-chambered heart, no lungs, capable of heart regeneration after injury as opposed to mammals). In some cases,mouse embryos will be used for analysis to study the effect associated with the development.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The health and welfare of the colonies and experimental groups will be supervised by PPL holder, incollaboration with the local NVS and NACWO.

For our breeding procedures, we can ensure that uniformly high standards of animal care andwelfare are applied since the staff involved have extensive experience in this field.

Early socialisation to human contact of mice in our care to reduce stress of being handled to anabsolute minimum. Tunnel handling is our method to pick them up.

Mice colony in breeding protocol 2 (moderate) will be kept as heterozygous since homozygousdeletion of gene may have deleterious impact. Homozyogous animals will only be produced forexperimental procedures.

Proactive management and care for animal welfare by limiting genetic drift and in turn increasesreproducibility through cryopreservation, SNP analysis and backcrossing procedures.

Before each and every experiment, we will submit a study plan to our NACWO which includes:A statement of the objective(s)

A description of the experiment, covering such matters as the experimental treatments, the sizeof the experiment (number of groups, number of animals/group), the duration of the experimentand the experimental material

An outline of the adverse effects, the end-point and special requirements.

For our experimental procedure, adult animals will be scored daily following basal characteristics relevant to every animal. This ensures a follow-up of the welfare of every animal. The animals will be humanely killed by Schedule 1 procedure if the scoring is inadequate. As further information is gainedon the pathology, we will refine experiments, e.g. by sacrificing and analysing animals at an earlier stage of the disease or by reducing the number of animals in the study.

Several experimental procedures in place for the assessment of the cardiovascular function will becontinuously refined:

Administration of tamoxifen for gene deletion:

Tamoxifen will be dissolved in corn oil, known to reduce abdominal inflammation. Peanut oil can also be used as an alternative.

Altered diet via Gel supplements such as ClearH2O's MediGel® Hazelnut and DietGel® Boost - the recommended dosage of 80mg/kg body weight/day for a maximum of 9 days. These highly palatable gels are used to mask the bad taste of tamoxifen, and add nutritional supplementation.

Atherosclerosis model:

Animals will be kept on the minimum amount of diet predetermined during our pilot studies. A maximum of 20 weeks will be observed.

Use of purified diets composed of highly refined ingredients.

Ageing model:

Most of the aged animals will be purchased from a certified supplier. They will be bought to

ensure they stay for the minimum amount of time in our husbandry.

Enhance monitoring after 15 months of age through extra characteristics in the score-sheet.

Animal husbandry and caging will be adapted where necessary to suit older mice after taking advice from the named persons.

Delivery of bioactive substances:

We will first use intraperitoneal injections. It is the most used method as the amount of administered compound can be better controlled.

Voluntary oral intake of substances avoids handling stress and can replace injections, and oral gavages (e.g. gelatine cubes, instillation with honey, Nutella and analgesia).

Ad lib water or food consumption must be determined before any compound is added to thedrinking water to prevent that water or food intake will not be reduced during the experiment. The mean daily (24-hour) water and food consumption vary between species, stocks, and strains. We will determine the ad-lib water or food intake for the same strain, sex, age, and weight rodents as will be used in the study. If rodents drink or eat less than 90% of their previous intake, become dehydrated, or lose weight, the researcher must adjust the drinking water or food with additives so as to make it palatable for the animal.

Certain reagents may require adenoviral or lentiviral delivery. In this case, new generation vectors (e.g. adeno-associated vectors) would be used to minimise the inflammatory response.

Advances in pain relief administration will reduce welfare costs (e.g. oral pain relief in jelly form replacing the necessity of daily injections).

Blood sampling:

The saphenous vein and tail vein are more refined and appropriate routes of sampling for most studies and strains of mice.

Imaging:

Investment in an appropriate mouse anaesthetic system to provide good anaesthesia control, and monitoring of animal vitals at correct timepoints by competent people. This will allow us to:

Reduce dead space allowing for refined anaesthetic control of depth which improves recovery and reduces risk of anaesthetic adverse events.

Fit for purpose warming module and temperature monitoring to ensure body temperature is maintained during any prolonged procedures under anaesthetic,improving recovery times.

Accurate monitoring of anaesthetised mice (e.g. heart rate and oxygen levels).

Transfer between facilities limited to one with 7 days recovery after the transfer. We will consider hosting the mice to the facility where the imaging device is whenever it is

possible.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

All the work undertake in this project will follow the University AWERB standards and the internal goodpractices through competency assessment and collaboration with Named persons.

In addition, we will follow the published best practice guidance:Animal Care, Health and Management:

“The Animals (Scientific Procedures) Act 1986 Amendment Regulations” from the Home Office.

"Guidance on the Operation of the Animals (Scientific Procedures) Act 1986" from the Home Office.

“Guiding principles on good practice for Animal Welfare and Ethical Review Bodies” from the Laboratory Animal Science Organisation.

“FELASA guidelines and recommendations” from the Federation of European Laboratory Animal Science Organisation.

“The Provision of Water and FooD for Laboratory Animals” from the Institute of Animal Technology.

Humane Methods of Killing:

Humane Methods of Killing Laboratory Animals” from the researchanimaltraining.com

Recognition of Pain, Suffering and Distress:

- “Recognition and Prevention of Pain, Suffering and Distress in Laboratory Animals” from theresearchanimaltraining.com.

- "Recognising Post-Operative Pain in Animals” from ahwla.org.uk.

Experimental Techniques:

- “Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines”.Nc3rs resource library.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Stay informed:

- Researchers and technical teams are educated, trained, assessed and supervised through the University’s Competency and Training for Animal Research. This program ensures good and up-to-date competencies to keep negative effects on welfare to an absolute minimum for every individual working with animal models.

- Assist to workshops/seminars/researcher group meetings organised by host establishmentthroughout the year to inform and share ideas to develop the 3Rs further.
- Internal and external newsletters .
- Information available on the 3Rs through different websites such as the Home Office, NC3Rs andthe many journals with relevant authority in animal biology.

Implement these advances:

- Prioritise grant opportunities from Research Councils advancing further improvements throughdevelopment, integration and promotion of the 3Rs approaches.

Collaboration with Named persons:

- NACWO: to develop the score sheet and discuss welfare of the animals.NTCO: to discuss new refinement and competency procedures.
- NVS: to implement new refinement procedures.

NUTRITION FOR SUSTAINABLE CATTLE PRODUCTION

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
    - Improvement of the welfare of animals or of the production conditions for animalsreared for agricultural purposes
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aimsmentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Cattle, Nutrition, Environment, Health, Rumen

Animal types	Life stages
Cattle	adult, pregnant, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description 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 reduce the environmental impact of cattle and improve their health, welfare and performance.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Products such as milk, cheese and beef have a high nutritional value as they offer a wide spectrum of essential nutrients such as high quality protein, minerals and B-vitamins. Additionally, cattle are able toupgrade by-product foods from the human food industry and convert forages such as grass that can begrown in areas such as the hills and uplands where it is difficult to grow human edible food. Central to this is the role of the first stomach, or rumen, in the cow. The microbial population in the rumen is able to break down fibre and convert low protein feed sources in to high quality protein that can then be used by the cow to produce milk and meat for human consumption.

However, the process of digestion of feed in the rumen of cattle results in the production of methane, and can result in high amounts of nitrogen, phosphorus and other minerals being excreted. Gases such as methane have a high global warming potential and can contribute towards climate change. Feeding excess nitrogen, phosphorus and minerals can result in environmental pollution and reduce biodiversity. It is therefore important to investigate means to reduce the environmental impact of cattlewhilst improving their health and performance.

This work aims to investigate means to improve the use of nutrients, particularly from forages, and reduce the environmental impact and improve the health and welfare of cattle. This requires a greaterunderstanding of the processes occurring within the rumen, digestive tract and whole body metabolism. The work also underpins the University’s strategic research aim to achieve ‘Net Zero’ in agriculture and food supply chains in concert with the wider requirements for ‘sustainable agriculture’ (e.g. rural economies, social justice, animal welfare, nutrient cycling and pollution).

What outputs do you think you will see at the end of this project?

This project will provide help to reduce the amount of methane and nutrients excreted by cattle, andtherefore contribute to the UK's commitment to reduce greenhouse gases and meet environmental legislative requirements. It will produce new information on the role of the microbes in the rumen in breaking down fibre and converting poor quality protein into high quality protein that can then be used by cattle to grow and produce milk more efficiently. This research will also provide valuable informationon ways to improve the health of dairy cows, particularly around the calving period when cows mobilisebody fat to meet the needs of lactation and are susceptible to metabolic disease. It will also be important in providing information to cattle farmers on how to reduce their costs of production.

Outputs will include publications in peer reviewed academic journals. This is important to ensure thatthe results are in the public domain and can be scrutinised by others.

This project will also provide information on means to ensile forages such as grass, red clover or maizesilage more efficiently and reduce the impact of yeasts and moulds that can produce spoilage productsthat can impair animal health and performance.

Who or what will benefit from these outputs, and how?

This project will provide 4 major benefits relating to purposes a, b, c and d of section 5C (3) of the Animals Scientific Procedures Act. The programme of work will advance the knowledge and understanding of the science underpinning ruminant nutrition and the interaction between the rumen microbiome, nutrient utilisation and physiological state. Such understanding will help reduce the environmental impact of cattle by investigating

dietary means to reduce methane production and improving the utilisation of nutrients such as nitrogen, phosphorus and other minerals. This will help thecattle industry contribute towards the UK's goal of reaching net zero greenhouse gas emissions by 2050, and reducing ammonia emissions by 16% by 2030. The research will also contribute to improvements in the environmental impact of cattle production in other areas such as nitrates, phosphorus and heavy metals.

This project will deliver greater knowledge on the quality, efficacy and safety of home-grown forages and alternative feeds (e.g. from the human feed or bioethanol industries) in the diet of dairy and beef animals, and as new forages become available as a consequence of climate change. The ability of ruminants to convert grassland herbage, forage crops and non-human edible by-product feeds into edible animal food of high biological value is likely to become of greater significance in terms of global human-food security as the population of the planet increases in future decades. Greater utilisation of high protein, home grown forage legumes such as lucerne or red clover will reduce the requirement forpurchased fertiliser and supplementary feeds such as soyabean meal, which will also reduce the environmental impact of milk and beef production. Improving the utilisation of forages requires a more efficient ensiling process and less aerobic spoilage at feed out, and a reduction in the negative effects of mycotoxins that represent not only a loss of nutritive value but also pose a risk to both cow and human health.

The beneficiaries of this work include cattle in the UK and further afield by improving their health. Dairyfarmers will benefit by improving the efficiency of feed and forage use (and therefore reducing costs), and society, by reducing the environmental impact of dairy cows whilst producing high quality, human edible feed from low quality, inedible feed. A greater knowledge of the digestion and metabolism in cattle will also assist in improving the welfare of animals by for example, better supplementation of cows grazing grass, or reducing health problems around calving.

World crop and livestock demand will increase by around 1.5% per year. This project will provide information on cost effective, environmentally sustainable dietary strategies to meet these targets. Greater demand for the use of cereals for human consumption and energy use will decrease their availability for animal use and increase the reliance on home grown forages and by-product feeds. Changes in climate and plant breeding will also mean new and novel forage crops become available.This project aims to provide basic information on how alternative feeds and forages can be incorporated into the ration of cattle to maintain or enhance performance whilst reducing their environmental impact, and improving cattle health and welfare. In the longer term, improving the sustainability of dairy and beef farming will contribute towards the maintenance of more viable rural communities which are essential for the protection of the rural economy.

How will you look to maximise the outputs of this work?

The findings of the work will be disseminated at national and international academic conferences such as those organised by the British Society of Animal Science or the Society of Feed Technologists in theUK, or the European Association of Animal Production in Europe and the American Dairy Science Association in the USA. Findings will also be disseminated at on-farm events organised by groups such as the Agriculture and Horticulture Development Board, grassland societies and farmer groups.

Results will be published in peer reviewed, academic journals so that the rest of the research community and industry in the UK and internationally will have access. Information will also be published in trade and farming magazines. Samples from the

studies will be banked so that they maybe analysed in the future for other metabolites without requiring additional animal studies to be undertaken. Similarly, data from the project will be banked for future analysis, and combined with results from other studies to reduce the requirement for animal studies.

Species and numbers of animals expected to be used

- Cattle: Mature dairy cows = 420. Calves and growing/finishing cattle = 200.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

As the objectives of this project are to reduce the environmental impact and improve health, then cattlefrom young calves to adults need to be used throughout their life-cycle. The effects of nutrition on the process of digestion and absorption, and how this the end-products are then metabolised by the animal need to be assessed using lactating and growing cattle to understand why the changes have occurred .

Typically, what will be done to an animal used in your project?

The cattle will typically be housed under the same conditions that they would experience on a commercial farm in the UK, and when grazing. The cattle will be fed different diets, additives or foragetreatments, and their performance (milk production, composition and growth) will be recorded. Blood samples will be taken from some animals to determine the effects of the diets on nutrient uptake and metabolism, and some may have a microchip inserted in their ear to monitor blood metabolites throughout the day. Some animals may also have a sample of liver collected by biopsy, as the liver is an important organ controlling nutrient use and health. Some animals may also be restrained in stalls so that all of their urine and faeces can be collected to determine nutrient excretion. Some animals willhave rumen fluid removed by a stomach tube, and a small number of cattle will have rumen fistulaes inserted which is a surgical procedure so that a sample of rumen fluid can be collected more regularly.This is important because the microbes in the rumen are very important in digestion in cattle, and it is important to understand how different diets, additives or forages affect their diversity, growth and metabolism.

What are the expected impacts and/or adverse effects for the animals during your project?

The diets, forages or additives that the animals will be fed will be similar to what they encounter undercommercial farming conditions. Intake and performance will be monitored and the treatments compared to a control group fed a standard diet. Dairy cows normally lose weight for the first 10 to 20 weeks in early lactation and the effects of dietary treatments will be compared against a control group.
Pain or discomfort when collecting blood, liver biopsy or collecting rumen fluid by the oesophagus islikely to be small and transient.

There have been no instances of infection following blood sampling at this institute over the past 15years. The risk of transient pain of blood sampling will be minimised by

reducing the sampling frequency to the minimum necessary, restraining animals in appropriately designed equipment, andusing trained and competent staff.

In the case of liver biopsy pain have been no instances of infection following sampling over the past 15years or other adverse effects (e.g. reduced intake, milk yield or growth rate). Aseptic techniques will be used to reduce infection and the risk of transient pain will be minimised by the use of local anaesthetic and pain killers for a period of 72 h.

Inserting rumen cannula in dairy cows is expected to cause pain and discomfort for a period of approximately 48 to 72 h. During this period the animals will receive appropriate pain killers and antibiotics to prevent infection. There is not expected to be any long-lasting pain, and any discomfortwill be minimised by regular inspection and cleaning.

Distress may be associated with restraint in individual stalls and infection during urine collection. In ourexperience the likely incidence of distress associated with restraint in stalls is low. Appropriately designed stalls permit the cows to view and hear other cows. Sterile equipment will be used to collect urine and faecal material will be collected from freshly deposited material. Competent staff will check the equipment at least twice daily.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The majority of the severties (98%) are expected to be mild, with the remainder (2%) being moderate.

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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The objectives of the project are to reduce the environmental impact of growing and lactating dairy cows and improve their health and welfare by manipulating rumen metabolism and the microbiome. Some information can be produced from *in vitro* models for rumen, hepatic and mammary gland metabolism. *In vitro* systems of rumen fermentation are used widely at this institute and will be employed as part of this research, but *in vitro* systems do not fully replicate what is occurring in the rumen such as re-cycling of nitrogen, and microbial populations such as protozoa are unable to be maintained in *in vitro* systems. Using models to determine intake and whole body metabolism has alsobeen considered, but they do not accurately represent the complex interaction between the rumen, liver, mammary and tissue growth, particularly for novel diets or additives. The primary data generatedin these studies can be used to inform and develop models in the future to reduce the requirement for animal studies.

Which non-animal alternatives did you consider for use in this project?

In vitro rumen fermentation systems were considered and will be used where appropriate to screen treatments and determine optimal dosages. Alternatives such as cell culture for mammary and hepaticmetabolism were also considered. Models for the intake, performance and nutrient utilisation in cattle were also considered, and will be used to formulate diets.

Why were they not suitable?

In vitro systems do not fully replicate what is occurring in the rumen such as re-cycling of nitrogen, andmicrobial populations such as protozoa are not maintained in *in vitro* systems. Using cell culture systems in isolation does not represent the complex interaction between tissues and organs on aspects such as intake, rumen metabolism, hepatic metabolism, muscle growth and mammary metabolism. Using models to determine intake and whole body metabolism has also been considered and will be used to formulate the study diets, but these do not accurately represent the complex interaction between the rumen, liver, mammary and tissue growth, particularly for novel diets or additives. The primary data generated in these studies can be used to inform and develop models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 have been estimated as the number that have been used in similar studies published in peerreviewed papers, and from similar studies undertaken and published in peer reviewed journals fromthis institute.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The experimental design was informed by results from studies published in peer reviewed journals andsimilar studies at this institute. Discussions on study design have been made with a statistician and use has been made of the NC3R's Experimental Design Assistant to calculate likely numbers based onthe variation reported in the literature and from similar studies at this institute.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Lactating and growing animals will be selected from a suitable commercial dairy herd and the performance and health status of the animals will be used to optimise the number of animals selectedfor study, along with their genetic status. Computer rationing packages designed for dairy cattle nutrition have been used to provide preliminary information on the possible effects of the diets on animal intake and performance. Pilot *in vitro* studies to

determine the effects on rumen fermentation and the microbiome will be undertaken when appropriate.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The use of cattle is necessary as the research is focussed on improving nutrient use and health of cattle. There are no non-animal based substitutes that accurately replicate the combined effects of dieton intake, digestion, rumen metabolism, liver metabolism, milk production and growth. Additionally, as some of the dietary strategies may alter aspects such as eating behaviour, the use of cattle is required.The animals will be kept under conditions similar to those encountered on well-managed commercial farms and commensurate with the Code of Practice. For example, some studies will require the cows to graze at pasture, and others to be housed in stalls or on straw over the winter. Where sampling is required, animals may need to be housed individually but will have sight and contact with others at all times. The use of appropriate pain killers will be used when required. If animals need to be restrained for example to measure urinary nitrogen output, they will have sight of other animals at all times and will be restrained for the minimum period to ensure that an accurate sample is obtained. The animals will be cared for at all times by trained and competent staff. All studies will be approved prior to commencing by the local animal ethics and welfare body. This body is composed of individuals with animal welfare, statistical design and animal health experience. The advice of a trained and experienced veterinary surgeon will be sought at all times during the planning, operation and at the end of the study.

Why can’t you use animals that are less sentient?

To reduce the environmental impact of cattle and improve their health and performance it is importantthat studies are undertaken using cattle so that we can determine the direct effects on their nutrient use, health and performance. Using other species such as sheep does not always relate to responsesin cattle (e.g. for methane production).

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Animals will be sampled in handling equipment that they are familiar with to reduce possible fear anddistress, and using staff that are trained and competent.

Pain management will include local anaesthetic when taking liver biopsy samples and provision ofantibiotics to reduce the risk of infection.

Rumen sampling by the oesophagus will be undertaken by trained staff and using appropriatelydesigned equipment, with sampling kept to the minimum.

Pain associated with inserting a rumen cannula will be minimised by the use of appropriate pain killersand antibiotics will be used to minimise infection.

When restrained in stalls animals will have sight and sound of other animals at all times, and will havefood and water available *ad-libitum*. As the animals are normally milked in the same stalls then fear from restraint is minimised. The stalls will be bedded with high quality mattresses to improve comfort and reduce discomfort. Cows will also be give break days within the collection period when possible when they can return to their loose housed accommodation.

All procedures will be undertaken using qualified and trained personnel, with advice sought from theNVS when necessary.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Guidance will be obtained from the Animals in Science Regulation Unit (ASRU) website. For example,guidance on the care and accommodation of animals will be sought from the Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes: Non human primates, farm animals and birds.

Harm benefit analysis has been assessed against The Harm-Benefit Analysis Process: New ProjectLicence Applications (advice note 05/2015).

Guidance to ensure that experiments are conducted in the most refined way will be informed by outputfrom the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs), and from reviewing the literature related to animal research procedures.

Guidance practice on general animal welfare will be obtained from the Animal Welfare Act (2006) andthe Welfare of Farmed Animals (England) Regulations (2007) and regular updates on the gov.uk website.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

To keep up to date with the latest 3Rs developments then the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) e-newsletter will be subscribed to, as wellas regularly reviewing their website and learning resources. The monthly newsletter updates provide information on funding opportunities, 3Rs events and publications. Attendance at NC3Rs events and workshops will also be undertaken. Registering for webinars, or watching the recordings of past webinars, will also be undertaken. Contact will also be made with the local NC3Rs Regional Programme Manager who can provide an informal route to 3Rs advice, developments and best practice. Information will also be obtained by being aware of the scientific literature relating to animal research and attending national and international conferences on farm animal research (e.g. British Society of Animal Science annual meeting) and discussing advances in implementing the 3Rs.
Use will also be made of the The EURL ECVAM Search Guide which has been developed to help users find information on alternative strategies and methods to animal-based research. The AnimalWelfare Information Centre also has tips for searching for alternatives, as well as links to relevant databases, and these will be used. Advice will also

be sought on good practice and alternative strategies and methods from other species using sources such as the Laboratory Animal Science Association (LASA).

UNDERSTANDING INFECTION PATHOGENESIS TO GUIDE THERAPIES

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Infection, Immunity, Pathogenesis, Therapy, Vaccine

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to understand and define the mechanisms involved in the host / pathogen interaction and to explore novel therapeutic approaches.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Infection remains a major cause of death worldwide. The latest available data from the World Health Organisation (WHO) shows that in 2019 infections accounted for 3 of the top 10 leading causes of death worldwide with a total of 6.1 million deaths annually (https://www.who.int/news-room/factsheets/detail/the-top-10-causes-of-death). As of 2019, infections were accountable for 6 of the top 10 causes of death in low-income countries. Even in the UK, lower respiratory infections are still in the top 5 leading causes of deaths each year.

Over the last 10 years, we have started to see a sharp rise in pathogens that are resistant to antimicrobials. These pathogens are not just resistant to one or two antimicrobials, but we are seeing the emergence of pathogens that are resistant to multiple antimicrobials with resistance in some pathogens, such as TB, to even the last few remaining first-line antimicrobials currently in use. This is confounded by the fact that there have been very few new antimicrobial products that have been developed over the last 15 years. A review by the UK government on emergence and economic cost of antimicrobial resistance can be found here (http://amrreview.org/sites/default/files/160525_Final%20paper_with%20cover.pdf).

What outputs do you think you will see at the end of this project?

We believe this project is in-line with the United Kingdom/global aim to use genomics to improve medical treatment and human health, and to develop therapies that will benefit the economy and human health.

We strongly believe that the work described in this proposal will both improve our understanding of infection related illness and help identify novel targets for new therapeutic approaches. We expect to characterise up to ten novel genes that are also in humans which influence infection, immunity and pathology. We will propose ways which these genes can be targeted for therapy when we have further defined their functions and, through publications, will make these findings available to the wider scientific community. We will identify pathogen genes or gene products that contribute to disease, and will define the way by which they escape therapy (resistance). We have a track record of using pathogen products in vaccine trials and in the formation of companies with the potential to take products to clinical trial.

We will share the data we generate with other researchers around the world through open access research databases and publications. Some of the bacteria we will be working on have no current vaccines and data we will generate here may lead to clinical trial and usable vaccines for public health.

All mouse lines used will be shared with other research groups.

Who or what will benefit from these outputs, and how?

We believe that the work described in this proposal will both improve our understanding of infection related illness and help identify new targets for new therapeutic approaches such as vaccines. In the short term we will publish data on novel genes associated with pathogen infection. We will identify novel pathogen proteins that either improve on current less effective vaccines or develop vaccine products for pathogens where no current vaccine is available. In the longer term we believe that products developed under this licence, be that either vaccine or immune modulators, will be used to prevent disease or enhance immune responses in humans. We will share the data we generate with other researchers around the world through scientific publications or with companies with the ability to produce therapeutics.

How will you look to maximise the outputs of this work?

This work will be part of a larger collaborative program including programs working on cells, fish infection models and human trials. The results will be shared at the earliest opportunity through publication and at conferences. Large data sets will be deposited on databases for external access. We will disseminate all findings of our studies, including unsuccessful approaches or non-significant data, through publication in peer-reviewed

journals, presentation at scientific conferences, and through meetings with other researchers.

Species and numbers of animals expected to be used

- Mice: 10680

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To better understand our body's ability to fight infection or to mount immunity, we cannot simply start experimenting on humans, but need to use a model system. The more similar the model is to humans, the more reliably we can make conclusions about humans based on the findings of our study. We have selected the adult mouse as a model animal for our work as it has a fully developed immune (bug defence) system similar to that of humans. In fact, the mouse immune system is made up of the same parts as the human one and uses the same strategies to get rid of bugs, bacteria and viruses in our body. The mouse is also widely used and very well characterised, which means many different scientific tests have been developed for it which we could make use of to investigate in our study. For example, the well characterised genetic code of the mouse allows us to look into the role certain genes and inheritance plays when it comes to vulnerability to infection.

Typically, what will be done to an animal used in your project?

Animals will be produced by traditional breeding methods. We will make a mouse mutant in a gene that we have identified from human immune studies that we think is important for controlling infection. This will allow us to study the role of that gene in a controlled way.

Animals in this project will typically be infected with a bug (virus / bacterium) or vaccinated. This will involve either an injection, for example intravenously (i.e into a vein), or administration intranasally (i.e into the nose) or by oral gavage (a tube inserted via the mouth into the stomach). Vaccinations will be given via injections into the muscle or under the skin. Samples of blood will be taken from a tail vein (blood vessel) at predetermined time points. Animals will be monitored during the infection period by weight and in some infections by sampling of faeces. We can also track some bugs over time by imaging the mice with imaging machines while the mouse in under anaesthetic. To better understand the role of some genes in the immune system we may need to irradiate mice (a way of killing only the immune cells of the mouse) to replace their immune system which means we can track cells to understand their role in infections.

At the end of the experiments all mice will either be humanely killed, or blood collected under deep, terminal anaesthetic where they will be asleep/unconsciousness throughout. The experiments will be typically 1 month long.

What are the expected impacts and/or adverse effects for the animals during your project?

As the mice will be infected with bugs they are likely to suffer some discomfort. In wild type mice all the infections we use cause disease but do not kill the mice. Mice infected with bacteria will have moderate weight loss of 5-15% over 7-14 days and will have mild clinical

signs of infection; slight piloerection (hair standing on end as a sign of pain or discomfort in mice), and hunched walking. With flu infections the weight loss will be 15-25% less than the starting weight over 10 days and mice may develop increased breathing rates.

In gene deleted mice where the gene is important in controlling infection, we may expect to see increased weight loss and more pronounced clinical signs. We will have pre-defined end points for our infections to capture animals and minimise any suffering.

Mice that are immunised can show mild signs of discomfort that should last no more than 24 hours. These mice are not expected to show any long term adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice

Mild 70%

Moderate 30%

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?

We only use live mice when we cannot use alternative approaches such as working with human or animal cells in a dish (these could be blood cells or cancer cells for example that have been taken from patients). It is not possible to mimic all aspects of the interactions between the different stages of a real infection process meaningfully outside of the whole animal. Additionally, it is not possible to study the complex interaction between bugs and the host that they infect (e.g. humans) outside of a whole and living animal. We recognise that our mouse model system has limitations and cannot reproduce all the conditions associated with parallel human infections or vaccinations. However, mice have similar immune systems to humans making observations in mice comparable to those in humans. As mice don’t always react the same way to bugs that attack humans, we use mouse-specific pathogens to evoke a more meaningful response that can be paralleled in humans. These mouse-specific pathogens include an adapted version of the flu virus that causes similar symptoms in mice to the ones humans experience from the flu, or an adapted bacterium that mimics stomach infections causing diarrhoea etc in humans.

Which non-animal alternatives did you consider for use in this project?

Cells and organoid systems (recreating organs or parts of them from cells in the laboratory, for example part of a gut) have been considered.

Why were they not suitable?

It is not possible to mimic all aspects of the interactions between the different stages of a real infection process meaningfully outside of 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?

By keeping our experimental conditions well controlled we are able to perform highly reproducible and statistically meaningful experiments using the minimal number of animals. The experimental approaches described herein have been vigorously evaluated over the past two decades. We have access to and have used an experienced statistician to help guide experimental design. Post-doctoral scientists in our group have been on experimental design courses to help understand sources of bias and variation and how best to reduce them. Where possible in our design we blind people to genotype/treatment groups with different people doing infections or analysis. Based on our experience with pathogen infected mice, we use 5-6 mice per group as this is a sufficient sample size given the differences between means and within-group variances we typically observe. We will share any new mouse models we generate with other researchers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have been running these infection models for the last 20 years and during that time we have generated thousands of data points in wild type mice in normal conditions. From this we know what the sources of variations are and have therefore been able to control for them. We have worked closely with Biostatisticians when setting up high throughput screens using these models to reduce the numbers of mice we need to use to get meaningful results. We have used the NC3R's experimental design assistant for work we have done on previous studies and will continue to use it in future studies. The PREPARE guidelines have also been consulted for formulation of this project, and these will be followed to ensure continued communication between the animal facility and our team.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The work is also a part of a larger program of work within the department where data will be generated for other experimental models including work in fish and humans along with experimental models in vitro such as cell and organoid culture. The data generated in these programs will also inform the work herein. When measuring something new or it is unclear what the correct dose will be to achieve our aim we will use a small pilot study to help guild decisions. Samples collected from any mice as part of experiments planned will be stored long term at -70oC. This will make the samples available for future analysis. There is potential that other groups may want to use post-mortem tissues that we do not take from mice that have been exposed to infections.

We are engaged with the local NC3Rs group who enable users to share surplus breeding animals to reduce numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have established that the mouse can be used to identify bug (also referred to as 'pathogen') and mammalian genes that influence infection and immunity. The wild type parental bugs that we use are almost exclusively disease-causing for the mouse and thus likely to yield important and relevant phenotypes and associated data. Although the pathogens we use are mainly mouse pathogens they are good correlates to human specific diseases. We believe that the similarities in the mouse and human genomes are such that we can infer between the two and we have closer links than ever before with patients in the clinic. Over the years we have gained tremendous experience with our infection models and, through careful observation, we are able to minimise the potential suffering of the animals. We have been able to identify key clinical signs that indicate illness in infected animals and consequently such animals can be quickly and humanely killed.

Why can't you use animals that are less sentient?

It is not possible to mimic all aspects of the interactions between the different stages of a real infection process meaningfully outside of the whole animal. It is not possible to study the complex interactions between the host (e.g. humans or mice) and infectious agents / bugs (e.g. the flu virus) outside of whole living animals or in animals that don't have a mammalian immune system. We recognise that our mouse model systems have limitations and cannot reproduce all the conditions associated with parallel human infections or vaccinations. However, mice have many similarities to humans, including in terms of their immune system. We also use mouse-specific bugs to evoke a more meaningful response that can be paralleled in humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice are monitored throughout all experiments and we collect daily scores composed of a set of physical signs of illness such as piloerection (raised hair as a sign of pain or discomfort), hunched walk and mobility along with weight loss. The cut-off for these physical signs lies within the guidelines for moderate severity, i.e. loss of pre-set percentage body weight being our main indicator, along with mobility (ability to feed and water). The scoring for piloerection etc. are also used as secondary indicators. My team are experienced in animal infection models and are trained to the high standards that I expect. The technicians that work in our holding facility and do the majority of the animal husbandry will also be trained by my team and will communicate abnormal behaviours in the mice early. At our establishment we have dedicated Named Animal Care and Welfare Officer who are impartial and can give advice/ make decisions on animals that lie outside of the normal adverse effects expected for the infections outlined within this project.

Potential refinements include increased monitoring if test animals show earlier clinical signs or weight loss. We will give wet mash food to animals that lose more weight quickly. Floor food will be given to animals that are to be infected to limit weight loss from the start of the infection or the use of medicated palatable substances for voluntary treatment such as flavoured jelly, paste or milk shake liquid where the use would not impact on the scientific aims.

All animals acclimatised to the facility before use and will be given environmental enrichment and be socially housed to encourage natural behaviours and reduce stress. Males will be monitored for increased aggression and additional enrichment added to combat or appropriate splitting of fighting animals if needed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use guidance from the NC3Rs website and the Laboratory Animal Science Association (LASA) to ensure experiments are conducted appropriately. In particular we will follow the 'Guiding principles on good practice for Animal Welfare and Ethical Review Bodies'.

We will follow the PREPARE guidelines for planning experiments and will follow the ARRIVE guidelines reporting of results.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep informed on advances through the NC3R’s website, Norecopa website, our establishment website and newsletter. We will discuss any advances with the relevant people at our establishment and implement them accordingly.

THE GENES AND BRAIN CIRCUITS OF SLEEP

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Sleep, Behaviour, Zebrafish, Genetics, Drugs

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

Sleep is of vital importance to our health, yet we do not understand the purpose and fundamental regulation of sleep. The aim of this project is to uncover the signals that convey sleep need and theregions of the brain where sleep need is sensed.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

How the brain works to regulate diverse functions, including eating, sleep, arousal, motivation, and other behaviours is still poorly understood. In particular, sleep problems occur in almost all neurodevelopmental (such as autism), psychiatric (such as depression and schizophrenia), and neurodegenerative (such as Alzheimer’s) disease, and often poor sleep exacerbates the symptoms ofthe disease. Therefore, understanding how the brain controls sleep may help to develop treatments that will impact a wide variety of clinical cases, not just specific sleep problems like insomnia.

What outputs do you think you will see at the end of this project?
At the end of the project, we will have a better understanding of how sleep is controlled and where inthe brain this happens. We anticipate that this project will result in multiple (2

per year) publications based on our recent track record. In cases where we uncover novel small molecules that regulate sleep, we will seek patent protection and explore avenues to develop these candidates into novel therapies.

Who or what will benefit from these outputs, and how?

On the short term, researchers who study sleep, including clinicians who treat sleep disorders, will benefit from our work on the basic underpinnings of how sleep is controlled. The tools we develop willalso be of benefit to the neuroscience community at large who are studying how the brain controls behaviours of all types.

On the medium term, our work will provide a new framework for developing drugs related to sleep byidentifying potential new drug targets that will be of benefit to pharmaceutical and biotech industries.

On the long term, understanding how the brain turns sleep on and off will allow us to develop better sleep aids and will help us understand how sleep is disrupted by developmental disorders, such as autism, and aging related disease, such as Alzheimer's disease. Knowing sleep's fundamental functions may allow us to develop novel therapies to treat these disorders by taking advantage of thehealthy aspects of sleep.

How will you look to maximise the outputs of this work?

We will publish all of our experimental outputs, even those that fail to produce significant results, andwe will make these available open access via the BioRxiv repository prior to publication. We will present our work at national and international scientific conferences to expand our reach. We have anextensive track record of collaborating with researchers around the world, including in Germany, France, the United States, Japan, and Canada, as well as an extensive network of collaborations within the UK.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 600,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Sleep is a complex behaviour that only occurs in intact animals. Some non-protected animals, such asfruit flies and worms exhibit sleep states, but the brain circuits that regulate sleep in these animals is not identical to humans. Since zebrafish is a vertebrate like humans, the regulation of sleep is more relevant for human disease.
We have chosen zebrafish because they are the least evolutionarily complex vertebrate species with clear sleep states, and sleep happens at an early developmental stage. That allows us to study sleep at a more basic brain stage. We also have direct visual access to the brain non-invasively, allowing usto watch brain activity and sleep behaviour at the same time.

Typically, what will be done to an animal used in your project?

A typical animal will have undergone a genetic manipulation that alters some aspect of sleep regulation. They, or their progeny, will be tracked with video cameras while they sleep over several days and the effect of the genetic manipulation will be assessed. Some animals will be given sleep drugs, which the fish take up directly from the water, and the effect on sleep will be assessed with avideo camera. For some animals, these drugs or other substances will be injected directly into the brain and the effect on sleep-circuits will be observed with a microscope for up to 24 hours. In otherexperiments, we will keep the fish immobilized with a muscle blocker or by embedding in agarose, allowing us to simultaneously observe brain activity and sleep state for up to 24 hours.

What are the expected impacts and/or adverse effects for the animals during your project?

In most cases, the only adverse effects on both larval and adult animals will be alterations in sleep, such as sleep deprivation or alternatively, too much sleep. The imaging of the brain of larvae is non- invasive; however, the animals are restrained and they find this mildly stressful as assessed by a briefperiod of more vigorous swimming, after which they return to baseline behaviour. They also show normal swimming after a period of restraint, suggesting they do not have long-term adverse effects.

Under restraint larvae engage in natural behaviors, like normal swims, responses to videos, hunting behavior, and even interest in other fish. In some experiments, the larvae are exposed to seizure- inducing drugs for no more than one hour, after which they recover fully. The larvae will also be sleep deprived for 2hr up to 24hr using a gentle handling technique, which can be done with exposure to lightor performed in shifts by researchers using a paintbrush to supply gentle visual, vibrational, and touch stimuli. Sleep deprivation is associated with higher levels of stress in a variety of species, including humans, rodents, and fish; however, humans and animals, including larval and adult zebrafish fully recover afterwards with an initial bout of increased, deeper (i.e. harder to arouse) sleep.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

98% of all animals will experience a mild procedure.

2% of all animals will experience a moderate procedure.
The likely level of severity for almost all procedures is mild, and any accidental harm can be swiftly detected to terminate the experiment. For example, in less than 10% of the small molecule screening and imaging experiments, animals exposed to small molecules or imaging protocols will exhibit moderate levels of severity, for example the induction of haemorrhage or seizure, at which point the experiments are terminated. All animals are killed at the end of the protocols and prepared for analysisof genetics and brain histology.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Because we are assessing the role of vertebrate brain structures in the control of complex behaviours,we must use living animals. Animal behaviour and brain function cannot be replicated in vitro. In particular, we are investigating sleep and wake behaviours, which are only observed in complex organisms.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives, as sleep is a property of whole animals. We have consideredusing non-vertebrate species including Drosophila and C. elegans.

Why were they not suitable?

Flies and worms do not have critical neural circuits involved in sleep regulation in humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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. Based on the number of genetically modified animals that are needed to test aspects of sleep, based on the breeding cycle of 18 months for most zebrafish strains, and based on the historical use of animals in my lab over the last10 years, I have estimated the number of animals that will need to be bred to adulthood as around 80,000 over 5 years. This is estimated as follows: Since larvae live in shoals, they are bred in groups of40-50 at a time. A total of 80,000 animals allows for 3 generations of 500 distinct genetically modified lines, which is in line with our needs over the past decade. We will also generate new genetically modified lines, requiring an additional 80,000 adults. Thus, 160,000 adult fish will be generated over the next 5 years.

However, most animals will be used in larval (5 to 15 days old) stages. We track larvae in 96-well plates to assess sleep behaviour in response to drugs, neuronal manipulation, and genetic modification. This is often done in a medium-throughput screening manner, in which hundreds or thousands of drugs are tested at multiple concentrations, or whole classes of human disease- associated genes (e.g. Alzheimer's Disease risk genes) are tested in parallel. This is where the powerof the zebrafish sleep model arises-- the ability to test quickly large numbers of drugs or genes, whichallows other researchers to focus their rodent or other animal work on just the most promising candidate biological processes. We project that we will track more than 1500 animals a week over the5 year period, requiring 400,000 animals.

In addition, we do a variety of experiments to image brain activity during sleep to pinpoint the neuralcircuits that are responsible for sleep. For these experiments, based on our planned work, we will require about 8000 animals a year, for a total of 40,000 over the next 5 years.

Thus, we need 80,000 adult zebrafish for the maintenance, 80,000 adult zebrafish for the generation ofnew genetically modified lines, 400,000 larval animals that will undergo sleep tracking experiments, 40,000 larvae for neural imaging studies, for a total of 80,000+80,000+400,000+40,000= 600,000 animals.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Before any experiments, we perform tests called power calculations that estimate how many animals are required for proper statistical assessment. For the monitoring of zebrafish larvae during sleep/wakebehaviours, we have extensive experience in the statistical analysis of very large behavioural datasets,and we have developed numerous statistical methods to handle maximally extract data from, which minimizes the number of animals required. However, behavioural variance for some sleep parameters,such as sleep latency, can vary by as much as 50% across zebrafish strains, which means that we need to observe more animals in order to be sure of finding an effect on sleep after an experimental manipulation. Because the analysis is highly quantitative, we are continuously updating our statistical measurements, for example by incorporating statistical models from computationally modelled datasets, and will appropriately adjust downwards the number of animals required when feasible.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

For the basic breeding and fish husbandry required to provide animals for our procedures, the fish facility has instituted many procedures to ensure that the numbers of animals generated is the smallestnumber required. Similarly, the efficiency of the generation of mutant and transgenic zebrafish, throughcommunity efforts, is now well established and high enough that the minimum number of fish are generated, fin clipped, and maintained in mutant backgrounds. For example, my lab has pioneered methods to generate large numbers of mutants efficiently by deep sequencing prior to 5 days post fertilization and only raising founder fish harbouring mutations of interest.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Zebrafish are the simplest vertebrate system that allows both forward and reverse genetic approachesto study behavioural processes, such as sleep, and many of these processes

are largely identical to those of higher vertebrates. Moreover, obtaining large numbers of eggs/embryos does not require invasive intervention as zebrafish externally fertilize their eggs. For these reasons, the zebrafish has emerged as a leading model system to study most aspects of biology, both developmental and behavioural, found in amniote vertebrates such as mammals.

We track the larvae continuously with video cameras to assess their sleep and wake state in responseto drugs and genetic manipulation and treatments such as sleep deprivation. Any behavioural indications of adverse responses to our manipulations are therefore readily detectable, allowing for intervention or termination of the experiment. In some cases, we will work with adult fish to track their sleep. However, all adult experiments will only expose fish to very mild stimuli, such as a visual cue oran acoustic cue.

Why can’t you use animals that are less sentient?

We have chosen zebrafish as the least evolutionary complex vertebrate that has a described sleepstate. However, to minimise suffering, the vast majority of experiments are performed at embryonic/larval stages at the youngest possible timepoints (pre-5 days old), although robust sleep/wake behaviours do not emerge until which is just after the zebrafish are capable of free swimming and feeding (post 5 days old). This is the most immature life stage at which sleep can bedetected.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The animals are continuously tracked with a video camera on computers that we can monitor remotelyfor adverse outcomes. We are investigating methods to create automated software that can detect particularly unusual movements that may be the sign of distress and will implement this technology if itproves feasible.

For breeding of genetically modified animals, we will investigate less invasive procedures for obtaininga DNA sample for screening. This is currently taken by fin clip, which regenerates in fish, but there aresome other methods that take skin swabs that are under development. 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 inthe most refined way?

We follow the PREPARE and ARRIVE guidelines, the 3Rs as elaborated by the NC3Rs, and theLaboratory Animal Science Association (LASA) guidelines.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We have regular meetings with an NC3Rs officer who keeps us up to date. We also have staff in our facility who have developed on-line and in person training refreshers, which we all attend at least oncea year.

GLIA-NEURON INTERACTIONS IN THE HEALTHY AND DISEASED NERVOUS SYSTEM

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, ortheir 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 aimsmentioned in paragraph (b)

Key words

Glia, nerve cells, neurodevelopment, myelin, neurodegeneration

Animal types	Life stages
Mice	neonate, embryo, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to investigate how different nerve cells interact with each other duringnervous system development, in the adult brain during learning and memory processes, in neurodegenerative diseases such as Alzheimer's and in psychiatric disorders such as autism and schizophrenia. One main focus is on finding out if manipulating different nerve cells can improve the symptoms of nervous system diseases.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 cells in our brain can generally be divided into two major categories based on their function - **neurons** and **glial cells**. Neurons are very important to our body because they process and transmit information in the form of electrical impulses to control our actions in daily life, while glial cells are seenas playing supporting roles to neurons.

There are three main types of glial cells in the brain oligodendrocytes, astrocytes and microglia. **Oligodendrocytes** ("multi-branched cells") enwrap axons (long filamentous extensions of neurons) with multi-layered fatty insulating membrane, called "myelin". Myelin allows electrical signals to travel along axons rapidly and energy-efficiently from one part of the brain to another. **Astrocytes** (star-like glial cells), the most abundant cells in the brain, provide structural and biochemical support and maintain a balanced microenvironment for other brain cells. **Microglia** are the resident immune cells inthe brain.

We used to think that our brain was controlled by neurons with a little help from glia. In recent years, with rapid advances in neuroscience, we have come to realise that glial cells are proactively engagedin brain activities. Therefore, study of glia and neuron-glia interaction is very important for us to understand how our brain works.

The purpose of our research is to understand how different types of brain cells work together to fulfilbrain function in health and disease.

What outputs do you think you will see at the end of this project?

Our work will generate new data on glial cells and neuron-glia interactions in physiological (e.g. duringdevelopment and during learning process) and pathological conditions (e.g. autism and multiple sclerosis). We will share the new information with our peers and the general public through presentations in conferences and seminars and through publications in scientific journals. In addition, we will apply for patents for any new findings that have the potential for commercial application.

Who or what will benefit from these outputs, and how?

Benefits to the academic community. Our outputs will advance our understanding of brain function andexpand the knowledge base for neuroscience research in the short term, as well as laying the basis fordeveloping new therapies for neurological diseases in the future.

Benefits to the public. Understanding how our brain works is of general interest to the public. Our workwill serve to raise public awareness of the roles of glial cells in neurological conditions and help to engage young people with the progress and outcomes of neuroscience research. In the longer term, our research will help find new treatments for neurological diseases, to the benefit of patients and the medical community.

How will you look to maximise the outputs of this work?

We have long-standing partnerships within the neuroscience field as well as cross-disciplinary collaborations. Moreover, we have ongoing collaborations with pharmaceutical companies and charities for brain diseases. These collaborations will continue to enhance the impact of our research outputs. In addition, leftover tissues (e.g. eye and liver) from our animals might be useful resources forour collaborators' research.

In addition to reporting our research progress at scientific conferences and publishing our findings inscientific journals, we will promote our work via our website and social media account and make ouroutputs openly available to the public wherever possible.

We will also share our negative results and unsuccessful methods in our social media account. In thisway, we will be able to help our peers avoid unnecessary waste of animals in compliance with the 3R'sand waste of time and money.

Species and numbers of animals expected to be used

- Mice: 20000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our project will depend on experiments using mice. Mice are a natural choice for neurobiology because 1) they are mammals, with similar brain structure and analogous behaviours to humans, 2) there are well-developed methods for genetic manipulation, electrophysiology, behavioural testing andin vivo imaging, 3) there is a wealth of information from previous studies about their brain anatomy andphysiology and 4) there is a large battery of transgenic and mutant mice available that will allow us to study the roles of particular genes and gene products in nervous system function, and to model certain human diseases.

For study of nervous system development, we will normally use animals less than 1 month of age. Forstudy of nervous system functions, we will normally use young adult mice around 2-4 months of age.
For study of age-related neurodegeneration, we will normally use older animals between 1 and 2 yearsof age.

Typically, what will be done to an animal used in your project?

Typically, we will use wild type and genetically altered mice in our project to investigate how different nerve cells interact with each other during nervous system development and in the healthy/diseased brain. We will administer certain substances such as chemical compounds, proteins, viral particles to mice via injection and through food or drinking water. These substances can interfere with the functionof brain cells and regulate nerve cell interaction, enabling us to deduce what effect they may have on various diseases . We will perform behavioural tests on some of the mice and terminally anaesthetize the mice before tissue collection for histological analysis, in order to examine the impact of abnormal nerve cell interaction.

Occasionally, we will administer substances including chemical compounds and viral particles directlyto the central nervous system by surgical methods.
An example of a typical behavioural experiment:

- substance administration on postnatal days 60-64 (P60-P64)

- recovery until P80, behavioural testing for up to 2 weeks P80-P94 followed immediately byterminal anaesthesia and perfusion-fixation

- in some experiments, longer term effects will be assessed by allowing mice to survive for varioustimes after behavioural challenge and re-analyzing by the same or a different test up to one month later P124-126.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect no or mild adverse effects for most mice. Some genetically altered mice, and some normalor genetically altered mice after substance administration, might unexpectedly develop adverse neurological symptoms. These might include unbalanced movement, tremor, weight loss or general discomfort evidenced by lack of grooming, inactivity or “hunching”. Substance-related adverse effectsusually recede within 2 weeks and mice that do not recover will be humanely killed.

We do not expect our surgical procedures to cause long-term adverse effects but should unexpected complications arise, such as infection following surgery, we will kill the mice humanely after consultingwith the Named 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 animaltype)?

Most genetically altered mice (>90%) will experience “mild” or “sub-threshold” severity levels. The remainder (<10%) might experience a “moderate” severity level. Similar proportions are expected forwild type or genetically altered mice with substance administration by non-surgical methods.

Mice with substance administration by surgical methods are expected to have a moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice are the only mammalian model that can be genetically modified with ease and have well- established databases and resources available. Mice have been widely used as an animal model for human nervous system, which is a complex network made up by different types of neurons and glial cells. There is no in vitro system that can accurately mimic the

in vivo architecture of the human nervous system. Therefore, to investigate the interaction between different nerve cells in the brain, weneed to use mice to manipulate cell function and acquire in vivo information.

Which non-animal alternatives did you consider for use in this project?

We considered using a variety of brain cell lines, such as neural stem cell lines, oligodendrocyte celllines, microglia cell lines and neuronal cell lines. We plan to use these cell lines for some in vitro experiments.

Why were they not suitable?

Brain cell lines are very useful but have their limitations.

Experiments using cultured cells can inform us how neurons or glial cells respond to specific stimuli butcannot mimic the response of the nervous system working as a whole. It is also difficult for us to study neuron-glia interaction using cultured cells.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals are based on my 20-year long and extensive experience on neuroscience research with animals and are estimated according to the NC3Rs regulatory guidelines and Experimental Design Assistant (EDA). In addition, this project will be a follow-up to our current project,which gives us a frame of reference for number estimation.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We have taken on board the advice of senior colleagues and local NACWOs.

We have used online experimental design tools from http://www.lasec.cuhk.edu.hk/sample-size- calculation.html and NC3Rs' Experimental Design Assistant (EDA, https://eda.nc3rs.org.uk) to optimizeour project design and reduce animal numbers.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Our animal facility has a good management system and database to facilitate efficient breeding andinformation sharing. We will optimize our mouse breeding to minimize numbers of litters and avoidover-production and over-production and waste. We will use unwanted in-house mice for negative controls wherever possible. We will follow NC3Rs *Colony management best practice guidelines* andarchive the strains that are just imported or not in use by freezing sperm or freezing embryos.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use normal and genetically altered mice. Most of our mutant mice carry conditional mutations,so that the mutations affect only a certain organ or cell type and/or take effect only after treatment withspecific inducers.

We will feed mice highly nutritious and tasty food (e.g. condensed milk or peanut butter) afteradministration of certain substances to prevent weight-loss and enhance their health.

For mice undergoing a surgical procedure, we will provide sufficient anaesthesia and administerpainkillers afterwards to relieve pain and suffering.

Why can’t you use animals that are less sentient?

Rodents are the most used mammals for modelling human nervous system. Non-mammals have simpler nervous systems and thus cannot replace mice as animal models for studying certain complexmechanisms in human nervous system and neurological disorders.

As part of our work relates to ageing and ageing-related neural degeneration, we need to use matureadult mice for behavioural tests to assess their cognitive abilities, and such tests cannot be done on terminally anaesthetised mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Our mice are maintained in group-housing with environmental enrichment (e.g. nesting roof, nesting material, chew block and hiding loft) to avoid stress wherever possible. The staff of our animal facilityand lab are experienced in animal handling and trained to spot signs of discomfort. Mice will be closely monitored and any mice showing signs of deteriorating health will be humanely killed immediately.

We will optimize our colony management and make sure that all breeding females retire before 8 months of age to prevent potential breeding-related health problems. When marking the mice by ear-punching, we will also use the removed ear tissue for genotyping.

We will choose the least invasive route (e.g. via feed) for substance administration wherever possible.We will perform pilot experiments to determine the minimum effective dose if needed.

After a surgical procedure, we will monitor the animals closely, providing post-operative

care/pain management and seeking advice from the Named Veterinary Surgeon (NVS) and Named Animal Care& Welfare Officer (NACWO) for any unexpected complications.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Before performing experiments, we will refer to the PREPARE checklist and make sure that valuabledata will be generated in the planned experiments.

When conducting experiments, we will follow the Laboratory Animal Science Association (LASA) guidelines. For instance, when conducting behaviour tests, we will follow Guiding Principles for Behavioural Laboratory Animal Science to minimize the changes of lab environment and keep home cage conditions the same; for performing surgery, we will use aseptic techniques by following GuidingPrinciples for Preparing for and Undertaking Aseptic Surgery.

We will publish our research data in scientific journals (open access) in accordance with the ARRIVEguidelines.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Newsletters from our university's Biological Services regularly inform us on advances in the 3Rs andhighlight seminars and other events relating to the 3Rs. I always keep myself updated on the new advances through these newsletters/seminars. I am also a subscriber of the NC3Rs newsletters.

I will consult with the NVS and NACWOs about implementation of new advances in the 3R during theproject.

Home Office

INVESTIGATING THE MECHANISMS OF COGNITIVE DEFICITS AND ANXIETY IN MODELS OF 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
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Alzheimer's disease,, anxiety, memory, novel therapy, pharmacology

Animal types	Life stages
Mice	adult, aged, 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?

Alzheimer's disease (AD) is the most common chronic neurodegenerative disease worldwide, affecting 5-8% of the general population over the age of 60. This disease is associated with symptoms such as progressive memory loss combined anxiety, for which there are no safe medication to treat these symptoms. Therefore, we will focus on discovering brain pathways that cause the symptoms of AD and facilitate developing new medications to alleviate anxiety and help with memory function.

Successful target validation in our pre-clinical models opens the possibility for clinical trials and the potential to unequivocally improve the quality of life for millions of patients and their carers worldwide.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

AD accounts for approximately 60 - 70% of cases of dementia, a global social and economic burden with 50 million people living with the condition, which is predicted to double by 2050 due to our ageing population and costing the global economy $818 billion. Early-onset of the disease can develop as young as 30 years of age and is seen in one in 20 people with familial fAD (ARUK, 2021). Along with cognitive decline 70% of patients diagnosed with Alzheimer's disease (AD) live with neuropsychiatric symptoms (NPS) including agitation, anxiety, behavioral changes including disinhibition and irritability. These symptoms affect patients' and caregivers' quality of life considerably. NPS are commonly treated with classical anti-anxiety medication, that are unsafe because they may be either ineffective or cause severe side effects, or both. To date, there are no effective treatments to prevent, halt or reverse the diseases. Hence, there is an unmet clinical need for targeted therapies to memory loss and manage the NPS associated with AD.

What outputs do you think you will see at the end of this project?

There is a global focus on curing dementia, and our expertise and a novel approach of normalising hyperactive brain circuitry in AD makes this project a timely priority area of research into dementia. The suggested work, based on our combined expertise, existing collaborations and track record can definitely enable feasibility and successful delivery of the overall objectives, which include:

reveal new data of the pathology of AD and other related forms of dementia that leads to loss of memory and neuropsychiatric symptoms such as agitation and anxiety associated with AD;

validate different target brain pathways responsible for memory loss to stop the progression of the disease at an early- and late-stage of the disease, that will allow us to design novel treatment strategies;

validate novel molecules that will alleviate neuropsychiatric symptoms of AD, that could be translated to new therapies to treat anxiety associated with dementia patients, but also the general population without the severe side effects of the existing treatment for anxiety.

The discoveries from these objectives will provide a better understanding of how to restore the memory loss and reduce anxiety in Alzheimer's patients and ultimately provide a novel approach for the treatment of these conditions. As such, this proposal represents an important first step in the process of making a significant impact within the field of psychiatry.

Who or what will benefit from these outputs, and how?

The outcome of this novel, unexplored basic research project will be to underpin mechanistic information of the brain and the possible causes of Alzheimer's disease at a brain network level that will lay the foundation for wide applicability to future therapeutic settings aimed at improving human health. Therefore, understanding of the underpinning biology that will be revealed from this project and developing of novel tool compounds will benefit both academic and pharmaceutical industry.

Successful target validation in our pre-clinical models will open the possibility for clinical trials and the potential to unequivocally improve the quality of life for millions of patients with Alzheimer's disease and their carers worldwide.

Apart from the existing academic collaborations, other academic beneficiaries will include industry partners as well as charity organisation. Researchers working to build mathematical and computational models of the interneuronal network to emulate the inhibitory functions of interneurons in network behavior in health and disease will also benefit from our single cell electrophysiological experiments.

To ensure the results of our research reach a very broad audience base (academic and non-academic users), we will present our data via seminars, lectures, posters and attendance at meetings both in the UK and internationally to scientific, clinical and lay audiences. The meetings we attend are often large international and national events (ARUK conferences, Society for Neuroscience, Federation of

European Neurosciences, the British Neuroscience Association, as well UK Physiological and Pharmacological Society meetings and our own Annual Neuroscience Symposium Day). We will also present findings at seminars by regular structured presentations made by our post-graduates to their peers and staff, enabling excellent communication between many laboratories.

Our finalised studies will be published in peer-reviewed journals, including those of the highest quality, with open-access publication allowing both researchers and interested members of the public unrestricted access to research data and outcomes. In addition, we are keen advocates of communicating our research to a wider lay audience by presenting at science festivals, exhibitions and to Schools. For example, during the recent dementia awareness week the PI hosted a fundraising event, Art for Dementia Research, raising over £5,200. Furthermore, we will present our work to the public in the form of workshops during Brain Awareness Week, with the aim of engaging and educating a new generation of neuroscientists and raising public awareness and interest in neuroscience research.

How will you look to maximise the outputs of this work?

To maximise the outputs, we will highlight our research findings, especially if of a topical nature, in websites dedicated to Neuroscience and Alzheimer’s research. New research findings will be disseminated to as wide a cross-section of the general public as possible via both a range of online and social media outlets, including regular news, press releases and via Twitter. We will also draw on research expertise to engage the public via traditional media including television, radio, and the popular press, as narrators, interviewees or as technical consultants, evaluating statements or publications relating to healthcare. We will also play an active role in promoting engagement in patient, public and carer in research and Pharmacy education.

We also have strong link with the various charities, Academic partners and various industry partners. We will also take part in European consortium for computational modelling. This is relevant when we feel our innovative biological findings have established the likelihood of a new therapeutic target that may lead to promising chemical leads for pre-clinical and clinical development pathways.

In addition, we will maximise the outputs of the work proposed here by working together with our widerange of a collaborators: i) translational research scientists and medical practitioners; ii) those in the general public who are directly or indirectly affected by memory related disorders and intellectual disability; iii) commercial entities engaged in drug development programmes such as pharmaceutical industry.

Species and numbers of animals expected to be used

- Mice: 1400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Experiments will be performed using control wild-type and genetically modified mice using preclinical mouse models of Alzheimer's disease, for example the knock-in amyloid protein precursor (App) knock-in mouse models, as well as other App or tau mouse variants.

Alzheimer's disease is displayed in these mouse models when the genes from both parents are expressed. The symptoms of this disease are expressed in later life, but with cellular pathology that may occur at birth. We are interested in discovering the development of the pathology from early stage to the late stages from birth to aged animals when the typical symptoms of memory loss and neuropsychiatric symptoms of AD are present.

Typically, what will be done to an animal used in your project?

Using an interdisciplinary approach, we aim to breed genetically modified mice that harbour 2-3 familial forms of human genes for Alzheimer's disease. During some procedures, animals may be used for behavioural experiments to measure their memory and level of anxiety before and after treatment with novel, potential medications to halt memory loss and alleviate neuropsychiatry symptoms (NPS) including agitation and anxiety. Some animals will undergo surgery to insert specific molecules or new gene therapy tools that will allow for the control of selected populations of neurons. These neurons can be later activated in the freely moving animal by administrating specific medication orally to the animals. This will allow us to probe specific pathways in the brain, allowing the investigation of their function and validation of novel targets for developing new therapies for memory loss and NPS.

What are the expected impacts and/or adverse effects for the animals during your project?

Analgesia will be provided to alleviate any pain during procedures where animals may experience any pain after surgery. Some protocols involving behavioural memory related tasks will involve positive reinforcements to motivate animals to find food treats located in a maze. This is to investigate whether certain novel drug treatments improve or halt neurodegeneration, alleviate anxiety and memory loss, all associated with Alzheimer's disease. Animals that will be aged to study memory loss and neuropsychiatric symptoms associated with Alzheimer's disease may display weight loss, formation of age-related tumours or hair loss. These animals will be continually monitored after the age of 12 months, and staff will be trained to be able to identify any adverse effects. The animals will be culled at the end of the experiment under a non-recovery protocol performed under general/terminal anaesthetic to ensure no pain is felt.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most of the research into AD mechanisms in this project will use breeding, ageing and behavioural protocols, with an expected severity level classified as “mild”. Approximately, 60 % of the animals will be used for functional electrophysiology or neuropathology experiments using ex vivo brain slice preparation, where brain tissue are kept alive under artificial conditions up to 6 hours. This involves procedures to obtain brain slices, which will be performed under general/terminal anaesthetic to ensure no pain is felt, with an expected severity level classified as “non-recovery”. Approximately, 10 % of the experimental procedure will involve surgery, with a severity level classified as “moderate”.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Most of the research into Alzheimer’s disease (AD) mechanisms still uses rodent experimentation with severity ranging from mild to non-recovery as there are currently no satisfactory alternative models for studying synaptic mechanisms in the mammalian CNS.

Mice are widely used in studies of anatomy, biochemistry, physiology and pharmacology and for studies of the intricate neuronal pathways in the brain. Rodents offer many advantages because of the large existing body of knowledge, ease of breeding, and availability of protocols for culture and transfection of primary neurones. All APP mouse models used are C57BlJ6. The APP KI mice carry the same mutation as human AD patients and are therefore relevant for the research into AD. We will use male and female mice at different ages for in vitro primary cell cultures, ex vivo brain slice recordings and behavioural experiments.

Which non-animal alternatives did you consider for use in this project?

Where possible, experiments will be conducted in cell culture from human stem cells and post-mortem human tissue. However, our parallel projects aim to address the need for in part, by establishing a sensitive and improved pre-clinical model using human brain cells to develop “dementia in a dish” model that is derived from human control and Alzheimer's disease donors (full replacement) which we would expect to mimic normal and abnormal brain circuit behaviour.

We also aim to build artificial computational networks, but the results obtained from this approach would still require verification in naturally developing brain tissue from mammalian species.

Why were they not suitable?

No computer model is currently available that can replace the use of animal tissue for this objective, as there is insufficient information on the network connectivity and circuit activity involved. Nevertheless, computer models will be used to assist the interpretation of the data obtained in experiments from animal tissue.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

From our experience, the number of laboratory animals bred to study mechanisms of Alzheimer's disease pathogenesis reported to the Home Office in for example, 2020, using male animals was ~230, which ranged from mild- moderate levels of severity. For this project, using both sexes, we predict to use 1400 animals over 5 years. This metric includes wild-type mice and two different mouse models expressing Alzheimer's disease - causing mutant genes.

Our parallel projects will also gradually implement reduction and Replacement, by developing in vitro human dementia models. Assuming that approximately 10% of this work could be reduced by uptake of the in vitro models developed in this project, this will replace ~140 mice in experimental procedures during this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The emphasis of this project is to instil the 3Rs principles throughout the project and provide multidisciplinary training for students and staff involving various neuroanatomical techniques, electrophysiology/pharmacology, and microscopy.

For this, experimental design has been optimised to obtain answers to the questions to be addressed, and statistical power analysis have been employed ahead of commencement of experiments.

(http://www.statsoft.com/Textbook/Power-Analysis#power_doe2 ; http://powerandsamplesize.com/). All students and staff are expected to attend a mandatory induction programme which gives them an overview of the PhD experience and introduces general training in professional research conduct, and cultural diversity training, short statistical/computing courses and offers a "help desk" for statistical advice, which includes appropriate training.

Based on the preliminary data obtained, we use power calculations in consultation with the NC3Rs representative, for e.g. ex vivo work, we expect 50-60% change in synaptic events recorded in brain slices after drug treatment in the disease model. This will result in a sample size of 11 experimental animals plus 11 control mice to reach statistical significance ($P<0.05$). For Behavioural experiments, we expect to see a change of 60-70 % in a sample size of 18 experimental animals in each cohort to yield statistical significance. All experiments will be carried out by researchers blinded (and allocation of all animals will be randomised) to treatment to prevent experimental bias.

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 committed to the principle of the three "R's" (Replace, Reduce, Refine) and will perform all experiments in this grant according to these principles. For example, we will reduce the number of animals by optimizing the breeding programme. Second, we will reduce the animals required for procedures by using the same mouse for different experiments and share tissue between different laboratory users. The largest number of animals will be used for breeding purposes.

To further optimise the number of animals used for the project, we are currently developing a patientderived, human neural cell model of sporadic Alzheimer's disease (fAD), enabling investigation of the impact of AD-associated pathology (funded by ARUK). We will disseminate these best practices via webinars and publication and hope that this model will allow further uptake of human cell-based models in other laboratories, particularly those that commonly make primary cultures from wild-type and transgenic mice, resulting in a 3Rs legacy. We are also working to build mathematical and computational models of brain networks to emulate the inhibitory functions of the brain in health and disease, that will also impact on “reduction” and “replacement”.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Familial App KI mouse models that display a time-dependent expression of AD symptoms that closely resemble human AD will be used during this project. Recently, mutant App and Mapt KI mouse models have been developed by Saito et al., (2014) that do not rely on non-physiological promoters and present characteristic phenotypical symptoms of AD including Aβ pathology, neuroinflammation and memory impairment in an age-dependent manner, these include AppNL-F/NF_F harbouring the double Swedish KM670/671NL and Beyreuther/Iberian I716F App mutations. Although fAD represent approximately 5% of the human population with AD, they share similarities to the idiopathic cases, and are commonly used to make predictions regarding idiopathic cases. These models appear phenotypically normal and do not show any harmful phenotype. Overall, these models and the methods will allow us to reach our scientific goals of advancing our understanding of the pathology disease to better develop cures that will halt, prevent or alleviate symptoms of memory loss and neuropsychiatric symptoms of AD, with the least amount of pain, suffering, distress to the animals.

Why can’t you use animals that are less sentient?

Mice are sufficiently close to humans to reveal principles of synaptic communication and are species that are much used in behavioural and cellular studies of the synaptic circuitry and the inhibitory system, which enables us to build upon a large body of research already carried out, and to relate our findings to previous results. Furthermore, non-protected species cannot be used in this study due to the lack of analogous brain structures.

Our primary model is stimulation and recording from a slice preparation in vitro. This is the most refined model that can be used for the study of synaptic communication of relevant architecture. We will employ state-of-the-art stimulation and recording techniques to maximise the information yield from each experiment. Where possible we will use animals at immature life stage for cell-culture work and implement partial replacement, and majority of our ex vivo experiments will include terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The student and staff working under this project will be given opportunities to gain further 3Rs training either by visiting animal units and/or meeting NACWOs to give a broader understanding of the 3Rs and how our replacement and reduction implemented in the project is aligned with other approaches. The personnel working under this project will be given full training on monitoring animal welfare, postoperative care, and pain management and will also be required to apply for a personal license.

Our primary model is stimulation and recording from slice preparation ex vitro. This is the most refined model because it allows us to investigate complex synaptic communication of relevant architecture to humans, by overcoming technical difficulties and minimising pain to the animal compared to in vivo experiments. It also allows sharing of tissue amongst various experimental users. Furthermore, our latest state-of-the-art equipment and experimental techniques will ensure that maximise information/data yield from each experimental animal.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All experiments will be conducted in accordance with current good practice including Refining

Procedures for the administration of substances and Guiding Principles for Behavioural Laboratory Animal Science, and guidelines included in using mice to model Alzheimer's dementia: an overview (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4005958/).

We will regularly follow published advice given on the MRC, and NC3Rs websites on experimental design and other published peer review work on the quality of experimental design, statistical analysis and reporting of research using animals as well as the LASA guidelines for the application of aseptic techniques during surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Students and staff working under this project will be encouraged to regularly attend virtual NC3Rs events in order to further understand the principles on which this project is based, and also learn more about reducing, refining and replacing animals in biomedical research, as well as the LASA guidelines for the application of aseptic techniques during surgery

(https://www.lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf).

PATHOGENESIS AND IMMUNITY OF MALIGNANT CATARRHAL FEVER VIRUSES

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

cattle, wildebeest, herpesvirus, vaccine, antigen

Animal types	Life stages
Rabbits	adult
Cattle	juvenile, adult
Sheep	juvenile, adult, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

Our main aim is to enhance understanding of the fatal disease of cattle, malignant catarrhal fever, andto develop useable preventative measures such as vaccines to reduce losses in the UK and worldwide.

We have developed a recombinant virus system based on the attenuated AlHV-1 that can express exogenous antigens on the virus particle. We plan to test whether such a system can induce protectiveimmune responses against other respiratory viruses of cattle, using ovine herpesvirus 2 as an initial model.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Malignant catarrhal fever (MCF) is a fatal disease of cattle, deer, bison and other livestock worldwide, caused by a group of related herpesviruses that are endemic in their reservoir species (such as domestic sheep globally and wildebeest in parts of Africa). MCF is also the most important viral disease of deer and bison, which die soon after incidents of contact with sheep and can only be managed currently by biosecurity – separating carriers from susceptible animals. In cattle, MCF is sporadic and appears to be more frequent following lambing/calving of reservoir species, suggesting alink with shedding of virus by newly infected animals.

Losses in some parts of the UK have compromised the beef aspects of sheep/beef farming and in Africa, MCF is seen as a significant threat to the well-being of the poorest cattle-keepers, who currentlyavoid the best grazing to avoid contact with MCF-carrying wildebeest. As a significant disease of bisonand deer, MCF is seen as a major threat to the farming of bison in the UK (and North America) due to the impact of infection from sheep on neighbouring properties.

What outputs do you think you will see at the end of this project?

The attenuated virus vaccine for wildebeest-associated MCF (WA-MCF) has recently been licensed formanufacture and it is hoped that this will be registered for use within the next few years. This research will continue to improve our understanding of this vaccine (helping to define dosage, stability, mode of action) and to develop candidate vaccines for sheep-associated MCF (SA-MCF), aiming to test the efficacy of such vaccines in laboratory or natural hosts.

Who or what will benefit from these outputs, and how?

The deployment of a working MCF vaccine in Africa should help both livestock and game ranching operations and the poorest cattle-keepers, for whom MCF is a severe limitation on their ability to grazecattle close to wildebeest – either captive or on migration routes. WA-MCF is also a danger in mixed wildlife collections, such as in zoos and game reserves, where a range of MCF virus carriers and susceptible species may be held in close proximity. The ability to do improved serological testing (developed by the applicant) and access to a vaccine for WA-MCF will improve options for such collections.

Development of candidate vaccines for sheep-associated MCF will be a benefit for livestock keepersworldwide where the threat to highly susceptible species such as bison or deer has impacted on the ability to farm these animals in areas with sheep.

Sporadic MCF in cattle could also benefit from the ability to protect high-value animals or herds atparticular risk

How will you look to maximise the outputs of this work?

The work done under this license will be balanced with collaborative engagement with vaccine producers and users in Africa and projects aimed at understanding the livestock-wildlife interface and ecological consequences of MCF vaccine deployment. Such collaborative links are currently in place and are expected to continue. These collaborative links will also be employed to enhance the impact ofthe work through dissemination to stakeholders, and to improve the potential for commercialisation of any successful sheep-associated MCF vaccine candidates, which would benefit cattle keepers worldwide.

Species and numbers of animals expected to be used

- Cattle: 125
- Sheep: 100
- Rabbits: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Using species that may be infected with MCF viruses and are susceptible to MCF (cattle, sheep, rabbits) is required for investigation of MCF virus vaccines and of the immunology of both disease andprotection by vaccines or therapeutics. Animals with developed immune systems are required for thiswork so that embryonic forms would not be suitable.

Typically, what will be done to an animal used in your project?

Animals will be inoculated with MCF viruses, infected cells, or inactivated viruses/virus components onone or more occasions. Studies of the resulting disease, or immune response, will be performed and the development of disease or protection will be analysed using animal samples taken during the experiment (blood, nasal secretions, etc) or after euthanasia (post-mortem examination and tissue sampling).

What are the expected impacts and/or adverse effects for the animals during your project?

Malignant catarrhal fever (MCF) is a fatal disease of cattle, deer, bison, pigs and other ungulates, caused by a group of related herpesviruses. There is currently no treatment or commercial vaccine. Our work is intended to support and enhance protective vaccines for MCF susceptible species and tounderstand the disease mechanism to allow therapies to be developed. MCF is fatal for affected animals and therefore animals subjected to experimental challenge in this work will be euthanized forwelfare reasons at or before the onset of moderate severity.

Immunisation of animals with attenuated MCF virus, or MCF virus antigens, does not result in adverseeffects and leads only to the development of specific immune responses in the animals involved.

For MCF-susceptible animals infected with pathogenic virus, the affected animals remain healthy and eat and drink normally throughout the incubation period, which generally ranges from 15-35 days depending on species and route of infection. The earliest clinical sign is generally fever (>40°C), wheneating and drinking are normal. Subsequently, between days 2 and 4 of disease, eating and drinking are depressed in rabbits, may be affected in cattle but animals are usually not seriously affected otherwise. Progress of disease is usually rapid, including depression and loss of condition, and animals are euthanized for welfare reasons within 2-4 days of the onset of fever (rabbits) or up to 7 days in the case of cattle.

Natural MCF infection of sheep does not cause disease, so any animals from this species

may bereturned to normal use after sampling.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

For immunisation of animals with attenuated MCF viruses, their derivatives, or antigenic fractions – mild. No adverse reactions are expected and have not been observed in multiple previous experimentsin cattle or rabbits during the course of previous PPLs.

For challenge of susceptible animals with pathogenic MCF viruses, previous work has shown that about 90% of cattle challenged intranasally with pathogenic AlHV-1 (under ASPA regulated protocols)succumb to fatal MCF, while vaccination protects about 80% of cattle from fatal challenge. The use ofdaily monitoring and a clinical scoring scheme for all animals challenged with MCF viruses will restrictthe actual severity of disease experienced by MCF-affected animals. This scoring scheme has been employed in previous PPL and ensures the euthanasia of MCF-affected animals at or before the onsetof moderate clinical signs.

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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The analysis of responses to vaccination, the performance of vaccine protection trials and the analysisof determinants of pathogenicity all require experiments to be done in appropriate animal species, under controlled conditions.

Which non-animal alternatives did you consider for use in this project?

For the proposed project, considerable progress has been made through use of cell culture systems,analysis of virus components and the use of diagnostic samples. Within the proposed work, animals will only be used where all alternatives have first been considered.

Why were they not suitable?

Cell culture systems are not able to replicate disease pathology or protective immune responses. Theanalysis of responses to vaccination, the performance of vaccine protection trials and the analysis of determinants of pathogenicity all require experiments to be done in appropriate animal species, undercontrolled conditions.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Previous work in this and similar projects provides good estimates of the efficiency of challenge experiments to induce disease (approximately 90%) and of the protective effect of vaccination (approx.80%). This allows us to make informed estimates of group sizes required for animal experiments with appropriate expert advice. Estimates given in this application are, however, based on an outline of the work to be done, which may be affected by changes to funding and research priorities.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

All experimental designs are required to be approved by a suitably qualified statistical advisor before ethical review. For this project, professional statistical advisors will be routinely consulted for advice onexperimental design, groups sizes and animal numbers required. Approval by the local Animal Welfareand Ethical Review Body (AWERB) is only given where the animal numbers have been demonstrated to be the minimum consistent with deriving statistically-significant results and where statistical advice has been demonstrated.

In addition, viruses propagated in tissue culture or purified from archived samples will be used wherever possible (e.g. for methods development, production of virus for infection of animals). Similarly, archived fixed and frozen tissues and samples will be used to avoid the need for new animalexperiments solely to provide material for analysis.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

In addition to the measures described above, the applicant will use data from published and ongoingstudies that are relevant to the conduct of this project to ensure that work is not duplicated where unnecessary and to access the most up-to-date information to inform power calculations where appropriate. This will include reference to previous work by the applicant’s team and collaborative partners.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project addresses pathogenesis and vaccine development for malignant catarrhal fever, a sporadic but fatal viral disease of livestock. For both of these purposes the use of animal experimentsis unavoidable to allow estimation of protective responses to vaccines and to study the detailed pathogenesis of disease. Established animal models of MCF virus infection, disease and vaccination in laboratory animals (rabbits) and in natural host species (cattle, sheep) will allow the selection of theappropriate species, based on experimental goals and containment requirements.

Why can't you use animals that are less sentient?

The animals involved need to be infectable by MCF viruses and mount an appropriate immunologicalor pathological response over the timescale of several weeks. Embryonic forms or non-vertebrate species would therefore be inappropriate.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Each experiment involving animals is critically examined in advance by the AWERB and an end of study report is required at the conclusion of study. These processes will be used to refine both proposed experiments and subsequent work to minimise welfare harms and to refine procedures asrequired. In addition, the scientists involved will be guided by the animal care staff involved with respect to day-to-day interaction with animals under study and will observe the animals regularly (atleast daily following infection with pathogenic viruses).

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

The applicant will use the Prepare guidelines and checklist in the planning of animal experiments (Smith et al., 2018. DOI: 10.1177/0023677217724823journals.sagepub.com/home/lan); and the Arriveguidelines and checklist (V 2.0) for the publishing of papers involving such studies (Percie du Sert et al., 2020 https://doi.org/10.1371/journal.pbio.3000410).

These guidelines also embody government advice, such as the European Union (EU) Directive2010/63 and related guidance materials, such as: https://ec.europa.eu/environment/chemicals/lab_animals/pubs_guidance_en.htm

Where possible, animals will be made available for re-use after work that does not include challengewith pathogenic MCF virus material.

Cattle will be used in experiments where there is a requirement to evaluate immune responses or MCFpathology in the natural MCF-affected species. A clinical scoring system (protocol 2) will be used to ensure animals suffering from MCF are euthanized at or before the onset of moderate severity.

Rabbits will be used as a model system in which MCF may be induced by nebulised intranasal challenge (a likely natural route of exposure) or via parenteral routes in some cases. This species maybe used where the amount of vaccination or challenge material is low or the required containment conditions makes it impractical to work with cattle. Subcutaneous transducers will be used in rabbits tominimise suffering due to use of rectal thermometers. Clinical signs, including temperature, will be evaluated at least daily after

infection.

In all cases, humane endpoints will be based on a limit of moderate severity, with consideration of weight loss, body condition, appetite, respiration, behaviour and dehydration under the supervision of the named veterinary surgeon and with approval of the Home Office Inspector.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

The parent institution has an active 3Rs group which promotes best practise, produces regular newsletters and organises events/seminars. The applicant is also a member of the Animal Welfare Research Network (https://awrn.co.uk/) and can take advantage of advances and best practise publicised through this route. All implementation of advances in 3Rs will be done with the participationand approval of the local Animal Welfare Ethical Review Body and the Bioservices department who have oversight and are involved in the execution of all animal experimentation.

DYNAMIC HOMEOSTASIS OF STEM CELLS FROM DEVELOPMENT TO AGING

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Stem cells, timing, embryo, aging, diapause

Animal types	Life stages
Mice	adult, embryo, pregnant, neonate, juvenile, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.
What's the aim of this project?

A long-held question in biology is how biological timing operates at the cellular level. Throughout the animal kingdom, the duration of development and lifespan vary hugely despite many species sharing equivalent sets of genes. Further, some species can halt development for extended periods of time in response to adverse nutrient conditions (diapause). This project aims to compare the dynamics of stemcells in development and aging to discover the molecular mechanisms that encode timing in the genome.

The key objectives of this programme of work are (1) to understand dynamic properties of cells in embryos and in adult stem cells; and (2) To obtain insights into the impact of developmental timing onlifespan.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
How biological timing is encoded in the genome to track time across timescales remains unknown. Understanding the rules that determine how cells can precisely initiate and terminate processes at specified times and how do they modulate the rate at which they

tick will help us explain when processes go awry. Examples of these are tissue overgrowth and deficits, or aging. Moreover, if we identify the molecular mechanisms that encode biological timing, we can harness them to speed up orstall in vitro stem cell differentiations to generate tissues in culture to study them in the lab or for transplantation therapy purposes.

What outputs do you think you will see at the end of this project?

The output from this programme of work will be in the form of new information (characterisation of developmental processes, differentiation and aging; whole genome profiling resources) that will bepublished and reagents, such as stem cell lines and transgenic mice to be used by our colleagues,other researchers and, potentially, the pharmaceutical industry.

Who or what will benefit from these outputs, and how?

The short-term benefits will be a better molecular understanding of how stem cells maintain their homeostasis from fertilization to death. Embryonic stem cell lines derived from mice, including those ofgenetically modified mice, will provide an ex vivo system with which to study the properties and potential of the tissue of origin. In the medium term, we expect to have generated a comprehensive understanding of the mechanisms that regulate the pace of development and adulthood, and the impact of lengthening development on lifespan. In the long term, we will increase knowledge about how can we slow down or speed up the pace of developmental and homeostasis processes. Ultimately, we expect to be able to translate our knowledge for application by us and others inregenerative systems and alternative systems that improve the health span of the organism.

How will you look to maximise the outputs of this work?

The aim of this work is to advance our biological understanding of dynamics in development and homeostasis, and the outputs from this research will include new knowledge and publications in peer-reviewed journals as well as in scientific conferences.

An important feature of the project is the association of mechanisms in development to homeostasis and lifespan, as these tend to be studied in separate fields. Moreover, the protocols and techniques wewill develop during the course of the work will benefit researchers studying similar problems. Our protocol on diapause is specialised, and we collaborate with one group in the UK and one in Germany to refine the protocols, avoid duplication, and maximize the output.

Species and numbers of animals expected to be used

- Mice: 14750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The use of mice is necessary to achieve the proposed research goals, which require analysis of intact embryos and adult mice. Unlike other experimental systems, the mouse offers the most relevant in vivomodel system, with the ability to genetically alter the genome, to address important biological questions that could impact on major advances in the stem cell field. This project will work with early developmental stages as well as with a small proportion of aged mice.

Typically, what will be done to an animal used in your project?

By far most of the work to be carried out under this licence involves breeding of genetically altered mice, for provision of early embryos from females culled using a Schedule 1 method. In some instances, females may undergo superovulation, diapause induction or embryo transfer. Some animalswill be maintained for a long period of time to study aging.

What are the expected impacts and/or adverse effects for the animals during your project?

It is expected that most animals will not experience greater than mild severity. Inducing agents will be used in some animals to control gene regulation/expression in cells and label molecules in vivo as required to meet our project objectives; it is not always possible to fully predict the phenotypic outcomein a novel environment.

In addition, some surgical procedures will be performed. Even though surgery is considered amoderate procedure, we expect the animals to promptly recover.

Ageing mice are more susceptible of distress. Regular monitoring will ensure that animals do not suffergreater than mild longer than 24h.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Five of the protocols (Protocols 1, 2, 3, 6 and 7) are classified as moderate because they involve routine surgical procedures such as embryo transfer or vasectomy. The proportion of mice exposed tothese moderate protocols is expected to be less than 15% of the total number of mice utilised under this licence.

Minimal animal suffering is anticipated as most animals undergoing administration of substances orsurgery are expected a mild severity as they may experience short-lived postoperative pain and discomfort.

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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We replace the use of animals with cultured cell lines whenever possible. However, we sometimes need to breed mice carrying particular genetic modifications to provide early embryos for short-term experiments, for stem cell derivation or to analyse specific phenotypes such as diapause. This is particularly important when we do not fully know how the in vitro system performs in relation to in vivodevelopment, as is the case for diapause. In addition, there is currently no culture alternative that exactly mimics normal development or aging.

Which non-animal alternatives did you consider for use in this project?

We will make use of stem cell models of development to minimize the number of animals used. Our team has developed 2D differentiation models to generate different primary non-dividing cell types fromestablished pluripotent embryonic stem cell (ESC) lines of mouse and human origin. Further, the lab will develop 3D stem cell models (namely mouse blastoids, gastruloids and motor neuron stem cell differentiations).

For aging, we may use terminally-differentiated neurons derived from stem cells cultured for long periods of time without any deterioration in survival. This has been successfully developed for humanmotor neurons, and a recent optimisation of the culture protocol that is applicable to mouse motor neurons demonstrated viability for at least 2 months.

Why were they not suitable?

Stem cell models are appropriate for the study of cell-intrinsic timing mechanisms but tissue development and homeostasis is multifaceted and an emergent feature for which we still do not haveintegrated models that recapitulate all of its entirety. We are developing more complex models that allow us to recapitulate in vivo processes. These are likely to further increase the rate at which we replace animal use with in vitro models. Nevertheless, we are still in the development and validation phase and it is essential to carefully compare and benchmark in vivo methods to the in vivo situation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers have been estimated considering the number of samples and embryos needed for the experiments proposed within the objectives set. These are informed by similar experiments we performed in the past, where we can estimate with accuracy the number of animals needed. For thoseexperiments that we have never performed, such as lifespan studies, we have checked in the literature, talked to peers and reached out to bio-statisticians to seek advice.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We started the process by reading relevant literature and studied how other researchers had performed similar experiments. We planned essential experiments only, and sought advice from experts. At the stage where we started designing the experiments, we made use of the NC3R's Experimental Design Assistant. We planned how would we group the animals, which variables wouldwe need to analyse and planned an unbiased statistical approach that would fit our experimental planfor our analyses. We also are in continuous contact with a bio-statistician that is helping us with the experimental design, and the whole licence was reviewed by an internal committee.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We minimise the numbers of mice we need to use primarily by making sure our breeding programmesare the most efficient for providing the genetically modified embryos we require. Our extensive experience in managing breeding programmes for the generation of embryos for research contributesconsiderably to the efficiency with which we can reduce the numbers of mice we maintain.

The methodologies for generating genetic modifications have now evolved sufficiently to enable us to delete specific genes in an inducible manner. Conventional deletion of the copies of such essential genes from both parents (known as 'homozygous null') in an embryo results in developmental failure. This means that homozygous null embryos can only be produced by mating male and female mice carrying one deleted copy of the gene each, and therefore, by Mendelian genetics, only 25% of embryos would be expected to be homozygous null. However, being able to breed mice carrying special modifications on both copies of the gene of interest that allow its deletion only when the embryo is exposed to a deleting agent means that all the derivative embryos from a mating have the potential to be made homozygous null. As a result, up to 75% fewer genetically modified mice need tobe maintained to generate as many homozygous null embryos as would be required from animals carrying only a single deleted gene.

For early pre-implantation stages, we use superovulation to generate large amounts of oocytes permouse which reduced the overall amount of mice that need to be used for oocyte supply.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse models are advantageous for biological discovery. They are small and easy to breed, reachingsexual maturity within two months from birth, and have the capacity to produce large numbers of embryos. Furthermore, mice are the best animals for our research because they can provide all the genetic modifications we require. Our gene alterations aim to induce the deletion of genes or label specific cell populations and

molecules, and we do not anticipate they will cause any adverse effect.

Most animals on this project are not expected to be subject to any pain, suffering, distress or lasting harm. A small proportion of our animals undergoing regulated procedures (~8%) will be subjected to surgery, which involves preparation for procedures required for making female mice receptive to transplantation of embryos, or for extending the period of development just before implantation (knownas 'diapause'). In both instances, the surgical procedures may be substituted for alternative non- surgical methods that are currently being piloted. All animals undergoing surgery will be provided with pain relief, and are not expected to suffer more than mild discomfort. If signs of ill health are apparent or animals experience more than mild discomfort, the animals will be humanely killed.

Some of the animals (<3%) in the project will be maintained for a long period of time to investigate ageing. There are no specific impacts or adverse effects expected on mice ageing healthily during thisproject, and regular monitoring of aged animals will prevent for any unnecessary animal distress.

Why can’t you use animals that are less sentient?

The study of biological timing in development and homeostasis aims to identify molecular mechanismsthat are relevant for human biology. Overall, the identification of physiological mechanisms that modulate timing and its translation to stem cell models may have important implications in the field of human assisted reproduction, regenerative medicine, and aging. As a proxy to human biology, we use mice for the in vivo part of our research because they represent the mammalian model system with thebest characterised development, most comprehensive availability of validated transgenics, small physical size, large litters and short inter-generational interval. Their husbandry is well established; highly trained and dedicated teams of technicians are already in place for their care and maintenance.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We will follow the latest advice for specific procedures, and we will bring in external experts whenneeded to refine our methods.

For diapause induction, we will liase with researchers that have recently refined the method for trainingand advice.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Refinements from the NC3Rs such as the ARRIVE guidelines on design and reporting; the LASA aseptic guidelines; LASA Diehl guidelines on volumes and frequency limits and the most up-to-dateveterinary knowledge.

Ronald L. Wasserstein & Nicole A. Lazar. The ASA Statement on p-Values: Context, Process, andPurpose. The American Statistician 2016 70:2, 129-133, doi:10.1080/00031305.2016.1154108

Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. PREPARE: guidelines for planning animal research and testing. Lab Anim. 2018 Apr;52(2):135-141. doi: 10.1177/0023677217724823. Epub 2017Aug 3. PMID: 28771074; PMCID: PMC5862319.

Festing MFW. The "completely randomised" and the "randomised block" are the only experimental designs suitable for widespread use in pre-clinical research. Sci Rep. 2020 Oct 16;10(1):17577. doi:10.1038/s41598-020-74538-3. PMID: 33067494; PMCID: PMC7567855.

Percie du Sert N, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, Emerson M, Garner P, Holgate ST, Howells DW, Hurst V, Karp NA, Lazic SE, Lidster K, MacCallum CJ,Macleod M, Pearl EJ, Petersen OH, Rawle F, Reynolds P, Rooney K, Sena ES, Silberberg SD, Steckler T, Würbel H. Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. PLoS Biol. 2020 Jul 14;18(7):e3000411. doi: 10.1371/journal.pbio.3000411. PMID:32663221; PMCID: PMC7360025.

Finally, protocols in this licence can be found in Manipulating the Mouse Embryo: A Laboratory Manual,Fourth Edition from CSHL by Richard Behringer, University of Texas, M.D. Anderson Cancer Center; Marina Gertsenstein, Toronto Centre for Phenogenomics, Transgenic Core; Kristina Vintersten Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto; Andras Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

I routinely monitor news and progress in the NC3Rs web page, and the Biological Support unit in the Institute also updates the researchers when it is needed. Further, I interact regularly with a member of Felasa.

SIGNALLING VIA REACTIVE OXYGEN SPECIES IN THE REGULATION OF INFLAMMATORY IMMUNE RESPONSES

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Respiratory infection, Inflammation, Neutrophils, Redox signalling, Reactive Oxygen Species

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

We aim to unravel the mechanisms that control and adjust host immune responses in the lung with focus on the function of highly reactive signalling molecules that are generated from inhaled oxygen,so called oxygen radicals. Therefore, we will elucidate if the production and localisation of oxygen radicals within cells and tissues affect the induction of inflammation, removal of pathogens and concomitant resolution of inflammation.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Overt inflammatory responses cause pathology and mortality. Especially during infections of the lung, for example with respiratory viruses such as Influenza A Virus or SARS-CoV-2 Coronavirus, the host immune response turns detrimental by causing tissue damage and impairing lung function. Moreover, during sterile and autoimmune diseases, such as Chronic Obstructive Pulmonary Disease (COPD) andasthma, uncontrolled inflammation exacerbates pathology and poses the primary driver of life- threatening symptoms.

Understanding the mechanisms leading to unwanted and disproportionate inflammatory reactions will enable us to control pathology. Reactive oxygen species represent microbicidal agents as well as critical signalling molecules adjusting host immune responses according to the pathogen encountered.Knowledge of the signalling molecules which attenuate inflammatory responses will allow the design ofimmune modulators and provide new avenues for the treatment of acute and chronic inflammatory andautoimmune diseases.

What outputs do you think you will see at the end of this project?

We expect that our studies will provide important new knowledge about the mechanisms which lead tothe development of excessive inflammation in the lung during acute viral and bacterial infections. We further anticipate to apply our findings to inflammatory sterile and autoimmune diseases. Excessive recruitment of innate immune cells as well as the production of oxygen radicals - called reactive oxygen species (ROS) - are key players in inducing detrimental immune responses during respiratory infections as well as in establishing chronic inflammatory lung diseases.

We aim to identify the role of key genes and proteins which are targets of reactive oxygen signalling inimmune cells and to understand the molecular basis of these events. Moreover, we wish to explore molecular factors that help or hinder these processes, for which an *in vivo* model is crucial to accurately recapitulate disease progression. The results of the study might lead to the development ofnew therapeutic approaches or novel drugs that control inflammation during adverse immune responses as experienced for example during respiratory virus infections with Influenza A Virus or Coronavirus SARS-CoV-2. Potentially, our findings could help to improve existing vaccination strategies against respiratory viruses.

Who or what will benefit from these outputs, and how?

Acute and chronic lung infections greatly contribute to the global health burden. We envision that theresults of the proposed work will be of benefit to patients with respiratory virus infection such as Influenza A virus and SARS-CoV-2, patients with bacterial pneumoniae, but also to patients with non-infectious inflammatory lung disease such as asthma or chronic obstructive pulmonary disorder (COPD).

The proposed project will deliver important new insights into mechanisms of lung inflammation and outcomes will be of interest to researchers on respiratory infection, immune regulation and inflammation. This is a discovery science project aiming to improve the basic understanding of the role of ROS production in innate immune cells. In future, the results of the proposed work could lead to the development of drugs that target affected signalling pathways and could increase effectiveness of established treatments. The localisation of ROS as a critical mechanism of immune regulation could bereviewed in many infectious as well as sterile diseases where an excessive immune response becomes problematic. In general, the proposed work has the potential to have a high

impact in the fieldof inflammation regulation and lead to the development of novel therapeutic approaches.

How will you look to maximise the outputs of this work?

Our lab has long-standing experience in bacterial, fungal as well as viral infection models in mice. Moreover, we have established collaborations with scientists specialised on Influenza A virus infection,bacterial lung infection and allergic asthma models in mice.

Completed work will be submitted for publication in peer-reviewed journals and presented to the scientific community on meetings and international conferences, via direct contact with other scientists,through teaching and training of the next generation of scientists - the project will provide excellent training for a junior scientist. We will capture non-academic audiences within public engagement events, for example to engage pupils at school. Further, we will publish negative results i.e. if pathogenclearance and inflammatory responses in specific infection models are unaffected by ROS signalling.

Our laboratory works in partnership with the National Health Service (NHS) to promote the translation of discoveries into medical advances. Clinical and discovery scientists work closely together to improvethe quality of life of patients and populations by translating basic scientific research findings into innovative therapies and promote their application in the NHS and around the world. Our department's multi-disciplinary approach tackles cutting-edge questions in areas of biology and medicine such as asthma, allergy, cancer, diabetes, arthritis, inflammatory bowel disease, organ rejection, and a range ofbacterial and viral infections. The ideal environment to extend our work and challenge the universality of the mechanisms regulating inflammatory responses through ROS localisation.

Further, the project's outputs may trigger more research in the commercial space. If the proposal results in findings of potential commercial interest, or directly in the design of immune-modulating drugs, our Commercialisation team works with staff to help make research commercially available innew businesses or companies, provide financial and commercialisation support for translational life science research projects in order to deliver improved patient benefit and accelerated impact.

Moreover, we have access to an excellent Communications and Engagement team which aims to communicate research of public interest. Publications produced within the department have receivedcoverage in major newspapers and television channels in the UK and abroad.

Species and numbers of animals expected to be used

- Mice: 5000 animals over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mammalian immune system is complex with many different cells and molecules

working in combination to produce a co-ordinated response. Thus, using less sentient organisms such as zebrafish or fruit fly is not feasible, as they do not possess the complex immune system seen in mammals. Similarly, *in vitro* models cannot give an accurate reflection of the complexity of the mammalian immune system. Therefore, the use of mice is crucial to study mammalian immunity. Thereare numerous discoveries made in mice that have led to the direct identification of similar mechanismsin the human immune system, with similar cells and molecules now being clinically targeted in disease.

We will be using 8-week-old mice as a model for the adult human immune response and a small number (<100) of 24+ week old mice to mimic the immune response of the elderly to respiratory virusinfections.

Typically, what will be done to an animal used in your project?

The majority of animals will be used in models of lung disease induced by respiratory virus infection or sterile inflammatory lung disease models of house dust mite-induced allergic asthma. Some animals maybe irradiated, injected with cells or administered substances to modulate gene expression, or modify the immune system and/or metabolic processes. In general, only one procedure or a maximumof 2 procedures will be carried out per animal. The majority of experiments will be finished after 3 daysfor analysis of innate, i.e. immediate immune responses. Some animals (~200) will be used in infectionexperiments with a low pathogen dose lasting 7 or 14 days for the analysis of adaptive immune responses. In some experiments (~200 mice), after animals have fully recovered from initial infection, they will be re-infected to identify cells and molecules that regulate immunological memory - the process by which we respond better and faster to infection the second time round. Mice will be monitored closely and culled immediately if symptoms reach a pre-determined threshold. Virus infection reaching moderate severity levels is necessary as it will allow to determine important ways of stopping such symptoms by using certain types of cells or drugs. Throughout studies, animals will be regularly monitored. If any animal causes concern, action will be immediately taken to alleviate this andif this is not possible the animal will be humanely euthanised. At the end of each experiment, all animals will be culled using humane methods and tissue will be collected for further analysis.

Approximately 1000 animals will be culled using Schedule 1 killing without undergoing any proceduresto harvest tissue and primary genetically modified cells for *ex vivo* experimentation in single cell cultures, co-cultures, air-liquid interface cultures and lung slice cultures.

What are the expected impacts and/or adverse effects for the animals during your project?

Some animals may experience reduced mobility, reduced lung function or short periods of respiratorydistress over a period of 1 - 2 days, but are not expected to show prolonged signs of breathing difficulties. In addition, some animals will experience weight loss over a period no longer than 3 days,which will be closely monitored not to exceed 15% of initial weight. Few animals will be kept for 7 and14 days after infection for the analysis of adaptive immune responses. These animals will be infectedwith a low pathogen dose to ensure a mild severity limit with minor weight loss.

Furthermore, genetically modified animals in this project are expected to be immuno-compromised butthese animals will be kept in specific pathogen-free environment and carefully looked after to minimisechances of infection.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

We expect 1000 genetically altered (GA) mice to be used for Schedule 1 killing, 2000 GA animalsunder mild severity for analysis of the immune system under steady state and low dose microbialchallenge and 2000 GA mice for microbial challenge experiencing moderate severity.

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The respiratory tract is extremely complex, with interactions between various different cell types and processes. This cannot be adequately mimicked using *in vitro* studies and, although *in vitro* and *ex vivo*experiments will be used, studying the respiratory tract in health and disease in a whole animal is crucial. Using mouse models allows us to investigate disease states *in vivo* in ways that would be impossible in human patients. Mice are invaluable for studying immune responses mainly because of the availability of immunological reagents, as well as inbred and congenial strains, gene knockout and transgenic mice. This makes the mouse the only species in which it is possible to perform the studies outlined in this application. The complete gene sequence of mice is available. Mouse and human genomes are very similar, each has about 30,000 genes of which only 1% is species-specific.
Equivalent mouse genes have been found for all genes known to cause human disease and 99% ofmouse genes have a human homologue.

Which non-animal alternatives did you consider for use in this project?

Additionally to *in vivo* experiments we will use primary mouse and human cell culture as well as airwayliquid interface (ALI) epithelial cell cultures and lung slice cultures to mimic the environment in the airway. These *ex vivo* and *in vitro* approaches will be investigated in parallel to *in vivo* experiments (see Action plan).

Further, we have considered using human leukaemia cell line HL-60 which differentiate into neutrophil-like cells.

Why were they not suitable?

We will use human ALI cultures as well as murine lung slice and ALI cultures. However,

these cannot assess systemic effects of inflammation and infection on the whole organism. Upon infection immune cells infiltrate the lung from the blood circulation. The priming cues and activation state of immune cellscannot be reciprocated in an *ex vivo* culture. Further, immune cells residing within the microvasculatureof the lung have the capacity to influence the immune response in the tissue. In conclusion, airway epithelial cultures cannot fully mimic the complexity of interactions in the whole organism.

Moreover, *in vivo* infection of genetically modified mice as well as using murine primary cell cultures provides perturbation methods which cannot be reciprocated in human ALI cultures. Further, neutrophils are notoriously short-lived cells which can be cultured for a maximum of 24 hours and therefore cannot be genetically modified. Human Chronic Granulomatous Disease (CGD) patients, which carry loss of function mutations of the enzyme investigated, often suffer from severe recurrentbacterial and fungal infections which limits the possibility to obtain sufficient blood samples for our research. Only our novel neutrophil-specific knockout mouse model allows to investigate the specificfunctions of neutrophil NADPH oxidase Nox2. We aim to use our results to design experiments in human cells using specific ROS source inhibitors, cell compartment-specific ROS scavengers or target-specific ROS inducers.

HL-60 cells proliferate into an incompletely differentiated state and do not acquire full functional properties of neutrophils. For example, HL-60 cells are unable to undergo formation of extracellulartraps, a killing mechanism of primary neutrophils which is closely linked to ROS production.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
How have you estimated the numbers of animals you will use?

We estimated the number of required animals based on preliminary data from full Nox2 knockout miceas well as pilot experiments with novel cell-specific Nox2 knockout strain to investigate Nox2 knockoutin the innate immune cell compartment. We calculated for the initial testing of different strains of pathogens, doses and read-out time points for the analysis of the immune response. This will allow to focus research on relevant conditions and reduce numbers of required animals.

Further, we require animals for isolation and *ex vivo* experimentation with mouse bone marrow- or lung-derived cells. Isolated cells will be used in cocultures and ciliated airway epithelial cell cultureswith different read-outs such as cytokine production and time-lapse microscopy in parallel. We calculated required animal numbers for *ex vivo* work on our lab's established experience culturing isolated cells *ex vivo*.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The number of times that an experiment has to be repeated, and the required group size, are a function of the variability within and between experiments. To minimise variability and improve the signal to noise ratio in all experiments, a number of steps will be taken.

These include weight matching,in addition to age and sex matching of animals. Care will be taken to use controls of the appropriate genetic background in any experiments that include genetically altered mice. A randomised block design will be used, in combination with factorial design, to further facilitate a reduction in animal numbers. Therefore, we make use of online tools such as the NC3R's Experimental Design assistant (https://www.nc3rs.org.uk/experimental-design-assistant-eda). Mice will be randomly allocated in control groups for mock infection or infection with pathogen. Where applicable we will share control groups by running experiments in parallel. This will inevitably increase the complexity and workload for the researcher, but numbers of animals are kept to a minimum.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Preliminary data from pilot experiments in full Nox2 knockout mice allowed us to perform power calculations to estimate the minimal number of required animals for each experiment. Further, we will analyse multiple tissues and organs in parallel to maximise the data obtained from each animal. We can measure immune cell numbers, cytokine levels, content of oxidized proteins in lung, bronchoalveolar lavage, lymph nodes, spleen and blood in parallel. Lung tissue can further be weighed and separated for isolation of protein and RNA from the same animal. Additional experimental groupsare required for the examination of physiological lung function and microscopy studies of immune cell distribution and colocalisation in the lung as well as histological scoring. The analysis of adaptive immune responses and effects on immune memory will require killing of animals at later time points.

Further, we will ensure efficient breeding of animals. We are maintaining a Cre-line to investigate cell- specific gene knockout. To avoid complications due to loss of function of the gene, whose promoter is used for Cre-induction, the line will be maintained heterozygous. However, Cre-negative littermates willbe used as control 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using 8-week-old mice as a model for the adult human immune response and a small number of 24+ week old mice to mimic the immune response of the elderly in models of lung disease induced by respiratory virus infection as well as sterile inflammatory lung disease models of house dustmite-induced allergic asthma.

To investigate the role of Nox2-mediated redox-signalling during lung immune responses we are usinga full Nox2 knockout as well as neutrophil-specific Nox2 knockout expressing Cre recombinase under a neutrophil-specific promoter.

To modify the immune system and/or metabolic processes we will administer substances that modulategene expression as well as labelling agents. To induce ablation of the immune system mice may undergo irradiation.

Mice are the species of lowest neurophysiological sensitivity that provide the necessary size to allow us to study lung infection and inflammation models with adequate resolution. The mouse models we intend to use have been chosen because they are widely accepted to be the most appropriate and relevant to the human condition they mimic. We have extensive experience with the chosen models, which allows us to reduce the number of animals required, to limit invasive procedures carried out and to limit the discomfort experienced by the animals. Throughout this programme of work, we will continue to monitor our own practices and the literature to look for ways to refine our procedures; thesewill be incorporated into our protocols wherever possible.

Why can't you use animals that are less sentient?

We work with mice because they have a well characterised immune system and well-established disease models that closely resemble human immune responses and disease states. Use of inbred strains controls against variability and a plethora of genetic tools allows the dissection of molecularpathways that control immune response *in vivo*. Investigation of a whole organism is necessary torecapitulate live body variables such as oxygen levels, blood supply, stiffness of surrounding supportive tissue or immune cell recruitment from blood stream.

We have considered less sentient animal models such as zebrafish and drosophila, which are currentlynot able to recapitulate the complexity of redox-signalling during antiviral and allergic lung inflammationin mammals or to dissect the contribution of different ROS sources. We will keep informed on advances in research of the lung immune response and models of respiratory virus infections carried out in zebrafish.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Animal harm will be minimised by:

A tight monitoring of the physiological state of the animal using a scoring system. Analgesia willbe used when appropriate.

In all infectious challenge experiments (protocols 3-4), animals that fail to overcome infection willbe humanely killed by a schedule 1 method.

Doses and strains of infectious agents (protocols 3-4) have been selected to analyse the immuneresponse in situation of immune resistance. This means that the pathogen doses we use are not lethal for C57Bl/6 mice. Our models induce a transient acute infection that involves the onset of transient lung inflammation and respiratory symptoms, possibly with partial but reversible weight loss. From preliminary data it is expected that Nox2 knockout as well as neutrophil-specific Nox2 knockout mice fare better during induced lung inflammation.

Majority of infection experiments will be finished after 3 days. Few animals will be kept for amaximum of 14 days.

Majority of animals will undergo one procedure only and culled by a schedule 1 method for

thecollection of tissue.

For the administration of substances routes, dosage volumes, frequencies and duration will bechosen that will result in no more than transient discomfort and no lasting harm and are the minimum consistent with the scientific objectives.

Irradiated animals will be held under barrier conditions and closely monitored to ensure cleanhealth status. Further irradiated animals will be reconstituted within 24 hours.

To ensure the rigorous implementation of human endpoints, all experiments will be performedby specifically trained scientists, holder of personal license and expert in their infectious/inflammatory model.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

All procedures will be carried out according to home office regulations as set out in the ASPA, Home office guidance on ASPA and AWERB. We will abide to the most recent advice of the NC3R website.We are following published research for the administration of substances in mice (Morten et al. 2001 Refining procedures for the administration of substances), for experimental design (Kortzfleisch et al. 2020 Improving reproducibility in animal research by splitting the study population; Smith et al. 2018 PREPARE: guidelines for planning animal research and testing) and for models of induced lung injury(Barnard 2009 Animal models for study of influenza pathogenesis and therapy).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

The project licence holder and personal licence holders working under this project will seek regular advise from the NC3R regional programme manager. We will further regularly consult Biological Service Unit website as well as NC3R websites and carry out scientific literature research as to new methodologies allowing combination of several readouts per animal, less invasive strategies and lesssentient models such as the use of zebrafish. Discussions on new developments and the implementation of the 3Rs with colleagues, during departmental meetings or conferences will further ensure project and personal licence holders stay informed. Finally, we will consult the NVS and NACWO to implement advances in our protocols.

PATHOGENESIS AND CONTROL OF BACTERIAL INFECTIONS IN FARMED 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, ortheir 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 animalsreared for agricultural purposes

Key words

Farm animal, Bacteria, Infection, Disease, Control

Animal types	Life stages
Cattle	neonate, juvenile, adult
Pigs	neonate, juvenile, adult
Domestic fowl (Gallus gallus domesticus)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

This project aims to control bacterial infections in farmed animals, with emphasis on *Salmonella*,*Campylobacter* and *E. coli*. We aim to understand the role of bacterial and host factors during infections and to use this information to design strategies to detect, prevent or treat infections.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

According to the World Health Organisation, foodborne diarrhoeal disease affects 1 in 10 people everyyear, causing an estimated 420,000 deaths and loss of 33 million healthy life years per annum.
Salmonella, *Campylobacter* and *E. coli* are the leading bacterial causes of foodborne disease and farmed animals are key reservoirs. They can transmit the bacteria through the food chain and environment to people and strategies to prevent this are lacking or ineffective. In some cases theseorganisms can cause disease in farmed animals, harming their welfare and productivity. By understanding the role of bacterial and host factors during infection, we aim to design strategies to detect infection and control it, for example by vaccination, selective breeding or novel therapies.

What outputs do you think you will see at the end of this project?

We aim to identify:

Bacterial factors that mediate colonisation of farmed animals and the production of disease. With this knowledge, we can design vaccines to prevent infection of farmed animals and thereby improve animal health and reduce the risk that they will transmit pathogens to people. Such vaccines may be based on isolated bacterial factors to train animals to recognise them and block their function, or bacteria that are weakened by removing one or more of their virulence factors so they can no longer cause disease but can prime immunity. Such studies may also identify bacterial markers of risk to aid improved diagnosis and risk assessment.

Host immune responses underlying natural or vaccine-mediated control of infection. With this knowledge, we can develop vaccines that are more effective because they elicit lasting responses ofthe required type(s) at locations that are relevant during infection.

Host genes underlying resistance to disease. With this knowledge, we can guide decisions to selectively breed for animals that are more resistant to infection or disease. We may also validate therole of specific genes in resistance by using genetically-altered animals and showing that they are more resistant.

Roles for microbes that live within farmed animals in health and disease. For example, we hope to understand the role that indigenous microbes play in maturing the immune system of farmed animals and resistance to infection or disease. With this knowledge, it may be possible to supplement animalswith specific microbes, or substances to encourage them to grow, to improve animal health.

We aim to generate high quality scientific publications and Intellectual Property, in some cases by working together with commercial partners. Freedom to publish findings arising from animal studies willalways be a priority in collaboration agreements and where Intellectual Property is generated, we will seek to protect it before timely disclosure of findings in the public domain. Many studies will address questions to understand host-pathogen interactions, but with a long-term view to develop products or processes that benefit animals and people.

Who or what will benefit from these outputs, and how?

Effective strategies for control of bacterial infections of farmed animals would benefit them directly by reducing the incidence and impact of disease. The scale of global animal production is vast, with the Food & Agriculture Organisation estimating live global stocks of 33Bn poultry, 1.7Bn cattle, 1.2Bn sheep and 1Bn pigs in 2020. In the same year, a total of

75Bn broiler chickens and 1.6Tn eggs were produced for food. Among the bacteria under study are some that cause severe diseases in farmed animals, as with avian pathogenic *E. coli* which is a major cause of disease and losses in all sectors ofthe poultry industry. As in people, *Salmonella* can cause life-threatening diarrhoea and typhoid in farmed animals. In addition to direct benefits to animals, the prosperity of farmers and the wider agricultural sector would be improved. Further, as many of the bacteria under study are able to transmit from farmed animals to humans via the food chain and environment, controlling them in animal reservoirs could reduce the incidence of human infections. *Salmonella*, *Campylobacter* and *E. coli* are estimated to cause over half a million infections in people in the United Kingdom every year, ata recurring cost of >£1Bn.

How will you look to maximise the outputs of this work?

We have a proven record of disseminating findings from our animal research, including the publicationof research where candidate strategies for control of bacterial diseases proved to be ineffective. We also disseminate findings via presentations at national organisations and international symposia. We have access to devolved funding to catalyse research on a path to commercialisation and have secured funding via schemes that leverage industry contributions to co-develop research to control bacterial diseases. We have embedded business development and legal teams that can assist in knowledge exchange and commercialisation to maximise outputs from our research.

Species and numbers of animals expected to be used

- Cattle: 100
- Pigs: 100
- Domestic fowl (Gallus gallus domesticus): 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We propose to use farmed animals (chickens, pigs and cattle) as they are natural hosts for the bacteriaunder study. They can incur disease due to *Salmonella*, *Campylobacter* and *E. coli* infection and are known to be important reservoirs of human infections by these organisms. By studying host-pathogen interactions using natural hosts, we believe that we will gain highly relevant knowledge required to control infections in these species. Indeed, we have shown that models based on cultured cells or rodents often provide poor surrogates for farmed animals. Further, we have shown that bacteria can deploy factors during infection in host-specific ways. The immune systems of farmed animals also differ in composition and functions, meaning that it is not always possible to extrapolate from model organisms to farmed animals. Our models have tended to focus on relatively early life stages where wecan reliably establish infections and, in some cases, produce disease that is typical of that seen in naturally exposed animals.

Typically, what will be done to an animal used in your project?

In order to define the role of bacterial factors during infection, we will typically inoculate animals by a natural route of exposure with bacterial strains that possess or lack the factor

of interest. This will typically be a mutant derived from a wild strain of the pathogen. We will analyse the magnitude and duration of colonisation and the induction of host responses and disease to determine if these differ between the wild and mutant strains. If it does, it may also be necessary to repair the defect in the mutated strain to prove that it was weakened as a consequence of the specific change made and not another change. Experiments will be of the minimum duration required to meet objectives, e.g. to detect a statistically significant difference in colonisation or host responses. It will not always result in disease and in the majority of cases disease will not progress to pain, distress or lasting harm. The timing and severity of symptoms is well understood, such that it is typically possible to meet objectives before disease occurs. While testing wild, mutant and repaired strains singly is a gold standard, we willalso employ novel strategies to reduce animal use when defining the role of bacterial factors. This has included methods to screen tens to hundreds of mutants simultaneously in a single animal and the useof genetic methods to follow the fate of each one. Additionally, we will use surgical models where manysegments can be created in the gut and each one inoculated with a different strain, such that we can define their ability to cause disease in one animal rather than testing each one separately.

In order to study immune responses, we will typically inoculate animals by a natural route of exposure with bacterial strains or constituents thereof. These may be wild strains that are associated with different outcomes (e.g. that may be virulent or avirulent in particular hosts). We may monitor immune responses following infection by sampling blood at intervals (e.g. to detect circulating antibodies). In some cases, it will be necessary to cull animals at intervals after infection, for example to study immune responses at internal body surfaces or in tissues that cannot be accessed in live animals. Thesame will apply when we test vaccines, to establish the nature, timing and magnitude of host immune responses following vaccination. Vaccines will often comprise an isolated constituent of the pathogen or a weakened form of it, and are not expected to produce disease or adverse effects. After vaccination, we will often seek to determine if the animals are protected against colonisation by wild strains of the target bacterium &/or against disease. This will involve inoculation with a wild strain at a dose adequate to cause colonisation &/or disease. We will compare colonisation and host responses in animals that received the test vaccine, or every other manipulation except the vaccine itself (mock vaccination). We will minimise the number of procedures that a given animal experiences to prevent cumulative harm.

To study the role of host genes, we will perform infection or vaccination studies in animals that vary in their genetic make-up (e.g. inbred or commercial lines that differ in resistance to bacteria) or which have been genetically-modified (e.g. by mutation of specific genes or to allow specific host cells to be visualised or removed). Where genetically-modified animals are used, these will have been bred underseparate project licence authority and we do not expect that cumulative harm will be any greater than when using normal animals.

To study the role of microbes that live within farmed animals, we will often examine the microbial communities that live in animals that differ in disease resistance including at different life stages andlocations in the body. Often this does not require licensed procedures. It is possible that we may testthe role of specific microbes, or strategies to expand or remove them, on the outcome of infection bypathogens. For this, animals may first be treated with microbes or substances followed by infection.

What are the expected impacts and/or adverse effects for the animals during your project?

Expected symptoms depend on the combination of bacterial pathogen and host. For example, differenttypes of *Salmonella* (serovars) can cause a spectrum of illness in a given host from no symptoms, through short-lived diarrhoea to life-threatening typhoid where bacteria leave the gut and grow in the blood and other organs. Further, a given serovar can vary in the disease it causes in different hosts.
For example, oral *Salmonella* Typhimurium infections in chickens produce no symptoms (except in very young animals) whereas in pigs or calves it can cause fever and diarrhoea. For *Campylobacter*, we envisage that the vast majority of studies will be in chickens dosed orally. Invariably we have found that this does not produce any disease. For *E. coli*, the outcome of infection varies across so-called pathotypes and their hosts. For example, avian pathogenic *E. coli* can cause disease affecting the lungs and internal organs following inoculation of birds whereas Shiga toxin-producing *E. coli* typically colonise calves after oral dosing without any symptoms. Uropathogenic *E. coli* cause urinary tract infections when inoculated into the urogenital tract, and in pigs we envisage that this will provide a highly relevant model of human urinary tract infections in which to test candidate therapies. Adverse reactions are expected to be rare and minor and may include transient discomfort at the location whereprocedures are performed. Where vaccines are tested these will be formulated together with substances that can boost vaccine-induced responses, but which have a good safety record in farm animals. We will seek to minimise the duration and severity of any symptoms to the minimum requiredto meet experimental objectives.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

We expect that the majority of animal use will involve chickens and be confined to the Sub-threshold orMild categories. In our preceding licence, of 1450 chickens used between January 2017 and December 2021, 97% experienced actual severity that was Sub-threshold (99) or Mild (1308), with just8 experiencing Severe symptoms.

For pigs and calves, we do not expect severity to exceed the Moderate limit. The nature, timing and magnitude of host responses are well understood in our models, such that it is typically possible to meet objectives while confining actual severity to the Mild limit. In our preceding licence, 21 pigs wereused and actual severity was Mild.

Where calves and pigs are used under terminal general anaesthesia the severity of procedures is Non-recovery. Such models avoid the use of conscious subjects when disease occurs and can substantiallyreduce the number of animals that would be required to assign roles to bacterial or host factors during infection, for example where many samples can be tested in discrete segments of the intestine of a single animal.

The use of relatively modest numbers of animals in procedures of mostly Sub-threshold or Mild severity should be set in the context of the scale and impact of animal and human disease caused bythe bacteria under study, and the vast number of farmed animals reared annually for food.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Farmed animals are naturally affected by the bacterial pathogens under study and key reservoirs of human foodborne infections. We believe that they offer the most relevant model to understand host- pathogen interactions in order to design and test control strategies. It is not currently feasible to reliablymodel the colonisation of the gut, spread to other organs, induction of pathology or the development ofcertain immune responses in laboratory-based models. Our past research has shown that bacteria can deploy host-specific virulence factors, and that events during infection of farmed animals can differfrom those detected in cell- and rodent-based models.

Which non-animal alternatives did you consider for use in this project?

Wherever feasible we will use immortalised cultured cells to replace animal use. These can be inoculated with bacterial strains in the laboratory in order to study adherence, invasion, intracellular replication and gene expression in both the pathogen and host cell. However, cell lines are lacking for some key species and tissues (e.g. to model the chicken gut lining). We have previously studied bacterial interactions with free-living amoebae and larvae of the *Galleria mellonella* (the wax moth), which can be useful in establishing differences between bacterial strains or mutants before animal useis considered.

Why were they not suitable?

While cell lines, ameobae and invertebrate models can be useful to detect differences between bacterial strains (e.g. to enter and survive within cells or to cause pathology in wax moths) they cannotfaithfully reproduce the complex environment of the intestines of farmed animals or the host responsesto infection. This is particularly the case in regard to immune responses, where replacement models cannot reproduce the development of antibodies specific to parts of pathogens, or the memory of past infections and recall of immune responses. Where strategies to prevent or treat bacterial infections in farmed animals are devised, these will need to be tested experimentally before they can be used in natural populations of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers have been estimated based on use of the species under seven preceding project licences held by the applicant, which have had similar objectives. They partly reflect funding for studies that hasbeen obtained, or will be sought, during the interval the licence

will be active. The number of animals tobe used for specific objective (e.g. to test if differences exist between bacterial strains or host lines, or if animals can be protected with a vaccine or treatment) will be the minimum expected to be required.

This accounts for variation between individual animals in the way they respond to infection, which is typically well-defined from preceding studies. Our ability to draw robust conclusions from the use of animals in experiments is evidenced by our record of publication of findings in peer-reviewed journals.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We make extensive use of methods to reduce the number of animals used to address our objectives.These include:

Strategies to simultaneously screen bacterial strains in animals and follow their fate. For example, we can co-infect animals with wild and mutant strains in equal proportions the study how they competewith one another by counting them in samples from animals. This is made possible by marking them with different antibiotic resistances such that they will grow on jelly media containing those antibiotics but all other microbes in the sample will be killed. We also use genetic techniques to identify all the strains or mutants present in a sample and define their abundance. By applying this to pools of strains or mutants given to animals, and pools recovered from them, we can identify strains or mutants that are less able to colonise animals or survive within them, presumably as they lack a factor that is required during infection.

Use of cells or tissues from animals as models to study how bacteria interact with the host. For example, we can isolate cells from the immune system of animals (e.g. by isolating them from blood) and study how they respond to bacterial strains or mutants in the laboratory. In some cases, cells isolated from animals can be cultured in ways that reproduce tissues of the host. For example, we have used miniature organs ('organoids') that can be grown from cells lining the gut in laboratory culture. Further, we have used pieces of whole tissue from animals which we can keep alive in the laboratory for long enough to study early interactions between bacteria and the host. In all these cases, animals are still needed as a source of the cells or tissues, however we can often obtain these from animals killed for other reasons that do not require a project licence (e.g. at abattoirs).

Use of surgical models that allow many strains or treatments to be tested at once. For example, in pigs and calves we have used a model in which animals are placed under anaesthesia (and not recovered) and separate sections of gut are created by tying ligatures around it. These segments eachact as a miniature gut and can be inoculated with different strains or treatments. We can quantify differences in the host response in each segment (e.g. inflammation), such that we can obtain information for many strains or treatments in a single animal, rather than needing to use separate onesfor each strain or treatment. Where cattle are used, we use calves that are routinely killed in early life (e.g. the males of dairy breeds that cannot produce milk).

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Every animal study conducted under this licence will be scrutinised by the local Animal Welfare & Ethical Review Body before consent is granted. This will include scrutiny of the

experimental design,group sizes and ensure that the 3Rs have been duly considered. Group sizes, expected symptoms and strategies for monitoring animals are well-refined from preceding studies. Where the outcome ofprocedures cannot be predicted with confidence, we will conduct pilot studies with small numbers of animals and enhanced monitoring. We routinely archive samples from animals used in experiments,such that we can return to these without needing to use further animals in the future. As described above, we will employ diverse strategies to reduce animal use wherever feasible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We propose to use models in which infections of farmed animals with *Salmonella*, *Campylobacter* or *E.coli* can be experimentally reproduced. This will typically involve inoculation of animals with bacteria viaa natural route of exposure. Animals may vary in breed or line and may differ in the microbes they carryor be genetically-modified under separate licence authority. They may also be given substances beforeor after inoculation in order to determine their impact on the outcome of infection (e.g. candidate vaccines or therapies). The expected outcome of infection will be a function of bacterial and host factors. We will use protocol 1 where it is expected that the intestines will be colonised and gastroenteritis may result. In chickens, gut colonisation by *Salmonella* or *Campylobacter* does not produce symptoms, except in young animals or those with weakened immune systems. In pigs and cattle, infection with some types of *Salmonella* and *E. coli* may produce fever and diarrhoea. We will use protocol 2 where it is expected that other internal organs will be affected after infection. In the caseof *Salmonella*, this may follow oral infection by some types that are able to leave the gut and grow in the blood and other organs to cause typhoid-like disease. For avian pathogenic *E. coli*, inoculation into the airways may cause lung infections that spread to other organs. We will use doses that result in reliable infection but with the minimum of pain, suffering, distress or harm. It is often feasible for meet experimental objectives before disease develops or to confine severity to the Mild limit. Occasionally, it may be necessary to maintain inoculated animals until they present with symptoms at the Moderate limit, for example to determine if a vaccine can prevent disease or to evaluate candidate therapies once disease has started.

We will use surgical models (protocol 3) as a means to reduce animal use when testing multiple strainsor treatments (e.g. by constructing discrete segments of gut that can be separately inoculated) or to beable to study how infection spreads (e.g. via insertion of tubes into vessels that drain blood or other fluids from sites of infection). Such procedures will be done under anaesthesia from which animals will not be recovered.

We will use protocol 4 to study of urinary tract infections in pigs and evaluate control strategies. Inparticular, we aim to understand how *E. coli* colonise the urogenital tract and to evaluate therapiesbased on viruses that kill *E. coli* (bacteriophages) and other alternatives to antibiotics.

The models are chosen to be representative of naturally-occurring infections in farmed animals.

Why can't you use animals that are less sentient?

While mice are widely used in research on *Salmonella*, *Campylobacter* and *E. coli*, they sometimes donot provide a faithful model of events in farmed animals. For example, while *Salmonella* Typhimurium causes diarrhoea in pigs and calves (both naturally and experimentally), oral dosing of mice does not cause gastroenteritis unless indigenous microbes are depleted with antibiotics. Further, in some lines of mice, *S*. Typhimurium causes typhoid-like disease involving bacterial growth in internal organs whereas in farmed animals it mostly stays confined to the gut. Mice can be useful to test the role of specific host factors (e.g. owing to genetic modification), where the equivalent is not yet available in farmed animals. If appropriate, we will seek an amendment to the licence for such use. We tend to useanimals at a relatively early stage of life, when infections can reliably be established but without the high cost of rearing them to full maturity. Where possible, we avoid using immature life stages as they can be more likely to experience severe disease (e.g. when their immune systems are not fully functional). We can make use of avian embryos before they are protected under the Act to study how virulent bacteria are, but inoculation via a specific route is challenging and infection spreads between organs rapidly in ways that may not be typical in chickens after hatch. We will use terminally anaesthetised animals in protocol 3, for example to study the induction of gut pathology to multiple treatments and reduce the need for separate conscious animals to test each treatment.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

In most cases, the nature, timing and severity of responses of animals to infection is well-understood from preceding studies. This reduces the potential for adverse effects and often allows us to time our analyses such that objectives can be met before animals progress to disease. We have well-refined procedures for monitoring animals, including plans to increase the frequency of observation in a stagedway if symptoms appear. Post-operative relief of pain will be applied when using our urinary tract infection model if required under the direction of veterinary surgeons involved in the work. We will typically not treat animals with substances that could control infection (e.g. antibiotics) unless this relates to objectives of the study. This is because we often need infections to proceed as they would naturally in order that we can faithfully model host-pathogen interactions.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will adhere to PREPARE guidelines when planning experiments (Smith AJ et al. 2018. PREPARE:guidelines for planning animal research and testing. Lab Anim 52:135-141) and to ARRIVE 2.0 guidelines when reporting our findings (Percie du Sert N et al. 2020. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol 18:e3000410). We will routinely review guidance and regulatory advice provided by the Animals in Science Regulation Unit (https://www.gov.uk/guidance/research-and-testing-using-animals).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

My laboratory has implemented many 3Rs advances, and was awarded an international prize for research that has replaced, reduced or refined animal use in the development of veterinary medicines. This recognised our use of strategies to screen pools of bacterial mutants simultaneously for phenotypes, our development of surgical models to study many samples in discrete segments of the gut, and our use of cell and explant models to study host-pathogen interactions to reduce or replace animal use. Where advances have been made by others (e.g. organoids that model the intestine or stem cells as a source of different cell types for infection in the laboratory), we have implemented thesein our research. We disseminate our own 3Rs methods via publications, symposia and collaborations. We learn of advances in 3Rs methods by reading academic literature and attending lectures and symposia. This includes an annual 3Rs day at our institution. The applicant has served on the grant assessment panel for the National Centre for the 3Rs and continues to review proposals involving theuse of animals in research and 3Rs approaches.

USING STEM CELLS FOR RETINAL REGENERATIVE THERAPIES

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Retina, Regeneration, Stem Cells, Extracellular Vesicles, Therapy

Animal types	Life stages
Rats	adult, juvenile, neonate, embryo, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

To investigate whether molecules produced by human Müller cells (a type of retinal stem cell in theeye) or extracellular vesicles released by these cells, can be used as a therapy to treat conditions affecting the back of the eye such as glaucoma, retinitis pigmentosa and age-related macular degeneration using rat models of these diseases.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 only prevent progression of these diseases, but do not reverse vision loss and in many cases patients do not respond to treatments. It is therefore necessary to develop new therapeutic agents to treat these diseases. Stem cell derivatives have much promise to treat ocular disease and we plan to investigate the use of retinal stem cell

derivatives. We expect the results of this study pave the way to generate a plan for pre-clinical investigations using retinal stem cell derived molecules or molecules mimicking cell products to treat patients with advanced glaucoma and other conditions that affect the light sensitive nerve cells of the retina. These include retinitis pigmentosa andage related macular degeneration. The identification and use of small molecules would potentially avoid the need of whole cell transplantation

What outputs do you think you will see at the end of this project?

It is expected that this study will identify the use of stem cell derived molecules and derivatives thereof,to restore visual function in models of retinal degeneration. This may lead to the formulation of plans toundertake pre-clinical studies to develop therapies to treat patients with advanced glaucoma and otherblinding conditions, such as retinitis pigmentosa or age related macular degeneration.

The research will be disseminated to Scientists working in the fields of Ophthalmology and regenerative medicine by presentations at international scientific meetings and by publications in peerreviewed journals. Through outreach activities, we also aim to disseminate our research to patient groups affected by the diseases we are investigating.

Who or what will benefit from these outputs, and how?

Short term outputs

To identify whether extracellular vesicles or their molecular contents may repair the retina, whichmay lead to restoration of visual function in rodent models of glaucoma and retinitis pigmentosa.

We aim to publish our work in peer reviewed journals and disseminate the information gainedwhich will be of benefit to scientists in the field of regenerative medicine, neuroscience and ophthalmology

Longer term benefits:

We hope that results gained will build upon previous research will lead to the formulation of planstowards pre-clinical trials to provide novel therapies to regenerate the retina and restore some visual function in patients affected by retinal diseases such as glaucoma and age related macular degeneration.

How will you look to maximise the outputs of this work?

We aim to publish our work in peer reviewed journals which will be open access. Information gained bythe study will be disseminated in local and international conferences.

Species and numbers of animals expected to be used

- Rats: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will be using normal strain rats and rats with inherited retinal disease (dystrophic rats). Rats will beused after 4-5 weeks of age when they are fully developed as we are investigating diseases that occurin adults. Genetic defects in rats to be used (RCS) usually manifest between 6-8 weeks of age.

Typically, what will be done to an animal used in your project?

Tolerisation in neonates. Part of the study may involve transplanation of human derived cells. To reduce inflammatory responses and rejection of transplanted human cells, pups will be injected in theabdominal cavity within 24hrs of birth, with the same type of cell that will be used in treatments in the adult rat.

Models of glaucoma: To induce experimental glaucoma, under general anaesthetic, a single eye ofeach animal will be injected with either:

A chemical to the inner eye to induce specific damage to nerve cells affected in this disease.

Magnetic bead or saline injection to the front chamber of the eye to increase pressure within theeye that leads to damage of the nerve cells.

After induction of experimental glaucoma, animals will be kept for a maximum of 6 months or to theexperimental endpoint. We will ensure there are at least 7 days between each ocular injection administered to the same animal to ensure recovery from procedures and anaesthesia.

Where the animals have undergone models to induce high pressure within the eye, a rebound tonometer will be used to measure eye pressure immediately after surgery and on a regular basis(initially daily, then weekly).

Genetic rodent models: Rats with genetic defects causing inherited retinal degeneration (models ofAMD or retinitis pigmentosa) will also be used in the study. These animals develop impaired visual function by 6-8 weeks of age.

Injection of Molecules: One-two weeks after induction of experimental glaucoma or at 4-7 weeks of age for rats with genetic defects, animals will receive transplantations of cells, cell-derived products (ieextracellular vesicles containing active molecules, or molecules mimicking cell derived products into the eye. Molecules mimicking cell derived products may include small molecules, pharmacological agents or synthetic molecules, which could avoid the need for cell derived factors. These procedures will involve ocular injections to a single eye on each animal.

Part of the study may involve the transplantation of human derived cells, therefore immune- suppressant drugs will be given one week prior to therapy transplantations at the minimum effectivedoses to last to the end point of the study when animals are humanely killed.

Visual function tests: Animals will be anaesthetised and flash lights will be shone into the eyes to determine the activity of the nerve cells in the retina. Contact electrodes will be placed on the eye and reference and ground needle electrodes will be placed under the skin. Procedures will last 45mins, andthe animals will be allowed to recover.

What are the expected impacts and/or adverse effects for the animals during your project?

Ocular injections of neurotoxins in normal rats will be administered to a single eye. Animals maybecome blind in the injected eye, due to the induction of glaucoma like damage. Some of the ratspecies to be used have genetic defects that mimic damage seen in conditions such as retinitis pigmentosa, that make them blind at 8-12 weeks of age, however this does not cause pain and animals usually adapt to their environment.

The animals will receive transplantations of cells or their derivatives into the eye. These may contain extracellular vesicles produced by cells in culture, or active molecules identified in these extracellularvesicles. There is a minor risk of infection and development of cataracts as a result of the ocular injections.

We expect minimum adverse effects to immune–suppressant drugs administered after cell transplantation, which will be administered, at the minimum effective doses. These effects may includesome weight loss for the duration of their administration. Weight levels will be monitored regularly throughout the study and animals will be humanely killed if it drops below 15% of body weight.

There is a very minor risk that visual function tests may cause light damage to the eye, however thishas not been previously reported.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

We expect moderate levels of severity to occur after ocular injections and visual response measurements . All animals will receive ocular injections, for which the majority of animals mayexperience some pain or discomfort as a result of injections to the eye.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The main outcome of our experiments will be assessed by examination of visual restoration, which canonly be achieved in living animals. This currently cannot be achieved in bench-based laboratory experiments on cells or donated tissues, which can only provide information on the ability of these molecules to attach, be absorbed or survive in these systems and does not provide information on the ability of such molecules to improve vision. This can only be achieved in vivo, as we are examining theactivity of nerve cells in the retina using electrophysiology.

Which non-animal alternatives did you consider for use in this project?

In the laboratory, we have used donated human retina as well as stem cell derived retina (known asretinal organoids) to model transplantation of our stem cell derived molecules. This has provided uswith information about the ability of molecules to attach, be absorbed, and promote cell survival in these model systems. Using these methods, we have generated preliminary data that can only be validated using in vivo models, and constitute the basis of this application.

Why were they not suitable?

Studies using these retinal organoids cannot provide us with information on the ability of these molecules to protect or restore vision. This can only be achieved by the transplantation of these products into living animals, in which we can only determine the functional activity of nerve cells in the retina using visual function tests, such as electro-retinograms. Where possible, we will ensure to use invitro based experiments before undertaking any animal based work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 NC3Rs experimental design assistant was used to help design experiments and help to reduce the number of animals involved through support and sample size calculations using a programme called G*Power. Advice has also been sought by a statistician, and are in agreement with our previousin vivo studies in the field.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We have performed extensive in vitro laboratory based experiments using donated human retina, rat retina as well as retinal organoids and will continue to develop these models. These initial studies haveallowed us to reduce the number of live animal models to be used in transplantation experiments to determine any improvements in visual responses.

In addition, we have sought the advice of a statistician, who has made power calculations to ensurethat the minimum number of animals are used to obtain meaningful data. We have also used the NC3Rs experimental design assistant to help reduce the number of animals used whilst gaining thebest possible result for meaningful data.

Our experimental design contains randomisation of rats to be used in the experimental procedures anddata gathering procedures to minimise selection bias, ensure animals are treated in a similar manner which therefore allows efficient data analysis and help reduce the number of animals needed for the study.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We have performed extensive studies in vitro and will continue assessing our methods. Based on our previous work with these models, we understand the outcomes from the nature of experiments we willconduct to ensure that the minimum number of animals will be used to obtain the most meaningful 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Although different species have different visual capabilities, fundamental mechanisms of vision are similar, therefore due to the size, availability and low neurological sensitivity, rats will be used in the current study. There are genetic strains readily available for this species that model retinal disease andin our experience there is an increased success rate of inducing experimental glaucoma in the rat eye when compared to mice.

Why can’t you use animals that are less sentient?
Maturation of the visual system is key to assess visual function, In addition, There are no reliable models of induced glaucoma in mice or fish, therefore the vast majority of the work will be undertaken in rats because of their low neurological sensitivity that results in lower pain severity. Our models mimicretinal degeneration that occurs primarily in adult and aged human population, therefore we cannot carry out procedures in animals at immature life stages to obtain meaningful data.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

All procedures will be performed under general anaesthesia, using topical analgesics where possible. The number of times an animal is under general anaesthesia will be limited and we will ensure there isa minimum of 7 days between any repeated general anaesthesia. To minimise infection, procedures will be performed using good aseptic techniques. Animals recovering from general anaesthesia will have access to wet food and a heat source to ensure good recovery. Throughout the study all animalswill be carefully monitored for signs of pain, distress or suffering and will be sacrificed by humane (schedule 1) methods should any suffering be observed.

Throughout the study animals will also have access to extra enrichment such as a ‘play pen’ which is alarger housed area (outside each cages) with enrichment toys in addition to enrichment provided in each cage.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We are committed to review and incorporate the 3Rs throughout the project duration to ensure best practices are followed. We are committed to constantly reviewing techniques to ensure that they are upto date and appropriate for our study. We will follow the PREPARE & ARRIVE and similar other GOODPRACTICE guidelines when publishing results, to ensure that other researchers may accurately replicate our studies if necessary.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will keep informed about 3Rs advances through our NC3Rs representative and also by consultingthe Nc3Rs website and understanding animal research website. Advice and involvement of local named people will be sought as appropriate.

GENETICS AND ENVIRONMENTAL INTERACTIONS IN THE DEVELOPMENT OF METABOLIC SYNDROME

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Obesity, metabolic diseases, interventions, genetics, diet

Animal types	Life stages
Mice	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The project investigates the underlining causes of metabolic syndrome and how this is affected bygenes and the environment with the long-term goal of identifying novel treatments.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 syndrome (MetS) is a combination of metabolic dysfunctions characterized by insulin resistance, impaired glucose tolerance, hypertension, hyperlipidemia and abdominal obesity leading togreater risk of chronic diseases such as type 2 diabetes and vascular diseases such as heart attack orstroke. In the UK, despite spending vast sums of

money, these problems are increasing. Currently, it is estimated that one in four adults (25%) have MetS [1].

Obesity affects 30% of the global population and causes changes, including MetS and mental health issues. It is currently the leading cause of morbidity, diminished quality of life, and mortality in the West[2].

While obesity is a significant contributor to MetS, it can also occur in the non-obese. Further, neither ahigh-fat diet nor obesity necessarily causes illness. We and others have shown that both the genetic background and gut microbes alter the chance of MetS, the likelihood of obesity and obesity-induced disease.

We have shown that a high-fat diet alters the main hormonal control system of the body (the hypothalamic-pituitary-adrenal-axis- (HPA)), and have demonstrated that neuro-inflammation is a potential cause of this change. A high-fat diet during pregnancy increases inflammatory markers in thenon-obese offspring's brain when they adult, which is exacerbated by a post-weaning high-fat diet. Wealso observed an effect of maternal obesity on offspring anxiety, behaviour and memory, which was associated with HPA changes.

Extending our work on inflammatory modulators and their relationship to disease, we have studied extracellular matrix-degrading enzymes called matrix metalloproteinases (MMPs). These show changes in their activity in numerous conditions, and are linked to systemic inflammation. Interestingly,we could show that certain MMP genetically altered (GA) mice have a far greater tendency for obesity and metabolic syndrome-like vascular changes even when on a standard diet. Furthermore, these mice become obese despite eating less than their control littermates. Finally, these modifications appear altered by the microbiological status of the animal's environment.

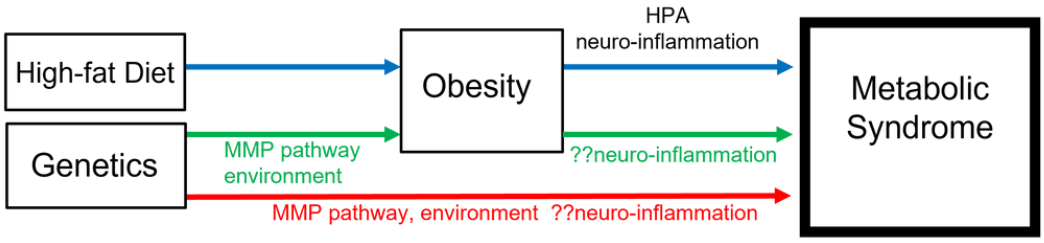


Figure 1: Susceptibility to Metabolic Syndrome

Hence given this disconnect between diet and susceptibility to MetS, our overarching question is whatdrives the predisposition to MetS? And, by modifying these drivers, can we alter the likelihood of high-fat diet-inducing MetS?

We wish to study

1/ the functioning of the MMPs, and related molecules, such as their inhibitors, in relation to obesityand obesity-related disease syndromes.

2/ the possible mechanisms, including neuro-inflammation, for why MMP pathway GA

animals havepoor cardio-metabolic outcomes.

3/ if modulating MMPs and/or neuro-inflammation offers potential therapeutic interventions in naturallyor high-fat diet-induced obesity-related disease.

References:

M.G. Saklayen. The global epidemic of the metabolic syndrome. Current Hypertension Reports(2018) 20:12

Obesity: preventing and managing the global epidemic. Report of a WHO consultation. 2000.World Health Organ Tech Rep Ser 894:i-xii, 1-253.

What outputs do you think you will see at the end of this project?

These studies will give information on how genetics and environment influence the development of metabolic syndrome (MetS) and obesity – by understanding this. We hope to provide novel ways toprevent or reduce the potentially devastating pathology caused by MetS.

We will use genetic and environmental models - that result in MetS to understand how neuro-inflammation and MMP function leads to pathology.

We want to pharmacologically intervene by changing both neuro-inflammation and MMP function to understand if we can alter the progress of MetS and produce better/ new treatments for humans withthese problems.

We will present our findings at scientific and medical conferences and publish these outcomes inpeer-reviewed scientific journals.

Finally, any changed procedures/methodologies leading to improved welfare developed throughthe project will be published and shared among the scientific community.

Who or what will benefit from these outputs, and how?

There will be multiple beneficiaries from our studies. We will publish and share our data widely to inform the scientific community and clinicians of our findings to stimulate more research on novel clinically applicable MetS interventions. Our results that MMP GA mice become obese and show MetSchanges even when on standard chow diets, unlike their wild-type littermates, suggest new therapeutictargets that interest the pharmaceutical industry. Ultimately understanding how MetS are related to MMP function and neuro-inflammation will open new avenues for treating patients.

How will you look to maximise the outputs of this work?

We aim to maximise our outputs and disseminate their use by presenting our findings widely at research conferences and publishing in open-access journals. These will fully disclose the associatedraw data, enabling our results to be accessible to all and include unsuccessful approaches.

Much of our work is carried out through collaboration involving other research groups and pharmaceutical companies. This approach ensures that we can rapidly maximise our outputs by sharing raw data, new knowledge, technical expertise and reagents such as

tissues. Protocols andresearch work will be communicated to new students and postdoctoral researchers, making information regarding the models widely available and fostering further collaborative projects. The team will acquire skills and expertise in metabolism, blood vessel physiology and endocrinology.
Training will be achieved through extensive multidisciplinary collaborations within the Establishmentand established national and international partnerships.

We will regularly present the study's progress and results at teaching events and public lectures. Wewill also communicate findings to the public through outreach activities and laboratory open days for GCSE and A-level students. Our press office will also publicise the results.

Species and numbers of animals expected to be used

- Mice: 1250 for five years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Whilst we will reduce the use of animals through cell-based laboratory methods, it is inevitable thatwhere the integration of whole animal metabolism is being studied, *in vivo* analysis is needed.

Complex interactions between cells, tissues and organs cannot be fully recapitulated outside the body.Here, interactions of genetics and the environment with the physiology of endocrine, gastrointestinal, muscular-skeletal, cardiovascular and neurological systems lead to pathology. It is necessary to use mammalian species to understand the changes occurring as these systems differ or are absent in invertebrates, and mammals allow meaningful interventions which may be transferred to humans.

We can determine the key factors of the agents being used pharmacologically to alter responses. Thiswill predict they are suitable for use in treatment. However, a mammalian system can provide evidence of systemic physiological effects. Animal models are essential to provide proof of concept in an amenable system that can be manipulated, which is impossible in humans.

Mice will be used for this work as they are the best-characterised mammalian model to study the physiological changes related to humans. Furthermore, mice are highly genetically tractable, allowingus to observe the roles of single specific genes in the pathogenesis of MetS.

The induction of human MetS often begins in early life. We have dietary mouse models where post- weaning high-fat diet induces MetS within six months. We propose using such models along with genetically manipulated mouse lines to understand the induction and prevention of MetS better. Usinginbred mouse strains ensures reduced variability in our experiments enabling valid conclusions to be drawn from the data obtained while minimising the number of animals used.

Typically, what will be done to an animal used in your project?

See Figure 4

Some mice may feed on a high-fat diet from 4 weeks old to 30 weeks old.

Mice may undergo interventions by injecting therapeutic substances or vehicles intraperitoneally.

Mice will have their body weights, glucose tolerance, blood pressure, behaviour and metabolicrate measured.

Their cognitive behaviour will be monitored during the last week of the interventions.

Mice will be fasted overnight for 14 hrs – a maximum of three times - when plasma glucose orglucose tolerance is being measured.

Mice will be killed by a schedule 1 or after terminal general anaesthesia for tissue collection.

What are the expected impacts and/or adverse effects for the animals during your project?

The GA mice are expected to show increased weight gain and fat deposition compared to wild-typemice on a non-obesogenic standard chow diet.

The mice on a long-term high-fat diet (from 4 weeks to 30 weeks old) may also develop mild cardiometabolic diseases and fatty livers. They may also show cognitive impairment. All these may decrease their quality of life, though we would not expect this to be a significant impairment. Any effectwould be expected to develop when they are ~25 weeks old, and we intend to end the experiments at or before 30 weeks of age.
It has been reported that a high-fat diet occasionally increased the occurrence of skin lesions in C57BL/6 mice [1]. But, we have not observed this pathology in our in-house produced C57BL/6 mice,nor in the MMP pathway GA mice during our studies using the previous project licence. If this pathology does occur, the NVS will be consulted. If the lesions are not healing, mice will be killed by aSchedule 1 method.

The metabolic analysis will require occasional blood sampling, and blood pressure measurements willneed to restrain the animals. The animals will be habituated to the restraining equipment to minimise the stress, which will only be temporary.

Behaviour analysis will be carried out and will require introducing the animals to novel environments;this may cause short-term stress.

The therapeutic substances will aim either to increase MMP activity (e.g. recombinant MMP) or to reduce it (e.g. recombinant TIMP, doxycycline - a synthetic tetracycline which inhibits MMPs, or tyrosine kinase inhibitor or metformin which also inhibits MMP activities). The chosen agents have been used in other clinical or preclinical settings, and we understand their potential toxicity. They will be used at dosages and routes to minimise this. We expect the therapeutic substances to lower bloodglucose, cardiometabolic changes and obesity. They will be administered intraperitoneally and requirerestraints; these interventions will be carried out at a maximum of 35 occasions (daily injection) during the five weeks of the experiment.

Reference:

[1] Yuwen Zhang, Qiang Li, EnyuRao, YanwenSun, Michael E.Grossmann, Rebecca J.Morris, MargotP.Cleary, Bing Li. Epidermal Fatty Acid Binding Protein Promotes Skin Inflammation Induced by High-Fat Diet. Immunity 2015;42:953-64

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

We consider all animals to be at an overall MODERATE level. This is based on cumulative harm asall animals will undergo repeat agent/vehicle interventions and testing.

Wild-type mice on a high-fat diet or GA mice on a regular standard chow diet may expect to show weight gain and a possible increase in neuro-inflammation markers, but they are very unlikely to showclinical disease. However, it is anticipated the GA mice (e.g. MMP pathway KO) receiving a high-fat diet may develop a degree of fatty liver diseases and short-term immune modulation, hence amoderate severity level.

Genotype of animals	Diet	Severity related to diet only
Wild-type (C57BL/6)	standard chow	sub-threshold
Wild-type (C57BL/6)	high-fat diet	mild
GA mice	standard chow	mild
GA mice	high-fat diet	moderate

The pilot dosing study in protocol one and the interventions in protocol two, either with active orinactive agents, will have cumulative effects due to the repeat intervention and restraints.

All mice in protocol 2 undergo a glucose tolerance test, which will require overnight fasting and repeatblood sampling, hence 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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

These experiments need to use animals because MetS is a complex disease affecting

multiple organs,altering the biochemistry and physiology of the animal. Such physiological changes cannot be studiedin isolation. Also, one of the goals of this work is to identify potential therapeutic interventions that need whole animal physiological readouts, such as glucose tolerance and cognitive behaviour changes, which test-tube studies fail to provide.

Which non-animal alternatives did you consider for use in this project?

We do employ non-animal investigation, which includes systematic reviews and statistical/ model dataobtained from those that have been published and from our previous studies. One can use cell lines from human/ animal tissue or commercially available cell lines to study potential cellular mechanistic pathways in the test tubes.

Why were they not suitable?

The non-animal experiments can establish preliminary data and hypotheses to design animal studies.They cannot replace animal studies examining the highly complex metabolic interactions between multiple organ systems, which can only be correctly observed in animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have calculated the number of animals used based on our past published studies. In most cases,approximately ten animals per sex will be required per treatment group.

From previous work, we will test the dose of 1mg/kg of the recombinant compound as the initial dosage intraperitoneally and study the effects on frequency and dosage. For protocol 1, we estimate three repeat experiments in this pilot study, using two GA strains (at 15 weeks old and at 30 weeks old)to find the dosage and frequency of the intervention.

We will use a small group number, i.e. n=3, in the initial experiments and may increase if necessary. This protocol will employ approximately 250 animals.

For protocol 2, where we have sex-separated, dietary and intervention groups, the estimated numberrequired is 1,000.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Use both sexes of animals to reduce the number of animals being used. As there are known sexdifferences in MetS outcomes/likelihood, the analysis will be stratified by sex.

All animals are on the same (C57/Bl6) background to minimise genetic variation.

We prepared our experimental design using the PREPARE guidelines to minimise the number of animals used by ensuring that the experimental design addresses the critical

scientific questions andstudy reproducibility to limit the required number of unnecessary replicates.
During the experimental design phase, we will use available *in vitro*, *in silico* and data in the public domain to limit potential data bias and variability. Standardised experimental procedures will be usedthroughout, such as randomisation allocation. The experimenter will be blind to the randomisation outcome until after data analysis. To limit further variability between data, all animals will be on the same inbred- C57/Bl6 background strain, of similar weight and age and will be housed in the same animal unit.

The experimental design we employ will allow the publication of our FAIR research data in open- access journals according to the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines. This design will maximise the information published and minimise unnecessary replicationof studies in our research group and elsewhere. Negative results will also be made public. The number of animals will reduce in the long run.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We use protocol 1 to perform a pilot study to investigate the dose and frequencies of intervention, sorequiring fewer mice in protocol 2.

We support the implementation of open science policy to strengthen knowledge. We are actively involved in tissue sharing with research collaborators, hence using fewer animals in research in thefuture.

As part of ongoing efforts to reduce animal usage, we will promote alternatives using non-animal methodologies, such as systematic reviews, data analysis *in silico, in vitro* studies, computer modellingand mathematical models to predict clinical outcomes.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Choice of species, models and methods

The project aims to investigate how environmental exposures (diet) affect the risks of specific diseasesin humans and develop appropriate intervention strategies that benefit subjects at risk from MetS later in life. It is, therefore, necessary to use mammalian species in which comparable early life exposures induce similar pathophysiological effects to those observed in humans.

We chose the mouse as the most appropriate animal model because they are the most studied species in terms of MetS and offer the best ‘functionally’ characterised and refined the mammalian model system to study the effect of environmental exposures (diet) and

genetic susceptibility on phenotypic outcomes. There is substantial data from mouse studies, including those conducted underour previous project licences, showing that the animals generally tolerated the high-fat diet well.

We showed that nutritional manipulation in wild-type or GA mice during pregnancy or early life couldinduce phenotypic changes later in life and in their offspring. The outcomes of the nutritional manipulation are similar to MetS in humans' cardio-etabolic changes and the likelihood of obesity.

Mice have a relatively short generation time, facilitating studies on the consequences of ageing in asomewhat shorter duration.

The enormous development of genetic manipulations and genomic tools for mice (i.e. well-characterised genome database and availability of transgenic animals) provides an unprecedented opportunity to take advantage of studying human diseases at a single gene level. Finally, protocols formouse husbandry and health management are well established.

Minimising suffering

We refined the last animal licence protocol by minimising animal distress during the energy expenditure measurements. Our current metabolic cages include housing boxes similar to a standardanimal cage. The new device allows bedding, nesting materials and nest boxes or shelters in the cageto recreate the mice's original housing conditions. The mice readily habituate and acclimatise to the metabolic cages, reducing distress and resulting in more representative results.

In the new licencing period, we want to improve the housing condition of the mice further. We may house two animals in the same cage during the energy expenditure measurement to limit the stress and anxiety caused by lone housing. However, further investigation or a pilot study to analyse the results obtained from pair-housing will be done and compared with lone-housing to see if any variableeffects of this change occur.

The research procedures in the project will not exceed the MODERATE severity level. All animals will be assessed daily for signs of distress or ill health to minimise suffering. Any animals exhibiting a rapidweight loss of 15% or showing signs of sickness, such as reduced movement and lethargy, will be killed by a Schedule 1 method.

Why can't you use animals that are less sentient?

We cannot use less sentient animals because the project aims to investigate how the effects of thelong-term consumption of a high-fat diet lead to the development of the MetS (with features of cardiovascular diseases, behavioural changes and obesity) later in life.

Our findings show that changes in certain MMP genes conserved between humans and mice but not present in invertebrates alter the likelihood of MetS. MMP pathway GA mice exposed to a high-fat dietsignificantly increased body fat percentage and vascular changes in older adults (30 weeks old) but notin younger animals (15 weeks old), so requiring the use of mature animals.

In summary, mice are the least sentient animal system. They allow using one model; defined dietary,genetic manipulation, MetS development, measurable metabolic cardiovascular and behavioural changes, and a system tractable to pharmacological

interventions relevant to the human disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We have instigated many refinements –

- to reduce stress - we routinely use tubing and hand cupping to transfer or examine animals.
- we acclimatise the animals to handling and restraint before beginning procedures to reduce stressand make them less nervous when manipulated.
- we refine our whole animal metabolic cage protocol from our last licence. The new device allowsbedding, nesting materials and nest boxes or shelters in the cage to recreate the mice's original housing conditions. The mice habituate and acclimatise to the metabolic cage and minimise distress. This also gives more reliable data. We may house two animals in the same cage during the energy expenditure measurement to limit any stress and anxiety caused by lone housing. However, further investigation or a pilot study is required to see if any effects occur from this change.

Any regulatory procedure, such as the injection of therapeutic substances and glucose tolerancetest analysis, will be carried out in the morning to allow longer monitoring time to ensure the procedures have caused no adverse effects.

We have instigated a single needle use policy.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We follow the PREPARE guidelines, which as well as minimising the number of animals used, giveguidance on sources of variability in experiments suggesting refinements resulting in greater reproducibility.

We follow the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) 2.0 guidelines (2019). These guidelines are for the specification of animals and husbandry methods when reporting the results of animal experiments and data (including negative results). They will maximise the informationpublished and minimise unnecessary duplication of studies in the animal research community.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We ensure to attend all the user meetings at our designated establishment (four times per year) that update any general advances in the 3Rs. We participate in national and international conferences andkeep up to date with the relevant literature in our field. Any advances in the application of the 3Rs will be disseminated through our research group and applied to our projects.

We register and subscribe to the NC3R e-newsletter to receive notifications regularly on new training and NC3R's latest developments, which inform experimental work, such as animal welfare in researchand the injection route, dosing, and frequent guidelines. There are E-learning, guidance, hard copy, webinar and video on the 3Rs relevant to our work available at the 3Rs resource library we can check on. https://www.nc3rs.org.uk/3rs-

resources.

The UKRI also publishes data, guidance and policies on research involving animals they support https://www.ukri.org/about-us/mrc/our-policies-and-standards/research/research-involving-animals/3rs/. In our case, the physiology and metabolism in animal information pages are shown in the BBSRC and MRC sites. We also regularly check the Physiological Society's website for updates on professionaldevelopment. These are the sources we stay informed about advances in the 3Rs.

DEVELOPMENTAL MECHANISMS OF MAMMALIAN CORTICAL DEVELOPMENT

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cortical development, Neural stem cells, Neuronal and glial diversity, Nervous system regenerationand repair, Neurodevelopmental disorders

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description 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 cerebral cortex, a part of our brain responsible for higher-order functions, contains multiple cell types that originate from a small number of stem cells during development in the womb. How do the limited number of stem cells generate this strikingly diverse array of cell types in the brain? To answerthis question, we will study a mechanism that produces different proteins from a single gene by including or skipping specific parts of the gene's messenger molecules. Our work will uncover how thismechanism results in the different cell types we see in the cerebral cortex, which ultimately contribute to the cures of neurodevelopmental disorders and 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?

Mammalian cortex, the control centre of our complex behaviours, thought, and intellectual

abilities, isbasically composed of neurons and glia that are generated sequentially over the developmental processes from neural progenitors

A deeper understanding of cortical development is essential to identify causes of developmental psychiatric disorders such as schizophrenia, bipolar disorder, and autism, and is also expected to develop strategies to restore function in damaged brains, which lack self-renewing capability due tovarious causes such as ischemia or neurodegenerative diseases.

Our studies aim to understand three issues of cortical development, 1) how the self-renewing or neurogenic capacity of cortical progenitors is regulated, 2) how a variety of neuronal and glial types aregenerated from cortical progenitors, and 3) how this knowledge of molecular mechanisms controlling cortical neurogenesis could develop strategies to repair the brain damage, and 4) how the neurodevelopmental genes are influenced on the connectivity that represents their brain function.

What outputs do you think you will see at the end of this project?

At the end of this project, we will expect the following outcomes:

To gain a molecular insight into how the stem cells (progenitor) in the developing cortex how theirpotentials to proliferate or generate neurons and glia to achieve the normal development.

To decode the molecular mechanisms how cortical stem cells give rise to a variety of differenttypes of neurons and glia in the cortex

To explore the molecular machinery that allows us to convert differentiated glia into cortical progenitors for the brain repair.

Our primary method for sharing our data will be through peer-reviewed scientific journals and at scientific conferences. This will allow our main findings to be scrutinised for accuracy and content. We will ensure that these data are published in journals that allow open access. In addition to publishingand/or presenting our data at meetings. We will disseminate our research to the general public through non-specialist publications targeting laypeople, online material and public outreach events.

Who or what will benefit from these outputs, and how?

Our research addresses the fundamental question of how stem cells excel the proliferation, neurogenesis and gliogenesis to develop the cerebral cortex. This is a key question not only in neuroscience, but broadly applicable to developmental biology and stem and cancer cell researchwhere scientists aim to utilise stem cell potentials for repairing the brains.

The primary beneficiaries of the proposed project in the short term, therefore, will be academics aimingto understand and control stem cell potentials. This topic is strongly relevant to broad areas of researchincluding brain size disorders such as micro- and macrocephaly. Thus, finding the genetic rules underlying such neurodevelopmental disorders would give us a foundation from which to identify drug targets.

In the long term, the use of stem cells offers enormous potential to develop novel

strategies to repair damaged nervous systems with limited regenerative capacity, including the human brain. Decoding themolecular mechanisms balancing cortical progenitor proliferation and differentiation may provide insights into the development of other tissues, thereby reaching beyond neuroscientists' interests to developmental biology and stem cell academic communities.

How will you look to maximise the outputs of this work?

We will maximise our outputs of this work through

- Sharing our knowledge and expertise to colleagues locally and externally to promote complementary research
- Promoting the collaborations with colleagues within our centre, the UK and overseas to complement the strength to facilitate the progress of the neurodevelopmental and related developmental disorders as well as regeneration research
- Disseminating the raw data and protocols widely to ensure the credibility of our work that can beinterrogated by other research groups

Species and numbers of animals expected to be used

- **Mice: 6500**

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

- The proposed research aims to decode the mechanisms controlling the development of the cerebralcortex, which is a unique anatomical structure in mammalian species.
- To dissect the developmental trajectories and the regulatory mechanism behind them, we will employthree strategies using the mouse as a model system.
- the generation of genetically altered (GA) animals,
- introduction of materials into embryonic brains either in utero or in the embryonic culture system and
- introduction of materials into postnatal and/or adult brains.

The mouse has served as one of the best model animals for brain research because of the mechanistic and anatomical similarities to humans, hence enriched knowledge and technical development made in the field offer optimised strategies and methodology which allow us to avoid unnecessary use of the animals. We will also apply alternative replacements such as ES-based corticaldifferentiation or primary cell culture systems where it is applicable.

Typically, what will be done to an animal used in your project?

In order to elucidate the functions of a particular gene in the brain development, we will manipulate thegene expression in vivo or in vitro mouse brain cells (i.e. neurons and glia). This will be done by eitherof three methodologies as described above (GA animals, introducing the genetic materials in the embryonic or postnatal/adult brains or cultures)

Genetically modified animals in which a gene of interest is disrupted will allow us to test possible functions of the gene of interest in neural development in the brain. Moreover, the insertion of proteins(for example, fluorescent proteins to mark up cells or proteins that are used to switch genes on) will beused to monitor the localisation and cells and how they develop as tissues grow.

Embryonic manipulations will give us more flexibility to assess gene function as well as the behaviour of developing nerve cells. To this end, we will inject genetic materials (DNA, virus or cultured cells) intoembryonic tissues in utero. We do this as part of a surgical procedure, where the pregnant mice are anaesthetised and we inject solutions carrying genes into the embryonic brain we are studying.
Embryos will receive electrical pulses after the injection of genetic materials and whilst under anasesthesia (i.e. electroporation). These strategies allow the introduction of genetic materials intocells that can increase or decrease protein production in cells which is useful for analysis of their growth and visualisation of neurons of interest. Manipulated embryos may be further used for the analysis of their postnatal brains.

In some cases, we will introduce the genetic materials in postnatal or adult brains of anaesthetised animals by injections of DNA genetic material combined with electroporation which moves the DNA intothe genome of the cells. This may also be achieved by injection of viruses or cells that have been modified to include genetic alterations.

In most cases, a typical mouse would undergo one surgical procedure. In some cases the animals will undergo two surgical procedures (i.e. where the first genetical materials are introduced in embryos andthey are then subjected to an additional injection as postnatal or adult mice).

What are the expected impacts and/or adverse effects for the animals during your project?

Most experiments in this project require mice to have at least one surgery, however, and these do carryrisks. Pain, anaesthetic overdose bleeding and infection are the most common side-effects. We work with the veterinary surgeon to make sure we give the correct dose of painkillers and anaesthesia meaning that with good surgical practices these happen only rarely. Mice are reviewed daily, so side- effects are picked up quickly and most can be easily treated with simple interventions (e.g. giving extrapainkiller medication) within 24 hours.

A very small number of mice in this project will be used to answer questions about whether creating new neurons can be a treatment for epilepsy. These mice have a specific injury that causes electricaldischarges in a part of their brain. They do not have convulsive seizures but sometimes they might show some abnormal behaviours (such as stopping mid-activity). These aren't harmful or painful for the mice but they can occur throughout life.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Approximately 70% of animals which will be used to obtain brain tissues from wild type or

geneticallymodified animals will experience sub-threshold severely as mostly related to general husbandry and breeding of the animals.

The other remaining animals will have at least one surgical procedure and these would be classed asexperiencing 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 studies focus on the development of the cortex, which is a unique anatomical structure in the mammalian species. For instance, other well established model animals such as chick or zebrafish do not have “the cortex” as defined by anatomical/molecular similarities to those of the human cortex. Therefore, the use of animals which have the “cortex” is absolutely essential for this study. In addition,normal development of the cortical structure is the temporal and spatial concerted processes of the number of cells, which is almost impossible to reconstitute in vitro in terms of correct establishment ofneural positioning, connectivity and maturation.

The proposed research is, however, preceded, informed and complemented by in vitro work including dissociation cell culture, slice brain culture as well as the usage of embryonic stem (ES)/ induced pluripotent stem (iPS) cell delivered neural progenitors. In this bottom-up approach, experiments on neuronal and ES/iPS cultures are used to plan the most crucial and effective experiments to perform invivo, and to provide effect size and variability estimates to allow power analyses for minimal group sizecalculations.

We will also continue to provide quantitative data to computational neuroscientists interested inmodelling our results, with the aim of producing accurate mathematical models of neuronal development.

Which non-animal alternatives did you consider for use in this project?

- Cell Culture including established cell lines and mouse and human iPS derived neuronal cells
- Brain organoids - 3-dimensional cell aggregates derived from iPS cells
- Computational modelling
- Human volunteers/patients

Why were they not suitable?

Cell lines have been adopted in culture environment and exhibit significant differences in gene expression and their characteristics. Moreover, they are isolated from other cell types and tissues such as meninges, microglia, vasculature cells, which play a crucial influence

on brain development.We will use these alternatives, therefore, in the cases where we investigate the intrinsic gene expression prior to testing the hypothesis in vivo.

Brain organoids are the newly developed 3D culture system that recapitulates many aspects of brainstructures with a certain level of complexity. However, they still fail to reproduce the developmental celltypes and their behaviours faithfully as seen in vivo. Also same to the cell culture, many surrounding cell types originated from other tissues influencing the brain development are lacking. Taken together, this system is currently not suitable for our aims of the project.

Whilst we apply the computational approaches to model certain aspects of brain development, currently there are no suitable models capturing the complex cortical progenitor behaviours generatingdiverse neuronal and glial types.

Our proposal to investigate brain development is using molecular invasive approaches, therefore notsuitable to apply to human volunteers and patients.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have been working with mice under the project licence. Our past usage of the number of animals on active grants was approximately 800 to 1000 procedures per year. However, the number may be increased if additional funding is available. Therefore, we estimate the number of animals requested forthis project will be 8000 during the next five years, which is the equivalent number of the current project licence. It is worth noting that many are used in breeding and colony maintenance.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Replacement

Our studies focus on the development of the cortex, which is a unique anatomical structure in the mammalian species. For example, other well established model animals such as chick or zebrafish donot have “the cortex” as defined by anatomical/molecular similarities to those of the human cortex.

Therefore the use of animals which have the “cortex” is absolutely essential for this study. In addition,normal development of the cortical structure is the temporal and spatial concerted processes of the number of cells, which is almost impossible to reconstitute in vitro in terms of correct establishment ofneural positioning, connectivity and maturation.

The proposed research is, however, preceded, informed and complemented by in vitro work includingdissociation cell culture, slice brain culture as well as the usage of ES/iPS cell delivered neural progenitors. In this bottom-up approach, experiments on neuronal and

ES/iPS cultures are used to plan the most crucial and effective experiments to perform in vivo, and to provide effect size and variability estimates to allow power analyses for minimal group size calculations.

Indeed, one project awarded by the BBSRC had employed the combination of mouse ES cell-based cortical progenitor differentiation screening, followed by the in vivo validation of genes of interest. Thisreplacement approach allowed us to reduce the number of mice being used.

We will also continue to provide quantitative data to computational neuroscientists interested inmodelling our results, with the aim of producing accurate mathematical models of neuronal development.

Reduction

A number of measures will be taken to ensure the minimum number of animals will be used in the proposed research. We will employ the following experimental guidelines to ensure maximum dataquality from every animal:

High quality animal welfare to minimise suffering and distress, reducing inter-animal variability.This includes refinement of surgical techniques where possible (see below)

- Randomised and blind group experimental design
- Analysis blind to the experimental group
- Appropriate statistical analysis

Our initial experiments will be pilot studies, informed by our in vitro data as to expected effects,variability, and group size by power analysis. All lab members should visit the NC3R's website (https://eda.nc3rs.org.uk/experimental-design)to use the various tools when designing the experiments. Statistical assistance for the power calculation in this will also be helped by the consultations with the department bioinformatician.

Also, in the case of embryonic manipulation, we can reduce the number of animals by comparing the transfected and untransfected sides of the electroporated brains, since the electroporation of genetic material usually targets only one side of the brain. Under previous project licence, we have developeda small electrode that allows us to target each cortical hemisphere. An alternative strategy would be touse half of the embryos from one pregnant animal experimentally and use the other half as controls.

This will reduce the number of animals to be used and reduce the variability of sampling, which also contributes to reducing the sample numbers. We have applied this bi-horn strategy and found that thisis useful not only for reducing the number of animals for the experiment but also for obtaining a propercontrol sample undergoing exact developmental stages.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Sharing data and resources (e.g. animals, tissues and data) between research groups and organisations can also contribute to reduction. KCL utilise the mouse colony management system thatallows us to identify the research groups that can share the same mouse lines.

Our centre groups encompass a variety of different techniques and protocols. Whenever we introducenew experiments, we will seek the research groups that can share their expertise with us, followed bythe small-scale trials to minimize the unnecessary use 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this project, we will use mice which is the simplest available mammalian model with a cortical structure. Using mice will enable us to build on a decent bank of existing knowledge concerning cortical development and cell connectivity and furthermore to include well characterised genetically altered lines. It will also enable us to use genetic technology to label proteins, cells and their connections in particular types of progenitors and neurons, technology that amongst mammals is mostadvanced by far in mice.

The methods outlined in the protocols below have been carefully chosen to fit these objectives while producing minimal possible animal suffering. The vast majority of animals will undergo a single procedure. The electroporation and injection system of embryos we are using has been shown to resultin a superior survival rate for animals, without the induction of cell death in the tissue. In combination with small and precise targeting of the cells, this will minimise suffering of the animals. Of course, as detailed in the protocols below, good surgical technique, animal husbandry and veterinary advice will ensure that all procedures cause minimal possible suffering.

We do not propose any “severe” protocols.

Why can’t you use animals that are less sentient?

The project focuses on the development of the cerebral cortex—a unique anatomical structure of the mammalian brain. Many non-mammalian species such as fish and chick possess significantly distinctcell types and structures, thus we cannot use other species for our study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We routinely review the updated protocols for anaesthesia and analgesia, and any drugs with other research groups and the veterinary surgeon to ensure that the route and dose of any treatments are optimised to a minimum pain, suffering and distress during the experiments. During the surgery, the animals’ status (body temperature, breathing reflex) are checked regularly to make sure their pain-freecondition. After the post-surgery, the animals will keep warm and being monitored until a full recovery before being returned to

their home cage. Animals are checked by the Biological service unit stuff dailyto make sure their health conditions. Additionally, the animals that underwent the surgical procedure will be checked daily by the researchers. New researchers are trained under supervision by experienced researchers to make sure they are competent with any new procedure before being allowed to work independently.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Researchers will be familiar with and expected to meet the criteria laid out in the latest publishedARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. We follow best practice advice laid out by NC3Rs at: https://www.nc3rs.org.uk/3rs-resources and expect new and experienced researchers to complete the e-learning resources and implement thesepractices. Additional useful guidance such as Guiding principles aseptic surgery:
https://www.lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf

Refining procedures for the Administration of substances:

https://doi.org/10.1258/0023677011911345https://doi.org/10.1258/0023677011911345 will be alsointroduced to the lab members.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Our Named Training and Competency Officer on our Establishment licence will advise us to ensurethat information about advances in 3Rs (and training opportunities) and help to the best methods tocause the least pain, suffering, distress, or lasting harm to the animals.

As well as completing all mandatory training, any new researchers in the group are expected to set aside time to visit the NC3Rs website to read/watch all aspects relevant to our protocols as publishedin our recent papers, as well as general best practice in husbandry, experimental design, euthanasia and surgical procedures.

DEVELOPING STEM CELL-BASED THERAPIES FOR INNER EAR 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, ortheir effects, in man, animals or plants

Key words

Stem Cells, Hearing Loss, Auditory Neuropathy, Vestibular Hypofunction

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, pregnant
Rats	juvenile, adult, embryo, neonate, pregnant
Gerbils	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The current project has the overarching aim of developing stem cell therapies for the treatment of innerear conditions.

Previous work has established the initial proof of concept that stem cells can be used to functionally replace damaged cells in the hearing organ, the cochlea. We have also explored the potential of cells to engraft into the vestibule, i.e. the part of the inner ear responsible for the control of balance. Now wewill expand on these studies, refining the identity of the cell populations to be transplanted and further exploring the combination of cells with cochlear implants.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could

be short-term benefits within 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 400 million people have disabling hearing loss (HL) worldwide, with this figure expected to nearlydouble by 2050. This epidemiology is only equalled by the global burden of diabetes (>400m) and outstrips conditions like rheumatoid- and osteoarthritis (~200m). HL has a devastating effect on patients' quality of life, causing significant direct and indirect socio-economic harm.

Despite the scale of the problem, there are no disease-modifying therapeutics for HL. Palliative medical devices like hearing aids (HAs) and cochlear implants (CIs) remain the only treatments. Thesedo not repair the damaged tissues, HAs simply amplify sound, while CIs bypass damage to stimulate neurons. This has resulted in considerable patient and healthcare system demand for disease- modifying therapies as this would transform the lives of millions of patients across the world.

The inner ear also harbours the vestibular organ that controls balance. The impact of vestibular failureon a patient's mobility and functional independence can be devastating. Patients with bilateral vestibular failure rarely recover. Traditional vestibular rehabilitation benefits a limited number of patients and has no significant effect on number of falls. Impaired vestibular function is a major risk factor for wrist and hip fractures from falls.

A cell therapy for inner ear diseases would be the first of its kind, and would offer a biological,restorative solution to conditions that are currently intractable.

What outputs do you think you will see at the end of this project?

The initial benefit of this work will translate in the generation of new knowledge on how transplanted cells engraft and integrate in a recipient. Data to be gathered will advance our understanding on how torepair the inner ear, although some fundamental principles could be beneficial to the neurosciences in general, and to the field of regenerative medicine in particular.

Besides the scientific benefit, this project could have a direct impact in the treatment of hearing loss and vestibular pathologies. Data generated will support the development of a stem cell-based product for hearing loss and advance our understanding of its efficacy. The information will be included into scientific publications and reports that will support applications to the regulatory bodies to progress intohuman clinical trials.

Who or what will benefit from these outputs, and how?

As described above, the population affected by inner ear diseases is vast. If a therapy is finallyachieved, the long term outcome of this project has the potential to benefit millions. In a more immediate time scale (3-4 years), the data to be generated here will support applications to theregulatory bodies (e.g. Medicines and Healthcare Products Regulatory Agency-MHRA) to seekauthorisation for clinical trials.

Moreover, the scientific community will benefit from advances presented at conferences andpublications in peer-reviewed journals.

How will you look to maximise the outputs of this work?

We are very actively driving the translational application of our work. We collaborate closely with industry and clinicians. In particular, we have close ties with a biotech company that is aiming to develop the stem cell strategy into a realistic, clinically applicable treatment. The output of this work ispart of a larger project, that includes developing the manufacturing of stem cells under industrial standards, overcoming the surgical challenges for the delivery of cells in humans, and aligning the advances with the appropriated regulatory bodies.

Species and numbers of animals expected to be used

- Mice: Approximately 800 mice are to be used for the entire project, with 160 allocated to eachexperimental protocol.
- Rats: Approximately 800 rats are to be used for the entire project, with 160 allocated to eachexperimental protocol.
- Gerbils: Approximately 800 gerbils are to be used for the entire project, with 160 allocated toeach experimental protocol.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We propose to transplant these cells into mature gerbils, rats and mice that have two different types ofdeafness and/or vestibular problems. In the first type of condition, the main deficit is generated by thedegeneration of their cochlear or vestibular neurons, the second will have primarily a loss of cochlear or vestibular hair cells. These resemble the more common mechanisms that produce deafness and vertigo in humans. In a third model, we will explore the interaction of stem cells with cochlear implants.Gerbils are a good experimental model with a human-like hearing range, while mice and rats allow us to study genetic defects that impact on the inner ear.

Typically, what will be done to an animal used in your project?

Cell transplantation and implantation will be done through a surgical procedure under general anaesthesia. Animals are expected to be deaf, or to have vestibular problems. We anticipate that thevestibular problems will be detected with behavioural tests (e.g. walking on a beam) but should not substantially disrupt their daily routine. The severity of the procedures is considered moderate. At theend of the procedure, the animals will be humanely euthanized.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals may be deaf. After the surgeries, they may walk in circles, roll or rotate, but these behaviours usually subside after a few days. They may also undergo some discomfort after the surgery, but this isalleviated by anti-inflammatories and analgesics. Moderate weight loss may occur (~10-15%). In theory, formation of tumours is a potential side effect, but it

has not occurred in the studies so far.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Approximately 75% of the animals are expected to reach a moderate severity, ~20% will be mild withthe remaining ~5% being subthreshold. The spread of different severities is likely to be similar for thethree different species.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The cells to be transplanted have been extensively studied in vitro, and we have evidence that they can differentiate into functional cells when treated with the appropriate conditions. We are performing more experiments in a test tube (in vitro), trying to understand their molecular and functional properties. However, interactions of cells with a live recipient are too complex to be modelled in the cellculture lab. Before transplanting them into a human patient, we need to study them in an animal modelto analyse the responses they may trigger, as well as their therapeutic effects. Gerbils are a good experimental model with a human-like hearing range, while mice and rats would allow us to study genetic defects that impact on the inner ear. Furthermore, there are strains of rats and mice that have a modifed immune system that would minise the rejection and would help to study the engraftment of the cells. The three different species are needed to achieve the scientific goals, as they provide different advantages depending on the parameters under study (i.e. functional restoration, impact of genetic mutations, etc).

Which non-animal alternatives did you consider for use in this project?

The interaction of cells with host tissues has been explored in vitro, using cell aggregates (known asorganoids) generated from human stem cells.

Why were they not suitable?

Although some useful information can be obtained from the in vitro models, the organoid systems arestill very rudimentary and do not provide the complexity that a whole organism possess. Critical parameters such as efficacy for functional restoration, biosafety and the interaction with the host immune system can only be studied in vivo.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals based on our previous experience of what is needed tomeasure a biologically and statistically relevant effect size. We have used these assumptions andestimates to determine the sample size using power calculations.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Each experiment is carefully planned, considering minimising confounding variables such as allocatinganimals of comparable age and equal proportion of both sexes to each experimental group.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We optimise the number of animals and aim to reduce variability by controlling other parameters thatcould impact on the experimental outcome. For example, each batch of cells to be transplanted is carefully assessed. We characterise them in vitro and capture batch numbers of reagents consideredto be relevant to the process. Pilot studies using small cohorts are implemented when a new experimental variable is introduced.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice, rats and gerbils are species widely used in hearing research. All of these species possess a cochlear and vestibular anatomy similar to that seen in humans. For this particular application we haveinitially chosen the gerbil, since their hearing resembles that of humans, with an ability to detect a similar range of frequencies. Models for pathology, primarily those that affect the neurons and nerve (neuropathy) are well established in this species and mimic the hearing impairments occurring in the human population. Several laboratories are now using the gerbil, creating a wealth of information that would help us to compare and put in context the functional data we would obtain from our transplants. Besides the gerbil, we are exploring the applicability of this potential therapy in other

species such as mice and rats. Both these species can be manipulated genetically, and transgenic models of inner ear conditions as well as models with reduced immunity are available. These would greatly facilitate the validation of a stem cell transplant and treatment. Ototoxic models are also well established, for instance strains like the Gunn Rat offer an excellent model of early onset jaundice, one of most common causes of auditory neuropathy in humans.

All surgical procedures are performed under anaesthesia and suitable analgesia is providedafterwards.

Work performed during previous licences has allowed us to refine the model in which the hair cells andthe spiral ganglion neurons, are ablated (to be used in protocol 3). To mitigate the systemic effects of the ototoxic treatments (e.g. ouabain and Kanamycin/furosemide), we now spread the procedure over the course of a week, allowing full recovery from the ouabain surgery before the kanamycin/furosemidetreatment is given. This regime leads to better survival rates with fewer associated health issues.

Why can’t you use animals that are less sentient?

The nature of hearing loss and vestibular dysfunction entail that it is a condition which primarily affectsolder adults. Consequently, it is appropriate to use adult animals as a model for this. Likewise, in orderto see if a potential treatment is effective, it is necessary to follow the animals' progress over a longer period of time.

Similarly, hearing loss is mostly caused by dysfunction within the cochlea - this organ is lacking in lesssentient vertebrates such as zebrafish.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Animals sedated for hearing tests (ABRs) are given a reversal agent to ensure a rapid recovery fromanaesthesia.

Animals undergoing surgery to induce neuropathy (by application of ouabain) are monitored regularlyin the days after the procedure to observe and log any incidences of temporary vestibular dysfunction
e.g. rolling, head tilt. Refinement strategies involve giving the animals a 'vet-bed' fleece lining to thecage, which is a better substrate for them post-surgery than sawdust. Hay is also provided as a distraction - an entertained animal recovers better than a bored one.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Advice from in-house specialists (AWERB, NACWOs, NVS etc.) will be acted upon as and when newpractices are adopted. We regularly visit the NC3Rs online resource library.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

There are regular opportunities to engage with in-house workshops run by the NC3Rs organisation. Any new, relevant information will be disseminated amongst the group and put into best practice wheresuitable.

SIGNALLING PATHWAYS IN CANCER AND INFLAMMATORY 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, ortheir 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 aimsmentioned in paragraph (b)

Key words

cancer, therapy, inflammation, metabolism

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aims of the Project are to advance the molecular understanding of the pathogenesis of cancer andinflammatory diseases and to use this information to develop better treatments and improve human health

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
Diffuse large B-cell lymphoma (DLBCL) is a serious form of blood cancer that affects the type of white blood cells that produce “antibodies”, which protect us against infections.

Each year, 5,500 people develop DLBCL in the UK and there are 28,000 new cases in the US, making DLBCL the second mostcommon blood cancer. Unfortunately, current treatments only work for some patients with DLBCL, while they are either ineffective or too toxic for many others. Thus, there is an urgent need for new therapies to treat patients for whom current treatments have not worked or who have seen their cancercome back following an initially successful treatment.

Studies of the genes that are activated in the tumour cells have identified two subtypes of DLBCL known as “Germinal-Centre B Cell (GCB)-like” DLBCL and “Activated B Cell (ABC)-like” DLBCL. Patients with the GCB-DLBCL subtype generally respond well to current treatments and many of them are ultimately cured. However, current treatments do not adequately block a key process, which mainlyoccurs in ABC-DLBCL and allows cancer cells to outlive their normal lifespan and continue on multiplying uncontrollably. It is believed that this is a key reason why current treatments are less effective in patients with ABC-DLBCL. As a result, only 4 out of 10 patients with ABC-DLBCL are alive three years past their initial diagnosis.

The research team has identified a new mechanism which they believe makes some types of DLBCLmuch harder to treat. With their proposal, they seek to verify how this new mechanism they found works in harder-to-treat forms of DLBCL. As part of their research, they plan to use state-of-the-art genetic mouse models to determine how this mechanism allows some DLBCL cells to carry on multiplying uncontrollably. By improving the understanding of this key process, the research team hopes to develop more effective treatments for patients with currently incurable forms of DLBCL.

What outputs do you think you will see at the end of this project?

The research conducted under this License will advance the understanding of the mechanisms thatcontribute to the development of cancer and chronic inflammatory and metabolic diseases, such as diabetes. Building upon this improved understanding of mechanisms of disease, we aim to develop better treatments for patients with both malignant and non-malignant diseases in areas where there are currently no effective treatments. We also aim to use the knowledge generated from these studiesto develop new laboratory tests that will help to identify benefiting patients and implement the new treatments into the clinical practice.

The discoveries from this research will be published in peer reviewed scientific journals and presented at scientific conferences, which will stimulate other scientists to pursue related research, building upon our discoveries, thus creating further scientific and clinical benefit. We will also disseminate our outputsvia websites directed towards patients and the general public.

Who or what will benefit from these outputs, and how?

Gaining a more complete understanding of complex diseases, such as cancer and inflammatory and metabolic disorders, are long-term outputs which can be difficult to fully appreciate, but these are keyto facilitate progress in clinical practices and the management of patients. Additionally, we seek, as short-term impact, to swiftly translate into clinical assessment and utilisation of novel medicines that are currently in clinical development, including new drugs we developed, along with new laboratory tests that will help to predict which patients will most likely benefit from these drugs.
Clinical impact: Patients with multiple myeloma (MM) and diffuse large B-cell lymphoma (DLBCL) will more directly benefit from this research, and we may be able to combine our

drugs with existing therapies to increase their clinical utility in select subgroups of patients for whom current treatments have not worked. The results from the Project will also aid in the development of new diagnostic tests for identifying these subgroups of patients who may benefit from the new drugs investigated under thisLicense.

Academic impact: We have established a wide network of clinicians to identify and study novel mechanisms underpinning the development of cancer, as well as to support our ongoing clinical trials. Our national multi-centre clinical network encompasses some of the busiest oncology clinics in the UK.As well as providing an efficient trial delivery infrastructure, this network supports the development of novel diagnostic and prognostic tests to inform the therapeutic strategy going forward. The research also supports a powerful alliance between university, industry, and the NHS, aiming at swiftly translating advances in the basic sciences into patient benefit. Academic researchers and clinicians willalso benefit from this inter-disciplinary collaborative approach to further their own research, accelerating the development of new treatments, devices, and diagnostics to address complex healthcare needs. Our numerous international collaborators, including oncologists, chemists, pharmacologists, and specialist pathologists, will also benefit from the scientific advances made in this Project. In addition, the benefits of establishing such a wide-reaching network include the rapid translation of bench-side results to bedside and the development and sharing of evidence-based best practice across Europe and worldwide.

Researchers in the field will also benefit from the animal models we already have or plan to develop.We expect the output from this programme of work to be published in peer-reviewed journals. To ensure free access for clinicians and scientists based in the poor regions of the world, we are committed to publish our findings in open access journals. The research will also be presented at national and international scientific conferences to inform doctors, scientists, and allied health care professionals of our findings. As the findings from this research reach an even wider audience, it is likely that our work will add to the broader knowledge base surrounding tumour development and mechanisms of inflammatory and metabolic diseases. Therefore, our findings will have relevance in the wider context of oncology and inflammatory and metabolic disorders, and as such be of interest to many other basic scientists and clinicians worldwide.

Economic impact: There could be substantial economic outputs from the project. In conjunction with the ongoing clinical research in the new drugs we developed, the translational projects advanced under this License will create opportunity for industry investment, commercialisation, and out-licensing.Moreover, since these drugs work via novel mechanisms of action, there is a possibility that they couldreach market registration as a result of academically led clinical trials. This would imply significant economic returns on UK public and private investments in biomedical research.

Public impact: We have made a significant contribution to public engagement and plan to continue these activities in the future. Our current plans for engagement, communication and awareness aim atmaximising the project impact and raise awareness on its outputs and results. The strategy is organised around two main aims: 1) To effectively communicate the progress of the project to a wide range of stakeholders, including researchers, scientists, and community-based groups, as well as engaging policy makers on a national, European, and international level. 2) To launch the Project anddisseminate the project results to the general public, while contributing to raise awareness on issues directly related to the Project.
We regularly update our website with results arising from our projects. In addition, our Press Office hasextensive experience in working with the responsible media to

disseminate scientific and medical outputs to both the scientific and lay public, and so has our Clinical Research Facility, which regularly provides updates on its research. We have extensive experience in disseminating the results from our research and in engaging with the public and with official bodies. Our research has already led to numerous feature articles and was featured in many online outlets, including blogs, patient chat rooms,and numerous websites. These outputs are likely to continue in the future as the research progresses further into the clinic. We will continue to keep the wider public informed on the nature and progress of our research and maintain public awareness of and interest in the project.

How will you look to maximise the outputs of this work?

We plan to collaborate with different research and clinical laboratories in the UK as well as abroad. This network will improve our ability to promptly share data and approaches, whether successful or not. We also have collaborations with pharmacological companies which will allow us to maximise thedevelopment of novel therapeutic strategies. Furthermore, we aim to publish our results in peer reviewed journals which will include negative results, and we will present our work in national and international scientific conferences.

Species and numbers of animals expected to be used

- Mice: 12,325

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 the project is to advance the molecular understanding of the pathogenesis of cancer and inflammatory diseases, in order to develop better treatments and improve human health. We plan to use several genetically modified adult mouse models, bought from accredited companies or derived from collaborators. The mouse is one of the model organisms that most closely resembles humans. The human and mouse genomes are approximately the same size, and display an identical number ofgenes, which are functionally conserved. Further, mice have genes not represented in other animal model organisms (e.g., nematode worm, and fruit fly) such as those involved in adaptive immunity.

Mice can be genetically altered, and there is extensive literature concerning the topics of our investigations. Thus, our studies can be enhanced by combinations with many complementary modelsdeveloped by others in the field. We will use mainly three classes of modified mouse models: immunocompromised mice, spontaneous tumour models, and knock-out/in models. Our experiments are guided by the current literature and analysis of available human cancer data. We aim to pre- validate our hypotheses in vitro, using primary mouse non-transformed cells, human and mouse cancer cell lines, and, whenever possible, primary patient material.

We aim to gain a better understanding of how the larger cancer-driving pathway governs cell survival, inflammation, and metabolism in both malignant and non-malignant human

diseases, also taking advantages of the fact that many of the mechanisms by which this pathway regulates these processesin cancer are essentially the same as in inflammatory and metabolic diseases.

Additionally, mice are needed as the specific cell-cell interactions involved in the complex process of tumour development cannot be accurately recapitulated in cell culture. Therefore, how these interactions affect tumour development and therapy response can only be effectively addressed usingmice.

For the experimental protocols we will use juvenile and adult mice to study the mechanisms importantfor tumour development and the efficacy of new drugs designed to stop them before they can be usedin humans. The use of aged animals is required in certain genetic tumour models in order to study thedifferent phases of tumour development.

Mice are a validated model to study the efficacy and mechanisms of action of novel therapies before they are used in humans. A review of the current literature has confirmed that there are no suitable alternatives to mouse models in research aimed at delineating the processes involved in inflammatorydiseases and cancer as required by this project.

Typically, what will be done to an animal used in your project?

When studying genetic alterations or specific aspects of disease, the most sensitive and specific method is to use genetically altered (GA) animals. In this scenario, animals will be bred to generate therequired genetic modifications. These animals may require an activation step, which can be accomplished in the form of injections or food/water administration at different life stages (e.g., embryonic, post-natal, or adult stages). Once the genetic alteration is activated, the animals can be used to progress different studies, from the characterisation of the phenotype (e.g., phenotypic cages, blood withdrawal, or body weight measurement) and detailed studies of the mechanisms of the disease (e.g., ex vivo analyses), to different therapeutic strategies (e.g., drug repurpose, diet changes), and testing novel compounds.

Genetically modified animals bred under this license will also be used to generate bone marrow chimeras to study the role of the immune system in the processes of tumour development. In this case,the animals will be subject to depletion of their own immune system by using radiations. The amount ofradiation used is the least possible to achieve the experimental objectives and will be adapted based on the specific mouse strain used. Subsequently, the animals will be reconstituted with immune cells derived from other animals. These animals will be then used to study the effects of different immune system changes on disease initiation, development, and/or progression.

The project will not aim to generate a mouse model for every single genetic alteration studied, as this is a lengthy and expensive process in terms of procedures and animal use. Rather, the project will use different mouse models to study certain alterations and investigate novels therapeutic strategies. In thiscase the animals chosen may be of an immunocompromised strain and will likely be obtained from reputable sources or bred under this license. As such, in some cases, tumour growth will be initiated bythe injection of malignant cells or tissues of various origins using an appropriate route, depending on the type of tumour being investigated. Tumour cells may be injected in up to two sites where the purpose of the experiment is to compare the effects of drugs or other variables on tumours of varying genetic background. In each case, the most refined route of administration will be chosen, according tothe cell origin and tumour type. Once engrafted,

the tumours will be closely monitored and used to study the development and/or response to compounds. In other cases, tumours will arise in genetically altered mice that have a genetic predisposition to tumour development.

The dosing of substances will be performed via one of the possible different administration routes (injections, minipumps, orally, or topically), and the route will be chosen based on the duration of the treatment (minimising in all cases the injection frequencies), compound solubility and stability, and theleast stressful condition for the animals.

Imaging will be used in order to monitor disease progression and/or treatments, as well as to reducethe number of animals used, while maximising data collection from each experiment.

Below, are examples of typical experiments conducted under this license:

Protocol 5: 1) First, total-body irradiation is administered to ablate the host haematopoietic system; 2) within 24 hours of the irradiation, donor bone marrow is transferred to recipient animals by intravenousinjection; 3) 6 to 8 weeks later, blood sampling is performed to confirm the bone marrow reconstitution;
4) mice are transferred to Protocol 6 or 7 OR killed to investigate the development of bone marrow cell populations that may contribute to the tumour microenvironment in experiments conducted under Protocols 6 and 7. Duration of the experiments: typically, 6 to 8 weeks; number of procedures: typically,4.

Protocol 6: 1) 6- to 8-week old mice are immunised by intravenous injection of immunogenic substances, typically sheep red blood cells (SRBC), once a month for 3 months (i.e., 3 injections) to activate transgenes that induce tumour development; 2) subsequently, mice are closely monitored andleft untreated for up to 700 days after the first immunisation OR are treated 200 to 500 days post-immunisation by intravenous, intraperitoneal, or subcutaneous injection of clinically approved and/or experimental anti-cancer drugs, typically once every other day for a period of approximately 6 weeks(i.e., 21 injections in total); 3) finally, mice are killed by a schedule 1 method to enable the analysis oftumour development. Duration of the experiments: typically, between 490 and 650 days (i.e., 16-22 months); number of procedures: typically, 2 OR 3.

Protocol 7: 1) Adoptive transfer of tumour cells by subcutaneous injection; 2) 1 to 2 weeks later (in the case of so-called “xenograft” and “allograft” models) OR 4 to 6 weeks later (in the case of so-called “patient-derived xenograft” models, also known as “PDX” models), treatment with clinically approved and/or experimental anti-cancer drugs, typically by intravenous or subcutaneous injection once a day for a period of 2 to 4 weeks for “xenograft” and “allograft” models (up to 28 injections in total) OR 4 to 6weeks for “PDX” models (up to 42 injections in total); 3) finally, mice are killed by a schedule 1 methodto enable the analysis of tumour development. Duration of the experiments: typically, up to 6 weeks for“xenograft” and “allograft” models OR up to 10 weeks for “PDX” models; number of procedures: typically, 3.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the adverse effects in this Project are of mild severity, with possible transient moderate adverse effects. We have in place several monitoring systems, including health and weight checks andimaging of tumours. Tumours implanted subcutaneously (under the skin) are not expected to give rise to significant adverse effects. With tumours developing

at internal sites, mice may develop clinical signs such as hind-limb weakness or paralysis, enlarged lymph nodes and/or spleen, and abdominal distension. Humane endpoints will be applied if these do not resolve within 24 hours.

Animals that fail to recover from surgery may exhibit signs of pain, distress, or ill health. Any animal that does not fully recover from surgical procedures within 24 hours (as shown by eating, drinking, andreturn to normal behaviour) will be killed by a schedule 1 method. In the case of wound dehiscence, uninfected wounds may be re-closed on one occasion within 48 hours of the initial surgery.

Some genetic modifications may affect gestation or parturition (the act of giving birth).

Some irradiated mice may experience weight loss, typically peaking 5 to 10 days post-irradiation, diarrhoea (due to damage of the intestinal epithelium, the outer cell layer of the alimentary canal), and general signs of ill health (piloerection, reduced movement, and reduced appetite). Irradiation may alsocause bone marrow depression at low doses and bone marrow destruction at high doses, resulting in increased susceptibility to infections, leucopoenia (a reduction in the number of white blood cells), and platelet deficiency, causing haemorrhages (bleeding) and/or anaemia (pallor). Further, irradiation may cause hyperaemia (erythema), especially in albino, SCID, and nude animals possibly resulting in dermatitis (inflammation of the skin). Irradiation commonly affects pigment production, leading to dark furred mice producing grey/white coats. However, this does not affect the animals' welfare.

Animals that do not recover from bone marrow reconstitution could show reduced activity, stiff movement/posture, and/or intermittent diarrhoea/blood stools and will be killed by a schedule 1 methodif these signs do not resolve within 24 hours.

Adverse effects of natural ageing may be seen in the animal population aged for a maximum of 700days (i.e., 23 months) after the start of the procedure (e.g., immunisation). These effects may vary depending on the genetic line and include alterations in bodyweight, reduction in activity, scoliosis (deformation of the spine), arthritis, cataract formation, loss of fur, dermatitis, respiratory disease, dental abnormalities, superficial tumour formation, and rectal prolapse (protrusion of the rectum).
Among the adverse effects of natural ageing, sudden death is expected to occur in aged animals witha frequency of about 5%. Once these signs occur, they are expected to persist and may trigger humane endpoints.

Any animal that exhibits signs of pain, distress or significant ill health (persistent hunched posture, no activity, prostration, no responsive movement when stimulated, bloody diarrhoea, laboured respiration (dyspnoea), neurological signs such as hind-limb weakness or paralysis, for more than 24 hours will bekilled by a Schedule 1 method. Any animals showing abdominal distension (ascites) causing a more than 10% increase in normal body weight, or a 15% loss in normal body weight compared to age and sex-matched controls will be killed.

Idiosyncratic (individual) responses may occur following the injection of foreign substances. Mice mayshow signs of sickness or distress (e.g., ruffled fur, hunched posture, reluctance to feed or move), or difficulty in breathing, typically starting within half an hour of the injection. Animals exhibiting these signs will be killed by a schedule 1 method if the signs persist for up to 24 hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Based on our prior experience with the experimental models used in this Project, we anticipate thatanimals will experience the following severities:

Subthreshold: 60%

Mild: 30-35%

Moderate: 5-10%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The mouse is one of the model organisms that most closely resembles humans. The human and mouse genomes are approximately the same size and display an almost identical number of genes and these are functionally conserved. Furthermore, mice have genes that are not represented in otheranimal model organisms (such as nematode worm and fruit fly), including those involved in the “adaptive” type of immunity. Indeed, mice mimic anatomically as well as functionally the immune system and the complex cell-cell interactions occurring within tumours. In addition, mice can be genetically altered, and there is extensive literature concerning the topics of our investigations using mouse models. Indeed, none of the scientific objectives of this License can be achieved without the use of animal models (such as using in vitro experimental models or by conducting human studies).

Which non-animal alternatives did you consider for use in this project?

The work undertaken in this Project will be integrated with the use of tissue samples taken from the biopsies of patients. We will culture these human samples to tests how new compound molecules workin vitro and study their mechanisms of action, as well as use them to develop so-called three- dimensional "organoid" co-cultures, which will allow us to recapitulate more closely the physiological and disease conditions existing in humans. We also work closely with clinicians and cancer patients, and the Principal Investigator (PI) of this License leads a multi-centre clinical trial of a new drug developed by the Project team in patients with different forms of blood cancer that have proven very difficult to treat. As such, the research team has ample access to clinical material to study the biological pathways and genetic alterations discovered using the animal models investigated under this License. We will also use different cell line models of diseases and gather information on the genetic mechanisms underpinning the origin and development of disease from the

analysis of publicly available patient datasets.

Why were they not suitable?

Primary human cells from patients are an invaluable research tool to study tumour development and progression; however, they lack a fully functioning immune system and tumour microenvironment, which involve many different cell types interacting in a highly dynamic three-dimensional environment and have a profound influence on both the development and aggressiveness of tumours. Similarly, tumour development and metastatic spread involve multiple interactions between cancer cells and theirsurrounding microenvironment, including signals originating from their immediate neighbouring cells and distant tissues. Further, the understanding of the early stages of tumour development and the impact of inflammatory and metabolic alterations resulting from drug treatments and/or genetic mutations are impossible to analyse without the use of animal models that replicate the complex interactions that occur between neighbouring and distal cells. As such, the study of the mechanisms underpinning the initiation and progression of tumours requires the use of living animals.

A review of the current literature has confirmed that, on the basis of the considerations outlined above,there are no suitable alternatives to mouse models in research aimed at delineating the processes governing inflammation, immunity, and oncogenesis as required by this project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated numbers of animals reflect the numbers necessary to achieve the scientific objectives outlined in the programme of work described in this Project. For experiments where outcomes in the experimental and control groups are compared, it is important that the group size is sufficient to demonstrate whether there are statistically significant differences between groups, whilst keeping the numbers of mice as low as possible. Typically, group sizes of 6 to 10 are used, with data from repeat experiments being combined to strengthen the statistical analyses. These numbers take into consideration the frequency with which any given gene alteration produces a measurable biological effect that can provide statistically meaningful results, whilst keeping the number of animals at a minimum. In all cases, we aim to minimise animal numbers consistent with achieving scientifically andstatistically robust results.

For breeding purposes, the estimated number of animals is based on prior studies with complex genetic breeding. This takes into account the fact that the genetically altered lines used in this Projectmay involve alterations of up to eight to ten different genes in a single animal.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We follow the ARRIVE and NC3R guidelines to help us in the design of experiments. We use statisticaltools and appropriate software to assist us in planning experiments with scientifically and statistically robust results, whilst using the minimum possible number of animals.

In accordance with the ARRIVE and NC3R guidelines, we aim to design experiments which are unbiased and adequately powered to produce statistically significant results with a wide range of applicability and which, at the same time, are simple and efficient. The implementation of these principles allows us to conduct well-designed experiments that aim to avoid the generation of bothfalse positive and false negative results and to produce robust data, with good levels of statistical significance.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We have taken the following measures to reduce the number of animals:

Mouse colonies are continually monitored to avoid excess animals. We have developed effectivebreeding strategies which can provide us with the required number of experimental and control mice.

In the majority of experiments, post-mortem tissues are harvested at the end of experiments toobtain results from molecular and immunological analyses in vitro.

In vivo experiments are carefully designed to use the minimum possible number of animals, whilstproviding biologically and statistically significant experimental outcomes.

Only inbred or congenic strains of mice are used in order to reduce the biological variability due togenetic factors.

Comparisons are made between strain-, sex- and age-matched groups of animals, and equalnumbers of mice are used in each group in order to avoid statistical artefacts.

Where no prior information is available, we conduct small pilot studies.

We collaborate with different research laboratories in academic institutions around the world andthe pharmaceutical industry and share information as well as protocols with them to help us to obtain more data and use the minimum possible number of animals.

We implement imaging techniques through the sequential analysis of biological endpoints in the same animal. Small-animal imaging allows us to monitor deep-seated tumours and metastases with orwithout treatment. In this way we maximise the data obtained from a single animal and thereby reducethe overall number of animals 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is currently the most refined animal model to achieve the scientific objectives of theprogramme of work outlined in this Licence.

All our protocols are designed to produce the least possible suffering to the animals and for the shortest possible time. To refine the production of genetically altered animals, whenever possible we will introduce gene modifications that only manifest in specific cells and at specific times, rather than inthe whole animal. This will limit the tissues and time window affected by the gene modification and thereby minimise any adverse effects on animal welfare that derive from it. We will use the same refinement to minimise the adverse effects of genetic alterations when choosing amongst mouse lines that already exist for conducting our experiments.

For the generation of new lines of genetically altered animals, the transfer of embryos into the reproductive tract of recipient females typically involves a surgical procedure. Every effort will be takento minimise any subsequent pain and suffering through the use of appropriate surgical techniques, general anaesthesia, analgesia, and perioperative care. Non-surgical embryo transfer methods may also be used whenever the success rate matches that of surgical embryo transfer methods or is satisfactory. Females from the same experiment will be housed together after the embryo transfer, thusavoiding having to keep social animals singly. In this way, the females will also help each other to raisethe joint litter. Wherever possible genetically altered sterile males will be used instead of males that aresterilised by a surgical procedure. In any case, the procedure will be performed by a scrotal sac incision, which avoids the incision of the abdominal body wall musculature and is, therefore, a refined and less painful method. In addition, animals will not be used for mating until they are regaining weight and they are showing no adverse signs following surgery.

Breeding and husbandry considerations will be used to refine the methods adopted for the productionand maintenance of genetically modified animals. As such, all genetically modified mice will be carefully monitored so that appropriate actions can be taken to minimise any harm, apply humane endpoints, and ensure that husbandry and care are optimal. Immuno-compromised mice will be maintained under strict aseptic housing and husbandry procedures to prevent incidental infection andthus minimise the risk of disease and death. Food and water will also be kept sterile. For genotype analysis, biopsies will be removed by using the least invasive method. Genotyping only requires a small amount of DNA, and therefore the amount of tissue taken will be kept to an absolute minimum.

For the production of bone marrow chimera, we will use the most refined procedure. Whenever possible (e.g., to study tumours of mouse origin), we will minimise the amount of radiation to achieve only partial ablation of the immune system. To avoid the risk of infection due to the ablation of the immune system, mice will be given antibiotics, typically in the drinking water, and maintained in a sterile environment (cage, food, and water). Strict aseptic housing and husbandry procedures will be maintained starting from a few days prior to irradiation, until full reconstitution of the immune system is achieved. Food and water will also be kept sterile. This will reduce the risk of bacterial contamination and potentially decrease the burden of gastrointestinal bacteria. Softened food and hydrogels will be provided to afford easy access to water and prevent dehydration. Animals will be inspected regularly toensure their general wellbeing. We will strive to cause the least possible suffering to the animals. We will follow the best practice, and the monitoring of the

animals will help to minimise the incidence of mild and mild-to-moderate clinical signs, and to ensure the rapid implementation of any humane endpoint as early as clinical signs are detected.

With tumours developing at internal sites, reliance will be placed on the clinical evaluation of the general condition of the animals, evidence of palpable tumours, and close observation of the animals. To refine the monitoring of tumour development and the therapeutic effects of drugs, we will use imaging techniques. This will help us to implement earlier and more refined humane endpoints. In addition, tumour burden will be limited to the minimum required to achieve a valid scientific outcome. Inthose case where we will be able to see the scientific outcomes prior reaching the humane endpoints, we will end the experimental procedure at that point. Similarly, drug efficacy studies will be terminated as soon as durable, statistically significant therapeutic effects are observed. In all cases, we will strive to cause the least possible suffering to the animals that is necessary to study the development of disease and the effects of drugs. LASA guidelines will be applied for all dosing of substances. We will use inhalation anaesthesia during imaging sessions in order have a humane way to immobilise the animals. Whenever possible, we will use previously published data, e.g., from papers, companies, and other sources, to assess the suitability of substances and minimise any unnecessary harms arising from their administration, given the particular strain used. If prior data are unavailable, we will perform pilot studies using a small number of mice. All staff will be appropriately trained and competent in the administration techniques. The volumes dosed will adhere to good practice guidelines and the most refined method will be used. Doses and duration of dosing will not exceed those found to have thedesired scientific effects.

Why can’t you use animals that are less sentient?

The immune system, and in particular its “adaptive” arm, in less sentient animals, such as Drosophilamelanogaster or Zebrafish, does not exist or has a different organisation, function and anatomy.
Therefore, these animal species do not provide a reliable model to adequately mimic the humanimmune systems in tumours or in inflammatory and metabolic diseases.

Where possible, we will use genetic models that allow the gene alterations to manifest only in specificcells and at specific times, rather than in the whole animal, to minimise the effects and time window ofthe phenotypes shown.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Imaging techniques (such as bioluminescence and ultrasound imaging, two non-invasive methods)allow the generation of serial data on the tumour and the immune and stromal cell interactions in a minimally invasive and non-destructive way that will result in a significant refinement. Overall, the tumour burden required for obtaining valid scientific outcomes may also reduce, allowing the healthstatus of the animal to be preserved.

To further refine the experimental conditions and to ensure minimal suffering, the following steps alsowill be taken:

Where possible, a short-acting general anaesthetic will be used;

Pre- and post-operative analgesia will be routinely administered;

Where possible, vasectomised males will be replaced by genetically sterile males;

Post-operative care will be implemented, and soft food, warmth and fluids will be administrated asrequired;

Singly housed animals will be used only if no other option is available (such as in phenotypingcages);

Enrichment will be provided in accordance with the need of the animal life stage.

In all procedures, as outlined in the individual Protocols, we will monitor the condition of the animalsregularly according to the protocol used. Furthermore, the monitoring of animals will be increased atany moment if required.

With any tumours developing at internal sites, reliance will be placed on the clinical evaluation of the general condition of the animals, together with an assessment of body weight, evidence of palpable tumours (e.g., by abdominal palpation), and the observation of specific clinical signs, such as hind-limbweakness or paralysis, enlarged lymph nodes and/or spleen, and abdominal distension.

If unexpected distress occurs, mice will be humanely killed by a Schedule 1 method.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow LASA and NC3R guidelines, such as the LASA Good Practice Guidelines on “Collectionof Blood” and “Administration of Substances”. We will also utilise references such as Workman et al. 2010 (“Guidelines for the Welfare and Use of Animals in Cancer Research”) and Ullman-Cullere and Foltz 1999. Welfare guidelines will be applied for all dosing of substances (Morton et al. 2000; BVAAWF, FRAME, RSPCA, UFAW; Wilkinson 2020).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

The 3Rs website will be the primary source of information. Furthermore, my research group and I willattend national and international conferences as well as 3Rs meetings organised locally, where new methods are presented and discussed. Also, advice from the NVS and NACWO will be sought whenever planning a new experiment.

REGULATION OF CELL FITNESS AND IDENTITY DURING MAMMALIAN DEVELOPMENT AND ITS DEREGULATION IN 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, ortheir effects, in man, animals or plants

Key words

Stem cells, Heart disease, Embryo development, Congenital heart defects

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The overall objective of our research is to understand how cell fitness and identity are regulated duringnormal embryo development and how they become deregulated in disease.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The project addresses the mechanisms that regulate the fitness and identity of the cells of the mammalian embryo and how these mechanisms become deregulated in disease. We aim to identifythe key factors and signals that regulate cell survival and cell identity during the different stages of development of the embryo. In the short term these studies will

allow an increased understanding ofthe development of the mammalian embryo. These studies will allow us to reveal novel genes, and novel pathways these genes act in, that regulate cell fitness in the early embryo, including the pathways that control heart formation.

The pathways regulating development of the embryo are used again in the adult, especially during processes involving tissue renewal. Consequently, our work will help not only understand the molecularbasis of birth defects, but also will provide insight into other pathological conditions, such as cancer and heart failure in humans. Additionally, the information harnessed in this project is essential to direct stem cells in a safe and efficient manner into cell types that are important for regenerative medicine.

Therefore, in the medium/long term our results will allow others to test the relevance of our findings inthe above settings.

What outputs do you think you will see at the end of this project?

The program of work covered by this project license will contribute to our understanding of the mechanisms that regulate cell fitness during embryonic development. Our work will primarily have twooutputs:

First it will provide new information regarding the pathways that control normal embryo developmentand how they become deregulated in disease.

The second output is the publication of our work in scientific journals and the communication of the results that we obtain via presentations at scientific conferences and events aimed at increasing thepublic understanding of science.

Who or what will benefit from these outputs, and how?

Our studies will help uncover the mechanisms that ensure the fitness of embryonic cells during the process where less specialised cells orm more specialsed ones and how they become deregulated in disease. The large promise that embryonic stem cells hold for regenerative medicine, where these cells are induced to form more specialised cells that can then be transplanted into patients with a range of diseases, from diabetes to heart failure, has carried with it a large degree of interest both fromthe academic and medical communities. Furthermore, the advances in our ability to reprogram adult cells into embryonic stem cells, have widened the scope of application of these embryonic stem cells, as patient-derived stem cells can now be generated to study and treat diseases. However, increased knowledge is required to be able to generate specific cell types that are fully functional before these stem cell therapies becomes meaningful clinically. Therefore, the results obtained in this study will be of direct relevance to producing these therapies. Given that understanding how cellular fitness is regulated is a key question in cancer, our work will also be of interest to researchers working in this field.

In the short term, the primary beneficiaries and users of this research are members of the academic sector - research workers, teachers, and students. These will be able to apply the knowledge that emerges to their own efforts to produce high quality stem cells for regenerative medicine. In the medium term to long term the beneficiaries are the general public and commercial sector. In particular,the data obtained in this project will benefit the companies developing stem cell-based assays for drugscreening as well as companies developing media for embryonic stem cell maintenance and differentiation, and pharmaceutical and biotech companies developing human embryonic stem cell- based cell

replacement therapies for treatment of degenerative diseases.

A further medium to long-term beneficiary of our work will be the lay public. Given the medical relevance and ethical implications of understanding developmental processes, and its impact on stemcell biology, our work will provide factual input to and therefore benefit the public discussion about theadvantages and risks of stem cell therapy.

How will you look to maximise the outputs of this work?

We will maximise the output of our work in the following ways:

We collaborate with a network of laboratories with different expertise to ensure an interdisciplinary approach to solving the problems we tackle. The results of this research will be conveyed to other researchers through the publication of findings in peer-reviewed journals, by reporting unpublished work at conferences and through personal communication with other scientists. Though the results willprimarily be disseminated through scientific journals, we will liaise with dedicated media teams at our institution to issue a press release when appropriate. We take seriously the responsibility of scientists to engage with the lay public, to raise awareness among them of the results of publicly funded research, to openly debate ethical issues relating to our research so that public opinion may be formedin an informed manner and to take the excitement of our research to the children of today, who will be the scientists of tomorrow. For these reasons we engage through our institution but also as individualsin activities aimed at the public dissemination of science.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the ideal animals for our studies because their development and physiology closely resemblethat of humans, and they are amenable to genetic manipulation. Additionally, mice are needed as the specific tissue interactions involved in the complex process of embryo development cannot be accurately recapitulated in cell culture. Therefore, how cell fitness is regulated during development canonly be effectively addressed using mice.

For the experimental protocols we will use embryos to study their development and adult mice to studyhow the mechanisms important for cell fitness in the embryo are conserved in adults.

Typically, what will be done to an animal used in your project?

Typically, two types of procedure will be done on the animals used in this project.
The first involves the breeding of genetically modified animals and these animals will be humanelykilled to obtain embryos for analysis. These mice will only experience one

procedure.

The second is aimed at analysing embryo development and heart function in adults and typically involves the injection of a substance to induce gene deletion and humanely killing the animals about 3-12 weeks after this injection. Blood samples may be taken from some animals, typically once a week for four weeks . Animals may also be imaged to analyse their heart function during this time, typically once. These mice will typically only experience one procedure, but about 15% will experience two and 3% will experience three.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not anticipate any adverse effects in the animals bred for embryo collection.

We only anticipate rare adverse effects in the animals used to analyse heart function in adults.These are associated with the addition of substances that could cause local inflammation or a temporary weight loss.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

We expect that overall 85% of mice will experience a sub-threshold severity, 12% of mice a mildseverity and 3% of mice a moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are required because the development of an embryo or the normal functioning of the heart cannot be replicated in a dish. This functioning involves the crosstalk of diverse cell types in 3 dimensions and suitable, properly validated models that recapitulate these interactions do not currentlyexist. Mice are required for our studies as their development more closely resembles that of humans than other lower vertebrate model systems, they are amenable to genetic manipulation and there are awealth of tools and resources available for their study.

Which non-animal alternatives did you consider for use in this project?

We routinely use embryonic stem cells and differentiate them into heart cells in a dish to understandthe process of embryo development and the functioning of the heart.

Why were they not suitable?

The complex cell to cell interactions that take place in 3 dimensions in the embryo and in the adultheart cannot be faithfully recapitulated in a dish. Therefore, we cannot study how genes affect cellfitness and cell identity without an intact embryo.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 mouse numbers used in this project based on the anticipated number of experiments, experimental groups, and number of animals per group. This number is based on the projected number of mice needed for our project aims and takes into consideration our experience ofprevious studies that have been conducted under our previous Home Office Project Licence in whichwe used similar experimental approaches and similar mouse models.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We use in vitro systems, such as assays performed in embryonic stem cell lines to identify importantfactors that regulate cell fitness. Although these in vitro systems have their limitations, they do help significantly to narrow down the list of factors needed to be studied in vivo. For example, only those factors showing robust effects in vitro are tested in mice. This helps us to prioritise our experiments and keep animal numbers to a minimum.

Also, the experiments proposed in this licence are primarily based on procedures we have longstanding experience with, which allows us to base experimental design considerations on a solidbase of prior data. We will continue to design and perform experiments following general principles ofgood experimental design and laboratory practice. Our in-house Statistical Advisory Service may be consulted if necessary. We routinely use G*Power software for Power analysis and GraphPad Prism for statistical analysis. To avoid bias and variability, experimental design will include randomisation, blinded assessment, and explicit inclusion and exclusion criteria.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We will take the measures listed below to optimise the number of animals in our project:

When breeding genetically modified mice we will use strategies to maximise the use of offspringwherever possible.

Whenever possible, procedures we will combine sequentially, and longitudinal studies on the samemice (including non-invasive imaging and serial phenotypic studies) as this will

reduce the overall numbers of mice required to reach the scientific end-points.

We will always strive to use the most refined techniques available in order to reduce animal numbersthrough improved accuracy of measurements.

To reduce animal usage, where it is anticipated that a genetically modified line will no longer be ofimmediate interest, embryos will be harvested and stored (frozen) to reduce the need to continue breeding.

Where possible, not used tissues will be frozen and stored for later analysis or to share with otherresearchers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use both wild-type and genetically modified mice. We will study mice with loss or gain of gene function mainly in specific cell types and using mutations that can be induced in specific organs and atspecific time-points. Targeting specific cell types in an inducible manner minimises the chance that thegenetic manipulations themselves have broad effects on animal well-being. The models we use causeno pain, suffering, distress, or lasting harm from the genetic manipulations themselves. We may administer substances to induce the indicated genetic mutations, using established treatments that donot themselves cause pain, suffering, distress, or lasting harm.

Dosing regimens will adhere to good practice guidelines and will follow our previous experience usingsimilar strains and/or available literature using the same or related mouse strain. Laboratory Animal Science Association guidelines will be adhered to, and the most refined method will be used. Local Animal Welfare and Ethical Review Body guidelines for administration of tamoxifen will be followed.
Doses and duration of dosing will not exceed those reported to have the desired scientific effects in theliterature. To reduce risk of accidental infection mice will be housed in sterilised cages and maintained on sterilised bedding/food/water.

Why can’t you use animals that are less sentient?

We study embryo development and heart function in mice and as they are the most appropriate animalfor this type of study for several reasons: mice are mammals and their development more closely resembles that of humans than other vertebrates like fish (which for instance, do not have a four chambered heart like humans and mice); the physiology of mice most closely resembles that of humans than that of lower vertebrates; mice are amenable to genetic manipulation so a large number of tools exist for their study, and mice have been studied for a long time by geneticists, so a large bodyof knowledge exists on which we can build.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We will take the measures listed below to refine the procedures to minimise the harms for the animals.Different measures will be applied depending on the animal experience.

Although we anticipate that the majority of our studies will not harm the animals in any way, where there is no prior knowledge of the outcome of the intervention (for example if a new genetic model is being used) and may have an adverse effect, pilot studies may initially be carried out on a small cohortof animals to determine appropriate dose rates/treatments/end points, being informed wherever possible by published data. Animals being used in such pilot studies will be closely monitored for signsof adverse effects and action taken to minimise suffering.

Anaesthesia, where used, will be of depth sufficient to prevent the animal being aware of painarising from the procedure.

Experimental protocols, including dosing and sampling volumes and frequencies, will be in accordance with current best practice, using sources such as the Laboratory Animal Science Association guidelines and the National Centre for the Replacement, Refinement and Reduction of Animals in Research guidelines, as well as by following the advice from the named veterinary surgeonand the named animal care and welfare officers.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We follow published Laboratory Animal Science Association guidelines as well as the Animal ResearchReporting of In Vivo Experiments guidelines for reporting of animal studies. We also implement new guidelines when published by the National Centre for the Replacement, Refinement and Reduction of Animals in Research and our local Replacement, Refinement and Reduction of Animals in Research Hub.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will consult with resources such as the The National Centre for the Replacement, Refinement and Reduction of Animals in Research database and the Replacement, Refinement and Reduction Hub at our institute. We will consult regularly with colleagues, animal technicians, vets and the Animal Welfareand Ethical Review Body about best practice and potential further refinement of our procedures.
Additionally, we will keep up to date with the scientific literature to follow the published best practice inmonitoring and assessing development and heart function in vivo.

To effectively implement the Replacement, Refinement and Reduction of animals in our work, we willdiscuss these issues in our weekly group meetings so members of the group who carry out animal work are kept informed on how to maintain best practice.

ROLE OF INFLAMMATION AND FIELD EFFECTS ON INTESTINAL NEOPLASIA

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

cancer, mutation, inflammation, risk, stem cells

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

We will investigate the role of chronic inflammation and acquired age related mutations in the onsetand development of intestinal cancers.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 now known that human tissues accumulate significant mutational burden with age in genes that are associated with the development of cancer, including colorectal cancer. Only a small proportion of such mutations ever develop into cancers. We want to understand how efficiently such acquired mutations predispose to the subsequent development of cancer to allow their relative risk to be estimated. Similarly, the risk of developing colitis associated colon cancers increases with the duration of chronic

inflammatory disease. Chronic inflammation generates local damage and adaptation that also leads to increased mutational burden through increased expansion of mutations that can be eitherpro-oncogenic or beneficial because they promote survival. Here we want to understand how to restrictor eliminate the former without impacting on the latter. Ultimately for both inflammation and age-relatedmutational burdens we want to be able to identify vulnerabilities that give maximum opportunities offer for prevention of disease.

What outputs do you think you will see at the end of this project?

We aim to generate:

An understanding of the nature and potency of synergistic interactions that cause tumours to arisefrom pre-existing pro-oncogenic mutations

An understanding of the extent to which it is possible to restrict the expansion of pro-oncogenicmutations or pre-neoplastic lesions while permitting growth of beneficial or neutral mutations

Data that will be shared with the scientific community (uploaded to open-access data repositories).

Who or what will benefit from these outputs, and how?

In the short-term (1-5 years), new basic scientific research will benefit the scientific community.

In the medium-term (>5 years), new information will result in translation of pre-clinical research tobenefit patients.

How will you look to maximise the outputs of this work?

All findings will be published in a timely manner and only in open-access journals to ensure free andcomprehensive access.

All reagents, data and expertise will be shared with collaborators. Published datasets will be depositedin open-access repositories. Expertise will be shared via detailed methods publications in open-accessjournals, or on our website.

Negative findings are included in our publications and will be rapidly disseminated at national andinternational meetings.

Species and numbers of animals expected to be used

- Mice: 21,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

- Mice are the lowest form of mammal that can be used to study most normal and

diseased states.

- Lower orders such as fish lack easily discriminated discrete organ compartments such asstomach, small intestine and colon.
- Fish also lack the diversity of epithelial cell types that in mammals may contribute directly orindirectly to the formation of cancer.
- Mice have many similarities with humans in terms of genetics, immunology, and cancer formation.
- Most current tumour and anti-tumour models were developed and have been applied in mice.
- Many genetically engineered mouse models are available to investigate the different stages ofcancer progression.
- We need to use adult mice because the tumours we study occur primarily in adults, as aconsequence of age or time related processes.
- Adult mice provided the appropriate ensemble of mature cell types that form cancers or thatinteract with them to promote or limit their growth and progression.

Typically, what will be done to an animal used in your project?

There are two lines of investigation:

To investigate the role of pre-existing mutational burden on the development of subsequent neoplastic disease (cancer) we will engineer intestinal specific activation of pro-oncogenic events and then initiate cancers by treating animals with a chemical mutagen. Commonly both of these proceduralsteps require one to three intraperitoneal injections with the timing between them varying from a few days to several weeks. Animals are then aged to allow tumour to develop. Latency times can vary from1 month to over a year. To alter disease outcome animals will be treated with drugs administered at different times during disease development.

To investigate the role of chronic inflammation on the development of intestinal cancer mice will be bred that are constitutively or conditionally predisposed to developing inflammation and inflammation associated tumours of the intestinal tract. Activation of additional pro-oncogenic events will commonly require intraperitoneal injection. Alternatively mice may be treated with colitis causing agents in the drinking water. Animals with inflammatory disease will be maintained to allow the development of pre neoplastic or neoplastic disease and may be treated with intraperitoneal injection of chemical mutagento promote a neoplastic outcome. The onset of inflammatory disease is probabilistic with around 80% of animals being culled by 8 months of age and the rest by 12-14 months. To alter disease outcome animals will be treated with drugs or immunotherapies adminstered at different times during disease development.

What are the expected impacts and/or adverse effects for the animals during your project?

Investigation of the role of pre-existing mutational burden on the development of subsequentneoplastic disease.

These experiments will cause short-term discomfort associated with the administration of reagents (i.e. injection of substance). In the medium (30 days) to long term (up to 12 months) intestinal tumour masses may form that can be associated with weight loss, rectal bleeding and diarrhoea. The duration of these effects is closely related to their severity from one or two days if they are pronounced to several weeks if they are mild.

Investigation of the role of chronic inflammation on the development of cancer.

These experiments will cause short-term discomfort associated with the administration of reagents (i.e. injection of substance). In the medium (30 days) to long term (greater than 12 months) epithelial inflammation or intestinal tumour masses may form that can be associated with discomfort, weight loss, rectal bleeding and diarrhoea. The duration of these effects is closely related to their severity fromone to two days if pronounced to several weeks if they 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 animaltype)?

- Many mice will receive no procedures and used for breeding (60% sub threshold)
- Many mice will receive treatments or be maintained and causing mild adverse effects (30% mild)
- Remaining mice will be recruited to procedures causing moderate adverse effects (10%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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cancers arise from a series of stochastic events (occurring with a certain probability but by chance) over life and from a multiplicity of complex interactions between different tissue constituents that are only present in a whole animal context. We are aiming to recapitulate these processes as closely aspossible. There are many similarities in the pathways that drive cancer, and colon cancer, between mouse and human that motivates mouse as the species of choice

Also, there are many shared reagents available for tumour biologists that work for mice,

includingantibodies for mouse proteins, or genetically altered mouse models that specifically focus on genetically modifying the intestinal epithelium and the immune and stromal cells that support it.

Which non-animal alternatives did you consider for use in this project?

- Cancer cell lines and primary organoid cultures from both mice and human (patient derived).
- More elaborate transwell and organ-on-chip type assays using the same organoid derived cells.

Why were they not suitable?

The repertoire of behaviours that can be read out from bioassays based on organoid cultures and theirderivatives is limited. For example, seeding and growth efficiencies can be measured but how these relate specifically to the behaviour of cancer as compared to normal cells is uncertain and does not relate at all to the more subtle and complex tissue behaviours of cells that are phenotypically normal but at increased risk of neoplastic transformation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissueand reuse.

How have you estimated the numbers of animals you will use?

Most mice that will be recruited to study will be compound genetically altered animals containing multiple genes that need to be interbred to provide both experimental and control groups. One consequence of this is that it can take several generations to assemble the alleles appropriate for breeding of the final experimental cohorts. Further there are constraints in how efficient breedings canbe: e.g. if the gene can only be inherited from one parent only half the mice will inherit it.

The number stated reflects the predicted maintenance and use of around 20 different genetic combinations, and so multiple parallel lines of investigation, that are generated or sustained by use ofbreeding pairs or trios with a breeding period of around six months.

Breeding to get desired genetic make up: 2,600 per year

Mice undergoing procedures or having tissues taken and anlaysed: 1,600 per year

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

- When designing experiments we will initially perform pilot studies to determine the observed effect sizes to ensure that we use the minimum number of mice per group that will be informative.

- We will consult with statisticians within the establishment before starting experiments andthroughout when required to ensure statistical confidence.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

- We meet as a team to review the number of lines being bred and the number of mice we have foreach one every 10-12 weeks.

- If one line serves more than one investigator then dialog is more frequent, around every one ortwo weeks

- Even within a given mouse line some new experiments require small pilot studies while others require larger numbers of animals that are roughly age matched. This means that animals can beappropriately matched to experimental need as breeding efficiencies naturally fluctuate.

- Breeding is optimised to make the generation of mice with the right combination of genes asefficient as possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

- Tumour formation models: These are the most accurate mouse models of tumour formation that allow the early stages of disease development to be studied because they are exploiting the same genetic events that drive cancers in patients. These mice will be humanely killed at an earlystage of cancer development, and therefore will not suffer significantly from cancer-related adverse effects; e.g. metastases will be rare events.

- Chronic inflammation. These models are designed to mimic a serious human condition (inflammatory bowel disease). We have selected a model and refined our protocols to cause theleast amount of inflammation necessary to model the human disease and that can be managed to minimise discomfort.

Why can’t you use animals that are less sentient?

- The development of cancer even if driven either by chronic inflammation or due to pre-existing gene mutations takes time and occurs over weeks or months. Therefore short term analyses interminally anaesthetised animals cannot be used.

- Adult cancers are also diseases of fully developed and aged tissues which precludes the use ofimmature life forms.

- The intestinal tissues of mice, unlike potentially less sentient species, have similar anatomy andfunction to humans and the cancers that arise have similar characteristics

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

- Foremost, we perform pilot studies to define the expected harms; we work closely with animaltechnical staff to implement comprehensive management and monitoring plans.

- We communicate continuously and effectively with animal technical staff to capture unexpectedadverse phenotypes or incorporate feedback into animal protocols.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Unless otherwise specified, the work in this project will be undertaken in accordance with the principlesset out in the Guidelines for the Welfare and Use of Animals in Cancer Research: British Journal of Cancer (2010) 102:1555-1577 (referred to subsequently as the 'NCRI Guidelines') and in the LASAGuiding Principles for Preparing for and Undertaking Aseptic surgery (2010).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Locally:

- A team member attends monthly meetings of animal users within the establishment.

- Active engagement with animal technical staff, and close liaison with technicians, NACWO, andNVS to refine our protocols.

- We receive guidances from our Animal Welfare Ethical Review Body (AWERB).

- Outwith the establishment:

- We consult with national and international peers using the same or similar models.

- Perform literature reviews to identify where more refined protocols have been successfullyemployed.

SAFETY TESTING OF MEDICINAL PRODUCTS USING SMALL ANIMAL SPECIES

Project duration

5 years 0 months

Project purpose

- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aimsmentioned in paragraph (b)

Key words

Regulatory, Small animal, Safety assessment

Animal types	Life stages
Mice	adult
Hamsters (Syrian) (Mesocricetus auratus)	adult
Rabbits	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

This licence authorises the conduct of studies in small laboratory animal species (rats, mice, rabbitsand hamsters) with the aim of evaluating the toxicity and tumorigenicity (ability to cause cancer) of medicinal products (human or veterinary/animal health). This is to aid in the development of new medicines, and to provide mandatory information to regulatory authorities to allow human/veterinarytrials or marketing approval.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Governments require (and the public expects) that substances we are exposed to are safe

or that theirpotential hazards are well understood and documented.
The data generated from the studies performed under this project will be used to inform decision- making processes on substances under development and, where appropriate, to satisfy governmentalregulatory requirements necessary to gain clinical trial approval, marketing authorisation or product registration.

This safety assessment is of immense importance along with other non-rodent and non-animal studiesin demonstrating to governments and the public the safety of these substances.

What outputs do you think you will see at the end of this project?

The overall benefit of this project is that it supports the development of safe, new medicinal products toimprove the health and quality of life of human and veterinary patients by generating high quality data that is acceptable to regulatory authorities and enables internal decision making within our clients' organisations.

Achievement of the objectives of this licence will enable safe drug development candidates to progressand will also help to remove unsuitable candidates from the development pipeline at an early stage, thus saving animals and resources.

Who or what will benefit from these outputs, and how?

Our customers will benefit, as the data we generate will allow them to progress their medicinal products under development and, where appropriate, to satisfy governmental regulatory requirementsnecessary to gain clinical trial approval or marketing authorisation.

Patients and animals will benefit from these studies as this work will contribute to the development of new drugs that help alleviate human animal 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 beused to develop further new drugs. The toxicity information obtained is important when planning futuretrials in humans and animals, to make sure any starting dose in a clinical trial is safe for the participants taking it.

How will you look to maximise the outputs of this work?

Where confidentiality permits, data, study design and best practice will be openly shared at conferences, workshops, webinars, blogs and publications.

As 3R's benefits are also realised under this project licence, these will be shared more widely withother establishments.

Species and numbers of animals expected to be used

- Mice: 58400
- Rats: 87900
- Hamsters (Syrian) (Mesocricetus auratus): 8050
- Rabbits: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Most of our experiments will be carried out on conventional adult mice and rats as these are the smallest relevant species that we can use that have body systems that are comparable to humans. Insome specialist cases we may use the hamster or the rabbit because what we are trying to find out is better performed in that particular species rather than in the rat or mouse.

The only other time we would use a species other than a mouse or rat is to continue work that has been previously done in that species. For instance if previous work, and results gained, had been carried out in a hamster, it would make no scientific sense to start the next stage of a programme ofwork in a rat or a mouse.

In some protocols we may use genetically altered animals. These animals may be more sensitive to developing tumours (which means fewer animals and a shorter study can be conducted), they may be immunosuppressed so that they do not reject the test substance (for example, biological test substances such as human stem cells) or these alterations mean the animals produce specific medicalconditions that we need to assess toxicity against. The use of genetically modified animals is low compared to conventional animals.

Typically, what will be done to an animal used in your project?

Typically on this project, animals are dosed over a period of time with test substances, and usually sampled (e.g. blood or urine) before having tissues taken after they have been humanely killed for extensive toxicology analysis. Studies would range from a single dose, to those which last a matter ofdays (much less than a month) although some can last for 1, 3 or 6 months, and sometimes up to 2 years (to specifically examine whether a test substance can induce cancer). Study durations are dependent on the specific regulatory test being performed. Some animals are left dose free for a few weeks after dosing is complete to see if any effects of the test substances can be reversed.

Dosing of animals is commonly done orally using a flexible tube, or by injection using a syringe and needle/removable indwelling cannula, maybe directly into a vein, into a muscle in the leg, or under theskin. Other common routes are used such as dermal (via the skin).

Blood samples are usually taken from easily accessible veins in the neck or the tail of rats or mice. Weare limited to how much blood we can take at once or, cumulatively, over a month. If we need a large blood sample, we would do this when the animal is anaesthetised and we would not let them recover consciousness.

Where possible, we try and take as many of the tissues and samples we need after the animals havebeen humanely killed after all dosing had been completed.

Some animals we use are genetically altered, so they better represent disease more applicable inhumans, and make toxicity testing more relevant (and often shorter).

In some protocols we also have to surgically prepare animals for testing, when a normal animal wouldnot be suitable. This maybe, for example, to implant a cannula into a vein for prolonged intravenous dosing, or for intravenous dosing over a period of hours. Surgery is only performed when there is no other way forward.

What are the expected impacts and/or adverse effects for the animals during your project?

When dosing an animal by injection or taking blood, the amount of pain an animal feels is similar to what a patient would feel having an injection done by a doctor. If we have to repeatedly inject animalsusing a needle and syringe, we would choose different sites to do this where possible. We can take blood samples when an animal is deeply unconscious. If we need to take repeated blood samples or need to dose repeatedly then we try and use different sites. Of course everyone who performs these procedures is trained to a high standard.

Animals undergoing surgery receive the same sort of care as a patient would in hospital. We discusstheir pain relief and use of antibiotics with a vet before we start and administer drugs as necessary.

The genetically modified animals we use are usually immunocompromised or modelling humandisease.

Routinely we need to take a urine sample for analysis, so we would then put an animal into a special cage which is smaller than their normal cage. The animal can still move around. Virtually every animalwill get used to their new cage within about 15 minutes and are fine.

Dosing with drugs and chemicals may cause adverse effects in some studies. Experience shows that (~45%) of animals are not expected to show any clinical signs of suffering (either no clinical signs or normal background signs expected of the rodent strain). A small percentage (~5%) may show transient subtle to mild clinical signs. Moderate signs of adverse effects may be seen in some animals(~50%), usually in the higher dose groups. Lethality and/or severe effects are not study objectives in any of the protocols within this licence, but for preliminary studies that may be the first animal studies with limited data available, a very small percentage of animals may inadvertently show severe findings before they are immediately and humanely killed.

We do observe our animals at least twice a day, and the people who do this know the signs when an animal is ill. If an animal is ill, we would check it more frequently, and get more senior staff involved inits care for advice, including vets.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

On the last project, about 50% of animals displayed mild severity, and around 50% of animals were classified as having displayed moderate severity. This is because these studies can last between a few days and weeks to up to 2 years, and although the individual procedures are usually mild in natureon their own, the cumulative effects make them moderate overall.

It's impossible to predict the proportion of severities expected on a service licence like this, as this willbe dependent on what study types we are asked to perform.

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 haveconsidered 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 regulatory and scientifically acceptable alternative to the use of rodents and rabbits in these studies. These studies are run to satisfy the regulatory requirements of governmentsaround the world to ensure pharmaceuticals are safe for humans and animals. These tests are very specific as to what they require in terms of testing in animals to ensure this.

We maintain a constant awareness of regulatory guidance and ensure that where non-invasive methods exist which fulfil the regulatory requirement they are used in preference to animal studies.

Which non-animal alternatives did you consider for use in this project?

There are no other non-animal alternatives for the work being undertaken on this project. The regulations we are following will not allow safety decisions to be made on non-animal systems alone.

In vitro and in silico methods (test tube or computer work not using animals) are used in combination with animal studies to inform study designs and assist in understanding of potential toxicity but cannotyet replace in vivo (animal) studies.

Why were they not suitable?

Although there are test tube tests that can model some parts of how drugs and chemicals get into ourbodies, and how our body deals with them, and can identify undesirable effects, for example, there isno series of test tube tests that brings all these complex happenings together, like we see in animals and humans.

That is why we need to test new drugs and medical devices in animals, as they have similar physiologyand processes as humans, and that testing gives us a good idea what may happen if they were ever tested in, or exposed to humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received from customers in the past. It is, however, impossible to accurately predict the number of studies that may be performed, inthe circumstances.

The regulatory guidelines we follow for each study usually indicate the number of animals in a study;otherwise, the number used is the minimum to achieve the aims of the study.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Studies are designed to provide maximal scientific value from the minimum number of animals, whilstusing sufficient animals to meet scientific objectives, and regulatory guidelines. Statistical input is sought, where appropriate, to strengthen the overall scientific quality and relevance of studies.

Where available, sensitive analytical techniques may be used to reduce animal numbers.

Wherever practicable, and by looking across studies, the combination of endpoints eg general toxicity,reproduction and developmental toxicity, mutagenicity etc in studies is considered, to reduce overall animal usage.

As most studies involve the examination of tissues following treatment, opportunities for re-use are very limited. Tissues are collected to support drug and in vivo developments from any surplus stockanimals.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We will try and get as many outputs as we can from a single animal where possible, without adverselyaffecting its welfare. So if we need to get several different samples, for example, we will often do that in the same animal, rather than use separate ones, when possible.

Before our main studies, we use smaller groups of animals to get an idea of the doses we need to usefor the main studies. These studies are important as it gives us confidence that the doses we are using are correct prior to testing them in bigger groups of animals required by global regulators.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Most of our models involve dosing animals with test substances or devices, and sampling them, withmany outputs taken after the animals have been humanely killed. This is generally the least invasiveset of procedures that can be done to give meaningful outputs to make scientific decisions about further tests, or to determine the safety of a test substance/device.

Throughout our studies, our animals are checked at least twice a day. This allows us to see over a period of time, whether dosing each individual animal is causing any adverse clinical signs. if this isthe case, we can take action: get veterinary advice, add food supplements and extra bedding if needing, and even reduce dose levels or stop dosing completely.

Why can't you use animals that are less sentient?

Rodents (rats, mice, hamsters) and rabbits will be used in all of the studies conducted under this licence. Rodents are considered to be of the lowest neurophysiological sensitivity (their brain functionand physiology) that will allow us to achieve the study aims and are considered suitable for predictingwhat's likely to happen in humans. Rabbits and hamsters will be used for specific studies where rodents are not a physiological or regulatory option, or if previous work has been carried out in these species.

The vast majority of tests on this licence will use either mice or rats.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Many of the procedures performed on our rodents like blood and urine sampling, cause only transientdistress to the animals. Blood sampling procedures are similar to and about as painful as having a blood sample taken by a doctor or a nurse. Blood volumes are kept to a minimum within rigid volume guidelines. Confining animals in special cages to allow us to take urine samples is similarly of little distress to the animals.

Dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm andwill be the minimum consistent with the scientific objectives of our studies. In addition, suffering will befurther minimised by implementing clearly defined humane endpoints.

Animal welfare is of utmost importance and Good Surgical Practice will be observed for any animal undergoing surgical procedures. Surgery will be conducted using aseptic techniques (to prevent infection) which meet at least the standards set out in the Home Office Minimum Standards for AsepticSurgery. Before we start surgery, we agree with a vet what pain killers or antibiotics the animals needboth before and after the surgery. When recovering from surgery, we give the animals extra heat and monitor them closely until they start behaving normally again. We then check them at least twice dailybefore they go on study.

In addition, care is taken to provide as much environmental enrichment as possible. This includes, butis not limited to, plastic shelters in their cages, wood blocks) and balls (short studies up to 13 weeks duration) to gnaw on and push around; mice are occasionally given swings, mice and hamsters aregenerally given extra bedding for warmth and food supplements are given as appropriate.

In some tests we use animals that are genetically altered, to mimic conditions seen in humans or morecommonly, transgenic mice because of their susceptibility to tumours. These animals are specially bred and don't display any harmful clinical signs due to their conditions.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

ICH Safety Guidelines

Guideline on the need for carcinogenicity studies of pharmaceuticals S1A

Testing for Carcinogenicity of Pharmaceuticals S1B

Dose Selection for Carcinogenicity Studies of Pharmaceuticals S1C(R2)

Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity StudiesS3A

Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies (S3B)

Duration of Chronic Toxicity Testing in Animals (Rodent and Non Rodent Toxicity Testing) S4

Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals S6(R1)

Safety Pharmacology Studies for Human Pharmaceuticals S7A

Immunotoxicity Studies for Human Pharmaceuticals S8

Nonclinical Evaluation for Anticancer Pharmaceuticals S9

Guidance on Non-clinical Safety Studies for the Conduct of Human Clinical Trials and MarketingAuthorisation for Pharmaceuticals M3(R2)

OECD Guidelines – see www.oecd.org or www.oecd-ilibrary.org

Notes for guidance on repeated dose toxicity. Committee for Proprietary Medicinal Products (CPMP),2010. CPMP/SWP/1042/99 Rev 1

Guideline on the evaluation of control samples in non-clinical safety studies: checking for contamination with a test substance. Committee for Medicinal Products for Human Use (CHMP), 2005.CHMP/SWP/1094/04

Note for guidance on carcinogenic potential. CPMP, 2002. CPMP/SWP/2877/00

CHMP SWP conclusions and recommendations on the use of genetically modified animal models forcarcinogenicity assessment. CPMP, 2004. CPMP/SWP/2592/02 Rev 1

EMA/CHMP/SWP/2145/2000 Guideline on non-clinical local tolerance testing of medicinal products(HAVE ADDED THIS).

LASA/NC3Rs: Guidance on dose selection for regulatory general toxicology studies for

pharmaceuticals. http://www.nc3rs.org.uk/downloaddoc.asp?id=1108
Notes for guidance on non-clinical local tolerance testing of medicinal products. CPMP, 2001.CPMP/SWP/2145/00

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery http://www.lasa.co.uk/pdf/lasa_guiding_principles_aseptic_surgery_2017.pdf)

Guidance on the conduct of regulatory toxicology and safety evaluation studies. UK Home Office 2005

Diehl et al. A good practice guide to the administration of substances and removal of blood, includingroutes and volumes. Journal of Applied Toxicology: 21, 15-23 (2001)

Gad et al. Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routes inmultiple species with notes on methods to improve utility. International Journal of Toxicology: 1-84 (2016)

NC3Rs: Recommendations from a global cross-company data sharing initiative on the incorporation ofrecovery phase animals in safety assessment studies to support first-in-human clinical trials (Regulatory Toxicology & Pharmacology, 2014)

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

This will be achieved by consultations with our Named Information Officer, colleagues in Animals Technology, and by attending appropriate training courses and conferences, or getting feedback fromsuch events.

PERCEPTUAL PROCESSES DURING COMMUNICATION IN FISHES

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

communication, mate choice, sensory processing

Animal types	Life stages
Xiphophorus hellerii (green swordtail)	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The overall aim of this program of work is to understand the role of perceptual processes during visualcommunication in fishes.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

All animals experience their environment through their senses, which shapes how they perceive and interact with their environment and others within it. Understanding how sensory organs such as the eyes process information is critical to interpreting animal behaviour and the evolution of animal signalsused during communication.

What outputs do you think you will see at the end of this project?

This research will primarily advance basic knowledge by tackling fundamental questions about animal perception and the evolution of animal signals. These questions are relevant to visual signals across allanimals, not just in the fish used in this project. We will generate

visual models in acuity (the ability to resolve detail), contrast sensitivity and colour sensitivity for multiple species of fish that can be widely used. We will also determine how similar perceptual processes involved in signal assessment are across related species, to understand how general the processes underlying animal perception are, and how they shape signals that are used to communicate. This information may help to mitigate issues in captive animals where dominance interactions between individuals may impact animal health.We will also incorporate our findings into existing mathematical models of signal evolution, which will explore the impact of how perceptual processes that occur during mate choice can have effects on evolutionary timescales. Finally, we anticipate gaining understanding in how environmental effects such as noise pollution can affect communication. This will help us to understand how human impacts on natural habitats may affect animal perception and communication.

Who or what will benefit from these outputs, and how?

This project combines behaviour, perception and evolution into a general framework of signal perception, meaning that we can understand perception at different stages: from how animals can seesignals, to how viewers respond to signals based on their perception, and how that behaviour ultimately shapes the evolution of signal appearance and signalling behaviour. Our outputs will be relevant to researchers working across diverse fields including ecology, behaviour and physiology. Wealso expect that providing more refined computer-based models of fish vision that can more accuratelydescribe the visual experience of fishes (the ability to resolve detail, contrast sensitivity and colour sensitivity) will benefit a broad range of researchers using fish in their experiments. It will enable researchers to refine experimental designs based on what their model species can perceive, and in some cases may allow live animals to be replaced with computer modelling approaches. In the longer term, we also hope that this research will have benefits for fish welfare, fish conservation, and aquaculture production, due to better understanding of fish perception and effects of social andenvironmental factors upon interactions.

How will you look to maximise the outputs of this work?

We will use the ARRIVE guidelines to maximise the quality and reproducibility of our published work. We will publish our outputs in open access scientific journals, allowing anyone to access the research,and findings will be further disseminated through presentations at scientific conferences and meetings.The findings of this project will also be shared with the general public through media outlets, social media and public engagement events.

We are also passionate about disseminating work to non-academics, for example through media, public engagement events and more targeted outreach. We have an existing collaboration with a localcompany that specialises in engagement and outreach, and we will seek to incorporate findings from this project into a specific public engagement activity.

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,

includingduration 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 primarily use adult green swordtails and their relatives (highland swordtails, Montezuma swordtails, southern platyfish, guppies and humback limia). Green swordtails have been extensively studied in mate choice research and male-male interactions, allowing us to build upon previous work on this species. We have previously investigated relevant components of the visual system of green swordtails, so we can create ecologically relevant tests of perception for this species. We will use the other species to determine how the perception of closely related species differs, and the ecological reasons behind such differences. The additional swordtail species are used because males vary in their sword length from very long (Montezuma swordtail), short (highland swordtail), to no sword (southern platyfish), which will allow us to investigate the interaction between female perceptual abilities/processes and the evolution of sword lengths. All swordtail species used have sexual signals that involve females assessing the length of a horizontally oriented trait; we will also usethree species (sheepshead swordtail, guppy and limia) with signals that differ in their form to determinethe generality of perceptual processes regarding signal assessment. Sheepshead swordtails, guppies and limia are used because they have colour/area-based signals and/or vertically orientated bars of varying numbers respectively which represents a different form of signal to the horizontal size-based signals of many swordtails. We will use adult fish throughout this project, as we are interested in behaviours demonstrated by mature fish such as mate choice.

Typically, what will be done to an animal used in your project?

Fish used will normally need to be individually identifiable as they will be subjected to repeated observations. In some cases, individuals may be uniquely identified from natural markings such as sword length and colour in male swordtails (preferred) but more typically they will need to be tagged toallow for reliable identification. This will be done once they reach an appropriate size (a minimum of 3cm), by injecting visible elastomer tags under the skin.

Fish are then subject to a series of non-invasive behavioural tests to measure their responses for various visual stimuli. For example, in a mate choice task a female will be presented with video footageof two males of different sizes, and her interest in each male is measured by the amount of time she spends near each one. These assays are typically under five minutes in duration to maintain fish motivation. Generally there are multiple trials within each assay and fish will undergo multiple assays. The maximum number of trials within an assay is 22, and the maximum number of assays a fish will experience is ten. We expect that most behavioural assays will require around 14 trials rather than 22, but this may vary based on how variable individuals within each species are in their behaviour. Some assays also involve only one trial, for example optomotor assays take less than one hour per fish, donein one trial. For longer assays, fish will have a recovery period of at least 48 hours between assay trials(that only last around five minutes each) and two weeks between assay types to minimise stress to theanimal and to encourage natural behaviour. A subset of fish may undergo training to train them to prefer one stimulus over another (for example a red disc over a green disc), with subsequent testing to determine if they have successfully learned the task. In these tasks fish may be individually housed (there will be no visual or chemical isolation as they will be next to tanks with other fish and on a shared water system), for a maximum of 42 days. Each fish may only experience isolation once. At theend of experiments, some fish (that have not

experienced the possible full testing schedule of 220 trials) may be used for breeding to maintain stocks of species that are difficult to source. At the end of the project, fish will be euthanised using Schedule 1 procedures.

What are the expected impacts and/or adverse effects for the animals during your project?

The primary expected adverse effect is mild stress related to tagging fish under general anaesthesia and netting them to move them into experimental tanks. We expect this stress to be short lived as fish typically exhibit normal behaviour within several minutes of being tagged and netted. As fish undergo multiple behavioural assays there is the risk that multiple netting events accumulate to create chronic stress that can last several days. Fish health is monitored daily using a set of criteria that assess demeanour, appearance, behaviour, skin changes and buoyancy. Signs of chronic stress include loss of appetite, abnormal behaviour (gasping at the surface or lying at the bottom of the tank) and change in colouration. We have not observed these issues in our previous experiments using green swordtailson a similar testing schedule. The subset of fish that may be socially isolated for an extended period oftime may also experience stress due to lack of social contact, although Poeciliids are not shoaling fish and we have not observed stress due to isolation previously. Any fish that demonstrate signs of stresswill be removed from testing and observed for 48 hours to confirm improvement.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

We expect that around 10% of animals will be sub-threshold, and the remaining 90% will experiencemild 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 haveconsidered 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 determine how animals process visual information. As we are interested inperceptual processes that occur in the brain, there are currently no suitable non-animal models that could be used instead as we need to study the intact animal itself. We need to use vertebrates as theyhave camera-type eyes (which differ in anatomy to insect compound eyes) so that we can use existingvisual mathematical models to objectively measure what they can see, and it allows our findings to be generalisable across all vertebrates.

Which non-animal alternatives did you consider for use in this project?

We considered a purely model-based approach based on existing models of vision, and an approach using invertebrates rather than vertebrate animals. We will also use our findings to model the effects ofperception upon signal evolution, which will replace using animals to set up real-time evolution experiments.

Why were they not suitable?

There are visual models available for colour perception in non-human animals, but no models for perception of size so we could not use a purely model-based approach. In order to develop accurate models for fish vision we need to collect data on multiple individuals of both sexes in six species to determine variation within and among species. We also considered using invertebrate animals such asstalk-eyed flies, but these have different types of eyes to vertebrates and so were not suitable for addressing questions regarding vertebrate signalling.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissueand reuse.

How have you estimated the numbers of animals you will use?

This estimate is based on our published data and pilot data from previous experiments. We have previous experience in using swordtails and can therefore accurately estimate the number of animalsneeded in each group to obtain statistically valid results. As we generate more results, we will continually assess group sizes to determine whether group sizes can be reduced from the number currently proposed. We will also use relevant statistical methods for data analysis to reduce sample size.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We have based our sample size on previous experiments using a similar protocol, and have identifiedthe effect size that we are trying to detect. We have also used resources such as the PREPARE guidelines and the NC3Rs experimental design assistant.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We are using previously collected data to determine a suitable sample size based on our expected effect size. We are using videos of fish as stimuli in the majority of behavioural choice trials, as green swordtails will respond to these in a similar way to real fish. This allows us to cut the number of fish used by two thirds and minimises variation in experiments by giving us full control over what a fish sees during a trial. We will also determine whether other species of Poeciliid also respond to videos in the same way, allowing us to minimise the numbers of stimulus fish required. In our behavioural trials we use automated tracking software to measure fish preference, which eliminates subjectivity and biases when measuring behaviour of known individuals. We are also using computer modelling to determine the evolutionary outcomes of our observed behaviour, instead of

establishing selection linesto directly determine the long term effects of our findings.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project uses a number of fish models. Primarily we will use the green swordtail, Xiphophorus hellerii. We will also use several other closely related species, including other swordtails where males vary in tail length (for example the long sworded Montezuma swordtail, the short sworded highlandswordtail, and the southern platyfish and sheepshead swordtail which have no sword), and severalmore distantly related species where females have no preference for swords such as guppies and limia.

We are using elastomer tags to identify fish as the adverse effects of the procedure are short-lived. Alternative options like individually housing fish are likely to be more stressful, and other identification procedures such as PIT tags often require fish to be captured to scan the PIT tag. We are using non- invasive behavioural testing throughout as our research questions relate to natural behaviour of animals such as mate choice and male-male competition. We are also using similar behavioural assays that we have previously refined to minimise stress and testing time (e.g. by priming females fora mate choice task prior to testing and by using videos of courting males rather than live males) whilst still providing robust data.

Why can’t you use animals that are less sentient?

There are no non-sentient alternatives for empirically addressing the research aims. We need to usevertebrates to address these aims as they have camera-type eyes (which differ in anatomy to insectcompound eyes), which allows us use and refine existing visual computational models of vertebrate vision to objectively measure what they can see. We need to use adults that exhibit behaviours not seen in immature individuals such as mate choice and male-male competition.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

At all stages of the project, we will monitor the health of the animals (using the lab's fish scoring and euthanasia criteria) to evaluate the impact of procedures, and we will regularly review procedures to ensure they are minimising harm. Fish are checked for general health (swimming and behaving normally, eating well, no physical signs of disease or damage) daily during feeding, and each fish will be checked prior to being netted for use in each trial. To minimise the number of mate choice experiments where we do not get a behavioural response, we isolate females in same-sex groups priorto testing to ensure they are not gravid. We may then provide them with visual and chemical cues of males immediately prior to the mate choice task to prime them for assessing males. Our previous workhas shown that this significantly reduces the number of trials where no data is

generated due to lack offemale receptivity, and thus will reduce the number of times a fish is netted. We have also increased the duration between assays from one to two weeks, to mitigate against possible effects of cumulative stress.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow the Association for the Study of Animal Behaviour's guidelines for the treatment of animals in behavioural research and teaching, the NC3R’s resource library, and PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

I carry out regular continued professional development, which includes staying up to date with developments in the 3Rs and taking statistical courses to develop more effective tools for data analysis. I subscribe to the NC3Rs newsletter and receive regular updates via our research ethics and governance team. The proposed project is divided into discrete objectives with distinct protocols so that any advances in methodology can be quickly implemented. I will also liaise with the NIO, NACWO,and NVS throughout the project.

RODENT REGULATORY GENOTOXICITY

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, ortheir 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 aimsmentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Genotoxicity, Pharmaceuticals, Agrochemicals, Industrial Chemicals

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required,and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this rodent regulatory genotoxicity project is to evaluate the potential of pharmaceutical (human or veterinary/animal health) or non-pharmaceutical (agrochemicals, food additives and industrial chemicals) compounds to cause genetic damage in rodents, principally the rat and mouse.

Genotoxicity is a term used to describe the ability of a compound to damage the genetic information within the cell causing mutations which may lead to cancer in the future. Thus, genotoxicity studies arepart of an overall work package as part of the safety evaluation

process.

A retrospective assessment of these aims will be due by 10 August 2028

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A key benefit of this programme of work is the provision of safety data to facilitate sound regulatory decisions when assessing the risks to humans when the test substances are produced, transported orused.

This work is vital to the development of safe substances such as pharmaceuticals (human or veterinary/animal health) or non-pharmaceuticals (agrochemicals, food additives and industrialchemicals) that people will come into contact with.

What outputs do you think you will see at the end of this project?

The output of this project will be the provision of safety data to facilitate sound regulatory decisionswhen assessing the risks to which humans, animals, plants or the environment are exposed when substances are produced, transported or used.

Who or what will benefit from these outputs, and how?

The public and animals will benefit from these outputs.

The data produced in this project allows the support of ongoing clinical trial programs and aids in the ability obtain a product licence to market a substance. The development of new medicines, veterinary/animal health products, food additives, agrochemicals and industrial chemicals is necessaryfor the continued success of efforts to combat disease, maintain food supplies and achieve improvements in the quality of life.

It is a fundamental expectation that such substances should not pose an unacceptable risk to the health and well-being of the human population or target animal populations, or to the environment. This project contributes directly to that expectation, and facilitates the development of products that willhave minimal adverse impact.

How will you look to maximise the outputs of this work?

Development and validation of new tests or modifications to existing assays will lead to an improved battery of tests for hazard and risk assessment. In addition, many new tests or modifications may allowmore thorough assessment of genetic hazard in one step, thus eliminating the need for extensive further testing and reducing overall animal usage.

Wherever possible, data from multiple end points will be obtained from the same animal.

Species and numbers of animals expected to be used

- Mice: 5500
- Rats: 10000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult rats and mice are the species specified in the regulatory test guidelines. Some studies also useadult genetically modified mice.

Typically, what will be done to an animal used in your project?

Animals will be exposed to test substances (as detailed earlier) via routes including inhalation, application to the skin, orally, in food or water and injection either into the skin, muscle or bloodstream.This may require anaesthesia or periods of restraint on a number of occasions.

Administration/infusions of substances can be performed using delivery devices such as catheters in blood vessels. Animals may be blood sampled to confirm exposure, or restrained to enable exposure.Animals will be humanely killed at the end of the study and tissues harvested for examination.

Most of the dosing techniques, manipulations or investigations do not cause any lasting adverse effects, but a small number of animals may show temporary moderate distress due to, for example,withdrawal of blood.

The vast majority of studies will last for days rather than weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

When dosing an animal by injection or taking blood, the amount of pain an animal feels is similar to what a patient would feel having an injection performed by a doctor. If we have to repeatedly inject animals using a needle and syringe, we would choose different sites to do this where possible. If wecan take blood samples when an animal is deeply unconscious then we do. If we need to take repeated blood samples or need to dose repeatedly then we try and use different sites. Of course, everyone who performs these procedures is trained to a high standard.

The genetically modified animals we use are specifically required by global regulators for a specifictest.

Following test material administration, adverse effects may be observed in some animals. This may include weight-loss, ruffled fur, subdued behaviour and breathing abnormalities for example. We do observe our animals at least twice a day, and the people who do this

are trained to recognise the signswhen an animal is ill. If an animal is ill, we would check it more frequently, and get more senior staff involved in its care for advice, including vets. Humane endpoints will be applied where required.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Administration of test substances may result in mild to moderate signs of toxicity, usually at the highestdose level. The rats and mice used in these studies may show effects at all dose levels, but they are expected to be transient. Experience shows that under the last licence (~80%) showed transient subtlemild clinical signs such as reduced weight gain or weight loss, subdued behaviour and fur ruffling.
Moderate signs (hunched posture and abnormal breathing) of adverse effects were seen in someanimals (~15%), usually in the higher dose groups.

Despite the close monitoring some animals (<5%) sometimes inadvertently experienced severe toxicological adverse effects such as repeated convulsions, persistent laboured breathing or indeed were found dead. Lethality and/or severe effects are not the desired outcome and animals will be closely monitored and promptly humanely killed at predetermined humane endpoints to minimise the likelihood of unexpected death as far as possible. Animals which exceed the project license severitylimits are reported to the Home Office.

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 10 August 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animalswere affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Regulatory guidelines require the use of animals to investigate genotoxicity. The in vivo tests (in animals) are conducted because some agents are mutagenic in vivo but not in vitro (in non animal alternative tests). The in vivo tests also include additional relevant parameters such as absorption, distribution, metabolism and excretion, which may modulate the genotoxic effects of a test substance.
You cannot fully model the complex interactions as seen in animals solely in non animal alternativetests.

We will however remain vigilant to the possibility of the development and emerging use of any nonanimal regulatory acceptable alternatives should they become available in future.

Which non-animal alternatives did you consider for use in this project?

The ECVAM database and other literature searches were conducted to determine if any non-animal alternatives were available. However, the animal study is preceded by an in vitro assay, the results ofwhich are used to optimise the design of the animal study.

All studies will be assessed to verify that there is a need to conduct the study and that there is no otherdata or approach that could avoid in vivo tests.

Why were they not suitable?

No non-animal alternatives are accepted alone by Regulatory authorities. However, if there is data orprevious test results available that mean in vivo tests are not required, procedures will not be conducted for that purpose.

A retrospective assessment of replacement will be due by 10 August 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These are based on the numbers used during the last five years and the expected demand for thisservice.

The Regulatory Guidelines usually indicates the design and number of animals included in a study therefore, there is lesser scope in genetic toxicology for reduction than in other fields of work. Attentionis paid to good study design to use the minimum number of animals in the most refined way to achievethe aims of the study.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Some study design decisions may have to be made based on data from bacteria or cell based assaysor from preliminary data in rodents, from which a progressive approach to the accumulation of information is adopted. This orderly sequence of data collection reduces the number of animals used and restricts the procedures to which they are subjected.

For studies that are being performed at a later stage, where studies in the rodent (and other species)may have already been performed, decisions on study design can usually be made with a higher degree of confidence leading to lower animal use. Scientists and Statisticians will be consulted at anearly stage as required, so that advice can be given on implementation of the 3Rs and study plans developed which minimise severity of procedures applied as far as possible.

ICH guidelines promote the assessment of genotoxic effects by including the relevant end-points into other toxicity studies that are required for regulatory submission. This has clear advantages in terms ofanimal reduction, however, the study designs must meet specific requirements, so that they are acceptable to regulators and that further animal studies can be avoided.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Regulatory guidelines define the minimum testing requirements for adequate data/statistical analysesfor the majority of assays and the study plans used generally adhere to these guidelines/recommendations. Where no guideline/ recommendations exist, animal numbers are selected on the basis of published literature and/or internal validation data that identify the minimum number of animals required for adequate statistical power.

We will always seek to minimise the use of control groups and multiple dose levels, where this isappropriate.

Where possible we will use the same animal to get as many of the required experimental outputs wecan.

A retrospective assessment of reduction will be due by 10 August 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rodents (either rats or mice) are used because their use is mandated by regulatory bodies who carry out the relevant risk assessments/safety evaluations for the studies in this project. There is considerable experience and background data for the species and studies in this project and the mostrefined methods will be used.

In a few studies we use a transgenic mouse (Muta™Mouse). This is for a specific test (looking atpossible gene mutations) and is to satisfy a global regulatory testing guideline.

Why can't you use animals that are less sentient?

The species used is generally the same as the rodent species used for the general toxicology studies.The toxicology and/or toxicokinetics from the general toxicology studies can then be used to inform dose selection, sample times etc for the genotoxicity tests, thus reducing animal usage. Species selection may also be driven by known absorption, distribution, metabolism or excretion (ADME) differences between rodent species.

The Regulatory test guidelines require the use of young adult animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Studies are performed in a stepwise manner, starting with preliminary studies using small numbers ofanimals where there is limited information, a so called 'pilot study'. This gives the highest prospect ofrefining and optimising the programme e.g. by optimising specific doses of substances given to achieve the desired scientific endpoints in the main study and also in consequence minimising the pain, suffering, distress or lasting harm for the animals used on study.

All animals are regularly monitored for signs of any adverse effects on their health or wellbeing, and to prevent unnecessary suffering, early pre-determined humane end-points are applied under appropriateveterinary guidance (e.g. modification/withdrawal of treatment with the test item, or humane killing of affected animals).

Veterinary surgeons are on hand at all times to give welfare advice on any concerns seen by technicalstaff looking after the animals.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

ICH (2011) EMA/CHMP/ICH/126642/2008. Guideline S2(R1): Guidance on Genotoxicity Testing andData Interpretation for Pharmaceuticals Intended for Human Use.

ICH (2018) EMA/CHMP/ICH/83812/2013. Guideline M7(R1). Assessment and Control of DNA Reactive(Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk.

OECD Guideline For The Testing Of Chemicals (2016): In Vivo Mammalian Alkaline Comet Assay, TestGuideline 489

EC Commission Regulation No. 2017/735. Method B.62: Mutagenicity – In Vivo Mammalian AlkalineComet Assay. OJ L 112/180.

OECD Guideline for the Testing of Chemicals. (2016) Genetic Toxicology: Mammalian ErythrocyteMicronucleus Test, Guideline 474.
EC Commission Regulation No. 2017/735. Method B.12: Mutagenicity - In vivo Mammalian Erythrocyte Micronucleus Test. OJ L 112/54

US EPA (1998) Health Effects Test Guidelines; OPPTS 870.5395 Mammalian Erythrocyte Micro-nucleus Test. EPA 712-C-98-226.

US FDA (Redbook 2000), Toxicological Principles for the Safety Assessment of Food Ingredients.
IV.C.1.d. Mammalian Erythrocyte Micronucleus Test.

Japanese Ministry of Agriculture, Forestry and Fisheries. Test Data for Registration of Agricultural Chemicals, 12 Nohsan No. 8147, Guideline 2-1-19-3, Agricultural Production Bureau, November 24,
2000.

Japanese Ministry of Health and Welfare. Evaluation and Licensing Division, Pharmaceutical andMedical Safety Bureau, Notification No. 1604, 1 November 1999.

OECD Test No. 475: Mammalian Bone Marrow Chromosome Aberration Test (2016).
OECD Test 488 Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays (2022).
For blood sampling and dosing then the following guidelines/literature will be used:
First report of the BVA/FRAME/RSPCA/UFAW joint working group on refinement, Laboratory Animals,27, 1-22 (1993).

A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routesand Volumes, Journal of Applied Toxicology, 21, 15-23 (2001).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Through the regular review of non-animal alternative developments / resources, attendance ofscientific conferences and animal welfare forums and reviews of scientific literature.

A retrospective assessment of refinement will be due by 10 August 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved forfuture work of this kind? During the project, how did you minimise harm to the animals?

THE REGULATION OF CORTICAL ACTIVITY

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Epilepsy, seizures, cortex, neurons, optogenetics

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged
Rats	neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

We aim to improve our understanding of how activity is processed in the cortex of the brain through different stages of life, how information flows through different sub-areas within the cortex, and how, in certain pathological situations, activity patterns change. A critical element of these studies is to characterise and understand how the various brain states, including pathological ones such as seizures (the defining symptom of epilepsy), differ from each other. A key aim of great clinical relevance is to understand the different types of epileptic activity, with the aim ultimately of finding waysto treat this condition.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.
Why is it important to undertake this work?

The World Health Organisation has estimated that over 50 million people worldwide are

living with epilepsy. This is a condition that impacts greatly upon people's lives. It is associated with raised mortality and morbidity. Currently the treatments available to us are inadequate, and many of thesepeople continue to suffer repeated seizures.

What outputs do you think you will see at the end of this project?

This work will greatly expand our understanding of the different functional states of the brain, withparticular relevance to how epileptic seizures develop. Two particular areas of interest are the following:

how the various cortical areas differ with respect to seizure susceptibility. This research will provideinsight into how seizures arise, and which routes they then progress through the brain, which will help guide the development of targeted surgical and medical therapies. For instance, by characterizing the expression patterns of critical proteins expressed in areas that are highly susceptible to seizure invasion, we might identify novel drug targets. The characterization of cortical areas is an enormous task, involving not just our lab, but many world-wide, and will be ongoing at the end of this project. However, we will publish interim reports of our advances regularly, as our work progresses, in peer-review journals.

how brain state varies through the daily cycle of rest / awake, and how this influences the epilepticstate. This work will shed light on what are the critical brain state factors that lead to seizures. We now know that virtually all people with epilepsy tend to experience seizures at certain relatively well defined times, and many people experience short clusters of seizures, yet the treatment is to take powerful drugs, that have many side-effects, continually. Improved understanding of the cyclical natureof epilepsy will help clinicians improve their management, will lead to better designed drug trials to takeaccount of this critical confounding factor, and this work, too, is expected to reveal novel targets for drugs.

Finally, we are investigating new ways of treating the condition, which might involve new medicines, and also technological solutions, including brain-machine interfaces, which work by using light or electrical stimulation to modulate brain activity. Again, we publish our ongoing work at regular intervalsthroughout the project.

Who or what will benefit from these outputs, and how?

Ultimately, the people we want to help are those living with epilepsy, their families and their carers.This will not be fully realised within the time frame of the project, although there will be continual incremental outputs, throughout. Our work seeks to develop new medicines and medical devices,which will also benefit Biopharma and associated industries. Finally, research will teach us fundamental things about how the brain works, and so will benefit anyone who is interested in howtheir brains work.

How will you look to maximise the outputs of this work?

Over many years, we have developed an extensive network of collaborators, with researchers at otheruniversities in the UK, in North America and across Europe. We will present our work at international conferences and workshops about epilepsy and related topics, and also participate in local and national outreach events, aimed at informing the wider community, including people with epilepsy and potential industry partners.

Species and numbers of animals expected to be used

- Mice: 6000
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

A large component of my work will involve tissue taken post mortem (living brain slices, and neuronal cultures), but some work can only be done on the whole living animal. The reason is that an epileptic seizure is the most intense form of activity seen in the brain, and it arises through interactions that are shaped by the complex connectivity of the intact brain. There are facets of the condition, that quite simply, cannot be replicated outside of the body. The rodent brain represents a good model of how thehuman brain is likely to experience seizures, and furthermore, the remarkable development of ways to modify the genetic code in mice (transgenic mice) has led to the creation of many different mouse models, incorporating both disease causing genes, and also genes that facilitate experimentation (optogenetics transducers, etc), available to the project. Fewer transgenic rats have been created, soour work will predominantly use mice.

Most of our work will be studying epileptic activation in adult animals. Some of these will have injections as pups, to introduce transgenes that will be used in the later (adult) experiments (e.g.biosensors, optogenetic actuators etc).

Some studies will test brain-machine interfaces –implanted electronic devices for recording and / or controlling brain activity. Mice will only tolerate very small devices, so some of these implant studieswill utilise adult rats, which are more able to tolerate such devices.

An important element of our studies will engage with memory loss associated with epilepsy, and howthis differs from memory loss in old age. For this reason, we will study small numbers of animals thatare aged (12-24 month mice).

Some studies can be performed on cultured neurons, sourced from embryonic animals. From a singlepregnant dam, we may create many neuronal cultures, thus providing a large return for the number of animals used.

Typically, what will be done to an animal used in your project?

The project will involve a range of experimental techniques, mainly using mice. Rats will only be used in cases where implants require the use of a larger animal, or for creation of neuronal cultures, typicallyusing embryonic or early postnatal brains.

Firstly, we will maintain a breeding programme of transgenic animals, most of which are for the purpose of introducing genes to facilitate particular experiments (eg. biosensors of calcium, chloride and pH; optogenetic proteins that allow neurons to be activated or inhibited by shining light on them);note that these genes do not affect the baseline state of the animal. Most of these mice will have a completely disease-free life until they are killed for preparation of the tissue. Some mice, though, willcarry disease causing genetic mutations, so that we can study the natural course of the epileptic condition.

Some mice will have viral vectors injected into the brain to introduce genes of interest (including biomarkers, optogenetics proteins etc), or synthetic dyes and tracers, to allow experimental investigations. Biomarkers and dyes allow the experimenter to visualise cell structure and function, whereas optogenetics proteins allow the activation or inhibition of neurons in isolation, even within aliving animal. The proteins typically need some time to be expressed at sufficient level, and so the actual experiments are done weeks to months after the injections. There are typically no adverse effects during this period, since the transgenes do not affect the animal but simply allow imaging or optogenetic manipulation at the time of the experiment.

In some mice, we will induce an epilepsy, either by injecting an epiletogenic drug, or making a surgicallesion in the brain, or using electrical stimulation ("electrical kindling"). Epilepsy is a chronic condition and these mice will remain epileptic thereafter. These mice will be studied in different ways, includinghaving brain implants (to record their seizures, or to stimulate the brains to treat the condition), cranial windows (to allow brain imaging experiments), receiving experimental drug treatments to modify their condition (either to test treatments, or to exacerbate the disease course - this latter may be done to test hypotheses about the pathological processes), behavioural tests to investigate their interictal (between seizure) state, or to provide brain tissue for post mortem studies. The duration of these experiments is dictated by being able to record a sufficient number of seizures, and then for experiments examining treatment options, to then record a period on the treatment to see if the seizureload has been reduced. These experiments will typically last about 8 weeks, but may on occasions last longer. We will have created a quick reference guide (a "welfare table") for use by the staff who care for the animals, which will enable the team to make rapid judgements in consultation with the vetsand the Named Animal Care and Welfare Officer (NACWO) regarding the treatment or the humane endpoint for the animals.

Additionally we will do behavioural studies, primarily aimed at understanding memory and general welfare (e.g. social behaviour and evidence of chronic pain or discomfort) in epilepsy. We will compareany behaviour deficits with those seen in aging animals.

We will also study normal mice in the same ways, in order to understand the differences betweenepileptic and normal brains.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the transgenic lines are used simply to facilitate experimentation, by expression of biosensorsor optogenetic proteins that have no phenotype (other than for their experimental purpose).

Epileptic animals will experience seizures, with varying severity and frequency. It is very difficult to assess the impact of seizures upon welfare, because they are distressing to witness, but it is importantto take account what we know of human epilepsy: when patients experience tonic-clonic seizures, theyinvariably have no conscious experience of these, or recollection of the event afterwards.
Observations of mouse behaviour immediately after a seizure support this view – animals recovertypically quickly after experiencing seizures, and may look no different from normal within a few minutes.

The mildest types of seizure are very transient (a few seconds), while more severe

epilepsy is characterised by protracted clonic-tonic seizures that can last many minutes. Rarely, a seizure can lead to death (less than 1% of animals with epilepsy). The great majority of seizures terminate naturally, without medical intervention. If an animal experiences seizures which cannot be stopped bymedical interventions, they will be killed humanely.

Some animals will be having their seizures monitored, in which case, we will know exactly how many they have, but in non-monitored animals, since the recovery is complete between seizures, we will alsomonitor general welfare of the animals. Seizures could impact upon social status in a shared living quarters, and the signs of this are best monitored, not by the occurrence of seizures per se, but rather by other metrics, such as weight loss and signs of distress (a full list of specific metrics is tabulated within the protocols). The most severe forms of progressive epilepsy may have associated co-morbidities, including memory loss and other behavioural effects, which in animals may manifest as a failure to thrive. Most epileptic animals though, will not show any other symptoms or signs.

In order to be able to assess treatment options, animals with epilepsy, that is not otherwise impacting on their welfare, may be maintained for many months. In order to be able to assess treatment efficacy(i.e. reductions in seizure frequency), it is necessary to achieve baseline levels of reasonably frequent seizures before the treatment is given; with lower seizure loads, monitoring will need to be done over longer periods to ascertain therapeutic effects. Animals with severe epileptic conditions will be maintained for the periods of epileptic investigation (typically 2-4 months), and for shorter periods, if there is evidence of weight loss (more than 15-20%) or other behavioural markers of distress.

The surgeries to implant cranial recording devices are performed aseptically, using anaesthesia and analgesia. Animals with implants typically recover very quickly after surgery, and beyond the first fewdays, show no behavioural changes or signs of stress. Very rarely, implants may dislodge, or show signs of infection at their margins (less than 5%). In cases where the implants are dislodged, the animal 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 animaltype)?

Transgenic breeding (only mice) – most lines (e.g. Cre-recombinase, fluorescent biosensors, opsinsetc) have no outward phenotype. The only transgenic lines that have a phenotype will be those with genetic epilepsies.

Animals with epilepsy (~1000 animals) will be experiencing seizures, by definition (moderate severity).We do not, however, allow the epilepsy phenotype to progress to a level that would be considered severe. All epilepsy (including in humans) carries a risk of increased sudden mortality, but this is estimated at less than 1% per year.

Rodents with cranial implants (~500 animals) / injections (~1000 animals) - moderate. The lightweight implants are well tolerated, and animals should not ordinarily show any adverse effects other than transiently as they recover from surgery (usually animals are behaving normally within 48hrs of cranial surgery).Very rarely (less than 1%) animals will experience neurological symptoms (limb weakness, movement disorders) caused by intracranial damage or bleeding at the time of the implant / injection. Intracranial surgeries in higher

animals do occasionally result in focal neurological deficits, but we havenot seen such deficits in our rodent studies.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Epileptic seizures are amongst the most complex disease conditions known, since they involve the combined activation of millions of interacting neurons. Our understanding of the cortical networks involved is still quite primitive, and we remain far from being able to simulate this using any artificial system (neuronal cultures or computer simulations). We need to study what happens at the single cell level during a seizure, and as well as at the level of large brain-wide networks, through the interactionsof neurons with each other, and with supporting cells (e.g. glia, immune cells etc).

Which non-animal alternatives did you consider for use in this project?

Some cellular neuroscientific questions may be addressed using cell culture techniques. We also collaborate actively with clinicians, to apply our knowledge gleaned from mouse work, to understandhuman clinical recordings, made from implanted electrodes in patients before they undergo epilepsysurgery. These recordings provide validation of the animal work, but also a means of translating ourfindings directly to clinical practice.

We will also explore our ideas further using computer modelling studies to refine our understanding ofthe network consequences of the cellular pathologies.

We will conduct parallel analyses of human epileptic recordings with our clinical collaborators, includingboth clinical EEG, and also studies of neurosurgical resected tissue.

Why were they not suitable?

Cell culture models are limited because certain patterns of brain activity, including seizures, arise as so-called "emergent properties" - that is to say, they arise because of how the individual elements areconnected together, and cannot be predicted easily without studying the elements (the individual neurons) in their natural environment (in real brain networks).

Computational models, while reducing the number of experiments that need to be performed, still depend upon biologically realistic data that can only be obtained from the experiments outlined in thisproject. They are important however for the refinement of the project analyses.

Clinical human data are typically extracellular recordings (no cellular imaging or

intracellular recordings), and invariably are “passive” (i.e. no manipulation of the neuronal activity), so are limited intheir extent, despite their evident importance for clinical translation of our knowledge. The recordings of resected tissue allow additional intracellular recordings, but are very small in size, so one cannot use these to study brain-wide network interactions. Furthermore, there is no natural expression of the genetically encoded biosensors and actuators, which have proved so useful for experimentation in animals (note: biosensors are fluorescent proteins that can be used to get a read-out of physiological processes in cells; actuators are proteins that can be used to manipulate neuronal and cellular activity.Both are not found naturally, but have been incorporated into experimental animal models (e.g. "transgenic" mice), but this cannot be done in humans for obvious ethical reasons).

We must stress that we use all these methodologies routinely – neuronal cultures from both animal andhuman source, computational modelling, studies of resected human brain tissue, and human clinical recordings – but it is important to understand their limitations, and that as such, these methodologiessupplement, rather than substitute, our animal studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These estimates are made in part from our experience of managing mouse and rat colonies on previous licences, and in part from projections of the key experiments. There will be multiple researchers working under this project licence (we have recently been awarded funds for an epilepsyresearch doctoral training programme, with 6 studentships, and additionally have multiple funded research grants aligned with this work) and the work involved will use a variety of transgenic mouse lines. The work within this licence is multifaceted, but we base our estimates upon the overall programme of work, and our prior experience of the minimal number of animals needed in each experimental group to provide statistically robust outcomes. Group sizes are constantly being re- evaluated and updated when necessary.

Breeding programmes of such mouse lines, inevitably leads to an excess production of mice beyond what is required for experimentation, since some animals will be unsuitable for use in experiments because they do not carry the gene of interest (e.g. heterozygotes in some instances) and when newlines are being generated – we will at all times work closely with the colony managers. We have accrued considerable experience in recent years, and have recently reduced the numbers of differentlines, and will endeavour to rationalise project management so that projects using the same mouse lines are aligned, to optimise their usage.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

For all new experimental work, we will perform pilot data collection, using 3-6 animals, and

perform power analyses on these initial data sets to estimate the minimal number of animals that will yield a well powered statistical test of the hypothesis. We will use these pilot studies, in tandem with experimental design planning tools (e.g. the NC3Rs' Experimental Design Assistant), to optimise thefollow-up studies.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Where specific transgenic lines are to be used, we will work with the colony manager, and across theresearch team, to coordinate the different studies that use the same lines, so that we use the mice asefficiently as possible and avoid excess breeding. Mouse lines will be maintained for the minimumperiod of time to complete the specific study.

All rats used on the licence will be wild-type adult animals that will be purchased from outsidesuppliers, only as 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use a variety of models of altered neuronal excitability, including both whole animals and also brain slices and neuronal cultures. Starting first with these latter models, the tissue is sourced from animals, but in most cases, these animals are wild-type in phenotype, although many will carry transgenes, such as optogenetic proteins, that are phenotypically neutral but which facilitate experimental studies. The brain slice preparations preserve many local neuronal connections, and importantly, sustain epileptic activity that looks remarkably like electrographic recordings from human patients. Indeed, study of brain slices have provided many important insights into epileptic pathophysiology. Since the mice have a completely disease free life until they are killed for preparationof the tissue, this represents a refined tool for studying the brain.

Where we need to introduce transgenes to facilitate experimentation, we will use breeding programmes if the appropriate transgenic animal exists (e.g. mice with Cre-recombinase cross bred with animals with floxed biosensors (eg. Ca2+ sensors) and actuators (eg channelrhodpsin). In othercases, we will need to introduce these genes, by injecting viral vectors.

For all surgeries, anaesthesia, and analgesia, we will use the most up-to-date procedures and will dothis in consultation with the veterinarian team.

Where we need to induce neuropathology (e.g. induce active epilepsy in a living animal), in order to study it, we will keep animals for the minimum time to be able to complete the study, when they will beterminated. The most persistent severity level will be for experiments where we are assessing an intervention, since in that case, one needs to

have persistent regular seizures in the control group if one is to assess whether a drug treatment or stimulation paradigm actually reduces this seizure load. In some instances, multiple grouped testing of different interventions may be conducted in parallel, using a single "control" group (this can help reduce the number of epileptic "control" animals required).Additionally, where the same model is used successively, for refinement of an intervention, we will useBayesian statistical methods to improve the power of the testing, so as to keep the animal use to a minimum level.

Why can’t you use animals that are less sentient?

Epilepsy in mammalian brains arises mainly within the neocortex and hippocampal structures. Whileless sentient animals, such as fish and insects, have been used for epilepsy research, they are primarily useful for drug screening assays, but because these lower species lack the same cortical organisation (or even cortices at all, in insects), they are very poor models of the emergent propertiesof cortical networks, which can only be studied in animals with cortices.

The incidence of epilepsy in people shows two peaks, very early in life and rising again in old age.Given the many differences between young, adult, and aging brains (e.g. differences in neuronal plasticity, synaptic function, ionic distributions (e.g. intracellular Cl-)), it is important to study all agegroups.

We will do some studies under terminal anaesthesia, but this may modify epileptic activity, and so likewise, so it is necessary also to study epileptic activity that arises naturally. Additionally we will examine how epilepsy affects memory - an important sequelae of epilepsy - which involves behaviouralstudies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We continually review our methodology, in order to reduce any unnecessary experimental variability, which helps to achieve statistically significant results while minimising the animal use. For instance, over the course of my previous project licence, we have assessed different cements for head implants,and sought external help with these difficult experiments, by visiting other laboratories and inviting visiting experts here. We have additionally examined how different anaesthetic regimes influence key metrics of cortical physiology. We have also adapted our peri-operative management, and further, altered the post-operative housing arrangements to minimise the risk of mice damaging, or even displacing, their head implants. This work is always done with help from the Veterinary team and the support staff within the animal facility. These are just some examples, but we continually review our performance, especially when developing new research techniques and methodologies.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow the published methodologies for the different experimental procedures. Of particular relevance to epilepsy research is the NC3Rs review of the use of animals in this field (Lidster et al, 2015); I was on the committee which co-authored this review. Further guidelines are provided by thefollowing

Code of Practice for Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes

LASA Guidelines

RSPCA Animals in Science guidelines

UFAW Guidelines and Publications

NC3R's and Procedures with Care I will also adhere to local AWERB standards for research animals, and where appropriate, support the development of new local standards for refinements discovered during the project licence. I will consultwith the Colony Manager to review genetic health, breeding practices and overall colony health and management at regular intervals.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

I constantly review best practice in our scientific studies, including regular discussions with all members of our research team and with other scientists both within the university and at other institutions. Additionally, the local Animal Welfare Ethical Review Board, the Named Animal Care andWelfare Officer (NACWO), Named Training and Competency Officer (NTCO), Named Information Officer (NIO) and Veterinary team regularly inform, and disseminate information regarding reduction, replacement and refinement, including new publications of guideline and research articles, and presentations and reports from collaborators, peers, and animal welfare bodies.

During the 1, 3 and 5 year reviews of the project licence, which are internally mandated and done incollaboration with the NACWO, NTCO, NIO and Veterinary team, we will review our work, includinghow we have implemented the 3Rs within the various research projects.

PATHOPHYSIOLOGY OF ACUTE LUNG AND ORGAN 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, ortheir effects, in man, animals or plants

Key words

critical care, physiology, inflammation, respiratory, ventilation

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The primary aim of this project is to better understand the disease processes behind the systemic inflammatory response syndrome and acute respiratory distress syndrome. These studies will allow usto identify possible targets for designing novel treatments for patients.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 systemic inflammatory response syndrome (SIRS) is a body-wide abnormal immune response, which is often fatal if it progresses to cause organ failure or poor organ function. When this occurs in response to infection specifically, this is termed sepsis. Alternatively,

SIRS may develop in response toa variety of ‘sterile’ (i.e. non-infectious) insults including trauma, haemorrhage (blood loss), and burn injury. One of the organs that most commonly fails during either sterile SIRS or sepsis is the lung, leading to what is known as acute respiratory distress syndrome (ARDS). Importantly though, ARDS isnot only caused by SIRS or sepsis, but can also be started by direct insults to the lung, both infectious (e.g. influenza/pneumonia) and sterile ones (e.g. smoke inhalation).

Together, sepsis and ARDS comprise the major cause of deaths within the intensive care unit. Depending on severity, 30-60% of people who develop sepsis and/or ARDS will die. It has been estimated that there are more than 40,000 deaths from sepsis per year in the UK, and ARDS is the main cause of death among patients with pneumonia (including severe COVID-19 infection), so these conditions have major health and economic implications. Unfortunately, few therapeutic options exist to treat them apart from antibiotics (which are no use if the cause is not an infection). Thus, treatment consists mainly of keeping patients alive in intensive care, usually receiving mechanical ventilation, until they either die or recover. To add to this complexity, mechanical ventilation itself is capable of making the situation worse through a process we only partly understand termed ventilator-induced lunginjury (VILI).

What outputs do you think you will see at the end of this project?

Our understanding of the precise mechanisms behind these conditions is still lacking (hence the absence of treatment options). Ultimately, our hope is that these studies will drive the development ofnovel therapeutic strategies in patients. This project represents one step along this path, by the end ofwhich we hope to better understand:

- how cells and organs communicate with each other during SIRS/ARDS
- mechanisms of ventilator-induced injury and inflammation
- pathways of cell death and organ failure during SIRS/ARDS

We anticipate a number of peer-reviewed publications and conference presentations as a result ofthese studies.

Who or what will benefit from these outputs, and how?

The main expected benefit of the current PPL is to improve our basic understanding of the origin anddevelopment of sepsis/SIRS/ARDS. The short term benefits of the work will therefore be a clearer picture of the complex functional changes accompanying sepsis/SIRS/ARDS, to allow us and other researchers worldwide to identify and explore potential novel targets.

In the medium term, within the lifetime of the PPL, we would hope to see some translation of our preclinical findings towards patients, starting by seeing if some of the changes we observe in our animal models are present in patients. This would ensure further confidence in our preclinical dataand potentially aid in identifying which patients may benefit from treatments, for example.

Finally, in the long term the data generated would potentially have direct implications for the treatmentof patients with sepsis/ARDS if we are indeed able to identify novel therapies.

How will you look to maximise the outputs of this work?

Our research team is composed of experienced basic scientists and clinical researchers, and has existing collaborative links with the pharmaceutical industry, and therefore is an ideal composition to exploit preclinical findings. Results would be disseminated in terms of publications and presentations at National and International Conferences. Discussion with clinical colleagues (informally and via moreformal routes such as Grand Rounds) will ensure timely sharing of relevant information.

Species and numbers of animals expected to be used

- Mice: 4920
- Rats: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The adult mouse is the least sentient species in which we can address our research questions. Its genetic make-up is well known, it is physiologically similar to humans, and numerous research tools are available. The use of genetically modified mice is also an invaluable tool, and thus most studies willbe carried out on mice. A small number of rats will also be used where this is necessary, for example where the larger size of the rat enables more efficient collection of samples, or where researchmaterials (e.g. antibodies) are only available for rats.

Typically, what will be done to an animal used in your project?

Most of the animals used within this project would receive either no, or minimal intervention (for example drug treatment) before being exposed under non-recovery anaesthesia to substances such as bacterial lipopolysaccharide (LPS) to mimic the response to infection, or hydrochloric acid (HCl) to mimic the response to a non-infectious injury. Some mice will undergo similar procedures, including administration of substances directly into the lungs under recovery anaesthesia so that the consequences of the injury can develop and be investigated. Animals may also receive substances tomodify the progression of inflammation/injury, which could be delivered directly into the lungs, into a vein, or into the peritoneal cavity (the space surrounding the abdominal organs).

Most experimental procedures carried out in the project would last no more than approximately 6 hoursafter the induction of injury. To explore more slowly developing forms of injury, or the process of organrepair after injury, some mice may continue for up to 30 days after receiving the injurious insult.

In addition, a small number of animals may receive altered diets for a number of months to induceobesity.

At the end of experiments animals will either be killed for tissue sampling or will be placed under non-recovery anaesthesia and surgically instrumented for mechanical ventilation and physiological measurements. A small number of animals which have not received any other form of injury will be exposed to a controlled burn injury under non-recovery anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Sepsis/SIRS and ARDS are associated with major deteriorations in normal bodily functions. Currently,there are no specific ways to confirm that ARDS or SIRS has been 'induced' other than signs of impaired organ function. ARDS for example is essentially defined by impaired lung function and decreasing oxygen in the blood, and published guidelines state that valid models should be able to induce these changes (i.e., if the model does not include signs of impaired lung function it is not truly amodel of ARDS). Therefore, it is somewhat inevitable that our animal models must have the potential to induce some signs of distress. These would include various signs of illness including reduced mobility, loss of appetite, weight loss and rapid breathing.

As far as possible we will design protocols in which such symptoms are minimised while still allowing our scientific goals to be reached. However, in some experiments, particularly those in which potentialtreatments are being evaluated, or where we are investigating how the lungs repair after injury, it is necessary that models incorporate such ‘clinical symptoms’. Most adverse effects would be expected to last no more than 24 hours. After this animals may continue to lose weight for up to 72 hours (whileshowing no other clinical signs) before body weight begins to recover. We have protocols in place which include refinements of the environment for animal comfort, such as keeping animals in a warmed chamber after induction of injury, to ensure that animals experience as little distress as is compatible with the necessary goals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

In this licence 100% of rats would fall in the non-recovery category. We expect that ~25% of micewould fall within the 'non-recovery' category, ~50% within the 'mild' category, and ~25% within the'moderate' category.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

At present, it is still impossible to produce a complete non-animal replica of such complex biologicalprocesses as SIRS or ARDS. Complicated interactions occur between different organs and the immune system which cannot be modelled without using animals. Where possible we will ask questions in cell-culture based systems, but ultimately any findings from these need to be confirmedusing animals.

Which non-animal alternatives did you consider for use in this project?

We will be using a number of non-animal approaches as part of our overall research program to address specific questions. These will include cell cultures using commercially available cell lines and cells recovered from volunteer humans and naive animals (animals that have not been injured or treated in any way). These cells will be grown and exposed to challenges using a range of increasinglycomplex scenarios, from single types of cells grown on their own (monoculture) to different types of cells grown together (co-culture) to explore how they may interact. Sometimes we will study cells grown in simple plates, and sometimes we will expose cells to fluid movement and cell stretch, conditions which are more like the environment they would be exposed to in the body. For some research questions we will use organs isolated from the body, which can act as an intermediate type ofexperiment between cells and whole animals. But ultimately none of these can answer our research questions on their own.

Why were they not suitable?

Sepsis/SIRS and ARDS are highly complex syndromes involving interactions between various organsand the immune system. For example, an insult to the lungs (such as pneumonia) may lead to injury to the kidneys. This seems to mainly occur when lungs are being ventilated and happens via the immune system. As another example, an insult outside of the lungs (such as a burn injury) may causeproblems inside the lungs. Until we understand exactly how these numerous complex interactions between systems occur, we cannot reliably or accurately model them in simple non-animal systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 substantial experience with most protocols within this project, which allows us to predict withsome accuracy the number of animals required to detect statistical differences. While we cannot predict the precise direction that our studies may take, the numbers quoted reflect what we believe to be the maximum for each protocol.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Experiments will be designed in such a way as to minimise the number of animals required to achieve our scientific goals, e.g. by careful consideration of appropriate control groups. Pilot studies and information from published literature will be/have been used to guide experimental design. We will alsoutilise our own extensive experience and tools such as the NC3Rs Experimental Design Assistant as appropriate.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

For any breeding of genetically modified mice that we carry out, we will do this as efficiently as possible, for example reducing the number of breeding pairs as much as

possible to avoid excess. During breeding it is very common to find that not all animals born inherit the genetic make-up required. Instead of discarding such animals as surplus to requirements they will either be used as controls (to compare with those animals that do possess the required genetic modification), or offered to other researchers. We will also carry out genotyping to identify the genetic make-up in house, whichis more efficient as it does not rely on accumulating large numbers to be sent for analysis. We will alsomaximise the amount of samples/information we can take from each animal, and store samples that are not immediately required. Finally, small pilot studies will be carried out to identify timings of endpoints, doses of substances to be administered etc.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

ARDS/sepsis/SIRS are essentially umbrella terms describing syndromes with common symptoms,rather than specific diseases. There are multiple different causes of ARDS for example which mayhave different mechanisms of initiation and progression. Just as there are multiple causes of disease/injury in humans, so there are multiple different experimental models, each mimicking particular aspects of the 'disease', and no model reflects human ARDS/SIRS/sepsis completely.
Importantly the research field places a strong emphasis on the comparison of multiple different models,both a) to evaluate whether biological processes/interventions have the potential to be effective regardless of the underlying cause of injury, and b) because identifying differences between models can lead to new understanding of mechanisms.

In the current project we will focus on a number of well-established models which can explore differentaspects of the disorders, with which we have substantial prior experience. These will include:

models of direct lung injury mimicking aspects of infection, injury and repair, and fibrosis (where therepair process goes wrong and tissues develop scarring)

models of SIRS and indirect lung injury, mimicking aspects of body-wide inflammation and lungdysfunction.

Crucially, we will not be utilising models of ARDS/sepsis/SIRS that involve either recovery surgery to induce injury or administration of live infectious agents. Rather, our models utilise dosing of non- infectious material to bring about host responses, as it is these host responses that actually cause thechanges in organ function. The models are a) less liable to unwanted effects due to unintentional or poorly controlled infection, b) more predictable in terms of severity and timing of onset and recovery and c) more controllable by manipulating doses. While they may not mimic all aspects of the diseaseprocess in humans, we believe they are the most controllable and thus most refined, inducing predictable degrees of injury in a relatively reproducible way.

Why can't you use animals that are less sentient?

The mouse is genetically well characterised, physiologically and immunologically like humans, and numerous research tools (antibodies, reagents etc) are available. Moreover, the use of genetically modified mice is an invaluable tool to dissect out the roles of genes of interest. Thus, the mouse is theleast sentient species in which we can address the questions raised in this Project. While many experimental studies in this project can (and will) be carried out on terminally anaesthetised mice, it can take time (days to weeks) for problems with organ function to become apparent in response to injury, and for repair to take place. For these studies it will be necessary for animals to be conscious, or to regain consciousness, after administration of agents to induce injury.

A small number of rats will be used, but not in studies in which there is induction of injury beforeterminal anaesthesia.

While less sentient animals such as zebrafish have been used to explore particular aspects of inflammation/immunity, which may eventually provide useful information relevant to SIRS for example,a crucial aspect of the current project is to explore the influence of mechanical ventilation on the immune system and organ function. The zebrafish (and other lower animals) is thus not sufficiently physiologically similar to humans to explore the questions raised within this project.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

For those models in which injury to the lungs or other organs needs to develop over days to weeks, wewill take various steps to minimise discomfort. These will include close monitoring, terminating experiments as early as is consistent with scientific goals, and options of administering pain-killing drugs, delivery of soft food, and maintaining animals within 'supportive' environmental conditions (e.g. warming and increased atmospheric oxygen). These are designed to allow organ injury to develop while the animal's wellbeing is less affected than it would be in the general environment.

In addition, within this project we intend to explore the potential use of oropharyngeal dosing as a lessstressful alternative to intratracheal dosing. Intratracheal dosing is a highly targeted approach, but requires relatively deep anaesthesia and has the potential to irritate the larynx and trachea. Intranasaldosing is commonly used (including by us) as an alternative method to introduce agents into the lungswhich requires less anaesthesia and tracheal manipulation, but it has been suggested that the rich blood supply of the nasal mucosa may lead to more systemic effects than intratracheal dosing.
Oropharyngeal dosing may potentially avoid pitfalls from each of the other techniques and therefore bea more refined way to introduce agents into the lungs.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow the ARRIVE guidelines for conducting and reporting experiments, plus publishedguidelines for animal models of ARDS/SIRS/sepsis.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

By following NC3Rs news and subscribing to newsletter; communication with colleagues in the research field and NACWOs, participation/attendance at relevant symposia/webinars, reading relevantarticles on the NC3Rs Gateway etc.

Home Office

NEUROBEHAVIOURAL MECHANISMS UNDERLYING MOOD, IMPULSIVE AND COMPULSIVE MENTAL HEALTH DISORDERS

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Behavioural neuroscience, Mental health disorders, Memory, Brain, Individual differences

Animal types	Life stages
Rats	pregnant, adult, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

To determine the neurobiological, neurochemical and molecular bases of psychological processes thatvary across the population and can go awry in mental health disorders.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Mental health disorders are a major health burden, both in the UK and worldwide. The cost of mental health disorders to the UK in terms of social, economic and health factors is extremely high. Although treatments for mental health disorders exist, currently available

treatments need substantial improvement, as they do not work for all patients. Treatment development for mental health disorders is a long-term goal for my laboratory and that of our collaborators, and we believe that treatment development will be facilitated by a better understanding of the psychological, neural and neurochemical mechanisms that underlie psychological processes that can become maladaptive or dysfunctional in mental health disorders. By understanding individual biological and environmental riskfactors, it might be possible to identify those at greatest risk of developing mental health disorders, to give them access to preventative treatments (or at least access to treatments sooner). By characterising the individual psychological profiles associated with mental health disorders, it might also be possible to identify patient 'subtypes' would benefit more from specific treatments, allowing better treatment targeting to the individual.

What outputs do you think you will see at the end of this project?

The primary output from this project will be the generation of new information about how psychological,neurobiological and neurochemical processes that are relevant to mental health disorders go awry.

These will be communicated mostly through publication in scientific journals and presentation at scientific conferences, but we are also committed to communicating our findings more widely with thegeneral public.

Who or what will benefit from these outputs, and how?

We expect our research to benefit the scientific community in the short to medium term, and in thelonger term to benefit people living with mental health disorders through the development of new treatments.

In the short term, we expect to gain insight into potential drug treatments for the mental health disorders of interest (mood, impulsive and compulsive disorders). This information can be used byother researchers to test these drug treatments in small-scale and, in the longer term, large-scale studies in humans.

We also expect, in the medium term, that our research will lead to the development of new behaviouraltreatments. The animal research allows these to be developed with an understanding of how these behavioural therapies can lead to changes in the brain, so that when they are translated to humans, we have a better understanding of how they work. We actively collaborate with researchers studying human participants and patient groups to ensure that our behavioural tasks work well and measure thesame processes in humans and non-human animals.

In the longer term, understanding the risk factors that predict whether individuals are more vulnerableto developing mental health disorders may allow early interventions to be made for these people.

Furthermore, it may allow for the development of personalised treatments based upon the underlyingdifferences in psychological and neurobiological functioning.

How will you look to maximise the outputs of this work?

We are involved in collaborations with pharmaceutical companies, which will support the impact of ourresearch, particularly with respect to treatment development.

I have a strong track record of publication and of speaking at scientific meetings, which will publicise the work. We are also fully committed to open science, and since 2017 we have made all supporting data for our publications freely available on the university repository, for other researchers to use as citable datasets. We also publish reliable null results, so that we contribute to a balanced interpretationof the scientific literature.

Species and numbers of animals expected to be used

- Rats: 4075

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will mostly be using adult rats. For some experiments, in which we are studying the impact of earlylife stress on behaviour in adulthood, we will use pregnant rats and their offspring so that we can control early life experience. As we are interested in adult behaviour, the majority of our behavioural tests will be conducted in adult animals. However, as one of our objectives is to predict which individuals are at risk of developing mental health disorders, for some experiments we will take
measurements (e.g. MRI images of the brain) in juvenile animals so that we can relate any differencesin their adult behaviour to their previous brain development.

Typically, what will be done to an animal used in your project?

A maximum of 4075 rats will be used for this project. The bulk of our experiments focus on measuringthe performance of rats engaged in complex behavioural tasks, motivated by rewarding stimuli. Depending on the complexity of the task, training duration ranges from a few days to several months ofdaily training (typically 5 days per week, but sometimes daily). A subset of experiments (approximately 25%) will involve phases in which aversive stimuli are used (e.g. nausea from a lithium chloride injection or exposure to avoidable electric footshock). Rats typically receive injections of drugs to modulate the function of the nervous system so that we can assess the impact on behaviour. Often rats will receive different doses of drugs or different types of drugs, so that we can compare the effectsof the drugs on the individual rats' behavioural performance. Typically, these types of experiment do not exceed 6 months in duration.
Approximately half (~45%) of rats will undergo surgical procedures in addition to behavioural testing. These procedures are necessary to allow us to directly record the activity of the brain, to deliver drugsdirectly to specific brain regions, or to experimentally control the activity of specific types of brain cell (either increasing or decreasing activity).

Approximately 12.5% of rats will undergo manipulations of early life stress, through the administration of drugs to the pregnant mother (e.g. to model stress during pregnancy) and/or periods of separation from the mother during early life (to model early life adversity). Early life stress is a prominent risk factor for developing mental health disorders in humans, and these experiments allow us to model thisin rats. We perform these manipulations to test the impact of early life stress on the behaviour of the offspring as adults.

Approximately 10% of rats will undergo brain imaging in addition to behavioural testing. The rats experience brain imaging under anaesthesia, and may undergo more than one scan, including somescans as juveniles. These scans allow us to measure brain changes between rats showing different types of behaviour, and the repeated scans allow us to track this across development.

A small percentage (~5%) of rats will be used to validate our brain manipulations (e.g. to check thatmanipulations are selective to the right type of brain cell). Approximately 1.5% of these rats will undergo surgery without recovery from anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the procedures will produce (at most) transient distress and no lasting harm. Extended or excessive distress would lead us to stop the experiment and either humanely kill the animal, or seektreatment under veterinary advice.

Some surgical procedures can lead to a short period of weight loss in rats, but this typically recovers within 10 days. Some of the drugs that we give to the rats may cause short-term side effects (e.g. hyperactivity) but most of the time the subtle effects of these drugs can only be seen on our sensitivebehavioural measures.

Early life stress produces extended (i.e. throughout the lifespan) effects on behaviour, but these are typically only measurable on our sensitive behavioural tasks and do not involve long-term changes inhome cage behaviour.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Non-recovery: 1.5%

Mild: 16%

Moderate: 82.5%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

- We need to use animals to achieve our research aims and objectives because:
- We need to manipulate early life experiences and determine individual predispositions that makeindividuals more likely to develop mental health disorders

- We need to understand the fundamental biological differences in the brains between individualswith and without mental health disorders
- We aim to develop new treatments for individuals with mental health disorders.

Our aims and objectives require long-term study of behaving individuals, where we have experimentalcontrol over their life experiences and can study both brain and behaviour.

Which non-animal alternatives did you consider for use in this project?

Humans, cell culture, and computational models.

Why were they not suitable?

Human behavioural studies are useful for some of our objectives, and we aim to design experiments where the behaviours can be directly compared between humans and animals (i.e. 'translational' and'backtranslational' research). However, we cannot fully understand the biological differences betweenthose with and without mental health disorders in humans, as human studies are limited to correlational approaches (e.g. brain imaging) when we need to understand causal mechanisms.
Furthermore, it would not be ethical (or possible) to conduct studies in humans where individuals aredeliberately put at risk of developing mental health disorders (e.g. early life stress studies).

Cell culture studies can be helpful for understanding fundamental biology, but they do not link readily tothe behavioural measures in which we are most interested.

Computational models do not yet fully capture the behaviours that are relevant to our research questions, because the models are not sufficiently advanced. They do not capture individual variation or early life environmental differences. However, we are beginning to use computational models to giveus better insight into the behaviour of each animal, and to increase the amount of data we produce peranimal in each experiment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals have been estimated by referring to the numbers used on my previous ProjectLicence, the numbers required for our ongoing funded research, and projections based on future funding at similar levels. These experiments have been planned following power analyses, either usingeffect sizes from previously published literature or from pilot data in the lab.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

I am well-trained in experimental design and statistics, and where additional expertise in

necessary I have support from statisticians within my institution. Our statistical analyses are determined during theexperimental design phase, and where possible we design experiments to allow us to measure within-subjects effects in addition to between-subject effects, to give us greater statistical power.

We refer to the PREPARE guidelines in designing experiments, with the design of each experiment checked by multiple researchers (including the Project Licence, PPL, Holder) and the Named AnimalCare and Welfare Officer (NACWO), to confirm that the animal facility is capable of supporting the experiment. We report our experiments in accordance with the ARRIVE 2.0 guidelines, and make ourdata openly available to other researchers by publishing datasets on our institutional repository at thetime of manuscript publication.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Where appropriate, we conduct pilot experiments to determine effect sizes, allowing us to perform power analyses where effect sizes are not available in the previous literature. We also design our experiments to collect the maximum number of behavioural measures from individual animals, including control measures to allow us to interpret our experimental effects in context. Our behaviouralmeasures are usually collected automatically on computer, allowing us to interrogate rich behavioural datasets for the animals.

We usually collect brains from the animals at the end of our experiments, to allow for further *post mortem* analyses that can be related to the individual behavioural data. We also participate in a tissue-sharing initiative run by our institution, to allow other researchers to access any unused tissues from our animals at the end of the experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use rats because they are the least sentient species that can model the behaviours, affect and cognitions relevant to the mental health disorders of interest. We do not attempt to model a mental health disorder 'in full' in the rats, but rather model specific psychological processes that have relevance to mental health disorders. This approach, called 'dimensional psychiatry', aims to understand the key processes that go awry in mental health disorders. These dysfunctional processescan be shared across different mental health disorders in humans, with different combinations of dysfunctional processes producing *different* mental health disorders. We take this approach to understanding mental health disorders, by studying these psychological processes in rats, often using behavioural tasks that can be used in both humans and rats (i.e. the tasks are both 'translational' and 'backtranslational').

As our behavioural tasks often require extensive training due to their complexity, we have both scientific and ethical reasons to maintain high standards of animal welfare. We are also committed, inour task development, to refining existing behavioural procedures and have previously published this work.

Why can't you use animals that are less sentient?

Our research relies mostly on sophisticated behavioural testing. The behavioural tasks that we use arereadily acquired by rats, but would be extremely challenging for mice. The majority of our experiments test complex behaviour in adult animals, and we only use neonatal and juvenile rats where this is necessary (e.g. in early life stress procedures). We could not conduct our behavioural experiments at amore immature life stage, because neonatal animals would not be capable of performing these tasks, which often take weeks to train.

We test that new experimental techniques are working as they should in unconscious animals whenever possible (1.5% of our experiments are conducted under non-recovery anaesthesia for thisreason) and when this is not possible (e.g. because the brain manipulation takes time to mature) wevalidate the procedures in animals in much shorter (typically 1-4 weeks) pilot experiments before committing to a full behavioural experiment.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The bulk of our experimental work relies upon sophisticated behavioural analysis, which typically involves daily contact (at least 5 days per week) with the animals. Any illness or pain would compromise the ongoing behaviour of the rats, giving us a sensitive measure of any welfare issues,often before any clinical observation could detect changes.

Where animals have undergone surgery, they are provided with pre-operative, peri-operative and post-operative pain relief. Post-operative pain relief is administered for several days (typically 3) following surgery, and if signs of pain are noted while rats are undergoing increased post-operative monitoring, this is continued for up to 5 days.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

There are no specific best practice guidelines for the specific behavioural procedures used on this project (many of which have been designed or refined for use in my laboratory). We refer to more general best practice guidelines provided by the NC3Rs, LASA, FELASA and other learned societies(e.g. the LASA/BAP/BNA/ESSWAP Guiding Principles for Behavioural Laboratory Animal Science), including for surgical procedures and other procedures such as blood sampling and administration ofsubstances.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

I subscribe to the N3CRs newsletter and my institution's 3Rs mailing list. I also actively participate in3Rs sessions at conferences (e.g. at the British Association for Psychopharmacology Summer Meeting).

Relevant guidelines are made available to all researchers working on this project through an onlinerepository, and are uploaded by the PPL Holder as they become available.

RETROVIRUS-IMMUNE SYSTEM INTERACTION IN MICE

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

innate immunity, adaptive immunity, virus infection, endogenous retroelement, cancer

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

Our aim is to study the various molecules and mechanisms, which trigger the immune response to achieve long-term protection from viruses, and understand the broader interaction between virusesand the human host.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Infectious diseases are a leading cause of death worldwide, and are increasing in almost every nation.Infection also directly cause 1 in every 5 cancers. They are also among the biggest disablers.
Protection against infection by viruses and other pathogens as well as the efficacy of vaccination crucially depend on appropriate activation of the immune system, a complex

and vital network of cellsand organs that fight invading pathogens. An understanding of these molecular pathways is essentialfor the design of vaccines for prevention and intervention in viral infections and cancer.

Host-pathogen co-adaptation has inevitably led to the evolution of many host resistance mechanisms,both innate and adaptive. Adaptive immunity is arguably a highly protective arm of host defence against both infection and cancer. Another important property of adaptive immunity is that it has memory, which forms the basis for vaccination. Vaccination is by far the most effective prevention strategy that has led to celebrated examples of pathogen eradication (e.g. Smallpox). However, vaccines against many chronic viral infections or cancer are still lacking. In these cases, natural infection or disease does not elicit an efficient enough immune response and, therefore, vaccination should aim to induce a quantitatively and/or qualitatively better response. This has not been achievedto date and further research into the basic aspects of immune-mediated protection, vaccination and immunological memory is needed.

Retroviral infection is an ideal tool to study host-pathogen interaction. Retroviruses are significant human pathogens with human immunodeficiency virus (HIV) and human T-lymphotropic virus (HTLV)currently infecting 30 and 15 million people, causing AIDS and leukaemias, respectively. Retrovirusesare also a significant part of our genetic constitution. Endogenous retroviruses (ERVs) make up 5% ofour genome and belong to a larger group of retroelements (REs) that collectively occupy 43% of our genome. These are genomic parasites that are replicating in our DNA to the extent that our genome has accumulated over 4 million copies. Comparably vast numbers of ERVs/REs are found in all other mammalian genomes examined, signifying considerable retrovirus-host co-evolution.

Furthermore, ERVs/REs have great potential to influence immune reactivity against unrelated immune challenges. There are multiple pathways and mechanisms by which ERVs may affect immune cell development and function. One such mechanism is whereby the activation of ERVs is perceived by theimmune system as a genuine viral infection. This triggers an antiviral interferon (IFN) response and general immune activation that inevitably heightens immune reactivity to unrelated antigens. By mimicking viral infection, ERVs may provide the necessary ‘intrinsic adjuvant’, an extra boost to potentiate the immune response. In addition to nucleic acids, certain proteins from ERVs have profound effects on immune reactivity. Thus, ERVs have a great potential to directly or indirectly influence host immunity, which warrants investigation of complete immune system development andfunction in response to a variety of other infectious challenges.

Another significant effect of ERVs on host biology is susceptibility and response to cancer. The documented ability of infectious retroviruses to cause cancer has led to the discovery of ERVs. Given the ability of ERVs and other retroelements to move in the genome, it is perhaps anticipated that these‘genomic parasites’ have been incriminated in the pathological processes leading to cancer, which is also viewed as a ‘genomic disease’. Understanding the nature of ERV association with cancer and elucidating the precise mechanisms underlying any such association will be particularly important.

Furthermore, adaptive immunity to ERV-encoded antigens is also relevant as it targets potentially cancer-specific antigens. ERVs are generally suppressed and controlled in healthy cells. However, thiscontrol is often lost in cancer, as part of overall dysregulation of gene expression during transformation.In turn, certain ERVs are reactivated specifically in cancer cells and begin to produce retroviral proteinsand other aberrant proteins, not normally found in healthy cells. These cancer-associated proteins derived from ERVs can be recognised by the immune system to attack cancer cells, and this will be carefully

examined in relevant mouse cancer models.

What outputs do you think you will see at the end of this project?

This project will support the discovery of novel virus-immune system interactions and the development of advanced methodologies for analysis of immune system function. It is therefore expected to generate both viral basic knowledge and experimental tools for the wider community. A longer-term aimalso supported by this project is the development of immune modulating therapies and vaccines, validated in the proposed animal models for further testing in clinical trials.

Who or what will benefit from these outputs, and how?

This project is expected to improve our understanding of the mechanisms that regulate the immune response during infection, autoimmunity and cancer. It should help with the development of strategiesthat improve immune therapies or inhibit immune pathologies. In collaboration with clinical scientists, this knowledge should help in the design of intervention therapies in multiple diseases where the immune system plays a major role. In addition, the proposed programme of research is to provide thebasic immunological foundation upon which rational vaccine design can be based. The potential benefit is an improved understanding of the immune response to persistent pathogens, such as retroviruses, as well as endogenous retroviruses. The knowledge gained from the vaccine and anti- retroviral experiments may be directly transferable to human studies or trials. The results of the research carried out under this project will be disseminated through publication in high quality peer reviewed journals and at meetings. Novel mouse strains created as part of this project will be transferred to other projects and reagents (including advanced methodologies for assessing immune system function) will be made available to the scientific community.

How will you look to maximise the outputs of this work?

Basic knowledge gained as part of this project and new discoveries will be rapidly disseminated in the form of peer-reviewed publications. Dissemination will also be accelerated by depositing the findings on pre-print servers and by presenting at conferences. The raw data will be made publicly available forwider use. Negative results will also be communicated (in publishing platforms such as the Wellcome Open Research) to reduce unnecessary work. Tools generated as part of this project (reagents, genetically-modified mouse strains or data analysis pipelines) will be made available to any interested investigators through public repositories or through appropriate agreements.

Species and numbers of animals expected to be used

- Mice: 33,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To achieve the objectives of this project, we propose to use the laboratory mouse as the

model organism. The mouse is the best-characterised model for these studies, with many features applicableto human infection. Their immune responses are well defined and the technology enabling sophisticated manipulations of the haematopoietic and immune system is highly developed. Mouse genetic alteration techniques are well established; mice have a relatively short generation time; its haematopoietic system has been extensively studied and, in addition to the accumulated knowledge, there exists a vast array of reagents that facilitate the studies to a level unknown for many other organisms. The laboratory mouse was second only to man to have its entire genome sequenced. Therefore, mouse genetics and genomics are as advanced as the human counterparts. Pertinent to this project, the repertoire of endogenous retroelements in the DNA of the laboratory mouse is mappedat the same degree of detail as those in human DNA, which surpasses all other species. To our knowledge, no other species of lesser sentience can fulfil the requirements of this project to the same extent as the mouse. Whilst viruses infect hosts of any age, including embryos or newborns, the complete picture of host immunity requires study of the adult host. For example, T cells, a central component of adaptive immunity are made by the thymus only after birth in mice. Thymic production inboth humans and mice sharply wanes before adulthood, and anti-viral and anti-cancer responses in the adult rely on T cells that were made earlier during development. This is particularly relevant for immunological memory, the basis for vaccination, which relies on long-term survival of antigen-specificlymphocytes, especially in long-lived species such as humans.

Typically, what will be done to an animal used in your project?

Almost all mice used in this project will have some form of immune function alteration, necessary for the study of the immune reaction to infection, vaccines or cancer. In many cases immune function alteration will be achieved by genetic modification and these mice will not undergo any further procedures other than selective breeding to achieve the correct genotype, at which point, mice will only be used to supply cells and tissues for ex vivo work. In other cases, immune function alteration will be achieved by injection of substances, such as vaccines or antibodies, or cells, such as hematopoietic stem cell therapy in recipients whose own hematopoietic cells are depleted by chemotherapy (e.g. myeloablative drugs) or radiotherapy (e.g. sublethal irradiation). These procedures are not expected to cause discomfort beyond that which is associated with vaccination or hematopoietic stem cell therapy in humans. Alteration of immune function can either result in a weaker or stronger immune response. Weaker immunity may lead to immune deficiency and increased risk of infection or cancer. However, since the mice are being housed in clean environments (free from specific mouse pathogens and manyopportunistic microbes), where even severely immunocompromised mice completely lacking adaptive immunity survive without overt infection, no adverse effects are expected from weakening of immune function. At the other extreme, a stronger immune reaction may lead to the development of autoimmunity, autoinflammation or immune-mediated pathology. Such symptoms, which are likely to resemble systemic autoimmunity, such as Systemic Lupus Erythematosus (SLE) in humans, will be closely monitored. In fewer cases, mice will be subjected to experimental infection with a virus (to mimic infection with HIV or SARS-CoV-2) or challenge with a tumour. Viruses are typically administered by inhalation or injection and tumour cells are transplanted by injection. All mice will be humanely killed at the end of the procedures.

What are the expected impacts and/or adverse effects for the animals during your project?

We are proposing to use appropriate mouse models for clinically-relevant viral infections

and cancer, inwhich the parameters of protective immunity can be studied and tested. In addition, genetic alteration of specific genes will reveal their precise role in the response to infection, autoimmunity or cancer. The most acute signs we expect to see in the experimental animals will be the result of tumour development or autoimmunity, with symptoms that range from mild to moderate. However, the development of moderate signs is expected only in a minority (~25%) of all the mice that will be used inthis project. In the case of tumours, potential symptoms depend on the growth pattern of each tumour type. Local tumours are expected to cause predominantly local effects, such as skin inflammation or more rarely ulceration in tumours that grow under the skin. Tumours that can spread throughout the body or metastasise in major organs can cause more generalised and less predictable effects, such assystemic inflammation, weight loss, reduced social interaction, lack of coat grooming, hunched posture and breathing difficulties (for tumours that have spread to the lungs). In the case of autoimmunity or immune pathology are likely to display chronic local or systemic effects. The effects of systemic inflammation caused by immune dysfunction are common with those caused by other conditions, including tumours and manifest as weight loss, reduced social interaction, lack of coat grooming and hunched posture. More specific effects may manifest as diarrhoea (in case of immune-mediated colitis), rashes of the skin, particularly around the eyes, joint pain (in case of arthritis), protein in the urine and enlargement of the spleen or lymph glands. Common to pathogenic murine retroviral infection or cancer models is the assessment of pathogenicity. This will be assessed most frequently as morbidity, which involves quantifying changes in physiological parameters relevant to each type of infection or cancer and associated clinical symptoms. When clinical symptoms reach a predefined and closely monitored level or the physiological parameters reach the predefined values, mice will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

We anticipate that the symptoms associated with these procedures will be sub-threshold or mild for thevast majority of animals. From prior experience we expect that from all mice that will undergo regulatedprocedures, 25% will exhibit moderate symptoms, with the remaining only showing mild symptoms (10%) or no symptoms at all (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 haveconsidered 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 dissect complex immune responses to viruses and cancer. This requires traffickingof all components (virus dissemination, cancer cell metastasis and immune cell infiltration into infected sites or tumours), within and between complex organs and anatomical locations. Similarly, virus vaccines or cancer immunotherapy need to be

tested in a living organism. To keep up with the ever- changing nature of pathogens, the immune system is the fastest evolving mammalian system, with many adaptations being species-specific. Whilst differences between mice and humans are noted (andsome are indeed studies as part of this project), the laboratory mouse represents the best balance of similarity to man, amenability to genetic manipulation and sentience level, compared with humans.

Which non-animal alternatives did you consider for use in this project?

Bioinformatics and in vitro approaches are routinely used in the lab to investigate certain aspects andinform the design of in vivo experiments. For example, the effects of immune mediators (e.g. viral or bacterial products) are tested on isolated immune cell populations and mixtures thereof in vitro.

Similarly, the effects compounds or genes that can affect virus replication or cancer cell growth is firsttested in established in vitro assays, including 3-dimentional assays and organoids. Gene or drug effects are predicted to a certain degree from mining existing data that are publicly available or generated in the lab.

Why were they not suitable?

Although in silico and in vitro approaches are used prior to in vivo work, ultimately, the immunologicaland immunopathological investigations cannot be completed without the use of animals. This is because the immune system is a vastly complex network of cells that cannot be entirely mimicked byany in vitro assay yet. Similarly, viruses exhibit in vivo tropism for specific cell types, the physiology ofwhich is not always maintained in vitro, and cancer cell growth in vivo is subject to many more variables that can be reproduced in vitro. Consequently, the protective capacity of the immune response to infection or cancer and effectiveness of vaccines or anti-cancer treatments necessitates invivo assessment, as potent in vitro activity might not translate into in vivo activity.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The design of quantitative experiments will be based on our extensive prior experience with the animalmodels proposed in this application and will additionally be tested against power and sample size algorithms. This combination will allow us to calculate with accuracy the minimum number of mice required to obtain a scientifically meaningful result. Using too few mice would lead to inconclusive results. Moreover, results will be reported according to ARRIVE (Reporting of In Vivo Experiments) guidelines published by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) (PLoS Biol 8(6): e1000412. doi:10.1371/journal.pbio.1000412).

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We have taken two main measures that reduce the total number of animals used in the project. The first relates to the breeding programme and the second to the experimental protocols. With respect to the breeding programme, we pay particular attention to the minimum number of crosses between distinct mouse strains that achieve the correct genotype of complex compound mutants. For example, for several X-linked immune genes (e.g. Foxp3, Tlr7 or Il13ra1), hemizygosity in male mice is often the most efficient way. Where mice are used as donors of cells for transplantation into secondary recipient mice, we expedite the genotyping so that we can terminate the breeding as soon as the required number of donor mice is reached and avoid surplus. With respect to the experimental protocols, we maximise the statistical power of our assays so that the number of mice required to reach a conclusionis, in turn, minimised. This is achieved by the following steps. Firstly, we minimise between-mice variability by using genetically identical (fully syngeneic) mice, matched for gender and age, and cohoused. This step reduces a considerable source of variability. Secondly, we optimise the readouts so that standardised and accurate phenotypic measurements are made. Moreover, we use multiple independent readouts to maximise the detection of effects. This also reduces experimental variability inthe readouts. Lastly, we endeavour to include a common control group to several experimental groups.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

The use of dedicated computer databases for mouse breeding and management has been implemented at our Institute. This allows us to carefully monitor the mouse breeding programme andalso share mice with selected genetic traits between investigators so that duplication is avoided.
Cryopreservation of gametes, embryos, tissues and cells is also routine at our Institute and will ensurethat the minimum number of mice is bred.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The project uses extensively mouse models for infection, autoimmunity and cancer. The aim is to enhance our understanding and treatment of these conditions, with the view to translating these to humans. As such, many of the relevant procedures are comparable with those that are applied to humans (for example vaccination by needle injection or adoptive cell therapy by intravenous infusion).Although certain procedures do produce some pain or distress to the mice (for example, pathogenic infection or cancer), these are kept to a minimum duration (not lasting) that are necessary to gain knowledge of the infection or cancer development and to evaluate appropriate treatments.

Why can’t you use animals that are less sentient?

This project involves the study of the immune response to viruses, including endogenous retroviruses, vaccines and cancer, in order to better understand immune responses in humans. Given the complexity and evolutionary divergence of the immune system in different animal classes, these studies require a mammal. Moreover, study of complex infection cycles or immune functions, such as in utero infection or transfer of maternal antibodies through the placenta or via milk, require the use of a placental mammal. Additionally, the project heavily relies on detailed characterisation and mapping of endogenous retroelements, on the availability of high-quality genome sequences, and the ability to carry out genetic manipulations. No species with less sentience than the laboratory mouse combines the features that are necessary for the completion of this project.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The procedures in all protocols have been selected for causing the least amount of pain and distress, whilst achieving the objectives. These procedures are continuously reviewed and will be refined basedon our improved ability to define endpoints and on developments described in the literature or communicated by colleagues performing similar procedures. For example, the use of reporter-based and reporter-free advanced imaging techniques may allow us to track solid tumours earlier, thereby reducing the length of tumour challenge experiments in the future. Similarly, the development and use of multi-parameter readouts as surrogates for tissue pathology, may offer the increased sensitivitynecessary to reduce the length and severity of infection models.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Percie du Sert N. et al. (2020) The ARRIVE guidelines 2.0: Updated guidelines for reporting animalresearch. PLoS Biology. 18:e3000410 (https://doi.org/10.1371/journal.pbio.3000410)

Smith A.J et al. (2018) PREPARE: guidelines for planning animal research and testing. Lab Anim52:135-141 (doi: 10.1177/0023677217724823)

NC3Rs/BBSRC/Defra/MRC/NERC/Royal Society/Wellcome Trust (2019) Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies, 3rd edition. London: NC3Rs (https://www.nc3rs.org.uk/sites/default/files/2022-01/Responsibilityin the use of animals in bioscience research 2019.pdf)

Guidance on the operation of the Animals (Scientific Procedures) Act 1986 (https://www.gov.uk/guidance/guidance-on-the-operation-of-the-animals-scientific-procedures-act- 1986)

Workman, P., Aboagye, E., Balkwill, F. et al. Guidelines for the welfare and use of animals in cancerresearch. Br J Cancer 102, 1555–1577 (2010). https://doi.org/10.1038/sj.bjc.6605642

Refining procedures for the Administration of substances (https://doi.org/10.1258/0023677011911345)

Guiding principles aseptic surgery

(https://www.lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf)

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will keep abreast of advances in the 3Rs through regularly reading the scientific literature, visitingthe NC3R's website (https://www.nc3rs.org.uk) and by discussing with colleagues. In addition, our Institute organises annual refresher courses and employs dedicated individuals who produce and disseminate regular newsletters.

STUDYING AND IMPROVING NATURAL TOOTH REPAIR.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Tooth decay, Tooth repair, Dentin, Collagen, Mineralisation

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

To study how teeth naturally heal the dentin that sits beneath the outer layer of enamel and surroundsthe pulp that contains the nerve and blood supply. The effect of an abnormal type I collagen protein, the main constituent of dentin, on the healing process will be studied.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Teeth can be damaged by trauma or tooth decay, which leads to toothache and infection. Treatmentsinvolve using fillings, the nature of which depends on various factors including the extent of decay.

Teeth have a natural ability to create new dentin and boosting this natural repair pathway could be analternative means to treat tooth decay, keeping teeth healthy, in place and in a natural state later intolife.

Type I collagen is a major protein constituent of dentine but an abnormal form of type I collagen is created due to genetic predisposition and in age-related disease. The influence of this abnormal, but common, type I collagen on natural tooth repair will be studied to understand how it is likely to affect, orinterfere with, the repair process.

What outputs do you think you will see at the end of this project?

Publications and new information on the natural tooth repair process and how it is affected by thepresence of abnormal type I collagen.

An established model with which to test a broader range of agents for their ability to promote naturaltooth repair.

Who or what will benefit from these outputs, and how?

The field of dentistry will benefit from this research in the longer term, after publication and dissemination of the results of the project.

The commercial sector may benefit via the development of new products to treat tooth decay in themedium term.

In the long term the general public would be expected to benefit via the development of new mainstream or specialised treatments for tooth decay that account for genetic and age-relatedpredisposition to abnormal collagen production in new dentin.

How will you look to maximise the outputs of this work?

We will collaborate with investigators who have successfully established the model within the UnitedKingdom. We will share any unsuccessful approaches via preprint servers such as ‘bioRxiv’ or ‘medRxiv’, and by full publication where possible. New knowledge will be shared via full publications,abstracts, conference presentation, scientific discussions and via patient involvement groups.

Species and numbers of animals expected to be used

- Mice: 1150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used as there are already established genetic models for abnormal type I collagen synthesis, and procedures have been established for producing molar defects and following the naturalrepair process in mice. The life stage used will be when normal developmental dentin formation is complete, once the teeth are large enough for dental

surgery and before there is significant erosion of the molar teeth; 4-8 weeks old (corresponding to early adulthood).

Typically, what will be done to an animal used in your project?

Mice will be bred to produce offspring that produce solely abnormal type I collagen, or which produceboth normal and abnormal forms. A dental procedure will be performed on no more than two molar teeth per mouse in which teeth will be damaged using a standardised drilling procedure and under general anaesthetic. The damaged area will be filled, pain relief provided after surgery and soft food provided subsequently. Mice will be sacrificed 4-8 weeks after surgery.

What are the expected impacts and/or adverse effects for the animals during your project?

The most likely adverse effect, though uncommon at <1%, is death due to complications of anaesthesia or surgery. This will be minimised by appropriate pre-operative acclimatisation, accurateweighing to ensure correct dosing, by good maintenance of body temperature during surgery and appropriate monitoring, followed by close post-operative monitoring and care.

Tooth drilling may cause short-term discomfort for which analgesics will be administered followingsurgery. Mice will be monitored for signs of discomfort requiring analgesia. Tooth drilling may alsocause bleeding which is likely to cease without treatment.

Some animals may experience subsequent pulp necrosis between surgery and the end of the experiment which will be minimised by using aseptic surgical technique. Pain relief will be applied asappropriate.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

A mild severity is expected for mice used for breeding only (~60%).

A moderate severity is expected for mice undergoing tooth drilling and recovery. (~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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Use of animals permits age matching between groups, possible inclusion of both sexes and reduction in biological variability by using an inbred strain. The tooth is a complex

tissue with both a nerve and blood supply, for which the pulp, dentine and enamel components cannot be reproduced in cell culture.

Which non-animal alternatives did you consider for use in this project?

Human tooth explant cultures and 3D culture systems

Why were they not suitable?

Human tooth explants suffer from much higher multifactorial variability which increases sample numbers to observe any effect and they are not amenable to controlled genetic modification. 3D culturesystems are not yet sufficiently developed and standardised to reproduce a living tooth.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used has been estimated based on the number of samples required to accomplish the objectives, as determined by size calculations to determine group sizes, based on thevariability in measurement parameters seen in previous studies. The total number also includes estimates for the required number of breeders and offspring to produce the specified group sizes.

Randomisation will be via Mendelian genetics for genotype and sex, and blinding will be carried out byusing animal numbers for identification.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The study design is similar to that which we have used previously, the design of which was guided by aco-investigator in the field of statistics. The experimental design was run through the NC3Rs Experimental Design Assistant, but choice of statistical test and sample size calculations had to be carried outwith the program due to inclusion of two factors (sex and genotype). Experiments will be carried out at the same time of day and by the same operator to reduce variability; otherwise such variables will be included as nuisance variables in the analysis.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Both left and right molars will be analysed. Tissues will be biobanked at the end of the experiment forfuture use where possible, or offered for use by other 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Molar teeth will be drilled to induce subsequent reparative dentinogenesis. Drilling will be carried out bya trained endodontal surgeon using standardised dental tools.

Why can’t you use animals that are less sentient?

More immature animals cannot be used because the teeth are not fully formed. There are no less sentient species with the appropriate genetic modification in which a suitable tooth repair model hasbeen established. Terminal anaesthesia cannot be used because the repair process takes several weeks following surgery.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Refinements will include pre-operative acclimatisation and handling, accurate weighing to ensure correct dosing of anaesthetics, good maintenance of body temperature during surgery, monitoring temperature, pulse, respiratory rate, capillary refill rate and reflexes, followed by close post-operativemonitoring and care.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

The PREPARE guidelines will be consulted to ensure all aspects of the running of the research have been considered and planned in advance. The NC3Rs website, newsletter, regional programme manager and academic community will be a source of information regarding relevant refinements andrecent improvements. The Laboratory Animal Science Association (LASA) publications will be consulted for best practice in aseptic surgery, avoiding mortality and other recent best practice guidelines.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Via the NC3Rs website, newsletter, regional programme manager and academic community.

DEVELOPMENT OF NEXT-GENERATION NON-VIRAL GENE DELIVERY PLATFORMS

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

vaccines, gene therapy, Nucleic acids, Formulations, Nanoparticles

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

To develop improved delivery systems for nucleic acids as therapies and vaccines for the preventionand treatment of disease.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The COVID-19 pandemic has emphasised the need for continued efforts to develop effective nucleic acid delivery systems (such as mRNA in the Pfizer vaccine). These platform technologies are valuablenot only as vehicles for antiviral drugs and vaccines against emerging viruses, but also as cancer therapeutics, approaches to treat monogenic

disease, and to augment regeneration. The key to using DNA and RNA in these 4 key therapeutic areas is 'delivery'. Where the nucleic acid drug gets into cellswithin tissue intact and can produce proteins effectively and have the desired effect on biology.

We have developed a series of formulations to deliver mRNA, DNA, siRNA and nanoparticle systems to apply to these wide 4 fields in future work. However these must be robustly tested and optimised for'delivery'. We will do this (and have been elsewhere in collaborations) by looking at biodistribution, efficacy, longevity and for immunogenicity (negative immune responses) with the goal that these will become enabling technologies. The new post-genomic era of genetic therapies, such as the exploitation of gene editing and augmentation still rely on expensive and inhibitory viral systems, to harness the gains in academic science delivery systems must be developed as enabling technologies.This is the focus of our PPL, to generate the tools we need to apply genetic medicines to these important issues for humanity and to have impact.

Our present non-viral technologies, like lipid based systems (such as Pfizer), although effective in delivery of RNA-based COVID-19 vaccines, will not address all applications where these approaches would be transformative for biosciences or medicine. Therefore, further development and optimisationof platform technologies such as ours are needed.

To be effective and to gain the most out of our research we argue that this should be done in-house under this proposed PPL. The impact on medicine is our long-term goal. However the work proposedin this application will facilitate application (via a number of delivery routes and techniques) to these 4key areas that could be revolutionised by non-viral nucleic acid delivery platforms.

What outputs do you think you will see at the end of this project?

The project is likely to generate publications in peer-reviewed journals and provide proof-of-concept data to enable additional funding to be secured for the further development of our delivery platforms,including applications in gene therapy, disease correction, regenerative medicine and antiviral drugsand vaccines.

The expected outputs for objective 1 are data demonstrating the biodistribution, expression level and longevity of expression via a series of different delivery routes and with different formulations. These including delivery of siRNA, mRNA, plasmid (p)DNA, synthetic DNA (minicircle) and these packaged inpeptide, polymer, lipid or bionanoparticles (phage). The data will confirm functionality and targeting of these formulations and will allow us to screen (using phage) for variants that allow us to generate targeted therapies against particular tissues or disease-states.

The expected outputs for objective 2 are data demonstrating the negative (or lack of) immunogenic potential of the formulations themselves, compared to the nucleic acids (mRNA, DNA) delivered as a proof-of-concept as novel vaccines. For this results will include titration of antibodies in serum samplesand measurement of T-cell activation (e.g. using ELISpot assays). Outputs expected for objectives 2 also demonstrate that novel vaccine formulations are effective for future challenge testing outside of this PPL.

The expected outputs for objective 3 are the isolation of candidates and the increased understanding of the variables in peptide targeting (screened by Next-generation phage display) that could be used atargeted, efficacious and non-self immunogenic delivery systems for gene therapies and vaccinology for many diseases

Overall, the project will provide proof-of-concept data about nucleic acid delivery formulations that willform the foundation for further development towards clinical application in both the human and veterinary medicine fields.

Who or what will benefit from these outputs, and how?

The academic community via publications arising from the studies. This will likely be in the medium tolong term.

New therapeutic applications and translation of these technologies will be significant in the long term.

How will you look to maximise the outputs of this work?

I have a strong track record in disseminating findings from my research via publication in scientific journals and lay articles and presentations to both scientific and lay audiences. I am engaged with industry and have experience of the requirements for licensing such technologies. I also collaborateextensively nationally and internationally.

Species and numbers of animals expected to be used

- Mice: 1000
- Rats: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are used because of the long history of gene delivery and vaccine development in the mouse model. This means that experiments to demonstrate efficacy of formulations and vaccines have been refined to the greatest extent in mice. Adult mice are used for formulation and/or vaccine immunogenicity testing because a mature immune system is required to generate antibodies and cellular immunity to the delivery system or vaccines. Testing the efficacy of novel therapeutics using these delivery systems requires that they are tested in wild-type and disease model states, for which the mouse is the most extensively used for vaccinology, regenerative medicine, cancer and monogenicdiseases. This means that the work here can be directly translated to specific models and that treatment outputs can be observed in model animals prior to translating the technology for clinical applications. Adult rats will be employed rather than mice for similar reasons except as they are larger rodents physical systems for delivery can be used which are not appropriate in mice such as pharmajetpressure mediated devices.

Typically, what will be done to an animal used in your project?

The majority of animals will have a delivery formulation (nucleic acid loaded or control, with peptides, lipids, polymers or as Phage particles) or methodology (gene gun/pharmajet) administered by one of several routes. The administration of the formulations is unlikely to impact adversely on the animals asthis would not be acceptable in the final product. The principles for protocols of minimal severity will befollowed. For most experiments a single

delivery is given and the mice monitored for expression. For immunological testing, the formulation/vaccines would be give as a single dose followed by one or two 'booster' immunisations. For serial delivery experiments formulations may require repeated administration over several days. The level of localisation and expression/function of the formulations will be assessed by terminal examination of tissues or live animal imaging techniques. For immunologyassessment circulating antibodies in the blood (the antibody titre) in response to delivery system or vaccination will be monitored by blood sampling at appropriate time points.

The typical experience of animals undergoing a non-recovery protocol for the collection of blood and tissue at the end of experiments would be exposure to an anaesthetic gas or the experience of an injection of an anaesthetic drug. The anaesthetic agents would be administered in such a manner as tominimise the risk of panic and the animals would gradually lose consciousness and enter a stage of deep surgical anaesthesia. At this point blood and/or tissues would be collected. The loss of blood during the procedure would ensure that the animals would be unable to regain consciousness; however humane killing would be completed and confirmed according to Schedule 1 of the Act.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will experience mild and transient effects when delivery formulations (as polymers, peptides,phage, carrying nucleic acids) are administered, the extent of which may depend on the route of administration with intramuscular or intravenous administration requiring a needle and syringe.
Intradermal administration using a ‘gene gun’ may also cause transient pain; intranasal administration is less painful but may cause some discomfort. Vaccine immunisation may lead to a period of mild lethargy for typically up to 24 hours. The recognised and characterised adverse effects of the adjuvantsused to stimulate the immune system may result in localised redness and inflammation for a period of typically 3 to 5 days. In some cases, a subsequent sterile abscess or granuloma will form at the injection site. These may not resolve during the time scale of the immunisation protocol but will be monitored by the PIL/technician and may be brought to the attention of the Named Veterinary Surgeon. When animals are blood sampled in a conscious state, use of refined techniques in line with LASA andNC3Rs guidelines will ensure that any pain is mild and transient in nature. On rare occasions (<5%) a bruise or swelling at the site of blood sampling may form, which should typically subside within 3 to 5 days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The expected severity experienced by animals during administration of formulations while conscious will be mild. This will apply to all animals on such protocols. There will be some delivery routes that will be moderate severity so will be administered under anaesthesia (intrathecal, cortical). The expected severity experienced by animals undergoing blood sampling while conscious will be mild. This will apply to all animals on such protocols. The actual severity experienced by individual animal will dependon delivery route and dose,

The expected severity of this work is non recovery for all animals from which blood is sampled underterminal anaesthetic.

What will happen to animals at the end of this project?

- **Killed**

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Efficacy testing of genetic delivery systems intended for use in humans or veterinary species involvesthe whole complexity of the body and immune systems of higher order animals.

Which non-animal alternatives did you consider for use in this project?

Due to the complexity of the tissue systems and immune response to delivery/vaccination, non-animal alternatives are not yet available for this project. However, laboratory work will be undertaken before moving into animal studies (e.g. to achieve optimal expression levels of protein from the formulations invitro).

Why were they not suitable?

They do not mirror the complexities of tissue delivery or immune responses needed for this project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers to be used are estimated by projecting the likely number of studies to be performed during the five year duration of this licence from the current demand, which is likely to be at a peak dueto the current COVID-19 pandemic situation. Typical experiments will have at least one, possibly up to five treatment groups and a control group with up to around 10 mice per group a likely maximum.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The number of animals being used over the course of this project was estimated following our previouswork to demonstrate efficacy and safety of our existing formulations. This has been added to employing literature searches examining experiments typical of those to be performed. Prior to designing individual experiments to be performed under this licence (in conjunction with using the NC3Rs Experimental Design Assistant) a systematic literature

review pertaining to the specific application will be performed (I have undertaken systematic review training) to identify relevant studies (e.g. studies using a similar formulation strategy and delivery route) in order to obtain the best data possible on effect sizes (for example) to inform study design and power calculations. We do however have a wealth of historical data which allows us to define the minimal effect sizes and numbers neededto demonstrate statistically significance in these types of studies.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Where animals remain naïve at the end of a delivery or immunogenicity study (i.e. control animals), thepossibility of sharing tissues with research colleagues will be explored. The facility electronic distribution list and internal intranet notice board can be used to alert colleagues in advance of the availability of fresh tissue for ex vivo use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Adult mice and rats will be used as they are widely used models of delivery and immunology with a broad range of reagents available to measure biodistribution, gene expression and immune responses.These will be wild-type inbred strains. Physical or nanoformulation methods will be used for delivery.
The extent of pain, suffering and distress will be correlated to the route of delivery, rather than the formulation used. We have chosen the most scientific robust and translatable routes of delivery, and also those which allow us to compare with the literature and previous work. These are the most refinedmethods, aiming to minimise the pain, suffering and distress to the animals employed.

Why can’t you use animals that are less sentient?

Animals at a more immature life stage, species that are less sentient or animals that have been terminally anaesthetised are not appropriate alternatives because assessment of delivery, gene expression and immunisation requires a fully developed and functional tissue and immune system forthe development of tissue specific expression and immune responses.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The welfare costs to animals will be minimised by the provision of group housing with environmental enrichment, bedding and or nesting material as standard. Animals will be handled and restrained withthe minimal effective restraint - the use of the tail for handling and restraint will be minimised.

The potential for pain and inadvertent infection will be minimised by use of single use sterile needles ofthe appropriate size for the procedure. Local or general anaesthesia will be applied, where appropriate,under the direction of the Named Veterinary Surgeon. Injection/delivery sites will be clipped or shaved and cleaned ahead of injection to minimise the risk of contamination and to allow regular monitoring of the injection site after the procedure. Veterinary intervention will be promptly sought as required and appropriate humane endpoints applied in the unlikely event that adverse effects develop and cannot becontrolled. Full records of procedures undertaken, daily monitoring and veterinary requests will be maintained using both an established electronic facility management software and / or hard copy records.

All licensees and animal care staff will be trained, supervised and signed off as competent for theprocedures they will undertake. We will always refer to more experienced staff and will bring in experienced staff from elsewhere if needed to have the most refined execution of the experiments wepropose here.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

LASA and NC3Rs best practice guidelines will be followed in respect to refining blood sampling and injection techniques. The NC3Rs grimace scales may be used to improve the monitoring of mice for potential signs of distress. Home Office and FELASA severity information will be used to ensure that the actual severity experienced by the animals can be recorded and limits within this licence adhered to. Body condition score charts specific to the mouse may be used to improve assessment. Home Office Code of Practice will be used to ensure animal care and housing is appropriate. This guidance will be used in conjunction with the advice available in the NC3Rs Resource Hub for both housing and handling of animals.

The NC3RS Procedures with Care resource will inform personal licensees of refinements in theconduct of the minor procedures undertaken in this licence.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

I receive regular e-mail updates from University colleagues including the Named Training and Competency Officer (NTCO). I am registered for alerts on the NC3Rs website and follow @NC3Rs onTwitter, this alerts me to the latest developments, for example in non-aversive mouse handling. I will continue to communicate with colleagues at the University and other institutes that are conducting similar studies to find out about the latest refinements they have introduced.

SAFETY TESTING OF CHEMICALS, PLANT PROTECTION PRODUCTS, BIOCIDES AND SUBSTANCES ADDED TO FOOD OR FEED PRODUCTS USING SMALL ANIMAL SPECIES

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animalsreared 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 aimsmentioned in paragraph (b)

Key words

Toxicology, Non-Pharmaceuticals, Regulatory, Safety Assessment, Small animals

Animal types	Life stages
Mice	adult
Rats	adult

Animal types	Life stages
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required,and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

This licence authorises the conduct of studies in small laboratory animal species (rats, mice and rabbits) with the aim of evaluating the toxicity, their ability to cause skin and eye

irritation, skin sensitisation and tumorigenicity (ability to cause cancer) of non-pharmaceuticals (agrochemicals, biocides, food additives /foodstuffs, ingredients of household chemicals (where legislation allows) andindustrial chemicals). This is to aid in the development of new chemicals, and to provide mandatory information to regulatory authorities to allow marketing approval (i.e. to show that they are safe when they come into contact with humans).

A retrospective assessment of these aims will be due by 03 September 2028

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Governments require (and the public expects) that substances we are exposed to are safe or that theirpotential hazards are well understood and documented.

The data generated from the studies performed under this project will be used to inform decision- making processes on substances under development and, where appropriate, to satisfy governmentalregulatory requirements necessary to gain marketing authorisation or product registration.

This safety assessment is of immense importance along with other non-rodent and non-animal studiesin demonstrating to governments and the public the safety of these substances or highlighting their known hazards and safe handling.

What outputs do you think you will see at the end of this project?

This project licence authorises the conduct of in vivo safety studies in laboratory small animal speciesto evaluate candidate molecules and novel and currently-registered substances in terms of systemic toxicity, toxicokinetics, irritation /sensitisation or the potential to cause or influence development of tumours.

The overall benefit of this project is that it generates high quality data that is acceptable to regulatory authorities and enables internal decision making within our clients' organisations. This project will alsoensure that chemicals and pesticides that the general population are exposed to are safe.

Who or what will benefit from these outputs, and how?

Our customers will benefit, as the data we generate will allow them to progress their substances underdevelopment and, where appropriate, to satisfy governmental regulatory requirements necessary to gain marketing authorisation.

The studies ensure that non-pharmaceuticals such as food additives, agrochemicals and

industrial chemicals that the human population are exposed to during their lives are safe or that their hazards areknown as that they can be handled safely.

How will you look to maximise the outputs of this work?

Where confidentiality permits, data, study design and best practice will be openly shared at conferences, workshops, webinars, blogs and publications.

As 3R's benefits are also realised under this project licence, these will be shared more widely withother establishments.

Species and numbers of animals expected to be used

- Mice: 19900
- Rats: 42900
- Rabbits: 4560

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodents (Rats and mice) will be predominantly used in this project along with Rabbits. Only adultanimals will be used.

Species choice and use of specific animal models is determined by the need to generate regulatory acceptable data. Where a choice of species is possible, care is taken to select the most biologically appropriate species, and the species which most closely relates to man. Studies to assess the types ofmaterial covered by this licence are usually performed on small animal species.

Generally the rat is the rodent species of choice in safety assessment. Rats are large enough to provide repeated blood samples, thus requiring significantly fewer rats than mice to achieve the sameobjective. Mice may be used when considered a more appropriate species, for example, if they more readily absorb the test material, are more relevant biologically or improved tolerance depending uponobjective of the study.

Rabbits may be used when considered a more appropriate species, for example non-pregnant range finding studies prior to conducting reproductive toxicology studies in pregnant rabbits or local tolerancetesting.

Typically, what will be done to an animal used in your project?

Animals will be given the "test material" under investigation in a way which mimics possible human exposure. As the most likely route of exposure is orally the majority of animals will receive the test material either mixed in their food or directly by insertion of a flexible rubber catheter/semi rigid plasticor metal cannula in to the oesophagus. For some test materials the oral route of administration may not be appropriate for example the material is more likely to come in to contact with skin or other bodymembranes (e.g. the cornea of the eye for example). Most animals are treated daily; occasionally studies may require

several doses within 24 hours or exposure to the test material for a number of hours each day for example by placing the material on the skin and covering it with a gauze dressing. The length of study depends on the likelihood of repeated human exposure and ranges from a single administration for example to assess accidental contact through to daily administration for 2 years to explore possible long term effects (when looking for the potential for a chemical to cause cancer).

Blood and urine samples may be taken to measure the level of test material or its metabolites with ananimal's circulatory system. These may also be analysed to detect any effects on body systems and organs for example liver or kidney function.

Study animals are closely observed at least twice daily by highly trained technologists who monitor forany signs of discomfort. Other measures such as food consumption and bodyweight may be used to closely monitor for treatment related effects. Veterinary surgeons are employed on a full- time basis and are available 24/7 to provide clinical treatment, guidance on animal welfare and the conduct ofprocedures including appropriate surgical technique, anaesthesia and analgesia.

The majority of animals are expected to have mild adverse effects of treatment such as reduced weightgain or changes in appearance or behaviour. A small number of animals (usually limited to the highest doses evaluated in early studies) may show more moderate adverse effects. The nature and type of effect varies dependant on the biological systems affected, however, these usually result in findings such as reduced food consumption, weight loss and changes in behaviour such as decreased activity. Humane endpoints will be adopted or dose levels reduced if animals show excessive effects. Longer term studies are expected to have progressively less adverse effects.

Many toxicological effects of the test material are not evident during the in-life phase of a study and donot impact the animals' wellbeing. Only through macroscopic and microscopic examination of the tissues from each animal, can evidence of all toxicological changes be fully assessed and the scientificvalue of each animal maximised. In order to undertake these evaluations the animals must be killed humanely at the end of a study.

What are the expected impacts and/or adverse effects for the animals during your project?

When dosing an animal by injection or taking blood, the amount of pain an animal feels is similar to what a patient would feel having an injection done by a doctor. If we have to repeatedly inject animals using a needle and syringe, we would choose different sites to do this where possible. On some occasions (eg jugular sampling in mice) we can take blood samples when an animal is deeply unconscious. If we need to take repeated blood samples or need to dose repeatedly then we try and use different sites. Of course everyone who performs these procedures are trained to a high standard.

Routinely we need to take a urine sample for analysis, so we would then put an animal into a special cage which is smaller than their normal cage. The animal can still move around. Virtually every animalwill get used to their new cage within about 15 minutes and are fine.

Dosing with chemicals may cause adverse effects in some studies. Experience shows that the majority of animals are not expected to show any clinical signs of suffering (either no clinical signs or normal background signs expected of the rodent strain). A small percentage may show transient subtle to mild clinical signs. Moderate signs of adverse effects may be seen in some animals, usually in the higher dose groups. Lethality and/or

severe effects are not study objectives in any of the protocols within this licence, but for preliminary studies that may be the first animal studies with limiteddata available, a very small percentage of animals may inadvertently show severe findings before theyare immediately and humanely killed.

We do observe our animals at least twice a day, and the people who do this know the signs when an animal is ill. If an animal is ill, we would check it more frequently, and get more senior staff involved inits care for advice, including vets.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

On the last project, about 70% of animals displayed mild severity, around 25% of animals were classified as having displayed moderate severity and less than 5% were in the severe severity category. This is because these studies can last between a few days and weeks to up to a year, and although the individual procedures are usually mild in nature on their own, the cumulative effects makethem moderate overall

It's impossible to predict the proportion of severities expected on a service licence like this, as this willbe dependent on what study types we are asked to perform.

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Rehomed
- Kept alive

A retrospective assessment of these predicted harms will be due by 03 September 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animalswere affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

At present there are no scientific and legally acceptable evaluations of systemic toxicity which will satisfy regulatory requirements and provide sufficient safety data other than use of animals. Validatedin vitro tests for specific organs and biological pathways are available and used to replace or refine procedures wherever possible. If new in vitro methods become available and achieve regulatory acceptance during the course of this project they will be validated and used to replace in vivo procedures. Where available, review of

scientific articles, non-animal methods and other animal data such as metabolism information will be utilised to reduce animal use.

Which non-animal alternatives did you consider for use in this project?

There are no other non-animal alternatives for the work being undertaken on this project. The regulations we are following will not allow safety decisions to be made on non-animal systems alone.

In vitro and in silico methods (test tube or computer work not using animals) are used in combination with animal studies to inform study designs and assist in understanding of potential toxicity but cannotyet replace in vivo (animal) studies.

Why were they not suitable?

Although there are in vitro tests that can model some parts of how chemicals get into our bodies, and how our body deals with them, and can identify undesirable effects, for example, there is no series of invitro tests that brings all these complex happenings together, like we see in animals and humans.

That is why we need to test chemicals in animals, as they have similar physiology and processes as humans, and that testing gives us a good idea what may happen if they were ever exposed to humans.

A retrospective assessment of replacement will be due by 03 September 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received from customers in the past. It is, however, impossible to accurately predict the number of studies that may be performed, inthe circumstances.

The regulatory guidelines we follow for each study usually indicate the number of animals in a study;otherwise, the number used is the minimum to achieve the aims of the study.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Studies are designed to provide maximal scientific value from the minimum number of animals, whilstusing sufficient animals to meet scientific objectives, and regulatory guidelines. Statistical input is sought, where appropriate, to strengthen the overall scientific quality and relevance of studies.

Where available, sensitive analytical techniques may be used to reduce animal numbers.

Wherever practicable, and by looking across studies, the combination of endpoints eg general toxicity,reproduction and developmental toxicity, mutagenicity etc in studies is considered, to reduce overall animal usage.

As most studies involve the examination of tissues following treatment opportunities for re-use are verylimited. Tissues are collected to support drug and in vivo developments from any surplus stock animals.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We will try and get as many outputs as we can from a single animal where possible, without adverselyaffecting its welfare. So if we need to get several different samples, for example, we will often do that in the same animal, rather than use separate ones, when possible.

Before our main studies, we use smaller groups of animals to get an idea of the doses we need to usefor the main studies. These studies are important as it gives us confidence that the doses we are using are correct prior to testing them in bigger groups of animals required by global regulators.

A retrospective assessment of reduction will be due by 03 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Most of our models involve dosing animals with test substances, and sampling them, with many outputs taken after the animals have been humanely killed. This is generally the least invasive set ofprocedures that can be done to give meaningful outputs to make scientific decisions about further tests, or to determine the safety of a test substance.

Throughout our studies, our animals are checked at least twice a day. This allows us to see over a period of time, whether dosing each individual animal is causing any adverse clinical signs. if this isthe case, we can take action: get veterinary advice, add food supplements and extra bedding if needing, and even reduce dose levels or stop dosing completely.

Why can’t you use animals that are less sentient?

Rodents (rats and mice) and rabbits will be used in all of the studies conducted under this licence.Rodents are considered to be of the lowest neurophysiological sensitivity (their brain function and physiology) that will allow us to achieve the study aims and are considered suitable for predicting what's likely to happen in humans. Rabbits will be used for specific studies where rodents are not aphysiological or regulatory option.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Many of the procedures performed on our rodents and rabbits like blood and urine sampling, cause only transient distress to the animals. Blood sampling procedures are similar to and about as painfulas having a blood sample taken by a doctor or a nurse. Blood volumes are kept to a minimum.

Confining animals in special cages to allow us to take urine samples is similarly of little distress to theanimals.

Where animals are planned to be restrained in tubes for longer periods (eg 30 minutes or greater), training of the animals occurs for increasing periods prior to treatment commencing to accustom the animals. Dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm and will be the minimum consistent with the scientific objectives of our studies. In addition, suffering willbe further minimised by implementing clearly defined humane endpoints.

For mice, sham dosing prior to the start of test article administration has been shown to allow the animal to be accustomed to the restraint and dosing procedure without actual liquid administration andwill be conducted for all oral gavage studies. Also handling in mice can be stressful if not conducted ina suitable manner. Therefore, mice will be cupped when handled where possible to reduce the stress,lifting via the tail should be kept to a minimum.

In addition, care is taken to provide as much environmental enrichment as possible. This includes, butis not limited to, plastic shelters in their cages, wood blocks and balls (short studies up to 13 weeks duration) to gnaw on and push around; mice are occasionally given swings, mice and hamsters are generally given extra bedding for warmth and food supplements are given as appropriate.

In some tests we may use animals that are genetically altered, for example, transgenic mice becauseof their susceptibility to tumours. These animals are specially bred and don't display any harmful clinical signs due to their conditions.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Regulatory Guidelines

OECD Guidelines

OECD 405 – Acute Eye irritation/corrosion

OECD 407 – Repeated Dose 28-day Oral Toxicity Study in Rodents

OECD 408 – Repeated Dose 90-Day Oral Toxicity Study in Rodents

OECD 410 – Repeated Dose Dermal Toxicity: 21/28-day Study

OECD 411 – Sub-chronic Dermal Toxicity: 90-day Study

OECD 417 – Toxicokinetics

OECD 420 – Acute Oral Toxicity – Fixed Dose Procedure

OECD 424 – Neurotoxicity Study in Rodents

OECD 429 – Local Lymph Node Assay

OECD 451 – Carcinogenicity Studies

OECD 452 – Chronic Toxicity Studies

OECD 453 – Combined Chronic Toxicity/Carcinogenicity Studies

Summary of Considerations in the Report from the OECD Expert Groups on Short Term and LongTerm Toxicology

Notes for guidance on repeated dose toxicity. Committee for Proprietary Medicinal Products (CPMP),2010. CPMP/SWP/1042/99 Rev 1

Guideline on the evaluation of control samples in non-clinical safety studies: checking for contamination with a test substance. Committee for Medicinal Products for Human Use (CHMP), 2005.CHMP/SWP/1094/04

Note for guidance on carcinogenic potential. CPMP, 2002. CPMP/SWP/2877/00

EU Directive 91/414/EEC – evaluation, authorisation, approval of active substances at EU-level and national authorisations of plant protection products (PPPs); EU Feed Hygiene Regulation (183/2005);EU Regulation 882/2004 on official controls for feed and food law (and animal health and animal welfare); EU Biocides Regulation 528/2012 and EC 1907/2006 REACH Regulations

Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008and Directive 2000/13/EC.

Dosing and sampling and other documents

Diehl et al. A good practice guide to the administration of substances and removal of blood, includingroutes and volumes. Journal of Applied Toxicology: 21, 15-23 (2001)

Gad et al. Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routes inmultiple species with notes on methods to improve utility. International Journal of Toxicology: 1-84 (2016)

NC3Rs: Recommendations from a global cross-company data sharing initiative on the incorporation ofrecovery phase animals in safety assessment studies to support first-in-human clinical trials (Regulatory Toxicology & Pharmacology, 2014).

LASA/NC3Rs: Guidance on dose selection for regulatory general toxicology studies for pharmaceuticals.

Guidance on the conduct of regulatory toxicology and safety evaluation studies. UK Home Office 2005

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

This will be achieved by consultations with our Named Information Officer, colleagues in Animals Technology, and by attending appropriate training courses and conferences, or getting feedback fromsuch events.

A retrospective assessment of refinement will be due by 03 September 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved forfuture work of this kind? During the project, how did you minimise harm to the animals?

PROTEOTOXICITY IN DEVELOPMENT AND AGEING

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

Proteotoxicity, Development, Ageing, Neurodegeneration, Therapy

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how proteotoxicity (pathology that develops due to damagedproteins) alters development and ageing.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Ultimately, this project will lead to the identification of potential treatment targets and therapies for proteotoxicity induced disorders such as neurodegeneration (disease caused by loss of function of brain cells or loss of brain cells). There is no cure for neurodegenerative disorders and thus, finding anew common target is at utmost importance.

What outputs do you think you will see at the end of this project?

A successful outcome would be the identification of potential treatment targets and therapies for neurodegeneration via targeting proteotoxicity. The dissemination of the results will consist of presentations at international conferences and publishing important discoveries in high impact publications. Additionally our lab will engage in communication and outreach activities for generalpublic in science fairs and by visiting schools.

Who or what will benefit from these outputs, and how?

It has been shown that proteotoxicity caused by protein misfolding and aggregation is connected with various types of health problems, including neurodegeneration, cancer (condition where cells in a specific part of the body grow and reproduce uncontrollably), muscle degeneration, cardiovascular disease (conditions affecting the heart or blood vessels), and autoimmune disorders (condition arisingfrom an abnormal immune response to a functioning body part). Therefore, it is important to characterise the underlying mechanisms of proteotoxicity that contribute to these diseases and to identify potential targets for their prevention and treatment.

One proteotoxicity induced disease is neurodegeneration. Neurodegenerative diseases are incurable and result in progressive loss of function or death of brain cells called neurons. Neurodegenerative diseases include Alzheimer's disease and other dementias, Parkinson's disease, Huntington's disease,motor neurone disease, Creutzfeldt-Jakob disease and multiple sclerosis. Dementia is a group of related symptoms associated with an ongoing decline of brain functioning that results in impaired ability to remember, think, or make decisions that interferes with doing everyday activities.

The dementias are responsible for the greatest burden of disease, with Alzheimer's disease representing over 60% to 70% of cases. Neurodegenerative diseases are strongly linked with age, andthe UK and other European countries have an increasingly ageing population. Currently 16% of the European population is over 65, with this figure expected to reach 25% by 2030.

Dementias are the biggest health challenge of our generation; one in three people born today will develop the disease. With one million people in the UK predicted to have dementia by 2025 and the current cost of £26 billion a year to the UK economy, there is a huge financial and societal impact, yetwe are still without treatment.

Short term benefits: if we successfully answer each of the research questions we will be able tounderstand how proteotoxicity alters development and ageing.

Long term benefits: ultimately, such testing steps will lead to the identification of potential drug targets and therapies for proteotoxicity induced diseases such as neurodegeneration via targeting proteotoxicity. A successful therapeutic strategy for the treatment of neurodegeneration would be at utmost importance for patients, enhancing both lifespan and quality of life. Apart from the scientific andsocietal impact, the economic value of effective neuroprotective therapies for neurodegeneration would be significant.

How will you look to maximise the outputs of this work?

We will provide open access to our publications, and we will have responsible management of research data in line with the FAIR principles. All information about results, tools, and codes required tovalidate the obtained conclusions will be completely available through public repositories such as GEO(https://www.ncbi.nlm.nih.gov/gds/), Github (https://github.com) and Mendeley Data (https://data.mendeley.com) in order to ensure the reproducibility and transparency of our findings.

Also, in line with the “early and open sharing” philosophy, the primary research manuscripts generatedduring the proposed project will be available from bioRxiv pre-print server (http://www.biorxiv.org/) as soon as we submit it to a peer-reviewed journal, which preferentially will be an open peer-reviewed journal, also to increase the transparency and reproducibility of generated outputs. Finally, we will implement a thorough process to ensure the robustness of the obtained results and conclusions by including all relevant negative results in our publications and performing robust and unbiased statisticalanalysis. The data will be disseminated by presentations at national and international scientific meetings and seminars.

Species and numbers of animals expected to be used

- Mice: 14000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project uses mouse as an experimental animal to study the role of proteotoxicity in developmentand ageing. Unfortunately, in-vitro studies (method to study the behaviour of animal cells in controlledenvironment that is outside of their normal biological context) cannot be used to understand how genetic modifications result in normal or abnormal physiological processes (the way in which a living organism or bodily part functions). We have created new mouse lines with specific mutations in genes that disrupt one of the stress response pathway that is called proteotoxicity. Preliminary data shows, that one of the mouse line with impaired proteotoxic stress response developed a movement disorder. The mice were otherwise healthy and without any body weight loss. As proteotoxicity is connected withvarious types of health problems, our aim is to understand how impairing proteotoxic stress pathway alters development and ageing. Therefore our aim is to phenotype (describe the set of observable characteristics or traits of an organism) these new genetically altered mice throughout their lifespan, from embryonic stage to adulthood to the age of 24 months.

Typically, what will be done to an animal used in your project?

Animals are expected to develop movement disorder (e.g. tremor with impaired posture and balance). Movement disorder will be permanent, although it is not expected to be fatal and animals will be killed before any symptoms become life-threatening. In summary, mice will be aged up to the age of 24 months. Typically mice will go through behaviour testing (e.g. object recognition to measure cognitive abilities and rotarod test to evaluate motor behaviour), non-invasive imaging (MRI) and have blood samples taken approximately in every three months, for example at the age of 1 month, 4 month, 7 month etc. Typically at the end of experiment, mice will be injected of cell labelling agents using standard routes (subcutaneous, intraperitoneal). And thereafter mice will be killed humanely at specifictime points and tissues will be collected for RNA and protein analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of animals are not expected to show harmful phenotypes that are more than mild andtransient.

Some animals will undergo surgical embryo transfer and they will experience transient post-operativepain and discomfort. Animals undergoing non-surgical embryo transfer will experience mild transient discomfort and no lasting harm.

Animals undergoing surgery for vasectomy will experience short-lived post-operative pain anddiscomfort.

Some genetically altered animals will develop movement disorder (e.g. partial hind limb paralysis,abnormal gait, involuntary movements, tremor, impaired posture and balance).

Some genetically altered mice will be maintained for ageing until they reach a maximum of 24 monthsof age. Animal that live beyond 12 months may experience conditions associated with ageing such asweight loss, loss of appetite, reduced movement, skin and eye problems, coat changes, mallocclusion(teeth are not aligned properly), and spontaneous tumours. Additionally, growth and development abnormalities may be observable for certain strains.

Motor behaviour and cognitive tests will place the mice shortly in an unfamiliar environment.

During non-invasive imaging e.g. MRI under isoflurane anaesthesia will take no longer than 30 minutes. Mice will have no more than eight anaesthetics throughout their lifetime and only one in any 24hr period. In case during MRI with isoflurane anaesthesia mice do not fully recover from the anaesthesia within 24 hours (eating, drinking and return to normal behaviour) they will be killed by aSchedule 1 method.

During blood sample collection from superficial vessels animals will experience mild and transientdiscomfort.

Animals will experience mild and transient discomfort from intraperitoneal and subcutaneous injectionof cell labelling agent.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Mice:

Mild 60%

Sub-threshold 20%

Moderate 20%

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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In-vitro research (cell culture, brain organoids (three-dimensional tissue cultures that are derived fromstem cells), brain slice culture) and computational methods are used in parallel with in vivo studies.

Although in-vitro assays alone cannot adequately model the complete array of molecular, cellular, developmental, physiological, and behavioural interactions necessary to fully understand how geneticmodifications result in normal or abnormal brain physiology and that can only be studied in whole organisms[1]. Zebrafish has become a model organism in neuropharmacology, although they are not mammals and thus are not as closely related to humans as a mouse is[2]. Moreover, nematode worm, fruit fly or other non-protected animal alternatives do not replicate system level neurological changes that occur during human neurodevelopment, ageing and neurodegeneration[3].

Barré-Sinoussi, F. & Montagutelli, X. Animal models are essential to biological research: issuesand perspectives. Future Sci. OA 1, fso.15.63 (2015).

Kalueff, A. V., Stewart, A. M. & Gerlai, R. Zebrafish as an emerging model for studying complexbrain disorders. Trends Pharmacol. Sci. 35, 63–75 (2014).

Arora, S. & Ligoxygakis, P. Beyond Host Defense: Deregulation of Drosophila Immunity and Age-Dependent Neurodegeneration. Front. Immunol. 11, 1574 (2020).

Which non-animal alternatives did you consider for use in this project?

We considered to use human stem cells derived organoids to study neurodevelopment, althoughorganoids cannot be used to study motor and cognitive behaviour.

In parallel to animal work we will use primary neurons that can be isolated directly from animal brain tissue. We will use primary cell culture to study the molecular and cellular consequences of impaired proteotoxic stress response. Although, primary neurons model immature (neonatal) neuronsand therefore they cannot be used to study neurodegeneration, motor and cognitive behaviour. To create mouse primary neuronal or mixed culture, neonatal mice are needed.

To define therapeutic targets we will use mouse brain slice culture, although for this mouse brainsare needed. However, the animals can be spared from pharmacological treatments.

Why were they not suitable?

Non-animal alternatives will be used in parallel to animal experiments, although they alone cannot be used to study neurodevelopment, neurodegeneration, motor and cognitive behaviour, this can only be done in whole animals. Moreover, cells are cultured in an

environment that is very different from that ina live animal. Furthermore, cells in culture experience a variable and abnormal oxygen tension, are usually cultured in high concentrations of glucose, growth factors, survival factors and secreted substances derived from cows. Although brain organoids can be used to study neurodevelopment, they are still unable to model complex interaction of multiple cell types.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 be studying at least 10 different mouse lines with different mutations in proteotoxic stress pathways over 5 years. Mice are needed to generate homozygous cohorts (two of the same mutant alleles are inherited).

Estimation of numbers will be informed by an analysis of published work and preliminary experimentsand with assistance from a statistician. We will use power calculations.

Variability between experimental groups will be limited by using closely related mouse strains raised in a controlled environment, free from specific diseases, fed a uniform diet and matched for age and bodyweight. Imaging and analysis will be performed by blinded and experienced users.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We will regularly consult websites such as Norecopa (https://norecopa.no/) and NC3Rs (https://www.nc3rs.org.uk/) and read the NC3Rs e-newsletter. We will take advice from dedicatedtechnicians within the animal units.

To minimise the number of animals examined to reach significance for any assay, we will minimise variance in phenotypic measures. This is done by using age and gender matched cohorts, born withina window of approximately seven days. Furthermore, genetic background is well known to influence phenotypic measures. Therefore, to minimise variance associated with segregating modifiers, mutations are generated and maintained on a defined and uniform genetic background. Before newly generated mice strains undergo behavioural assessment, analysis will be carried out on the target gene(s) to ensure it is appropriately modified.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Where there is a need to use alternative genetic backgrounds for certain tests, pilot studies may be employed to provide baseline data and experiment feasibility. Where new strains are created; a smallcolony will be generated on the breeding protocol of this licence initially to assess for lethality and welfare prior to larger cohorts going through the developmental/phenotyping steps of this protocol.

Wherever possible, multiple experiments will be performed on the tissues collected from an individualmouse. For example, in addition to the brain, the spleen can be used to isolate T cells for toxicology assays (to understand the harmful effects of the environment), heart can be used to study changes incardiomyocytes (heart cells), the bone marrow can be flushed and frozen down, multiple tissues can be collected for DNA/RNA/protein extraction for expression analysis and embryos harvested from females for establishing embryonic stem (ES) cell 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mouse as an experimental model because the genome of the mouse has been well characterised. This all allows us to genetically manipulate the genome and thereby study the role ofproteotoxicity in brain development and ageing, induced by individual gene mutations.

In order to define the consequences of impaired proteotoxic stress response in newly created mice brain physiology, we will perform motor behaviour tests such as: Catwalk, Rotarod, Grip strength to asses motor and cognitive behaviour. Additionally to define which brain region is affected, we will perform in vivo MRI analysis. All previously planned experiments are non-invasive and therefore causethe least harm. Additionally we will collect blood to test biomarkers and we will inject cell labelling agents before culling mice. Blood collection and dosing will be done using the minimum possible volumes and frequencies.

Similar studies investigating the role of proteotoxicity in brain development and ageing cannot beperformed in humans or in other non-animal models

Why can’t you use animals that are less sentient?

There is detailed knowledge and comparison of mouse physiology and genetics which allows to compare mouse physiology to human physiology. Moreover our aim is to define the consequences of impaired proteotoxic stress response to motor and cognitive behaviour. This cannot be done using lower vertebrates such as zebrafish because they are not mammals and thus are not as closely relatedto humans as a mouse is1. Thus, we will use mouse brain to model proteotoxicity of human brain. The mouse represents the lowest level of sentience to investigate the role of proteotoxicity in development and ageing in mammalian brain.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The procedures carried out in this protocol will be refined to a high extent, prioritising the

welfare of theanimal.

We will ensure that the animals are regularly monitored by experienced workers so as not to exceedpre-determined endpoints that might increase the amount of distress caused.

Animals are housed according to the best recommendations and enrichment, and nesting material willbe added to cages and where possible mice will not be singly housed. We will ensure there is acclimatisation to handling and procedures and optimal handling and interaction with the animals to maximise their welfare.

Mice will be monitored using a scoring system. Mice will be monitored weekly, although when needed e.g. after detecting weight loss, mice will be monitored daily. When needed, there will be intervention toprevent further worsening of body conditioning such as providing food on the cage floor, including mash and gel packs. Mice cages will be required to display health observation cards to notify technicians of scoring to ensure counter measures remain in place. Where weight loss is not improvedby these measures, mice will be culled. In all cases, weight loss is expected to be gradual.

All procedures will be continually evaluated, reviewed and refined to minimise and reduce experimentalduration, animal numbers and suffering while maintaining or improving scientific benefits. To facilitate this, we will have regular meetings within the lab after every series of experiments, and we will also have similar discussions with our close collaborators.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will refer to LASA guidance for surgery and aseptic techniques https://www.lasa.co.uk/current_publications/. We will use guidelines (PREPARE) prior to initiating anyexperimental study to aid in the planning of each stage (https://norecopa.no/PREPARE), and guidelines (ARRIVE) to help in the design, analysis and reporting of all studies (www.arriveguidelines.org).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will regularly consult websites such as Norecopa (https://norecopa.no/) and NC3Rs (https://www.nc3rs.org.uk/) and read the NC3Rs e-newsletter. We will take advice from NVS, NACWOand technicians within the animal units. Additional support will be given by ATLA (Alternatives to Laboratory Animals) Journal: https://journals.sagepub.com/home/atla, and the LASA Guidelines: https://www.lasa.co.uk/current_publications/.

PROTECTING HEARING AND VESTIBULAR SYSTEMS

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

hearing, vestibular, hair cell, deafness, balance disorders

Animal types	Life stages
Zebra fish (Danio rerio)	adult, embryo, neonate, juvenile, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aims of this project are to reduce, protect and/or alleviate hearing loss and balance disorderscaused by aging, genetics or the environment by identifying new routes and/or compounds for therapeutic intervention

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 inner ear, located behind the eardrum, comprises two parts: the cochlea and the vestibular system. Our cochlea detects vibrations made by sound, translating these into electrical signals our brain perceives as noise, while our vestibular system detects body movement; translating these into signals our brain uses to maintain balance and orientation. Both structures contain hair cells, specialised cells with height ranked hair-like projections on the top end of the cell that are deflected bysound and movement transferring this movement into electrical signals. In addition to the hair cells, there are specialised gelatinous accessory structures that either transmit the sound waves and body

movements to the hair-like projections (stereocilia) or act as structures against which the projections can push. Defects in the hair cells and the accessory structures can result in deafness and/or balance disorders.

The hair cells of the inner ear are lost with age; as a result of disease; and due to environmental conditions, such as excessive noise or as an unwanted side effect of certain life-saving medication. It isestimated that 40% of people over the age of 50 have hearing loss. While one in 2000 babies is born deaf due to one of a number of genetic defects.

Vestibular disorders, like hearing loss, can again result from age; or as an unwanted side effect of certain life- saving medication but these are less well understood as they can, to some degree, be compensated for by the visual system. As we age, however, our eyesight also fails. It is suggested thatdefects in the vestibular system may underlie balance problems in the elderly with the NHS estimating that falls cost the taxpayer more than £2.3 billion per year.

The proposed research will use zebrafish as a model system to understand hearing loss and balance disorders and to discover new and refine existing compounds that will protect human hearing and balance. In using zebrafish as a model we can also aim to answer fundamental questions in fish biology regarding the structure and function of the lateral line compared to the inner ear. The lateral line is a series of discrete sensory patches running along the fish's body that sense vibrations and pressure changes in the surrounding water. These sensory patches have hair cells and accessory structures similar to those in the mammalian inner ear, though in the fish they play an important role in shoaling, prey avoidance and have overlapping functions with the inner ear in orientation. Studies have shown that damage to the lateral line results in fewer fish swimming in the same direction and collisions between neighbours increasing resulting in physical damage and the risk of infection.

What outputs do you think you will see at the end of this project?

The outputs at the end of this project will include new information that would lead to publications or future research proposals. This information will help us to better understand 1) how some clinically important medications cause the loss of hair cells from the inner ear, leading to deafness and balancedisorders, and identify compounds (products) that could protect these hair cells 2) how either age of mutations in certain genes leads to defects in balance and orientation.

Who or what will benefit from these outputs, and how?

In the short term (2-3 years) this project will advance our understanding of how certain medications and mutations in our genes cause deafness and balance disorders benefiting the research community.This research may also lead to the discovery of drugs and strategies for preventing and/or managing hearing loss and balance disorders benefiting people at risk of these conditions (5+ years). Balance disorders are particularly under researched with few viable interventions for those who experience them, therefore this research will be beneficial to other researchers (2-3 years) and people experiencing balance disorders (5+ years)

In addition to the benefit this will have for humans this research will also benefit fisheries research as itwill improve our understanding of the genes responsible for balance and orientation in fish and the roles played by the inner ear and lateral line in shoaling, prey avoidance and orientation. This information could be used by researchers to improve fish

welfare in commercial fisheries and/or aquaria.

How will you look to maximise the outputs of this work?

Our data will be readily available to the research community. We maintain regular communication with the research community in the UK with meetings, collaborations and email correspondence with other hearing researchers, as well as zebrafish researchers and technicians to exchange good practice in animal care and welfare. We attend and present data at meetings with ENT clinicians and audiologists to exchange information on the relevance and need for research in human health it is these discussions that have led to our research into balance disorders. We endeavour to disseminate our research at relevant international meetings such as the Midwinter Research Meeting of the Association for Research in Otolaryngology in the USA, the largest scientific meeting in hearing research, Inner EarBiology meeting and relevant zebrafish meetings. We will publish in open-access journals or traditionaljournals that offer the option of making content freely available. In addition to this we will present at public engagement and stakeholder events, for example RNID organise events for donors and people with hearing loss to meet the scientists and discuss their research we have presented these several times.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 18800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This research project will use zebrafish as the model species. Zebrafish provide advantages over mammals as many of the experiments can be done on larvae under the protected age as the hair cells and accessory structures are formed and fully functional early in development. In addition, this project seeks to understand the contribution of the inner ear and lateral line (unique to fish and amphibians) to hearing and balance in fish therefore zebrafish make an ideal model system. Protocols conducted on the protected life stages are the production and maintenance of genetically modified animals and also behavioural tests. Non-animal alternatives are not possible, as functional hair-cell lines do not yet exist.The literature has some examples of organoids (organ-like structures derived from stem cells) and mouse cell lines derived from hair cells but these do not respond well to sound-like stimulation and are less sensitive to drugs that cause hearing loss compared to fully functional hair cells. In addition to this some of the research in this project seeks to investigate how altering hearing and balance genes effectanimal behaviour, this cannot yet be done using synthetic methods, particularly given that balance disorders are multifaceted and can to some extent be compensated for by environmental cues.

Typically, what will be done to an animal used in your project?

Protocols conducted on the protected life stages are the production and maintenance of geneticallymodified animals and behavioural tests.

Animals involved in the production and maintenance of genetically modified animals will be

naturally mated to produce larvae for experiments (before protected life stages) or larvae, juveniles and adults for behavioural experiments. Animals may also have small amounts of tissue removed for genotyping or in the unlikely event that natural mating is not possible be used for production of gametes for in vitrofertilisation.

Behavioural tests will involve wild type or genetically modified animals being placed in an observation chamber and videoed free swimming, swimming in a current (assessing rheotaxis) or when stimulated (assessing the startle response). The time in the observation chamber will not exceed an hour. Animalsmay undergo this procedure up to 10 times in their lifetime to understand how behaviour changes with age.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of the animals on this project are expected to show no adverse effects. Those animals with mutations in hearing and/or balance genes may show abnormal behaviour, for example occasional spinning or circling.. This may occur throughout the life of the animal though it is possible some of the animals may adapt and show no abnormal behaviour. To minimise any distress caused tothe fish we have developed a number of mitigations. As defects in the vestibular system can be largelycompensated for by the visual system mutant fish showing abnormal behaviour are housed with wild type companion fish to aid in shoaling, are provided with appropriate environmental enrichment (e.g. plastic plants and or housing/refuges) and also provided with tank adornments that simulate the riverbottom allowing the animal to calculate tank depth and therefore aid in orientation.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The expected severity of the majority of animals for both the production and maintenance of geneticallymodified animals and behavioural tests is expected to be either mild (max 50-55%) of subthreshold (min 25%). Some moderate phenotypes are expected in approximately 20-25% of cases of those animals with mutations in hearing and/or balance genes. .

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Non-animal alternatives are not possible, as fully functional hair-cell cell culture lines do not yet exist.In addition to this some of the research proposed seeks to investigate how defects in hearing and balance genes effect animal behaviour which cannot yet be done

using synthetic methods.

Which non-animal alternatives did you consider for use in this project?

We have looked in the literature and considered organoids (organ-like structures derived from stemcells) and mouse cell lines derived from hair cells.

Why were they not suitable?

Organoids and mouse cell lines are not suitable as these do not respond well to sound-like stimulationand are less sensitive to drugs that cause hearing loss compared to fully functional hair cells.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissueand reuse.

How have you estimated the numbers of animals you will use?

Numbers are based on actual numbers from similar zebrafish experiments conducted on a previous project licence with adjustments made for the increase in the number of anticipated behavioural tests,genetic variability, and environmental variability. The number of animals required for production of GAlines varies greatly depending on the gene mutated or the DNA inserted. In addition, pilot data suggests that not all homozygous animals will show the phenotype as it is anticipated that some animals may adapt to vestibular defects and therefore a larger number may be needed.
We expect to use up to 3000 fish for the generation of 40 new strains. This is calculated based on theassumption that for 25 strains only 1% of the animals will be able to transmit inserted DNA to the nextgeneration, for the other 15 strains we calculated a 5% success rate of producing a desirable gene mutation able to be transmitted to the next generation.

We expect to use up to 10,000 GA fish for breeding purposes to supply embryos for experiments and for maintenance of the strains. This is based on 50 different strains, the addition 10 being combinationsof strains. We also expect to use 5,000 of those 10,000 fish for testing vestibular function and rheotaxis.

Zebrafish are shoaling animals and would ideally be housed in groups of 10-20. New generations areraised when adults are between 6-10 months the optimal age for successful reproduction. Older generations are also required for aging experiments. For some combinations of strains, only a small proportion of the fish are the correct genotype. Over a 5 year period, this adds up to 10,000 fish. A further 800 fish will be used for the production of gametes.

The calculation of 18800 fish is the maximum number of fish used for all experiments the number is actually lower as the 5000 fish used in behavioural experiments and 800 for production of gametes arethe continued use of the GA fish.

What steps did you take during the experimental design phase to reduce the

number of animalsbeing used in this project?

The experiments were designed in consultation with a statistician and lecturer in experimental design who is familiar with the research. A calculation of the number of animals needed is based on pilot datafrom a previous project licence analysed by the statistician.

We can preserve sperm to reduce live animals, negating the need to recreate strains or combinationsof strains, and reduce genetic drift.

We aim to trial genotyping embryos < 5dpf using either the protocol outlined in Wilkinson et al 2018 (https://doi.org/10.2144/000114509) or the Zebrafish Embryonic Genotyper, which gently removes cellsfrom the skin for genotyping. If these are successful then only fish of the required genotype will then beraised to adulthood, reducing numbers.

Finally, as these experiments use zebrafish as a model, we have the opportunity to answer questionson the role played by the lateral line and inner ear in maintaining balance and orientation in fish, with only a few extra experiments as we can use data from the inner ear mutants produced in other experiments.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Pilot studies have been undertaken on a previous licence. These pilot studies were conducted usingzebrafish and mice with a mutation in a gene expressed in the vestibular system of the inner ear in both animals. This data showed that only 20-25% of animals showed an adverse phenotype.

To keep the number of fish to a minimum the GA zebrafish used for breeding will also be used for behavioural experiments. Optimisation of experiments will mean that tissue will be taken from existingexperimental animals so that no additional animals will be needed for tissue collection e.g. animals nolonger needed for breeding and/or behavioural experiments will be culled and tissue used for analysisof morphology.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

This research project will use zebrafish as the model species with most of the experiments doneon larvae under the protected age as the hair cells and accessory structures are formed and fully functional early in development. Protocols conducted on the protected life stages are the production and maintenance of genetically modified animals and also behavioral tests e.g. videoing zebrafish in a viewing chamber both free swimming and in a current, testing alertness (startle reflex).

Why can’t you use animals that are less sentient?

Production and maintenance of genetically modified animals must be done on fish that have reached sexual maturity. We will be using a number of life stages to conduct behavioural assays to understandhow balance changes with age both for human health benefits and fish welfare therefore other less sentient species or terminally anaesthetised animals can’t be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

To minimise any distress caused to the fish by the procedures we have developed a number of mitigations. As defects in the vestibular system can be largely compensated for by the visual systemmutant fish showing a moderate phenotype are housed with wild type companion fish to aid in shoaling, are provided with appropriate environmental enrichment (e.g. plastic plants and or housing/refuges) and also provided with tank adornments that simulate the river bottom allowing theanimal to calculate tank depth and therefore aid in orientation.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

I will follow the RSPCA Guidance on the housing and care of zebrafish, the NC3R refining proceduresdocumentation on their website and also check the Norecopa website as they publish links to articles showing best practice and refinements for animal welfare.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

I am on the NC3R mailing list and have attended relevant workshops. If possible I attend zebrafish husbandry association meetings, if not possible I discuss the meeting with colleagues who have attended. I am in regular contact with the NACWO who keeps me updated of any advances and wediscuss how we implement them.

STUDYING THE OUTCOME OF INFECTIOUS DISEASE FOLLOWING CHANGES TO THE HOST

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Pigs, Infectious Disease, Prevention, Intervention, Microbiome

Animal types	Life stages
Pigs	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Disease-causing microorganisms can cause devastating illness in pigs. The aim of this license is to devise and assess new intervention strategies to combat infectious disease in pigs. The strategies for disease intervention include assessing the effects of genetic variance, either natural or through genome engineering, the impact of vaccination or treatment, or the prevention, for example through probiotics or other microbiome manipulations.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Infectious disease in pigs not only has significant economic but also severe welfare impacts. Of major interest to our research are viral diseases. As mandatory intracellular parasites, viruses rely on the host for their replication. It is also the host's immune response that determines severity of illness.
However, viral disease rarely occurs in isolation since modifications to the immune system allow for bacterial disease to take hold as an opportunistic infection. Both enteric and respiratory illness in pigs are known to frequently be polymicrobial and in the majority of cases of respiratory disease, viral pathogens are known to be the primary infectious agents. This increases the use of antimicrobials even for primarily viral illness.

Devising new intervention strategies to prevent and treat infectious disease in pigs are paramount not only to combat viral and complex disease in pigs but also prevent potential zoonotic transmission. In this project, we aim to investigate the impacts of the following intervention strategies:

1) The impact of microbiome manipulation on enteric viral disease severity and prevention

Coronaviruses emerge relatively often in pigs, and most commonly cause severe enteric disease in young pigs. Enteric diseases that cause diarrhoea in young pigs impact their welfare and are a major source of loss to farmers as there is a high rate of mortality, and those that survive often have reduced growth for the duration of disease and recovery. Often farmers treat young pigs with antibiotics to protect them from bacteria that can cause diarrhoea, however this does not protect them from non- bacterial sources of enteric disease, such as viruses, or from disease caused by antibiotic resistant bacteria. There is evidence that commensal microbes that inhabit the guts of animals, the gut microbiome, play a role in priming the immune system to more quickly and efficiently respond to pathogens. Young pigs lack a mature gut microbiome, and treating them with antibiotics further depletes their microbiomes. Given the microbiome's role in priming the immune system to respond to pathogens, introducing microbes that are capable of priming the immune system prior to exposure to pathogens could lead to a more robust immune response to these pathogens and lead to a milder disease, or no disease, as is seen in adult pigs.

Here, we will manipulate the microbiomes of pigs to change the diversity of species present in the gut prior to challenging with Transmissible Gastroenteritis Virus (TGEV). The pigs will be cannulated to allow for access to their ileum microbiome throughout the trial. Cutting edge technology will be used to understand how the introduced microbes change the gut microbiome and the pigs' immune responses and identify the functional potential in the microbiome that may contribute to changes in the immune response.

2) The impact of genetic variation on disease severity or prevention

3) The impact of drug intervention strategies on disease severity

4) The impact of vaccines on disease prevention

Overall, this research directly benefits pigs and, in the long run, will result in healthier pigs thereby reducing usage of antibiotics and contributing to pig welfare, a healthier environment and better meat quality.

What outputs do you think you will see at the end of this project?

- Novel information on basic host-pathogen interactions and mechanisms of pathogen pathogenicity will be generated.
- Novel information will be provided on the composition of the pig microbiome, gut-resident microbial species, the relationship between faecal and resident microbial species, and the impact of manipulation on the microbiome and immune response.
- Novel information on the impact of (mucosal) stimulation of the immune system on disease.
- Peer-reviewed publications on the investigated topics and outcomes will made available to other researchers but also the general public via open access.
- Possible products of this research include novel probiotics for the prevention of severe viral enteric and complex disease

Who or what will benefit from these outputs, and how?

Livestock

This research directly benefits livestock in the long term in several ways. By investigating the consequences of important pathogens on a livestock and by identifying and testing control measurements against these pathogens this project will result in controlling diseases and ultimately healthier livestock. For example, understanding the consequences of microbiome manipulation can lead to targeted, scientifically-based, beneficial manipulation to increase overall health of animals and reduce the use of antimicrobial agents. This benefits not only the animals but also the consumer.

Consumers

One of the main aims of this research is to develop better husbandry strategies and improve animal health, which ultimately reduce reliance on antimicrobials. This in turn will reduce the risk of animal agriculture contributing to antimicrobial resistance, a major concern for consumer health. Healthier animals with improved welfare and better meat quality are important considerations for the public.
Furthermore, reduction of animals with reduced weight gain and premature deaths reduces the food waste in the production chain, improving the environmental footprint of animal protein production and reducing costs.

Veterinary Practitioners and Farmers

This research will provide a better long term understanding of the pathogenesis of infectious livestock diseases which ultimately will lead to improved intervention strategies to prevent and treat pig infectious disease. Novel intervention strategies can prevent disease or improve disease outcome.

This has a strong impact on animal welfare but also an economic impact for farmers through improved weight gain, reduction of food waste in the production chain, and better feed conversion. Veterinary practitioners and farmers can make use of novel intervention strategies, such as drugs, vaccines, or probiotic feed additives.

Scientists

Publications that become available during the course of this project will contribute to scientific knowledge immediately.

Enteric viruses in pigs are causing major economic losses. Coronaviruses (CoVs) are emerging and re-emerging worldwide and are often associated with severe disease in particular in suckling and weaned piglets. The outputs from this research project will benefit a wide range of academics with research interests in virology, immunology, microbiome interactions, antimicrobial use reduction and replacement and infectious diseases in livestock in general and pigs in particular.

How will you look to maximise the outputs of this work?

The outputs of this work will be maximized by collaborations with other investigators within the Establishment but also across other UK institutions and other nations. Any new finding will be presented at farm animal producer and/or veterinarian meetings at the UK, EU and international levels. Knowledge obtained in this research, regardless of success, will be published in international journals and presented at international meetings as we have done in the past.

Species and numbers of animals expected to be used

- Pigs: 35

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Juvenile/weaned pigs: Most commonly pigs will be used at weaning or shortly afterwards as vaccines or treatments are often given to young pigs around this time. Furthermore, at weaning pigs are often leaving their farm of origin and are co-mingled with pigs from other sites, as a standard practice in pork production. Comingling frequently results in transmission of pathogens between pigs. Therefore, studying this crucial phase of an underdeveloped and highly susceptible immune system with the high risk of exposure to new pathogens during a stressful phase in piglets lives is highly important to developing new strategies to combat disease burden.

Adults: Sows will be used as faecal microbiome donors. Both their age and immunological experience make their microbiomes highly diverse and ideal for enrichment in juvenile pigs.

Typically, what will be done to an animal used in your project?

For typical investigations on conventional sourced pigs, they will be obtained from a commercial farm at weaning age and will be randomly divided into several groups and rooms. After an appropriate acclimation time, animals may undergo prevention treatment, such as vaccination or microbiome manipulation, prior to infection with a disease-causing infectious agent. In some cases, animals may undergo surgical placement of an access cannula to allow for repeat sampling at a specific locus, e.g. collection of gut contents to monitor the microbiome of the small intestine through an ileum cannula.
Sampling may start as early as two days after placing a cannula and as frequently as every second day. Other samples collected may be blood, nasal swab, or faecal swabs/samples. Blood samples will be collected on the same dates as cannula samples and may only be taken every second day.

What are the expected impacts and/or adverse effects for the animals during your project?

Haemorrhage during blood collection. The duration of the direct impact of haemorrhage on the pig is commonly 10-20 min. In very rare cases, the pig may be weak for up to 3 h.

Epistaxis as a potential adverse effect of nasal swabbing

Wound healing disruption at the cannulation site: The pig needs to be caught restrained and held still while opening the cap of the cannula. This can all result in wound healing issues at the surgery site progressing into septicaemia or abscesses.

Clinical signs after challenge: Fever, diarrhoea, weight loss, respiratory signs, lethargy. The duration of these clinical signs depends on the pathogen involved but typically, clinical signs may start as early as 1-2 days after challenge for enteric pathogens such as TGEV.

Expected severity categories and the 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 of the surgically treated (cannulated) pigs to reach a moderate severity. Control animals are expected to reach mild severity.

Pigs: Up to 65% moderate, 35% mild

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?

We are working with the target species and will investigate diseases and intervention strategies in pigs. Studying the disease in the natural host is essential in order to inform management and production decisions which are immediately translatable.

We aim to generate as much data as possible on any intervention strategies using non-animal alternatives, including cell lines, stem cell-derived complex models, such as organoid or air-liquid interface systems, and primary cells derived from surplus research animals, surplus tissue from animals culled for other purposes, or slaughterhouse samples.

Some of our research explores highly complex host-pathogen interactions that rely on systemic interventions that cannot be modelled in less complex systems, such as the modification of an organism's microbiome or a systemic change to disease susceptibility.

Which non-animal alternatives did you consider for use in this project?

Non-animal derived alternatives including cell lines, organoids and tissue sections have been identified and will be used whenever possible to generate data.

Several immortalised cell lines of different tissue origin of the pig are available to us, including PK-15 (kidney), IPEC-J2 (jejunum), IMI-2 (ileum), ST (testis), NPTr (trachea), NSK (kidney). Furthermore, we have developed small intestinal (ileum) organoid systems (2D and 3D) relying on adult stem cell technology, that can be used to model simple infections and stimulations through cytokines or (inactivated or cultivation supernatant) bacteria in vitro. Induced and embryonic stem cell technology is available to us and we are using iPSC-derived macrophages wherever possible. We also have primary monocyte and macrophage cells stored from surplus animals or to make use of tissue from animals on other studies following culls where these tissues were not used.

For the initial projects proposed on this license, namely the impact of the microbiome on TGEV infection, we have used cell lines to cultivate and assess TGEV infections in vitro. We will furthermore use the more complex cell line models of IPEC-J2 and organoids to validate findings reducing the need for in vivo work. However, as the microbiome, especially with regards to mucosal immunity is very complex, a complete replacement is unfortunately not possible.

Why were they not suitable?

It is not feasible to quantify organ colonisation, systemic spread, tropism or immune responses mediating pathology or protection solely in cell-based assays or organ culture systems such as lung or liver slice cultures. Non-animal derived alternatives as listed above, are not suitable to study the development of a disease, infectivity of a certain pathogen, determining if vaccines protect an animal and the impact of systemic modulation of the immune response following microbiome manipulations.
Cell culture systems are not suitable to study interactions between the animal’s immune system and the disease-causing agent. Similarly, antibody responses (“titre development”) can only be studied in pigs /ruminants and not in cell culture systems.

The decision to use animals will only be taken once other avenues of research, such as in vitro studies have been exhausted. Alternative routes will be considered whenever possible and feasibility of using alternatives will be assessed for each separate study. Cell culture studies are being done and results will inform future animal studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number is based on planned funded experiments and experience doing similar studies in the past. For each of our projects, approval by the local Animal Welfare and Ethical Review Committee is only given where the animal numbers have been demonstrated to be the minimum consistent with deriving appropriate scientific data.

Whenever possible, we will use protocols that refine methods and reduce numbers. For example, the ileum cannulation model significantly reduces animal numbers (x*samples collected) whilst improving the sample quality, thereby reducing the numbers of animals needed to reach statistical significance.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

- The Experimental Design Assistant (https://www.nc3rs.org.uk/experimental-design-assistant-eda)
- Relevant literature on this topic including published studies for the pathogens investigated.
- Advice from experts in the area of investigation.
- A statistician has been consulted when planning individual studies to determine the minimal number of pigs needed to have enough statistical power to determine significance if there are true differences between groups.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- Initial small-scale studies will be done whenever no or limited data are available.
- Animal studies that could be shared with other researchers to investigate two scientific questions at the same time will be preferred.
- Computer modelling to investigate aspects such as infectivity speed will be used in the optimization phase for the animal project.
- Tissues and other biological samples collected post-mortem, or residual blood will be shared if not needed to achieve the project aims.
- Whenever feasible biobanks of tissues or blood will be established.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Pigs are the primary host of the investigated infectious agents. Studying the disease in the natural host or a host that is very similar to the natural host is essential in order to inform management and production decisions which are immediately translatable. Information gained on the host and pathogen used in this study, i.e. pigs and TGEV, will have wider impact on the role of the microbiome, mucosal immune stimulation, and mucosal viral disease. All these aspects can be translated to other hosts, whether this is human, ruminants, or other livestock.

Other groups have reported using rodent models with many of the infectious agents we intend to investigate in pigs. However, the outcomes of published studies vary between different groups, emphasising that while rodent models may be useful for analysis and characterisation of immune responses against selected pig pathogen vaccine candidates, for more complex host-pathogen interaction studies, such as microbiome manipulation, they are not a suitable alternative. Furthermore, rodents used in research are inbred and often immune-deficient or impaired lines, which do not reflect the complexity and variability of the immune response in an outbred species, such as pigs.

A clinical scoring system refined under preceding studies will be used to monitor the severity of infections and identify animals requiring increased monitoring, treatment or euthanasia. Commonly we measure rectal temperatures in pigs to identify animals that develop a higher than expected fever for a prolonged time.

When vaccines or drugs are used, these are typically commercially available and licensed products for pigs. While injection site reactions are not expected we typically monitor the reaction sites for up to three days for any reactions. All pigs are group housed, whenever possible, and refined models are being used.

Why can’t you use animals that are less sentient?

Pigs are the target species for the pathogens investigated. Other common laboratory species such as mice or rats cannot be used.

More complex host-pathogen interaction investigations are furthermore species-specific. Including the genome and expression of genes, the microbiome, and the immune response to pathogens. Therefore, lower species do not represent a model.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animals will be closely monitored tailored in frequency and type to the pathogen and intervention procedures. A plan for adverse effects is in place for each pathogen and model, detailed in the individual protocols. Pain management will be provided perioperatively for surgical procedures and if needed within the infection studies.
We aim to minimise any stress or discomfort to experimental animals. Pigs will be kept in social groups whenever possible. If this is not possible, pigs will be able to see each other through transparent partitions. All pig rooms/pens will be equipped with environmental enrichment. Other refinements that are in place include usage of real-time PCR cycle threshold numbers or virus genome calculation to inform endpoints. This information can help inform the severity of infection prior to development or worsening of symptoms. Furthermore, this can allow to set earlier endpoints where a certain level of infection and / or immune response has been reached.

Animal husbandry, surgery and anaesthesia will take place at our Establihsment's fully-equipped centre for large animal experiments. Anaesthesia and surgery will be overseen by the highly qualified vets with specialised knowledge.

Daily welfare checks will be carried out by the NACWOs or trained PIL holders. Where a potential welfare issue is identified the frequency of these checks will be increased.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3R guidelines (https://nc3rs.org.uk/the-3rs) will be followed.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Continued information through literature searches and reading of relevant information on model development and refinement, as well as specific developments for the study goals on hand.

Animal welfare will be prioritised throughout the study by incorporating all permissible refinements identified by the establishment’s Named Veterinary Surgeon and other veterinary colleagues.

The NC3Rs website (https://www.nc3rs.org.uk/) will also be consulted and full advantage taken of the annual seminar day organized by the Establishment to identify changes in best practice and methods to improve animal welfare.

Any adaptations will take place following discussions with the NVS and NACWOs before incorporating them into the study plan.

Home Office

SAFETY TESTING OF MEDICINAL PRODUCTS USING DOGS AND MINIPIGS

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

Regulatory, Safety Assessment, Dogs, Minipigs

Animal types	Life stages
Beagles	adult
Minipigs	neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Uses cats, dogs or equidae

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aims to test pharmaceuticals (for human use) to determine the scientific and/or regulatory endpoints in non-rodent (dog or minipig) toxicity, pharmacokinetics and metabolism for submission to regulatory authorities, to satisfy governmental regulatory requirements and for safety assessment purposes.

These studies are run to satisfy the requirements of UK/EU (and sometimes international regulatory authorities) who are independent of governments) which require the testing of pharmaceuticals in a non-rodent species. Study designs are based on OECD and ICH guidelines for Pharmaceutical and medical device testing

A retrospective assessment of these aims will be due by 09 September 2028

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence? Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

New medicines have the potential to be of benefit in new or improved disease treatments. Before potential new medicines are administered to humans or animals their safety must be evaluated. This testing is a mandatory legal requirement and provides information on risks to people taking new medicines. Often, the new pharmaceuticals we test in this programme will be designed to be better than existing treatments, possibly with fewer or less severe side effects.

At present there are no alternatives that don’t use animals that are scientifically, ethically or legally acceptable as replacements for systemic toxicity or safety assessment. Most new medicines are tested in rodents (mainly rats and mice) before being tested in a second, non-rodent species like the minipig or dog.

What outputs do you think you will see at the end of this project?

The overall benefit of this project is that it supports the development of safe, new medicines to improve the health and quality of life of human patients by generating high quality data that is acceptable to regulatory authorities and enables internal decision making within our client's organisations.
Achievement of the objectives of this licence will enable safe development candidates to progress and will also help to remove unsuitable candidates from the development pipeline at an early stage, thus saving animals and resources.

Study reports will be included in regulatory submissions to allow regulatory authorities to make judgements on whether to permit clinical studies or to licence a drug. Global guidelines recognise that the justification for animal-based regulatory toxicology and safety testing is the need for regulatory authorities to have sufficient information to assess the risks to which humans or animals are exposed by the use of new drugs. Supporting studies, including preliminary studies, will enable appropriate dose selection and appropriately focussed observations and investigations in the definitive regulatory studies.

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 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 can be dosed safely to humans. These 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 effect. This is important when planning future trials in humans or animals, 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 for regulatory purposes or to support regulatory purposes (e.g. to support drugs progressing to clinical trials). Where appropriate, we collaborate with our customers to share data we have produced in the form of scientific publications that are in the public domain.

We are able to advise our customers on which studies are required in their development programme and on suitable study designs, based on our experience and on knowledge gained from previous post-registration feedback from customers and/or regulators, leading to focussed and effective studies.

It is difficult to predict how the benefits of any work done on this project will be seen in the future due to confidentiality issues. However, this work will contribute to the safety of 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

- Beagles: 4800
- Minipigs: 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 dog or minipig are used in these studies, as they are well characterised species with a lot of background scientific data available over many years. They also satisfy the requirements of global regulatory authorities for safety evaluation in non-rodent species, which is required by law prior to testing in humans.

As stated earlier, most pharmaceuticals are tested in a rodent species prior to testing in a non-rodent species, as covered by this project.

It is a legal requirement in the UK that dogs (or cats or equidae) may only be used in a programme of work involving regulated procedures when the objectives of the work cannot be achieved by using another species. In this project, the dog will only be used when use of the minipig would not achieve the aims of the experiment, or satisfy regulatory authorities. All requests for studies using dogs are assessed by means of an internal review process; the review panel, including scientists, project licence holders and responsible persons under ASPA, consider the information presented to reach a consensus decision, and will only approve the use of dogs where there is robust justification that the study could not be successfully performed using minipigs instead.

Typically, what will be done to an animal used in your project?

Animals are dosed by the intended/likely route of human or animal exposure (for example oral administration, injection or infusion), and observed regularly to monitor appearance, behaviour and clinical health. Routinely, animals can expect to be blood sampled and undergo ophthalmoscopy.

Some animals may undergo a surgical procedure under general anaesthesia, eg placement of a deep vein catheter for intravenous infusion, or implantation of a monitoring device or minipump. Investigative

procedures carried out in these studies are similar to diagnostic procedures that might be used medically to monitor progress of a human patient and include, for example, collection of blood and urine samples for laboratory investigations, or ECG monitoring to assess heart rate/function, or examination of the eyes using an instrument similar to those used by opticians. Animals undergoing surgery receive the same sort of care as a patient would in hospital. We discuss their pain relief and use of antibiotics with a veterinary surgeon before we start. We administer drugs as necessary and give them plenty of time to recover from surgery before we use them in experiments. These surgical procedures are carried out only for essential purposes.

If we need to take a urine sample for analysis, we may put an animal into a special collection cage which is smaller than their normal cage. The animal can still move around.

Other more unusual tests might include assessment of neural function, taking small samples of tissue under general anaesthesia, collection/examination of body fluids, collection under general anaesthesia and examination of lung washings or spinal fluid, body temperature by rectal thermometer. A minimal degree of restraint or confinement may be required for some procedures. Where appropriate, positive reinforcement training (using treat rewards) is used to encourage co-operation in (and minimise any stress of) handling/procedures.

Some animals may be used on procedure on more than one occasion (re-use); such re-use is limited and strict criteria are applied, eg veterinary examination indicates that it is appropriate to do so. Some animals (dogs only) may be re-homed via the establishment’s rehoming scheme if it is in their best interests, but most animals are humanely killed at the end of the study to allow detailed examination of the organs.

What are the expected impacts and/or adverse effects for the animals during your project?

When dosing an animal by injection or taking blood, the degree of pain or discomfort an animal feels is similar to what a patient would feel having an injection done, or blood taken by a doctor.

Animals undergoing surgery receive the same sort of care as a patient would in hospital. We discuss their pain relief and use of antibiotics with a vet before we start and administer drugs as necessary.

Dosing with drugs may cause adverse effects in some studies. Experience from the last licence shows that roughly half (43%) of animals display only mild severity with the remaining 57% displaying moderate severity. Lethality and/or severe effects are not expected to occur, in any of the protocols in this licence.

We observe our animals at least twice a day, and the people who do this know the signs when an animal is ill. If an animal is ill, we would check it more frequently, and consult vets and other senior animal care staff for advice and guidance in its care.

Most animals are expected to experience no, or only mild, adverse effects during the course of the study such as slight weight loss. A small percentage of animals may show more significant adverse effects, such as more marked weight loss, reduced activity, vomiting or tremors. No animals would be expected to die or to suffer prolonged adverse effects as a result of the procedures, and where necessary early humane end-points are applied, under veterinary guidance as necessary, to prevent this; such end-points might include interventions to discontinue dosing, or to provide supportive treatments, or if necessary to humanely kill the animal.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

On the last project, about 50% of animals were classified as having experienced mild severity, the rest were classified as moderate. The moderate severities in the last project would have been due to treatment-related signs of moderate severity (mostly in prelims) or because a surgical procedure e.g. cannulation was involved. It's impossible to predict the proportion of severities expected on a service licence like this, as this will be dependent on what study types we are asked to perform. However, a distribution between ‘mild’ and ‘moderate’ severities similar to those in the last project are anticipated.

All protocols on this licence are classified Mild or Moderate only, there is no intention to perform any procedures that are Severe in nature.

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Rehomed
- Used in other projects

A retrospective assessment of these predicted harms will be due by 09 September 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Pharmaceutical testing in animals is a mandatory legal and regulatory requirement and provides information on risks to people and animals taking new medicines. At present there are no alternatives that don't use animals that are scientifically, ethically or legally acceptable as replacements for systemic toxicity assessment.

We maintain a constant awareness of regulatory guidance and ensure that where non-animal methods exist which fulfil the regulatory requirement, they are used in preference to animal studies.

The regulatory requirements are mainly for UK/EU regulators, but occasionally other regulators in other countries like the US for example. If the requirements for these non-UK/EU tests are over and above the requirements for a UK/EU regulators, or the test required is more severe, then we consult the Home Office to ask for prospective authority to run such tests.

Which non-animal alternatives did you consider for use in this project?

There are no other non-animal alternatives for the work being undertaken on this project. The regulations we are following will not allow safety decisions to be made on non-animal systems alone.

In vitro and in silico methods (test tube or computer models work not using animals) are used in combination with animal studies to inform study designs and assist in understanding of potential toxicity but cannot yet replace in vivo (animal) studies.

Why were they not suitable?

Although there are in vitro tests that can model some parts of 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 (animal or human) organism.

That is why we need to test new drugs in animals, as they have similar physiology and processes as humans, and that testing gives us a good idea what may happen if they are subsequently used in humans.

A retrospective assessment of replacement will be due by 09 September 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received from customers in the past. It is, however, impossible to accurately predict the number of studies that may be performed, in the circumstances.

The numbers of animals used in each study are in some cases specified in the regulatory guidelines; where not specified, numbers are based on established minimum regulatory expectation, or on scientific estimates of the minimum numbers required to meet study objectives.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Studies are designed to provide maximal data and statistical power (where appropriate) from the minimum number of animals considering that it is better to increase the number of animals used to achieve the objective than to use too few animals and risk having to repeat the study.

For regulatory studies, guidelines require the number of groups and animals per group to be adequate to clearly demonstrate the presence or absence of an effect of the test substance; core study designs are based on international guidelines where these exist. Otherwise reference is made to standard study designs with input from statisticians, where appropriate, to identify the optimum number balancing the need to achieve study objectives while avoiding excessive animal use. These internal designs are reviewed and updated in line with changing external guidelines and internal refinements that either minimise numbers or reduce severity.

Whenever possible, common species of animals are used such that a large amount of control background data is available. This reduces the need for large control groups.

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.

Before our main studies, we use smaller groups of animals to get an idea of the doses we need to use for the main studies. These preliminary studies are important as they give us confidence that the doses we are using are correct prior to testing them in bigger groups of animals as required by global regulators.

A retrospective assessment of reduction will be due by 09 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use juvenile and adult dogs and minipigs. We only use dogs when minipigs are unsuitable for scientific reasons.

The models we use are the least invasive procedures, for the least amount of time necessary to get the information we need. They are carried out using standard and recognised techniques by fully trained staff. We also have veterinary clinicians on hand for advice and on the occasions we have to anaesthetise the animals and for general advice on animal welfare.

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.

For all surgical procedures pain relief will always be provided. Surgical procedures will be carried out aseptically and to at least the Home Office minimum standards for aseptic surgery, and in accordance with the principles set out in the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017) (LASA is the Laboratory Animal Science Association). Any animals that undergo surgery will get the same standard of care as a patient who needed surgery in hospital.

Why can’t you use animals that are less sentient?

There is a scientific and regulatory requirement for safety/toxicity data in non-rodent species such as dogs or minipigs to supplement rodent data and enable a complete risk assessment. We use minipigs in preference to dogs wherever possible; (a legal requirement in the UK), and dogs are only used where necessary to achieve the study

objectives, ie when the minipig is unsuitable (for example due to species-specific differences from humans, confounding pharmacology or toxicological responses, or practical limitations due to anatomy or physiology).

Most of these studies require repeat dosing for days, weeks or months, to assess potential adverse effects in man, so it is not practical to perform them under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal welfare is of utmost importance and Good Surgical Practice will be observed for any animal undergoing surgical procedures. Surgery will be conducted using aseptic techniques (to prevent infection) which meet at least the standards set out in the Home Office Minimum Standards for Aseptic Surgery. Before we start surgery, we agree with a Vet what pain killers or antibiotics the animals need both before and after the surgery. When animals are recovering from surgery, we give them extra heat and monitor them closely until they are fully recovered and showing normal behaviour. We then check them at least twice daily before they go on study.

During dosing and restraint, animals are constantly and closely watched for signs of distress. If equipment is used to enable us to achieve the scientific aims of the study (e.g. confinement in a metabolism cage for urine collection), then we would habituate animals to this equipment prior to dosing. Most animals habituate well to this equipment, but if they don't (rare) we remove them from the study

If we have to repeatedly inject animals or withdraw blood using a needle and syringe, we would choose different sites to do this where possible to minimise local adverse effects.Where appropriate we place temporary cannulas in blood vessels to reduce the number of needle punctures necessary. If we can take blood samples when an animal is deeply unconscious, then we do so. All personnel performing these procedures are trained to a high standard to minimise adverse effects.

All procedures are subject to ongoing assessment and technique improvement and we participate in cross-company working parties on best practice. Animals are regularly reviewed for general health and veterinary staff are on call at all times to assess any adverse events and provide supportive care and treatment as appropriate.

Animal welfare costs are minimised by the careful selection of dose levels to reduce the likelihood of unexpected toxicity, and the application of rigorous and comprehensive humane endpoints.

Socially compatible species are routinely group housed with environmental enrichment which encourages species specific behaviours without adversely impacting study outcomes.

Individual studies are designed to cause the least possible suffering by frequent review of practices, provision of highly skilled technical staff and veterinary support, purpose built facilities and a clear focus on animal welfare. Any confinement or restraint is restricted to the minimum required to achieve the scientific objectives of the study and all study plans/protocols are reviewed for adherence to welfare guidelines and best practices by the site’s Animal Welfare and Ethical Review Body (AWERB).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

ICH Safety Guidelines–see www.ich.org/products/guidelines/safety/article/safety-guidelines.html
Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies S3A (1994)
Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies (S3B) (1994)

Duration of Chronic Toxicity Testing in Animals (Rodent and Non Rodent Toxicity Testing) S4 (1998) Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals S6(R1) (2011)
Safety Pharmacology Studies For Human Pharmaceuticals S7A (2000) Immunotoxicity Studies for Human Pharmaceuticals S8 (2005) Nonclinical Evaluation for Anticancer Pharmaceuticals S9 (2009)

Guidance on Non-clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorisation for Pharmaceuticals M3(R2) (2009)

OECD Guidelines – see www.oecd.org or www.oecd-ilibrary.org

OECD 409 – Repeated Dose 90-Day Oral Toxicity in Non-Rodents (1998) OECD 417 – Toxicokinetics (2010)
OECD 452 – Chronic Toxicity Studies (2009)

Summary of Considerations in the Report from the OECD Expert Groups on Short Term and Long Term Toxicology (2006)

Notes for guidance on repeated dose toxicity. Committee for Proprietary Medicinal Products (CPMP), 2010. CPMP/SWP/1042/99

Guideline on the evaluation of control samples in non-clinical safety studies: checking for contamination with a test substance. Committee for Medicinal Products for Human Use (CHMP), 2005. CPMP/SWP/1094/04

LASA/NC3Rs: Guidance on dose level selection for regulatory general toxicology studies for pharmaceuticals. http://www.lasa.co.uk/pdf/lasa-nc3rsdoselevelselection.pdf

Notes for guidance on non-clinical local tolerance testing of medicinal products. CPMP, 2001. CPMP/SWP/2145/00

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017) http://www.lasa.co.uk/wp-content/uploads/2017/04/Aseptic-surgery-final.pdf

Diehl et al. 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)

Gad et al. Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routes in multiple species with notes on methods to improve utility. International Journal of Toxicology: 1-84 (2016)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This will be achieved by regular discussions with our Named Information Officer, colleagues in Animal Technology, and by attending appropriate training courses and conferences, or getting feedback from such events.

A retrospective assessment of refinement will be due by 09 September 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?

REGULATION OF THE MUCOSAL IMMUNE RESPONSE DURING INFECTION WITH PATHOGENS OR PATHOBIONTS AND INFLAMMATION IN RODENTS

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Infection, Immune response, tuberculosis, peritonitis, Inflammatory bowel disease

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required,and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The over-arching aims of this Project Licence are to study the regulation of the mucosal immuneresponse to infection. Specifically, the aims are to:

Identify molecular pathways regulating resistance and susceptibility to Mycobacterium tuberculosisinfection, the causative agent of the disease tuberculosis.

Identify molecular pathways regulating mucosal immune responses in the intestine and the peritonealcavity.

A retrospective assessment of these aims will be due by 13 September 2028

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

An individual responds to infectious agents and other foreign substances to control and block diseasefrom progressing. An understanding of the immune response is thus critical for control of infectious disease and conversely to block the immune response from over-responding to microbes or foreign substances and causing damage to the individual.

Mycobacterium (M) tuberculosis studies

Tuberculosis (TB), caused by infection with the bacterium Mycobacterium (M) tuberculosis, remains a major cause of death from infectious disease, with 1.5 million deaths in 2020 1.6 millions deaths estimated in 2021 and a major need for new drugs. In addition to new antibiotics, therapies to enhance the individuals' immune response are badly needed to help eradicate this disease. It is knownthat a quarter of the world's population are estimated to have been infected by the pathogen, M. tuberculosis, but only approximately 10% of those individuals go on to develop TB disease. The individuals that remain healthy appear to control and eliminate the infection and so are resistant to the disease. These individuals only show evidence of exposure to M. tuberculosis, by a specific skin test or blood test, but remain healthy. The mechanisms underlying progression to active TB or control of the infection are unclear. An understanding of the early immune response in the airways which determineswhether an individual controls the infection or progresses to disease is critical to identify mechanisms of disease outcome. We are currently studying this in the bronchoalveolar lavage (BAL) of human TB patients and their contacts funded by a large grant and will compare and test findings from these studies in our experimental mouse models. It is necessary to test molecules, cells or pathways that areidentified during our studies of human TB, in experimental in vivo models to help to identify the mechanisms determining the outcome to M. tuberculosis infection. There is a need to understand why in some cases the infection is controlled whilst in other cases active TB disease develops. The aim is to identify the early immune events which determine disease progression or control, and thus to identify new host-directed therapies to enhance the effects of antibiotics in human TB. Currently, a combination of antibiotics is required, many of which can cause severe side effects, and they have to be administered for many months, leading to people not taking the drugs and to drug resistance.
Hence other therapies to enhance the protective immune response are badly needed.

It is essential to study the immune response to M. tuberculosis in vivo in experimental mouse models,since there is evidence from new highly-sensitive radiography of TB patients and their contacts, that the immune response starts in the immune organs and only later is evident in the infected lung tissuewhere immune cells migrate in to kill the pathogen. Thus, testing of molecules, cells and pathways in mouse models of TB resistance or susceptibility in vivo is essential to define targets for immune intervention and for the development of new host-directed therapies.

The experimental model that most resembles the human infection resulting in a controlled immune response or the development of active TB disease, that we can use in the UK and with which we can perform advanced immunological analyses, is the mouse model. Importantly, there are TB resistant and susceptible mouse strains, which when infected with M. tuberculosis, show similar lung pathologyand blood gene signatures of the immune response to human TB.

Intestinal immunity studies

Infection of the intestine via the oral route can be controlled or lead to intestinal inflammation, in somecases inflammatory bowel disease (IBD), and if there is dissemination it can cause systemic disease, such as peritonitis, which is a disease that can occur if microorganisms escape the gut and cause inflammation in the inner lining of the abdomen or other conditions. It is essential to study the intact mouse model since immune cells migrate into the tissue from immune organs during infectious challenge. In addition, we complement and replace some of the work in animals with epithelial organoids, which can be cultured with some of the immune cells represented in intestinal infections, which could be isolated from humans or the experimental model, for in-depth mechanistic studies reducing the number of mice used.

There is a high global burden of intestinal infection. The pathways of protection or pathogenesis, wherethe disease progresses or is controlled, are not clearly understood. Moreover, the burden of IBD (Crohn's Disease and Ulcerative Colitis) is rising globally, with substantial variation in levels and trendsof disease. Why some individuals develop IBD, whereas the majority of the world do not, is crucial for formulating effective strategies for preventing and treating IBD and other associated intestinal disorders resulting from infection. Understanding how individuals make an inflammatory and immune response to microbes in the diet or environment, pathobionts (microbes which invariably infect immunocompromised individuals) or pathogens which can infect and cause disease in otherwise healthy individuals, is crucial. This will enable the formulation of effective strategies for preventing andtreating intestinal infections and associated pathologies, such as IBD, to block inflammation and disease development and promote control of the pathogen and also understand how in some cases some infections can disseminate to the peritoneal cavity causing peritonitis.

Mice have a comparable physiology to humans and the inflammatory and immune response in the gut triggers a similar cascade of events as in humans. In particular, the human immune-system's reactionsto gut microbes and diet, and pathogens which infect otherwise healthy individuals or pathobionts which are microbes which invariably infect immunocompromised individuals, are highly recapitulated inmice. An understanding of the molecules and pathways of control and disease are an essential focus of our work, which will identify targets for therapeutic intervention.

What outputs do you think you will see at the end of this project?

The expected benefits of the work will be mechanistic understanding in the immediate future and therapeutic benefit in the longer period. This project could answer key questions about the immune response to infection or foreign substances that lead to control/resistance or development of disease.

They can be summarized as follows:

We expect the mouse models used in this project to generate new insights regarding the

development of the immune response to infection in the lung with the bacterium M. tuberculosis in models of resistance or susceptibility, which resemble active TB disease or asymptomatic controlledinfection.

The TB infection models will assist in defining infection response stages for early intervention duringthe development of TB disease. The models will also assist in the design of future anti-TB therapies, since we aim to use these models to test the effects of molecules on the immune response, that we identify in human TB. It is important to study lung microenvironment and the draining lymph nodes in the mouse since the lymph nodes have been shown in human TB to show an early immune response to the pathogen. The long-term aim would be to identify molecules/pathways tested in the experimentalmodels which can efficiently be targeted to control M. tuberculosis infection in humans and stop the progression to active TB disease, and to define molecules during early stages of infection which determine disease outcome and guide earlier therapeutic intervention than is currently possible.

We expect mouse models of intestinal and/or peritoneal infection used in this project to generate new insights regarding the development of the immune response in the gut to infection with different microorganisms, which would lead to control of infection, or lack of control of the infection, intestinal inflammation, or IBD, and additionally if there is dissemination which could result in systemic diseaseand/or peritonitis.

By studying the involvement of certain inflammatory components and systemic signals in models ofperitoneal and intestinal infection and IBD, we aim to clarify the mechanism of controlled responses inthe peritoneal cavity and in the intestine or those that contribute to disease and so help to identify targets as potential host-directed therapies to control disease.

Finally, this work will benefit the basic research community by increasing our knowledge of theimmune response in health and disease.

At the end of the studies supported by this licence, the outputs will include publications and seminars at national and international institutions, as well as presentations at national and internationalconferences. In some cases, the results of the work will be presented to the public at public engagement events.

Who or what will benefit from these outputs, and how?

These outputs will play a major role in informing other researchers nationally and internationally of key findings defining the molecules and pathways of the immune response that: (i) control disease or contribute to disease outcome in experimental models of TB resistance and susceptibility to resemble the spectrum of human TB; and (ii) experimental models of intestinal and peritoneal infection leading tointestinal damage and/or peritonitis due to infection, or in more extreme cases of intestinal diseases such as IBD. In the long term these findings may lead to the development of biological therapies to treat TB disease, where there is a great need due to a shortage of effective antibiotics particularly due to drug resistance, with around 1.6 million deaths per year world-wide. By defining molecules during early stages of infection with M. tuberculosis, which determine disease outcome, these findings may guide earlier therapeutic intervention than is currently possible due to difficulties in diagnosing TB. We may also define new targets for potential host-directed therapies during intestinal inflammation and/or peritonitis and in IBD.

The output of this work will initially be mechanistic information on the immune response in infection (TB) and intestinal infection, including IBD. In the mid and long-term the benefits of defining potential host-directed therapies, will reach the pharmaceutical industry and clinicians, resulting in improvement of care for patients, to guide future potential treatments, by identifying new potential drug targets for either disease. The work will also provide valuable information as to the early immune events determining disease outcome to guide doctors in the clinic for the management of TB or intestinal infections/peritonitis and IBD with disease, that may have spread to other sites in the body and in somecases cause systemic disease.

Within the duration of this licence, we expect short-term benefits such as the publication of discoveriesin scientific journals and at conferences. We also expect that the identification of novel molecules, cellsand pathways of protection or disease progression, together with large databases of gene expression in TB, or in intestinal and/or peritoneal infection and resulting intestinal diseases such as IBD that are generated will be of use for the wider scientific community.

Where we make large datasets, defining the immune response in the different models, these will bemade accessible to researchers and industry.

As all the studies supported by this licence fall within the category of basic research, we expect thatthere mostly will be longer-term benefits. The knowledge gained will be used in further studies to investigate new pathways of resistance and susceptibility in either TB or intestinal or peritoneal infection and IBD.

How will you look to maximise the outputs of this work?

We will look to maximise the outputs of any significant findings through interactions with the scientificcommunity through presenting at seminars and conferences. We collaborate with researchers in Portugal, Spain and other parts of the UK and share data with our collaborators at regular meetings and with our in-house colleagues at institute-wide seminars and lab meetings.

New findings resulting from mucosal infection of genetically altered (GA) animals and mouse models ofdisease will be made available to other researchers, multiplying the impact of our work. Most importantly all big data regarding gene expression underlying the immune response during the differentimmune responses obtained from these studies will be made available to other researchers and the public, using accessible tools and thus contributing to reduction, refinement and replacement of animals by minimising any unnecessary repetition in their future studies. For example, publication of large datasets arising from our studies, of gene expression changes in tissue or blood during early stages of mucosal infection with different doses of the infecting pathogen, will help refine our own continuing studies to limit discomfort to animals, and help others in refining their studies to understand the outcome to infection with reduced numbers and reduced suffering to animals.

Species and numbers of animals expected to be used

- Mice: 30,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our area of research is the immune response to infection of the lung, modelling TB disease, and toinfection of the intestine/gut and peritoneal cavity. We also need a model organism to model humanoral infection, intestinal inflammation such as inflammatory bowel diseases (IBD) and if there is dissemination systemic disease and/or peritonitis.

The reasons why mice are the best choice as experimental models, for TB and inflammatory boweldiseases, can be summarized as follows:

the physiology of the immune response in TB, intestinal inflammation, IBD and peritonitis in mice isconsistent with the human diseases.

The genome of a mouse is easy to modify allowing the study of particular genes in the immuneresponse process. Mice can also be modified to be genetically prone to infection (genetically engineered mouse models) to mimic these diseases.

In mice, there are many models that are either commercially available or available from academic depositories, as well as well-defined techniques for production. There are also many scientific tools likeblocking antibodies etc that are largely available, minimizing the need for use and breeding of genetically altered mice. Adult mice will be used.

Typically, what will be done to an animal used in your project?

For TB, mice will typically be infected by aerosol with different strains of M. tuberculosis on the first day. They may be treated with drugs or antibodies by injection (weekly, or more regularly, as required)to test immune pathways and targets of resistance or susceptibility to the infection and progression of TB disease. To alter gut bacteria, diet may be modified or antibiotic treatments may be administered. We are interested as to how the changes by drugs, antibodies, diet, or any immune modulators (thesecould be antibodies against immune molecules/targets that we define and wish to test) will impact the host response and the outcome to infection with M. tuberculosis, and its response to therapy.

The animals will have their disease monitored over time using symptoms, for example weight loss, andpossibly non-invasive imaging techniques, since they will have internal disease. Extra care will be taken when infection is with highly virulent strains of M. tuberculosis which can result in more severe disease at an earlier stage, and/or if M. tuberculosis infection is accompanied by administration of immune modulators or is performed on genetically altered mice or strains of mice e.g. C3HeB/FeJ, which are TB-susceptible. The duration of the experiments will vary depending on the mouse model and M. tuberculosis strain, or if immune modulators are co-administered, and will consider many features of the disease specific to the experiment. We always aim to maintain duration of experiments to the minimum amount of time required and the minimum symptoms and extent of disease required toaddress the scientific need, particularly resembling human TB.

For diseases of the intestine or peritoneal cavity eg IBD or peritonitis, mice will be orally infectedby oral gavage with the infectious agent (some infections on the first and second days), or by intra- peritoneal infection. They may be treated with drugs or antibodies by injection (weekly, or more regularly, as required) to test immune pathways

and targets of resistance or susceptibility to the infection and progression of disease (inflammatory bowel disease/peritonitis/wasting disease). To altergut bacteria, diet may be modified or antibiotic treatments may be administered. We are interested as to how the changes by drugs, antibodies, diet, immune modulators, genetic alterations in the mice, willimpact the host response to infection of the gut or the peritoneal cavity, and its response to therapy.

The animals will have their disease state monitored over time using symptoms, for example weight loss, diarrhoea; and possibly non-invasive imaging techniques, since they will have internal disease. Extra care will be taken when infection is accompanied by administration of immune modulators (thesecould be antibodies against immune molecules/targets that we define and wish to test) or in genetic altered mice. We always aim to maintain duration of experiments to the minimum amount of time required and the minimum symptoms and extent of disease required to address the scientific need, particularly resembling human intestinal inflammation after infection, peritonitis and IBD. The duration of the experiments will vary depending on the model and will consider many features of the disease specific to the experiment, including the nature of the infection. We always aim to maintain the durationof experiments to the minimum required to address the scientific need.

What are the expected impacts and/or adverse effects for the animals during your project?

In most cases animals used in this license will either develop symptoms of the lung disease TB; or inthe gut symptoms of intestinal inflammation such as inflammatory bowel disease; or in the peritoneal cavity symptoms of peritonitis.

Lung infection: The models of TB will vary with respect to possible adverse effects in the lung, and potentially weight loss, however the majority of mice (TB-resistant C57Bl/6) will develop only very mild disease, while others (up to 5%) (TB-susceptible C3HeB/FeJ) may develop severe disease, which is essential to be able to model human TB disease. The symptoms may be affected by the strain and dose of M. tuberculosis and/or by co-administration of immune modulators and/or if performed in genetically altered mice, and the duration of each experiment which can range from 14 days to a maximum of 100 days. Infected mice kept for the longer time periods (over 26 days) will be those infected with very low dose of a low virulence strain of M. tuberculosis, where the duration is needed to obtain developed lung pathology resembling the different stages and heterogeneity of human TB. In allcases mice are monitored daily and mice will be humanely killed if reaching or before reaching the humane endpoint.

Intestinal infections: The gut symptoms of IBD; or in the peritoneal cavity symptoms of peritonitis; willalso vary according to the dose and strain of the infectious agent (pathogen eg. Escherichia [E.] coli, Citrobacter [C.] rodentium, Enterococcus [E.] faecalis, or the pathobiont Helicobacter [H.] hepaticus).
Experiments will be conducted usually over a few days to 2 weeks, but in some cases may need to bekept for up to a month to resemble human disease. If immune modulators and/or genetic altered mice are used, this may accelerate or delay the time course of disease, but all mice are monitored daily andmice will be humanely killed before if reaching or before reaching the humane endpoint.

We have much knowledge and experience of all the models that we use, both for lung infection and forthe gut and peritoneal cavity infections (for the latter we also seek additional expert advice if needed from our collaborators and experts on experimental

mouse models of IBD in new experiments). We aremostly able to predict well the time frame over which the animals will not show adverse effects as we discuss in more detail above for either lung or the intestine or peritoneal cavity. The use of certain interventions with molecules of the immune response, or antibodies directed against them, can make this slightly less predictable, but the animals are monitored closely in this case to minimise any suffering and we aim to terminate experiments before the onset of potential adverse effects and humanely kill mice before they reach the humane endpoint. Undesirable affects could be limited to the site of the infection (for example, lung – pneumonia or TB; or gut – ulceration or IBD) or by general signs of poor health, such as weight loss, piloerection, hunching, increase breathing rate (especially when infection has spread), but in cases may become systemic resembling human disease. Again, mice developing such symptoms will be monitored carefully and humanely killed if reaching or before reaching the humane endpoint.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The expected severities predicted by this proposal are mainly mild and moderate, with one severe protocol. Only up to 400 mice are expected to approach this severe level, in order to reflect the extreme of the lung pathology in human TB disease. Mice will be monitored carefully and humanelykilled if reaching or before reaching the humane endpoint.

For phenotyping we anticipate that mice will if anything only reach mild symptoms since although theycarry genetic alterations in genes involved in immune pathways, the mice are housed in a facility with pathogen-controlled measures.

We anticipate that only 10-20% of mice may reach moderate severity for the immunomodulation Protocol, the rest being mild, since although the mice may be administered immune modulators or blockers of the immune response, the mice are housed in a facility with pathogen-controlled measures.

We anticipate that mice infected with the bacterial strains, E. coli, C. rodentium and E. faecalis, will only show mild to no effects in wild type mice. However up to 50% of the mice may reach moderate severity upon infection with these pathogens, if they are either genetically altered mice with changes inan immune molecules, or if they are wild type mice administered with immune modulators or blockers of the immune pathways during infection.

Wild type mice infected with H. hepaticus show no symptoms, however, up to 50% of the mice may reach moderate severity upon infection with this pathogen, if they are either genetically altered mice with changes in immune molecules, or if they are wild type mice administered with immune modulatorsor blockers of the immune pathways during infection.

During infection with M. tuberculosis of wild type mice or genetically altered mice, or mice co- administered with immune modulators or blockers of immune pathways, it is expected that most will only reach mild to moderate severity, with only up to 200 400 mice approaching severe symptoms. Since this is a chronic infection, where although immuno-modulation may manifest adverse effects, careful monitoring of mice will allow clinical predictions to facilitate humanely killing of the mice beforethe humane endpoint is reached.

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

A retrospective assessment of these predicted harms will be due by 13 September 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animalswere affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The experimental model that most resembles a human controlled immune response to M. tuberculosis, or the development of active TB disease, that we can use in the UK, and with which we can perform advanced immunological analyses, is the mouse model, where there are TB resistant and susceptible mouse strains, which when infected with M. tuberculosis, show similar lung pathology and blood gene signatures of the immune response to human TB, supporting our need to work in in vivo TB mouse models. It is essential to study the immune response to M. tuberculosis in vivo in experimental adult mouse models, where the response is detectable in immune organs as well as infected tissue, since highly-sensitive radiography of TB patients and their contacts has shown that theimmune response starts in the immune organs and only later is evident in the infected lung tissue where immune cells migrate in to kill the pathogen. Thus, testing of molecules, cells and pathways in mouse models of TB resistance or susceptibility in vivo, and dissecting the early airway and immune organ response to M. tuberculosis is essential to define targets for immune intervention and for the development of new host-directed therapies.

We are using adult mice which are necessary for dissecting and testing mechanisms underpinning the immune response to M. tuberculosis and to infections of the gut or peritoneal cavity with other infections. Lower organisms and cell culture do not capture the complexity of the immune response that determines outcome of infection, specifically protection or progression to disease. Therefore, thesestudies must mainly be performed within the animal as the complexity of these changes and the number of players involved cannot be modelled in the laboratory. Experimental models of TB resistance and susceptibility have been defined which most closely resemble human disease, at the level of pathology, gene expression and the immune response which may help in identifying targets to therapies in human disease and also identifying immune mediators of disease outcome which could help in guidance for earlier therapeutic intervention.

Intestinal and peritoneal cavity infections: In the past we had used cellular assays of immune cells to define genes, molecules and pathways of the immune response that had

potential to help to maintain stability or homeostasis in the gut and provide protection against inflammation during system perturbations such as infection. However, these cellular assays are not able to model migration of cellsfrom other immune organs which are likely to play an important part in these mechanisms. It is therefore essential to study the intact mouse model since immune cells migrate into the intestinal tissue from immune organs during infectious challenge and can contribute to host damage or regulation of the response. Thus, to test cells, molecules and pathways that we have defined as potentially important in regulating or controlling damage to the host, it is essential to use the intact mouse models of gut and peritoneal infection and inflammation, to support the development of new host-directed therapies.

Which non-animal alternatives did you consider for use in this project?

Lung infection and TB: In the past in research leading up to the current programme, we had used cellular assays of immune cells to define molecules and pathways of protection or disease progressionwith M. tuberculosis and other infections and control of the infection by immune cells. Our research of the past years has identified the cells, genes and pathways that potentially regulate the molecules of the immune response to protect against TB or result in progression to the disease using mouse modelsand analysis of human TB disease. It now is necessary to test these molecules, cells and pathways as targets for immunotherapy using experimental mouse models.

We have studied the literature and have gained experience from our own studies and found no non- animal alternatives which could address the questions of the immune response to M. tuberculosis in cell culture or organ culture. Although lung-on-a-chip models of M. tuberculosis infection have been published, these models will not capture the immune response to the pathogen which is initiated in theimmune organs with immune cells which once activated migrate back to the infected tissue to kill the pathogen. The experimental model that most resembles a human controlled immune response to M. tuberculosis, or the development of active TB disease, that we can use in the UK, and with which we can perform advanced immunological analyses, is the mouse model, where there are TB resistant andsusceptible mouse strains, which when infected with M. tuberculosis, show similar lung pathology and blood gene signatures of the immune response to human TB.

Intestinal infection: We will additionally use long-term expansion of epithelial organoids, which contain some of the immune cells represented in intestinal infections as well as epithelial cells of the gut. Cells used for organoids could be isolated from humans or the experimental mouse models and could help to replace as well as reduce the number of mice used. However, these can only be used forin-depth local mechanistic studies as they do not reflect the involvement of immune organs where immune cells are activated and then migrate to the mucosal/intestinal tissue.

In both cases, given the limitations of these in vitro approaches, it is essential to use a whole mousemodel of lung or gut disease to test these immune molecules or their antagonists since the immune cells once taken from animals and used in in vitro in culture cannot recapitulate the whole immune response in the body, which is complex, and cell culture is no longer useful.

Why were they not suitable?

In vitro cell culture cannot capture the complexities of the physiological immune response since afterinfection, for example of the lung by aerosol, cells in the lung become activated

and then in turn activate immune cells that traffic to the lymph nodes become further activated and multiply and then return to the lung to kill the infectious agent. It is not possible to model these complex multi-organ events in the in vitro assays. The same is true for infections of the gut through the oral route, or invasive infection such as through the blood or peritoneal cavity.

Even the most sophisticated lab-based model systems cannot convey the complexity of the microenvironment of the immune response to infection, or the impact that this infection can have tocause disease either locally or systemically on an animal. The complexity of the whole animal is required to investigate the impact of infiltrating immune cell types alongside both the local and systemic response of the infection.

Although lung-on-a-chip models of M. tuberculosis infection have been published, these models willnot capture the immune response to the pathogen which is initiated in the specialized lymphatic immune organs where immune cells become activated and migrate back to the infected tissue to killthe pathogen.

For study of intestinal infections, we will additionally use long-term expansion of epithelial organoids, which contain some of the immune cells represented in intestinal infections as well as epithelial cells ofthe gut. Cells used in these systems can be isolated from humans or the experimental mouse models, which could help to replace the number of mice used. However, these can only be used for in-depth local mechanistic studies. It is still essential to study the intact mouse model since immune cells migrate into the intestinal tissue from immune organs during infectious challenge and can contribute tohost damage or regulation of the response.

We have considered multiple options of non-animal approaches, however, given the limitations of invitro isolated organ/cell culture approaches, in both lung and intestinal/peritoneal infections, non- animal alternatives are not useful for our current and future studies.

A retrospective assessment of replacement will be due by 13 September 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used has been estimated on the basis of the previous 20 years of work ofmy lab and the current landscape of projects of the lab going forward, staffing in my lab and the funding I have for the next 5 years.

We use statistics and past experiments to define the minimum number of mice required for statistical significance to obtain robust reproducible data to inform the immune response during infection of micethat determines outcome.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We employ several strategies to try to limit the number of mice in the study:

We will always aim to maximise the amount of data (pathology information and gene expression) weget from each mouse, for example by obtaining multiple tissues or experimental read-outs from the same mouse.

Lung: We always maximise the data from the animals we use. For example, when taking lung tissue for a histological estimate of pathology, one lung lobe is taken for RNA/DNA genomics analysis or flowcytometry. Similarly, when performing bronchoalveolar lavage on euthanised mice (to compare to human TB bronchoalveolar lavage samples), we also obtain the paired lung tissue from the samemouse for parallel analysis.

Intestine: We always maximise the data from the animals we use by taking tissue from the gut of eachmouse for histological estimate of pathology, for RNA/DNA genomics analysis, and for flow cytometry or other analyses.

Also, we will limit the use of genetic models (that often require many generations breeding) by treating the mice with chemical agents or antibodies to either to block immune-system components or enhancethem.

We use(d) statistics and past experiments, including pilot experiments, to define the minimum numberof mice required for statistical significance to obtain robust reproducible data to inform the immune response during infection of mice that determines outcome. We can always rely on our in-house statistician for any additional advice whenever we need.

For most of the experiments, quantitation is required and we will use the minimum number of animals to provide an adequate description, generally on the basis of previous experience (our own or from the literature). Pilot experiments will use approximately 5 mice per group, which should be sufficient if a significant result is obtained. The experiment will be repeated to obtain further significance if: (a) there are only small differences; in this case it may have to be repeated with larger numbers of mice and/or modifications; (b) does not work; in this case it would be repeated in the presence of candidate molecules/modulators; (c) to obtain statistically reproducible results which are set using power analysis, generally using a significance level of 5%, a power of 80% and at least practicable difference between groups of 20%. Once a desired effect has been obtained it may be necessary to use a greaternumber of mice per group in order to facilitate obtaining rare immune cells involved in the response and to analyse their function (e.g. phenotype by flow cytometry, cytokine production and other immune parameters). For example, numbers of cells like dendritic cells, or small populations of T cells are limiting, so these experiments may need to use between 10-50 mice per group in order to be able to purify enough cells for subsequent genomics and transcriptomics analyses. Cryopreservation of gametes, embryos, tissues and cells is routine and will ensure that the minimum number of mice is bred.

Each experiment (including the pilot experiments) will include a small group of animals that are susceptible to infection with the respective pathogen or pathobiont and develop a well-

defined course of disease in response to a given dose of pathogen. This group will serve as an internal control for theexperiments involving infections, allowing comparison of results from different experiments (performedat different times, with different doses and/or batches of pathogens etc.), in wild type mice, mice of a different genetic background, mice genetically altered in a molecule under test, or mice administered an immune modulator. This group will also provide the quality assurance for the virulence of the innocula, which is necessary for comparison of results across experiments.

We will use the PREPARE guidelines: https://norecopa.no/PREPAREand the Experimental Design Assistant: https://eda.nc3rs.org.uk/.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Pilot studies have been performed for all experiments in the past and will be executed when neededfor future experiments, however, with care to still ensure robust and reproducible data is obtained.

The majority of experimental mouse numbers per group are decided based on years of experience, statistics and our publications (or those of others when investigating a new experiment regimen). Our aim is to use to the minimum number of mice per group to produce reproducible results within a group(eg. this can depend on the dose of infecting pathogen) which shows a statistical difference to control animals. In the past we have used power calculations when required and will do so again if needed.

We obtain all tissues possible from the same infected/challenged mouse, for procedures including immune assays, histology to inform the level of inflammation, and gene expression changes to inform the immune response determining outcome. This enables the maximum information to be gained fromthe minimum number of mice. Whenever possible, we will share animal tissue from experiments to enable multiple studies ex vivo. We are committed to improving education and training for those working under this project licence.

We also use gene expression data from our own published studies and studies from others to understand the patterns of the immune response, which will help inform the necessary experimental timepoints for the study, thus reducing mouse numbers by avoiding inclusion of unnecessary timepoints. These data are available to the research public and so help to reduce mouse numbers needed for experiments at large by providing an immense database and resource of the immune response in infection and diseases. In our publications we have made the data highly accessible via acomputer app that we constructed.

Regarding breeding of mice, we share mouse colonies with other researchers, which are obtained bycentralised breeding, minimising numbers used and avoiding duplication.

We breed many of the genetically altered animals ourselves in order to promptly adapt colony sizes torespond to the experimental need and reduce wastage from overbreeding.

We also take lead from HO Assessment Framework of Efficient Breeding of Genetically Altered Animals (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773 553/GAA_Framework_Oct_18.pdf) and take decisions to archive lines by cryopreservation when not required over a period of time.

The NC3Rs have also recently published a guidance document about the sharing and archiving of GAmice which is of use: https://www.nc3rs.org.uk/3rs-resources/breeding-and-colony- management/sharing-and-archiving-ga-mice

Obtaining wild type mice from in house facility-shared breeding allows better efficiency for largercolonies.

A retrospective assessment of reduction will be due by 13 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use wild type mice and genetically altered mice lacking components of the immune response tostudy the immune response to infection with: 1) M. tuberculosis, via aerosol) to model the spectrum of human TB (from asymptomatic, resistant to progression to TB) and; 2) the pathogen C. rodentium, andthe pathobionts H. hepaticus, E. coli and E. faecalis (via intraperitoneal or oral gavage) to model intestinal infection, peritonitis and human IBD.

Lung infection: We will use wild type mice and genetically altered mice lacking components of the immune response to study the early immune response in the airways to infection with the pathogen M. tuberculosis, via aerosol to model the spectrum of human TB (from asymptomatic, resistant to progression to active TB disease) using models which we have previously refined and published. Our studies of these early immune events in the airways (and sometimes blood) will mainly concern early time-points which we have carefully determined from the literature and our own previous studies wheremost mice will show only mild disease.

Only TB-susceptible mice at later time-points after infection may start to show symptoms which could lead up to severe disease. This is essential to present a model which most resembles human TB disease, at the level of lung pathology including granuloma and cavity formation, but will only occur in asmall percentage/number of mice. In these experiments, mice are monitored daily and mice will be humanely killed if reaching or before reaching the humane endpoint.

The models of TB will vary with respect to possible adverse effects in the lung, and potentially weight loss, however the majority of mice (TB-resistant C57BL/6J) will develop only very mild disease, while others (up to 5%) (TB-susceptible C3HeB/FeJ) may develop

severe disease, which is essential to be able to model human TB disease. The symptoms may be affected by the strain and dose of M. tuberculosis and/or by co-administration of immune modulators and/or if performed in genetically altered mice, and the duration of each experiment which can range from 14 days to a maximum of 100 days. We have a very clear knowledge from our past experiments and our publications, of the strain and dose of M. tuberculosis in the different genetic strains of TB resistant and susceptible mice which results in the different extents of disease. These generally range from mild to moderate, with only up to5% approaching severe symptoms. Infected mice will only be kept for the longer time periods (over 26 days) when infected with very low doses of a low virulence strain of M. tuberculosis, where the durationis needed to obtain lung pathology resembling the different stages and heterogeneity of human TB. Mice are monitored daily and mice will be humanely killed before reaching the humane endpoint to minimise suffering.

Intestinal infections: The gut symptoms of IBD; or in the peritoneal cavity, symptoms of peritonitis, will also vary according to the dose and strain of the infectious agent (pathogen e.g. C. rodentium, or pathobiont e.g. H. hepaticus, E. coli, E. faecalis). Experiments will be conducted usually over a few days to 2 weeks, but in some cases may need to be kept for up to a month to resemble human diseaseif the invading pathogen or pathobiont only leads to mild disease as can be the case with H. hepaticus.Mice are monitored daily, and mice will be humanely killed if reaching or before reaching the humane endpoint. If immune modulators and/or genetic altered mice are used, this may accelerate or delay the time course of disease, but mice are monitored daily and mice will be humanely killed if reaching or before reaching the humane endpoint, to minimise suffering. We monitor the mice carefully within eachexperiment to ensure reduction of the suffering experienced by the animals.

Why can’t you use animals that are less sentient?

Other animal models have been published studying the immune response to mycobacterial infection ofthe lung, or intestinal infection, but these lack the organ and physiological complexity needed for translation to humans. This complexity needs to be recapitulated in vivo in adult mice to make the investigation of infection to the microenvironment of the lung, peritoneal cavity and intestine possible and to resemble the major burden of human TB disease and intestinal/peritoneal disease, which is in adults. The requirement here is to have a physiology which is as close as possible to adult humans, and only mammalian organisms such as mice have the same complex immune system as humans, including at the immune organ level e.g. lymph nodes and immune cell activation and migration to tissue, which is only fully developed in adult life.

Modelling human TB disease: It is necessary to test molecules, cells and pathways that we have discovered from research on human TB and experimental mouse models as targets for immunotherapyor to define early immune determinants of disease outcome, using experimental mouse models where outcome can be determined and linked to the immune response. Therefore, terminally anaesthetised mice will not capture the immune events related to outcome. Models of TB have been published with infection of zebrafish with Mycobacterium marinum, but these models again do not exhibit a physiologywhich is close to that observed in humans. This model does not capture the innate and adaptive immune responses in the immune organs which resemble human TB disease and model the mechanisms behind why some individuals infected with M. tuberculosis will control the infection while others will progress to active TB disease. Mouse models of TB resistance and susceptibility have been published however, which do recapitulate the spectrum of TB in humans and thus offer the most accurate model of human TB disease.

Intestinal and peritoneal cavity infections: In the past we had used cellular assays of immune cells isolated from animals that have been humanely killed, to define genes, molecules and pathways of the immune response. This allowed us to discover immune molecules and cells that could potentially offer protection against infections, which but on the other hand could potentially cause damage to an individual, or regulate the immune response to limit host damage. However, these molecules and pathways need to be tested in intact adult experimental mouse models of intestinal infection to define new targets for the design of host-directed therapies to block inflammatory diseases of the intestine and the peritoneal cavity. Infection of the intestine via the oral route can be controlled or can lead to intestinal inflammation, and in some cases IBD. Where there is dissemination, systemic disease such as peritonitis may occur, which is a disease that can occur if microorganisms escape the gut and causeinflammation of the inner lining of the abdomen, or other conditions. In both of these instances, immune cells migrate into the intestinal tissue or peritoneal cavity from immune organs during infectious challenge and can contribute to host damage or regulation of the response. We will additionally use long-term expansion of epithelial organoids, which could help to reduce the number ofmice used, since these organoids are isolated from either animals which have terminally anaethetised or from human biopsies. These organoids can be cultured with some of the immune cells representedin intestinal infections and can only be used for in-depth local mechanistic studies. These organoid systems are not able to model dissemination or cell migration. Hence, it is still essential to study the intact adult mouse model since immune cells migrate into the intestinal tissue from immune organs during infectious challenge and can contribute to host damage or regulation of the response. Thus, to test cells, molecules and pathways that we have defined as potentially important in regulating or controlling damage to the host, it is essential to use the intact adult mouse models of gut and peritoneal infection and inflammation, to support the development of new host-directed therapies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We have access to cutting edge techniques and experts within various fields of medical research. We actively share refinement and improvements in techniques and seek to constantly improve our modelsto ensure that we are minimising any harms to the animals, as this also helps to improve the accuracyof our study and reduce artefacts caused by stress. For example; we are vigilant to ensure careful monitoring of experimental mice within each experiment and use our previous experience and experiments to guide us, as well as advice from other experts when needed.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Unless otherwise specified, the work in this project will be designed using the principle outlined in thePREPARE guidelines for planning animal research and testing (2017) and from the following links:

https://view.pagetiger.com/RSPCAAvoidingMortalityResearchReport/RSPCA
https://www.dropbox.com/s/wls05epsbykinhh/administration_substances.pdf

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We regularly receive updates on advances in the 3Rs from within our establishment from

NC3Rs. Where we are placed to refine techniques without impacting the scientific validity of our work we aim toimplement advances. The NC3R Regional Project Manager keeps our institute informed on key advances and knowledge. As experts in the field we are up-to-date with all the latest publications and are up to date with the latest research from conferences and collaborations, thus we are fully aware of any refinements published, that could help us in refining our experimental mouse models further. The fact that we ourselves are researching the cellular changes and immune signatures during the early airway response in TB patients and their contacts – most of whom control the infection whilst 10% progress to active TB disease, allows us to model closely the experimental mouse models and only test genes and molecules that show a potential role in human TB disease or control. We use the literature of human IBD and other intestinal infections and inflammation to again identify potential genes and pathways of protection or disease, and always refine our experimental mouse models totest potential targets relevant to human disease.

A retrospective assessment of refinement will be due by 13 September 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved forfuture work of this kind?
- During the project, how did you minimise harm to the animals?

UNDERSTANDING AND MANIPULATING AUTOPHAGY 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, ortheir effects, in man, animals or plants

Key words

Autophagy, Mitophagy, mitochondria, Neurodegeneration, Parkinson's

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

Autophagy is your body's process of reusing old and damaged cell parts. We aim to understand when,where and how the process of autophagy happens in the body. We particularly want to know if autophagy is altered in diseases where nerves become damaged such as Alzheimer's and Parkinson'sDiseases and if it is whether there are treatments that we can give to reverse any changes.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Dysfunction of this autophagy has been linked to many diseases. This is because the process of autophagy prevents the build up of toxic or damaging cellular components that are harmful to our cells.Impaired autophagy has strong links to neurodegeneration (where

nerves stop functioning properly), for example in Parkinson’s disease, where nerve cells may be particularly sensitive to accumulation of these damaging cellular components. As there are currently no cures for Parkinson’s and related disorders, information on why cell processes like autophagy aren't working properly in these patients iscritical in designing much needed therapies. Our mouse models let us look at these processes to workout what is going wrong. It is our goal to find ways to enhance autophagy and so improve clearance from cells of the toxic products. We hope that this may provide new disease treatment opportunities.

What outputs do you think you will see at the end of this project?

The main output from this project will be new information on how autophagy works in normal and diseased cells. This information will be shared via scientific papers that are published in freely available(Open Access) journals and will also be presented to scientific colleagues at meetings and conferences.

Who or what will benefit from these outputs, and how?

While we understand a great deal of how autophagy is regulated in isolated cells under lab conditions, very little is known about the process in a living body (*in vivo)*. The main benefit from our work will therefore be to increase knowledge of the process. This will help other researchers working in this areaof science to design better and more relevant studies by giving better information on what happens in cells and tissues in different circumstances. We are able to do this because we have developed a way to see whether the mitochondria (the "engine" in the cell that drives the autophagy process) are working by modifying the genes in our mice so that we can identify which mitochondria are operating properly and which aren't. This is done by a non-harmful genetic change that makes functioning and non-functioning mitochondria in cells fluoresce different colours under a microscope.

If we can show autophagy only occurs in certain cell types, or when particular environmental conditionsare met (for example, when a particular protein or sugar molecule is present), this should allow future research to focus on the right types of cells and on the changes in environment in the body that are most important. How quickly we can spread this knowledge will vary but we will publish and disseminate of our data as quickly as we can. This licence will continue ongoing work so we hope to see some benefit from new work within 1-2 years, but it could take longer. We will continue to produce data from the work after the end of this project from stored tissues and will continue to share our results.

In addition to research scientists, pharmaceutical companies who are looking to find ways to alter autophagy in order to treat patients will also benefit from the information we provide. We already work with multiple companies who are interested in this area, and we will share data as and when it is obtained with them. This will hopefully allow these companies to better target their programmes of drugdevelopment for relevant diseases.

How will you look to maximise the outputs of this work?

We aim to share as much information and material as possible. Firstly, we will publish all our data as open access, thus increasing accessibility of our work to as wide an audience as possible. Secondly, we will make the mice we have genetically changed available to the whole of the scientific community.Indeed, we have made our mouse line that is engineered to allow us to see if the mitochondria are working available to researchers internationally via the European Mutant Mouse Archive (EMMA).

Thirdly, we always take as many tissues as we can from any mouse we use. We will give away anytissues that we don't analyse ourselves to any researchers who can use them, thus preventing wastage and allowing other research programmes to benefit directly from this work.

In addition to the types of benefits we describe above, we also do whatever we can to take part in patient and public outreach, to make knowledge gained from our work available to the general public.My department works extensively with Parkinson's charities, and I have been involved in forums for patients to explain the science we perform and how this adds to our understanding of their disease.

Species and numbers of animals expected to be used

- Mice: 12500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The process of autophagy is believed to be the same across all mammals. Although we work with clinicians and use human tissue when it is available, this is limited and also isn't appropriate for someof the experiments we need to do in this programme of work. We will therefore perform most of our work in mice because this lets us monitor autophagy and its effects within complex tissues and physiological conditions (the changing environment provided by the body) that cannot currently be created artificially. This is important, especially when we need to work out what happens to change theautophagy process in multiple different cell types, for example to see what happens in the different tissues of the body when we give a drug that is being trialled as a new treatment for a disease. The mouse also allows us to study changes in autophagy that might happen during ageing or exercise so we can see if these improve or worsen the process. Ageing is particularly relevant to study because it is a key risk factor for neurodegenerative disorders such as Parkinson's.

We usually carry out or research on adult mice. Occasionally we will need embryos or young animals to let us answer specific scientific questions, for example to allow us to obtain fresh cells from particular tissues or organs. Usually we use cells that we grow continuously in the lab, but this isn't possible for some types of cell such as nerve and heart cells, or the cells that make fibrous tissue in the body. Taking these tissues means that we can do studies in the lab that investigate different autophagy mechanisms. The tissue can be used for many studies, so we use fewer animals and it alsomeans that we can safely test the effect of compounds that might be harmful to an animal.

Typically, what will be done to an animal used in your project?

The primary use of animals is to generate tissue for postmortem analysis. As described above, we usemice with a genetic change that allows us see if the mitochondria are working or not, using a special system (call a fluorescence-based reporter) where we can see what colour the mitochondria in tissuestaken from the animals fluoresce under a microscope. We need to use a special treatment ("fixing") to stop the tissues changing

after they have been removed and to prevent other florescent chemicals forming from the compounds that are naturally made when cells die. To "fix" tissues we need to put the animal under an anaesthetic that it doesn't wake up from and then to flush the blood out of the tissues by using a special pump that, through a small needle into the heart, replaces the blood with saline or achemical solution (this is called trans-cardiac perfusion).

If we need to look at proteins in the cells or grow cells in the lab, the animals do not go through the perfusion process and instead we take tissues after they have been humanely killed by an approvedmethod.

Wherever possible, nothing is done to the live animal and the studies are performed in tissues in the lab. However, sometimes we need to change the cell's environment in the body to see what happenswhen the cells are interacting with other cells, organs and systems in the body. When this is needed,there are several methods we might use.

We will breed our "reporter" mice with mice who have had one or more genes altered so either the gene doesn't function any more ("knock-outs"), or there has been genetic information inserted ("knock-ins"). We do this where these genetic changes are predicted to alter autophagy, or where the gene alteration is seen in diseases where changes in autophagy are thought to be involved in why or how the disease occurs.

We will dose the animals with compounds that we think could be used to change autophagy. To givethe compounds, we will use the method that causes the least discomfort to the animal possible. Whenever we can we put the compound in food or water, but sometimes this isn't possible and we need to either give it by injection on one or more occasions or by gavage (where a small tube is gentlypassed down the throat into the stomach). We will use the smallest volume we can and if we need to repeat treatments, we will limit dosing to the fewest occasions possible and we will always stay within good practice guidelines.

We may try and change autophagy by changing the environment in the body (the physiological state). We may do this by making dietary changes, such as changing the energy content of the diet forup to 12 months (animals will always get enough nutrients to keep them healthy), or by fasting the animals for one or more periods of up to 24h (animals will always have access to water). As well as, orinstead of changing the diet, we may change the amount of exercise the mice take by providing running wheels that they can chose to use as much or as little as they want for up to three weeks.

Sometimes, as well as the above-mentioned treatments, we may also need to take blood samples fromanimals from time to time to measure responses to the treatments/pharmacological (drug) interventions, or to measure the amount of a compound in the blood. We take as little blood as possible on each occasion and the sampling procedure does not cause the animal any more than brief discomfort, like for human blood sampling.

What are the expected impacts and/or adverse effects for the animals during your project?

For most of the animals that have genetic alterations, we do not expect to see any outward signs to show this; mice will look and behave normally. A small number of the genetic changes are designed tomodel human diseases such as Parkinson's Disease, whereby such animals could develop signs of illness over time. However, we don't require the animals to show any symptoms as we think that autophagy acts at an early stage of the

disease before clinical signs are seen. These animals will either be used before any signs of disease are seen or will be humanely killed at the first sign of any symptoms. Sometimes breeding different lines of mice together can cause unexpected effects. Where we are creating a new line by breeding different types of mice together, we monitor these animals particularly carefully and if we see anything unexpected it is immediately discussed with the vet to make sure that the animals aren't suffering. Where we need to age animals we monitor animals for anysigns of changes in their well-being due to age and humanely kill any animal that appears to be unwell.Animals undergoing procedures such as injections or blood sampling are not expected to experience anything other than minor, transient discomfort. Any animals showing signs of suffering that are greaterthan minor or transient, or in any way compromises normal behaviour, will be culled via an approved humane 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 animaltype)?

Most animals studied will simply live to specific ages and then be killed humanely. Where these animals are genetically altered, we do not expect them to look or behave in any way differently from a normal mouse. These animals will therefore either experience a severity that is below the threshold thelaw sets for regulated procedures (sub-threshold) or, if anaesthetised and not allowed to recover, will only have the brief discomfort of the induction of anaesthesia (non-recovery).

Two of the licence protocols are classified as "moderate", however, we do not intend for animals to suffer moderately. For protocol 2, which allows us to age animals that could have signs of disease laterin life, we always aim to use these animals before onset of noticeable clinical signs. However, sometimes symptoms can be very subtle or can occur rapidly and therefore the animal has experienced some degree of adverse effects before we can intervene. We estimate that this will occur for no more than 10% of mice on this protocol and these will be classed as mild or moderate depending on the clinical signs seen. For Protocol 3, mice will undergo regulated procedures such as injections. The majority will only undergo one regulated procedure, hence will only experience minor or transient discomfort and so will be classed as having undergone mild severity. However, some may undergo multiple steps in this protocol and while each treatment may be classed as minor and transient, cumulative harms over the lifetime of the animal must be considered, meaning up to 10% might reach a cumulative severity of moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered 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 currently not able to reproduce the complex tissue architecture and cellular make-

up of multipleorgans in the lab, nor are we able to mimic the changes caused by ageing or exercise in cell culture.
We know that one type of cell can change the behaviour of other cells in the body. This means that processes that happen in cells, such as autophagy, may occur at different rates or at different times *invivo* (in a body) as opposed to *in vitro* (in the lab). This is due to the influence of other tissues, so we have to look at processes in a whole body, at least some of the time, to check what we see in the lab mirrors what is seen in the body. As well as this, some cell types which we need to study can't be grown in the lab, or grow poorly. This includes cells such as nerve cells, which we are interested in because they stop functioning properly in disorders such as Parkinson's disease.

Therefore, the only way to currently study autophagy accurately, in a true physiological ("whole body")setting, is to use animals. We do not yet have an understanding of the amount of autophagy that occurs in tissues and what influences this. Once we have this knowledge, some of which will hopefullybe gained from this project, we will try and replicate it *in vitro* and this should help us to move away from using live animals.

Which non-animal alternatives did you consider for use in this project?

We already make extensive use of *in vitro* cell-based systems to help us to work out the molecules thatare needed in the cell for autophagy to work properly, in parallel to the mouse work. The information from the *in vitro* work is used to inform which experiments we chose to do *in vivo.* We only take forward planned animal studies where the work in cells lines confirms that the work we plan in the mice will bevalid. This reduces how many animals we use but stopping us from doing experiments that would be unlikely to give informative results.

A promising technique is the development of tissue organoids (3D structures made from multiple cells types) that can be grown from cells grown in the lab or from stem cells (cells from which all other cells with specialized functions are generated). This will mean that in many instances detailed investigationson how the process of autophagy works can be carried out in a complex biological system without the need for mice.

Why were they not suitable?

These techniques are still in their infancy and require great expertise that is only available in a very small number of labs worldwide. Most organoids are still very basic and only mimic a few functions compared to the organ that they are trying to replace. The needs to be more comparative work for us to reliably say what processes can truly be represented in organoid culture. Also, where you need to look at the interactions between different body systems as we do (for example interactions between thenervous system and the immune system), this would require growth and use of multiple organoids together in a single system, which is currently not possible.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of mice is based on my previous project licence, which covered a broadly similar type of work as the new licence, and discussions with the vet and animal care technicians on how many mice are needed for breeding, as the vast majority of animals are used within the breedingprotocol, with subsequent scientific investigations postmortem.

We regularly review our breeding strategies to ensure that they are appropriate and use as few animals as possible. Strategies are tailored to individual lines and we prioritise animal welfare. This means that some lines are bred in a way that uses more animals overall to achieve the genotypes that we want, but that avoids producing ill animals or animals that might otherwise have breeding problems.For example, we have one line of mice where we only use males carrying the altered gene (a mutationin the mitochondrial DNA polymerase enzyme) to continue the colony, mating them to normal female mice. If we didn't do this, and mated the males to genetically altered females, toxic products would build up in the cells of the offspring and would make them ill. Males are only mated with females with the genetic alteration to produce experimental animals, who are all humanely killed before the toxic products build up to a level to have adverse effects.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

I am registered with the National Centre for Refinement, Reduction and Replacement (NC3Rs) and willconsult their online resources to aid in designing breeding strategies for our colonies. I will also use their resources to help with optimising experimental design and the procedures we use so that we can perform the best possible experiments that will generate reproducible data. I will also have access to expertise in the animal facility to discuss experimental design.

We anticipate that most of our experiments will be comparing two variables (treated and untreated). However, sometimes we collect information needs more complex designs because we may be comparing a number of different genotypes and several different experimental changes that have all been done together. For such studies, we will consult specialists in data analysis to make sure that thedesign of the studies lets us get the most information in the best way.

We have already generated datasets that mean that we know the "base-line" (starting) level of autophagy in many different tissues. This information can be used in a particular type of calculation known as a power calculation, to more accurately determine the number of animals we need for our experiments so that we can measure if what we have done has had an effect with no more than a 5%chance of the result giving a "false positive" (so we think there is an effect when there isn't one), and an 80% level of certainty that we don't have a "false negative" (so we think there isn't an effect when there is one).

For each experimental group, animals will be put into the group using a technique known as randomisation (this means that the animals are assigned to a particular group by chance), as this helpsmake sure the results are reliable (it reduces the risk of bias). A lot of the data we collect is in the form of images (from pictures of cells we take down the microscope), which we then use to work out how many or how few mitochondria are working in the tissue. The calculation for this is automated, as it is done using image analysis software, so that the experimenter can't influence the result. Should counting need to be performed manually, this will be done blinded (meaning the researcher doesn't

know which experimental group the tissue came from) and suitably randomised (so the tissues appear in any order rather than all from one group then all from another or time about). All experiments will be planned and reported following the NC3Rs' "Animal Research- reporting in vivo experiments" (ARRIVE) guidelines, which are a checklist of recommendations to improve the reporting of research using animals.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

The only animals we use are mice. Currently all the background lines we need exist, either in our establishment, at commercial suppliers or at a collaborator's research facility, but we expect to need tocross different mouse lines in order to add the mitophagy/autophagy reporters (markers telling us about the autophagy process) in different lines where we need to look at changes in autophagy and haven't so far. We estimate a requirement of approximately 200 mice per year per line for maintenanceof each colony. To help minimise numbers, mice will be kept for no longer than 6 months of age, unlesson a specific aging study plan. We will also cryo-preserve ("freeze down" by freezing sperm or embryos in a special way) all lines, so breeding will be restricted to those lines being actively workedon.

We also note that in most experiments we will harvest and store as much tissue from each mouse as possible, even though we may not be analysing all of it. We can then go back to tissue at a later date ifneeded, or we can send spare material to other researchers, without the need to breed/ship additional mice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the chosen species for all experiments, primarily because of the ease with which it can be genetically altered. There is good pre-existing evidence (generated by ourselves and others) for themouse being a good model system to investigate autophagy pathways that are believed to be important in pathophysiological (disease) conditions in humans.

Our interventions will be mild to moderate in severity and all these will be described in specific study plans, to be discussed and lodged with the animal facility and Named Veterinary Surgeon (NVS). Weuse postmortem analyses and aside from compound dosing or blood sampling, the mice will not undergo any invasive procedures.

Our studies will not require animals to exhibit signs of advanced disease, indeed the majority of the animals will live apparently normal lives and will be killed humanely before tissues are harvested foranalysis. All mice will be very carefully monitored to minimise welfare costs, for example, monitoringsigns of reduced weight loss, neglect of grooming and reduced activity levels.

Why can’t you use animals that are less sentient?

Due to the nature of our work that involves understanding the regulation of autophagy pathways in neurodegenerative disorders, a mammalian model is the closest to humans. This means that our results will be more relevant for identifying possible approaches for new treatments for drug companiesthan if we were using less sentient species. Given that most neurodegenerative disorders, including Parkinson’s disease, are associated with ageing, we will therefore need to study the pathways in adult animals as we simply can't get the right information from immature animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

All procedures to be performed on mice will be described in specific study plans, to be discussed andlodged with the animal facility and NVS and will be made available to the Home Office inspector on request.

When we use mice that can develop clinical signs we describe in detail what these might be, how theywill be monitored for and when an animal must be humanely killed for welfare reasons (the humane endpoints) in the study plan. Before we bring in or generate a new mouse line that we want to use we will also undertake a "harm benefit" assessment of their use to consider the likely scientific benefits against the potential harms. This is performed locally via a system approved by the AWERB (currentlyperformed by consideration of the NVS of relevant study plans).

For phenotypic animals (animals that could show clinical signs) detailed information on how to house and breed the line will also be considered by an appropriate NACWO involved in the animal care. We hold an up to date list of the mouse lines we are breeding and this will contain information on any expected adverse welfare effects of the genetic alterations and the steps that will be taken to mitigate them. Where we believe the harm of a particular line is too much, we would consider generating a more refined mouse model by using technology that limits the genetic changes to specific tissues/cells (such as nerve cells within the brain) rather than occurring in the whole animal so that potential harm is minimised.

In certain experiments, we will administer compounds to examine their effects on autophagy. We willonly use compounds that have already been shown not to cause toxic effects in mice by the administration route we will use, which will always be the route that causes the least discomfort.

Where we need to do longer-term studies (5 days or more), our preferred method will be to combine compound with food or water. In this instance, we may need to do a small pilot study to check that we are getting the level of compound expected into the tissues. Likewise, if we have studies where compounds need to be given to a large number of animals and we haven't used that compound beforeourselves, we may run a small pilot study at the dose and duration planned for the large study to confirm compounds are working as they should and that there are no harmful effects.

We may take blood samples at intervals in order to measure responses to administered agents. Thesewill be of the smallest feasible volume. Blood vessels may be dilated gently warming the animal (in a warming box or on a heating pad), or by immersing the tail in lukewarm water to make the collection ofblood samples cause the least stress possible.

All animals that are aged will undergo regular weighing and body condition scoring to

monitor general well-being from the age of 6 months. This will be carried out by a trained and competent person, who isindependent from the ongoing study, to reduce any bias.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow the guidelines published by the NC3Rs and the Laboratory Animal Science Association.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We work closely with the NVS, Named Animal Care and Welfare Officers (NAWCOs) and the staff of the animal facility, to ensure that we're always up to date with any advances in the 3Rs and these areimplemented effectively. Furthermore, in our Institution, all researchers involved in animal work (whether as the project lead or performing procedures on animals) are required to complete Continuing Professional Development via relevant training mandated by the Animals Welfare and Ethical Review Body (AWERB), including local refresher training and training by recognised providerssuch as the online training modules on the Research Animal Training website.

IMPACT OF SCHISTOSOMIASIS ON HOST IMMUNE CELL DIFFERENTIATION AND FUNCTION

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

helminth, schistosomiasis, bone marrow, haematopoiesis, vaccine

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

To identify how schistosome infection alters host immune cell differentiation and examine the consequences for immune responses to both the parasite and unrelated immune stimuli such asvaccines

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Parasitic worms (helminths) infect ~1/4 of the global population. Schistosome parasites are the causative agent of the disease schistosomiasis (bilharzia, also known as snail fever) that infects 200- 400 million people, of which 90% live in sub-Saharan Africa. Schistosomiasis causes a substantial health burden leading to the annual loss of more than 3 million healthy life years (disability-adjusted lifeyears, DALYs). Disease pathology develops in response to parasite eggs lodged within host organs, such as the liver and bladder, which causes inflammation and fibrosis. Disease control relies on a single drug (praziquantel) that does not prevent reinfection nor reverse existing pathology, and to whichresistance is thought to be developing. Chronic schistosome infection also weakens

immune responses against other pathogens (including malaria, TB and HIV) and vaccines (e.g. BCG). This reflects the ability of these long-lived parasites (years-decades) to manipulate and dampen host immune responses to promote their own survival. We do not fully understand how the parasite achieves this. Greater knowledge in this area will inform strategies to boost anti-pathogen and anti- vaccine immune responses for people living in areas where these infections are endemic.

What outputs do you think you will see at the end of this project?

We expect this project will give a greater understanding of the mechanisms that allow parasitic wormsto modify host immune responses, promoting their own survival, and at the same time weakening responses to unrelated challenges. This knowledge will inform future strategies to stimulate better immune responses (to both infections and vaccines) in people who are infected with parasitic worms. This project will lead to new information in these areas in the form of scientific publications.

Who or what will benefit from these outputs, and how?

In the short term (1-5 years), the scientific community will gain additional understanding of how schistosomes modify host immune responses. These findings will be relevant to those working on schistosomiasis, other parasite infections (e.g. human nematode infection, >2 billion infected) and allergic diseases (e.g. asthma). This is because worms and allergens induce similar types of immuneresponse. The findings will also be relevant to those working on the impact of viral and bacterial infections on immune cell differentiation, and to those working with human patient groups from low- middle income countries that often have high levels of parasite infections. In the longer term, better understanding of the pathways by which worms dampen immune responses will potentially benefithuman health to (1) promote stronger immune responses in helminth-infected people and (2) limit detrimental immune responses in people who have autoimmune conditions (>5 years).

How will you look to maximise the outputs of this work?

Findings will be communicated through publications in open access scientific journals and at scientificconferences. Large datasets (e.g. global gene expression in immune cells) will be made available through open web interfaces and repositories

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 pre-clinical model to study the impact of schistosomiasis on host immune responses.This is because mice are a natural host for the types of schistosome parasites that infect humans (i.e. mice are a zoonotic reservoir for this disease) and are fully permissive to infection; they develop pathology similar to humans; there is greater availability of immunological tools compared to other models; and there is extensive

conservation between mouse and human immune systems. We use adult mice of either sex with or without genetic modification. We have optimised infective doses of parasites to levels that mice tolerate well for sustained periods (we have tested up to 20 weeks post-infection).

Typically, what will be done to an animal used in your project?

Experiments follow the PREPARE guidelines, A typical experiment will assess the impact of schistosome infection on immune cell development and function. This will be used for ~50% of animalsin this project and involves anaesthetising mice (standard laboratory mice = wildtype mice, or genetically manipulated animals) then infecting with ~40 schistosome parasites. This will result in the development of 10-15 adult worms. Infection will be allowed to proceed for different periods of times.
Disease symptoms are caused by schistosome eggs that cause inflammation and damage to host organs. This peaks around 6-8 weeks post-infection (acute disease) and settles down in the chronic phase of infection (10 weeks+). In a small number of experiments (<5% of animals), mice will be infected with the intestinal worm Heligmosomoides polygyrus instead. This allows us to compare immune responses that occur following infection with different parasitic worms. Some mice will be injected with substances that modify immune cells (such as growth factors or antibodies to deplete certain cells) or given other immune challenges such as vaccines. Mice may also be given drugs thatkill parasites to see if infection-induced changes revert to normal. Mice will be humanely culled and immune responses measured. Together, these experiments allow us to test how infection alters immune responses. These experiments also generate cells that can be transferred into irradiated mice (<20% mice used). Irradiation removes immune cells from the recipient mouse, creating space for transferred cells to grow and establish. These mice are then given vaccines or other immune stimuli to test their immune responses. In some experiments, mice will be given non-living immune challenges (e.g. parasite material or vaccines) in the absence of live infection (~30%). This allows us to determine the signals that drive immune cell changes in infection. A small number of mice (5-10%) will be infectedwith relatively high numbers of parasites (~100 parasites) to generate parasite material (worms, eggs) used in other experiments. High dose infection maximises recovery of parasites and so reduces the number of animals required. In these instances, animals will be humanely culled at an earlier point (~7wks) to prevent excessive disease pathology.

What are the expected impacts and/or adverse effects for the animals during your project?

We have optimised our infection doses so most of the animals (>95%) used in this project show no outward signs of distress or suffering. Infected mice develop enlarged livers and spleen (hepatosplenomegaly), which mirrors human schistosomiasis. A small number of infected mice (<5%)develop overt ill health characterised by weight loss, pallor, and hunched posture. These animals arereadily identified (by body conditioning scoring and/or weight loss) and are humanely culled.

Irradiated mice lose some weight in the first week after treatment but soon recover. These mice aresupported with mash diet and oral antibiotics (to prevent opportunistic infections).

Injections of anti-parasite drugs and substances that modify immune cells are not expected to causemore than transient discomfort at injection site.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

~50% of mice will be infected with live parasites and will be of moderate severity.

~20% of mice will be injected with parasite material. Of these mice, 1/4 will be of moderate severityand 3/4 of mild severity.

<20% of mice will be irradiated (moderate severity).

The remaining mice will be used as controls for interventions (mild severity or sub-threshold).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our research is focused on how schistosome parasites modify host immune cell development that occurs primarily in the bone marrow, but also at non-bone marrow sites. We also study how these changes alter the function of resultant mature immune cells in multiple organs (including bone marrow,liver, spleen and others). Additionally, schistosome parasites migrate through several different tissues (skin, lung, liver, intestine) as they mature and develop. This complexity with host and parasite cross- talk occurring across multiple tissues cannot be recapitulated with cell/organ culture systems or computer modelling approaches.

Which non-animal alternatives did you consider for use in this project?

We perform cell culture studies with mouse and human cell lines where possible and informative (e.g. macrophage cell lines). We also use human cells from both non-infected and schistosome-infected individuals, and results here inform animal studies as we identify shared pathways between mouse and human. We use existing global gene expression datasets in the public domain and through collaborators to inform our animal experiments (e.g. from non-infected animals and alternative infectionmodels).

Why were they not suitable?

These approaches do not allow us to address our primary aim – how do schistosomes alter immunecell development, and what are the consequences of this for our immune response?

Reduction

Explain how the numbers of animals for this project were determined. Describe

steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This estimate is equivalent to 200 mice per year over the course of the project. It is based on the design of experiments we wish to carry out, the funding we have secured, and our staffing. Group sizesof experimental mice are based on our previous studies that take into account expected differences within and between experimental groups.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Experimental design was aided with design tools such as NC3R's Experimental Design Assistant. We use statistical analysis to estimate group sizes based on mouse-to-mouse variation and expected effect sizes (informed by our previous studies, small scale pilot data and published experiments, whereappropriate). We will carry out longitudinal in vivo measurements including tail bleeds to analyse circulating immune cells. This allows paired analysis of data from the same animal, which reduces variation and group sizes necessary to reach statistical significance. We will also make extensive use of mouse cell cultures to generate a large amount of data from small numbers of mice (here the unit of analysis can be as low as a single cell).

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We create a large tissue bank from all animal experiments that is archived and frequently used to test new hypotheses (e.g. frozen tissues for imaging studies; frozen cells for functional tests; parasite eggsare recovered for use in other experiments). Parallel human studies ensure we prioritise pathways andmolecules most relevant to human disease in animal models. Tissues from a single experiment are often used to answer related experimental questions. We reduce breeding of genetically modified strains by careful planning experiments to ensure we match breeding to requirements.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the most commonly used animal model for schistosomiasis studies as they are the lowest mammalian species in which the parasite can develop. The unrivalled collection of immunological toolsand extensive prior characterisation ensures that mice are the best placed model to characterise the mechanisms of infection-induced changes to immune cell

development and function. We cannot reduce infective doses further without substantially increasing the number of mice that fail to develop patent infection (i.e. contain egg laying adult worms). This is because stochastic variation in the numbers of male and female worms would result in more male-only or female-only worm infections (which do not develop schistosomiasis pathology). As such, lower infective doses would mean we need to substantially increase our group sizes. We cannot harvest experiments earlier as our aim is to assess the impact of chronic schistosomiasis on mice (i.e. 10 weeks infection and beyond). Chronic infection best reflects human disease as curative drugs such as praziquantel are only administered annually (at most). Our animal facility enforces a robust health policy and all protocols have well defined end-points. Animals displaying symptoms of ill health are culled humanely using approved methods.

Why can’t you use animals that are less sentient?

Mice are the lowest sentient mammalian species to study schistosomiasis. Whilst larval zebrafish havebeen used by others to assess inflammatory immune responses to experimentally injected schistosome eggs, it is not possible to use this approach to test the impact of chronic infection on mammalian immune cell development and function. In this regard, the mouse immune system is muchmore similar to that of humans than that of less sentient species. We use adult mice (6 weeks+) as they have fully developed immune systems most similar to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Procedures are continually refined through discussion with researchers, animal facility staff and the Named Veterinary Surgeon (NVS). One example is our optimisation of parasite infective doses to prevent excessive pathology caused by higher infection burdens. We have regular training to ensureresearchers remain highly competent in experimental procedures and are aware of new refinements(e.g. new tube handling technique, policy on single-use needles). As weight loss is the best non- invasive measure of disease, we regularly weigh animals and also assess animal health using body condition scoring measures.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We follow PREPARE and ARRIVE2 guidelines for designing, conducting and reporting experimentalresults. The NC3Rs website is used to ensure robust experimental design for in vivo studies where appropriate.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We are part of an active research community that includes (amongst others) large numbers of parasitologists and haematologists who carry out animal work and share new approaches through shared lab meetings. We have a regular animal facility user meetings where internal and external speakers share new findings and discuss best practice. We also keep up to date with latest NC3Rsdevelopments through their newsletter.

INVESTIGATING THE ROLE OF BILATERAL ALTERNATING STIMULATION IN AVERSIVE MEMORY MODIFICATION

Project duration

3 years 0 months

Project purpose

- Basic research

Key words

posttraumatic stress disorder, memory, therapy

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of the project is to confirm that alternating stimulation of the left and right hand sides of thebody (otherwise known as bilateral alternating stimulation; BAS) facilitates the updating of fear memories, and to investigate the mechanisms by which it does so.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Eye movement desensitisation and reprocessing (EMDR) critically depends upon the use of bilateral alternating stimulation and is a recommended therapeutic intervention for posttraumatic stress disorder. However, it is also used for other conditions without having formal recommendation. Much ofthe lack of consistency over its use and application is due to a lack of understanding of why it works. This also impacts upon the understanding of why it works for some people, but not others. We wish first to test the hypothesis that bilateral alternating stimulation facilitates memory updating, and then follow up by exploring the precise biological mechanisms involved. This will help to establish how

EMDR works, and may underpin personalisation of its implementation.

What outputs do you think you will see at the end of this project?

The project will realise new information concerning whether bilateral alternating stimulation facilitates memory updating, and does so through the memory reconsolidation process. Memory reconsolidation is known to be an important process that re-stores memories that have been reminded; reconsolidationis believed to be a major mechanism of memory updating. Therefore, if bilateral alternating stimulation facilitates updating, it is likely to do so via reconsolidation. This information will be disseminated via peer-reviewed publication.

Who or what will benefit from these outputs, and how?

In the short term (1-3 years), the outputs will inform further work undertaken by researchers in the fields of PTSD, memory reconsolidation and eye movement desensitization and reprocessing (EMDR).This will be work in both experimental animals, and healthy human participants.

In the medium/long term (4-10 years), the expected outputs would likely impact clinical research inPTSD patients. This may lead to refinement of the EMDR protocol or influence the development ofalternative therapies.

How will you look to maximise the outputs of this work?

We are already in collaboration with influential UK Clinical Psychology researchers with expertise in human memory reconsolidation research. These collaborators will have early sight of any outcomes inorder to facilitate translational collaborative research.

We will disseminate outputs in interdisciplinary peer-reviewed journals and in practitioner publications.Evidence contrary to our working hypothesis will be equally valuable in progressing the understandingof the mechanism of action of EMDR.

Species and numbers of animals expected to be used

- Rats: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The objectives are focussed on a change in fear memories relevant to human psychiatric conditions. This involves inducing a fear memory that produces a fear response. It is the reduction in this fear response that we aim to achieve. We are using rats because the objectives involve behavioural assessments of fear memories that cannot be modelled effectively in lower animals. The fear responsethat we measure is freezing (i.e. a cessation of movement, except for breathing) in response to a feared sound. This freezing response is much more reliable in rats than in mice, and we also see equivalent levels of freezing in male and female rats.

We will use adult rats because there are important developmental changes in memory processing, andEMDR therapy is typically applied to adult patients.

Typically, what will be done to an animal used in your project?

Rats will be exposed to a mild electric footshock in order to condition a fear memory. This memory willthen be reminded, by exposing rats to sound cues that were present at the time of the original conditioning; this involves returning the rats to the same place and playing a sound that was present during conditioning (the footshock would not be re-experienced). Rats will have their whiskers brushedalternating on the left and right sides; this is hypothesised to place the reminded fear memory into a state that allows it to be updated. The memory will be updated behaviourally through rat tickling (an action shown to be a positive experience for rats). Subsequently, the memory will be tested using computer-scored behavioural observations in response to the sound cue, with the expectation that in experimental conditions the memory is weakened to become less fearful (control conditions would remain at the original memory strength). In some rats, post-mortem examination of molecules in the brain will allow the study of the mechanisms of memory updating.

The injection of drugs will be used only as a treatment control. We know that these drug injections cause a beneficial reduction in the fear response if the memory is conditioned and reminded appropriately. Therefore, it will be used to evidence that the memory can be updated, if the whiskerstimulation and tickling do not have the expected beneficial effect.

The duration of the study for each rat will be 1-2 weeks. During this period, rats will experience 1footshock, repeated exposure to the sound cues and a maximum of 2 drug injections.

What are the expected impacts and/or adverse effects for the animals during your project?

The footshock experience induces pain that is felt acutely during the learning session. While the pain results in an enduring memory, the pain itself only lasts for seconds. Later, the rats will be reminded ofthe painful learning, through re-exposure to the sound cues. This reminder will induce fear and expectation of further footshock (although no further footshock is experienced). This fear will last for the duration of the cue exposure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

100% moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you

have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The objectives are focussed on the updating of fear memories, and so require behavioural memoryobservations in awake animals. The hypothesis in this project involves focussing on a very specific process in memory (memory reconsolidation). This can only be achieved in animals using validatedexperimental manipulations and post-mortem analyses of brain tissue; it is not currently possible toachieve this in human participant studies.

Which non-animal alternatives did you consider for use in this project?

We considered human participant studies and in vitro preparations that allow for the study of synapticplasticity (a likely biological mechanism underpinning memory).

Why were they not suitable?

Human participant studies can update memories, but cannot demonstrate the mechanism by which this happens, which is a critical aim of the current project. The post-mortem analysis of molecules in the brain is a necessary procedure to determine the mechanism of the change in fear response. Oncethis is confirmed in the current project, some follow-up studies will be able to translate the finding into the updating of human memory in human participants. The aim of demonstrating memory updating cannot be modelled in simplified in vitro preparations.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have conducted several experiments showing the enhancement of memory destabilisation usingdrug treatment. We assume that bilateral alternating stimulation will have an effect of similar magnitude. Therefore, the same number of animals will be needed to detect the group differences inbehaviour.

We have also conducted many experiments showing drug impairment of memory reconsolidation, aswell as fewer focussing on true memory updating. Again, we assume that the updating methods usedin this project will have effect sizes of similar magnitude.

The estimated total number of animals is based upon these effect sizes and the design of the plannedexperiments.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We routinely use the NC3R's Experimental Design Assistant to refine our experimental design. Wecarefully plan the necessary control groups. This allows us to minimise to those

that are required todraw the anticipated interpretative conclusions, while ensuring that we do not need to repeat experiments with additional controls.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We will use pilot studies in order to maximise the experimental refinement of our procedures. This willallow us to minimise inter-animal variability and thereby reduce the number of animals used. Our previous studies have taken a similar approach and so we do not anticipate further reductions in the required numbers of animals.

We will use equal numbers of male and female animals. As fear memory expression has been shownreliably in the literature to be sexually divergent in rats, this requires fully-powered independent experiments in males and females.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Due to the nature of the scientific question, a fear memory model is necessary. The model needs to have reliable behavioural measures of fear memory, but is refined to use the minimum necessary aversive experience. Rats will be conditioned to learn that a stimulus (typically an auditory tone) is followed by the delivery of a footshock. We have reduced the intensity of the footshock to the thresholdneeded to elicit a memory that lasts for the duration of the study. This ensures that the aversive experience itself causes the least possible pain, suffering and distress. Our footshock intensity of 0.4 mA, presented for 0.5 s, is lower than typically used in studies of fear memory in rats, representing a refinement in the procedure. We have experimented with reducing the intensity further, but the lower footshock does not produce a reliable response across rats. It is equivalent in experience to a strong static electricity shock (both in intensity and duration). There needs to be a lasting impact of the aversive experience, in the form of the fear memory that is expressed behaviourally days later; when the rat is presented again with the tone, it displays an alteration in behaviour. However, no other signs of lasting harm are seen within the model. The alteration of behaviour is quantified via automated computer analysis of video recordings of the behavioural sessions. This maximises the quality and consistency of the scoring.

Other fear memory models are available, but are less refined as they expose animals to repeated orprolongued stress. This stress can be in the form of footshocks or predator (scent) exposure.

We will use behavioural interventions to update the memory. The use of drug administration is restricted to the possible implementation of positive control experiments only in the event that thebehavioural interventions do not have the predicted effect.

Why can’t you use animals that are less sentient?

We cannot use less sentient species, because the relevance of our scientific outcomes to human clinical therapy necessitates a mammalian model that shows similarity to human behaviour and underlying biological mechanisms. As EMDR therapy is normally applied to adult patients, it is necessary to use adult rats. We cannot conduct our studies on terminally-anaesthetised animals, aswe will be observing behavioural measures of the fear memory.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The negative experience (footshock) that is part of the model lasts typically for no more than 1 second.Rat behaviour during and immediately after the footshock involves jumping to escape the floor that delivers the shock. Shock intensities are monitored such that vocalisation is not observed, and we have extensive evidence that shock intensities in this range (between being noticeable and not inducing vocalisation) are sufficient for our scientific needs. We monitor behaviour within each cohort inorder to note to any change in the sensitivity of our outbred rats. This allows us to adjust the parameters of the negative experience in order to keep the welfare costs to a minimum, while maintaining a robust scientific model.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will adhere to LASA guidelines (dosing section) to minimise harms, use the PREPARE guidelinesin the planning of our experiments, and publish protocols and data as recommended by the ARRIVE 2.0 guidelines to ensure reproducibility of data.

We will continue to monitor the fear conditioning protocols of publications, in order to ensure that ourprotocols are always of the highest refinement and least severity possible.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We are kept up to date on 3Rs via periodical emails from our animal unit and from the NC3Rs newsletter, and have incorporated these in to our protocol; in particular, to use rat tickling, rather than positive drug administration, as the positive experience. We will also monitor advances in the scientificliterature that might be adopted to refine our procedures further.

We will review each experiment upon completion to determine any refinements that can be applied tofuture experiments.

MODULATION OF THE IMMUNE RESPONSE IN CANCER TO DEFINE NEXT GENERATION IMMUNOTHERAPEUTICS

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir 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 aimsmentioned in paragraph (b)

Key words

Cancer, Immunology, Therapy, Stroma, Adoptive cell therapy

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate new drug targets, drug combinations and treatment approachesfor awakening and harnessing the power of the immune response to identify and attack cancer.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

One in two people are affected by cancer at some point in their lives. Further drugs are

required to tackle the disease. The immune system is an incredibly powerful collection of cells and soluble molecules that work together to detect and kill abnormal cells in the body, such as cancer. However, therapeutic approaches which raise or harness the immune response against cancer still do not represent cures for all patients. One reason for this failure is that cancer is able to suppress the immune system. This project will investigate approaches to therapeutically block pathways through which cancer suppresses immune responses as well as approaches to harness the power of immunecells/system to support the eradication of the disease. If we can identify new drugs and approaches which achieve this, it would lay the foundation to new clinical trials in patients.

What outputs do you think you will see at the end of this project?

Outputs from this project will include new insight into a rapidly evolving field which will be crystalised inpublication in peer reviewed journals. Due to the translational nature of the project, and my teams research focus, we are also working towards delivering this insight and new drugs/combinations into Phase I trial.

Who or what will benefit from these outputs, and how?

In the near-term, it is anticipated that the insight from this project will inform those within the field through peer reviewed publications to guide thinking and further our understanding of cancer and itsability to shut down the immune response to preserve its survival. The long-term value of this projectwill be in translating the insight into new Phase I trials in patients with the disease. My team is well placed to deliver on both of these goals and benefits.

How will you look to maximise the outputs of this work?

All outputs from the project, positive and negative, will be brought into papers for peer reviewed publications. Also, as a collaborative group, we will be focused to present and discuss our finding at external scientific meetings and within our networks to inform others within the field. My group will alsoseek patents on key observations to preserve the long term translational journey of the drugs and combinations to later stage clinical trials and wider clinical use.

Species and numbers of animals expected to be used

- Mice: 33,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice have been selected as the model for this project as the murine immune system closely resembles that of humans. We will be using adult mice as we are looking to explore the mature, ratherthan developing, immune response against cancer in these animals and approaches to harness this.

Typically, what will be done to an animal used in your project?

The intended study requires the use of spontaneous and implanted murine models of cancer. In mostcases within this project we will look to develop established cancers prior to starting drug treatment which might be small molecule drugs or cellular therapies (or a combination of both) to study the effects of these approaches on the immune response and its ability to control tumour growth. Such drug treatments tend to last for up to 28 days, however where we see significant control of cancer growth, we may also in a small number of animals stop treatment and resume (or switch drug treatment) to understand the long term impact from the treatment and optimal drug treatment approaches which could last up to 1 year.

What are the expected impacts and/or adverse effects for the animals during your project?

This project is focused on testing and developing new drug approaches for treating cancer which will improve the immune response to enable it to control tumour growth. As such, the key models being tested will be those associated with cancer. As such, the majority of mice on a non-breeding protocol will have cancer or cancer-related metastases. The models being used are highly validated, and previous experience has informed us how to monitor these animals and identify our safe/reliable windows for treating these mice. Also, in our experience, using the human endpoints outlined in theseprotocols of this licence, the mice do not show signs of pain or ill health as a result of their tumours.

Signs of toxicity associated with the cancer or drugs will result in the mice being humanly killed as thekey output from this project is a control of tumour growth in the absence of significant toxicity. This is due to the fact that we are looking to develop drug approaches for movement into clinical trials.

Although the drug treatments will typically last for around 28 days (for the majority of animals), ifsignificant toxicity arises the experiment (or cohort in question) 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 animaltype)?

It is expected that around 60% of the mice used under this project will be on protocols classed as 'mild'severity, where the remaining 40% will be used on protocols that would hold a 'moderate' severity category.

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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice have very similar immune systems to our own, and murine models of cancer, which have alreadybeen developed, share many characteristics of the human disease. The complexity of the immune response and its suppression in cancer cannot reliably be modelled in the laboratory and, as such, theuse of mice is an absolute requirement to address these important questions.

Which non-animal alternatives did you consider for use in this project?

The closest non-animal alternative for addressing these questions would be *in vitro* based assays suchas spheroid/organoid cultures which involve either malignant cells alone, or in combination for select stromal cells, cultured in a 3D format. Also, there is an emerging use of organ-on-a-chip approaches tomodel the tumour microenvironment.

Why were they not suitable?

The use of spheroid/organoid cultures and organ-on-a-chip approaches have proven useful for implementation of drug screens and could still be used to some extent to screen for targets. However, for the research questions were are asking, in particular, relating to harnessing the immune response in cancer, studying the tumour in the context of the complete murine immune system still represents the only feasible route to addressing these questions. Immune cells work together in cancer, and we are still understanding how these cells interact with one another to facilitate cancer growth. We have found that HO-1 inhibitors and chemotherapy elicit a change in the recruitment of immune cells into the tumour, which is an important part of the mechanism of tumour control but one that cannot be replicated using in vitro approaches. As such, as our therapeutic approaches rely on the immuneresponse (in its entirety) in cancer, we believe our most accurate evaluation of a drug, or drug combinations, efficacy will require the use of murine models above that of other available assays.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 calculations for our use of spontaneous tumour mice have been estimated based on Mendelian genetics for the likely transfer of the genes from a heterozygous male (PyMT Tg+/-) breeding with a wild type female as it is not possible to generate a homozygous PyMT+/+ male mice as the females carrying the gene develop tumours). We have, as such, assumed 50% of pups will befemale and 50% of these will carry the PyMT Tg to guide these numbers.

For therapeutic studies, experimental cohort sizes of 8 mice to be used which is a number derived fromPower calculations and previous experience using the model (10 years' experience). Most studies that will be conducted under this licence will involve 4 cohorts as they will involve the testing of combinationtherapies (for example a HO-1 modifying agent and standard of care chemotherapy or alternative immune checkpoint blockade). As such,

single agents will need to be tested alongside the combinationand a control vehicle treated group.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

As the spontaneous models of cancer are enrolled on n=1 (as tumours arise spontaneously) it will provide us an opportunity to continue to refine our experiments to ensure that cohorts are correctly Powered in ongoing studies. Past experience with the models in question has taught us much about the required cohort sizes for correctly powering experiments. Past experiments and those moving forward will continue to be guided by Power calculations conducted by statisticians to guide our preclinical studies involving mice. We also randomly assign mice to treatment groups and gender andage match to reduce experimental bias and limit variables which may impact response, and as such, limiting cohort sizes required as far as possible.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Our research group has established internal and national collaborations with which have shared tissuefrom animals that we have humanely killed and will continue to share with on the current project.
Equally, we share tissue across group members by timing experiments to ensure that we get the most from each mouse used on the project. Where possible, we use homozygous knock out mice for key genes of interest to limit the number of negative/heterozygous pups we receive. Where feasible, pilot studies will be used. However, as the majority of our tumour studies are conducted using spontaneousanimals it provides an ability to stop experiments early where it is clear no effects on tumour growth is being observed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The tumour models chosen for this study are robust and generate reproducible growth curves, backed by a significant body of published literature. The reproducibility of these models greatly decreases the number of mice required in each experimental group to achieve the desired significance. Where possible, pilot studies will be employed on a smaller number of animals, to test the effect of the therapies on tumour growth, based on which, group sizes will be refined to achieve the required level of statistical significance with the minimum number of mice. In addition, the least harmful tumour modelwill be utilised to address the experimental question. For validating approaches, subcutaneous tumours, as the least harmful, will be utilised where possible. When we have identified and validated the therapeutic strategies to be tested, spontaneous models of cancer will be used. The tumours in these mice develop over longer periods of time and are not a

homogeneous collection of cells as they continue to transform as they develop, which closely mimics the human disease. We have selected a use spontaneous murine models of cancer for this work as these models have been demonstrated to most accurately recapitulate the response of therapies in patients in the clinic (Singh et al, Nature Biotechnology, 2010), and as such, for a translational project/programme they represent a gold standard approach for reliably predicting efficacy and relevance of the test therapies and combinations.

Why can't you use animals that are less sentient?

The use of mice, and not a less-sentient species, is required based on the existence of well validated mouse models of cancer, which have been proven to share many similarities with the human disease. In addition, the murine immune system, and immune response, is closely related to that of our own. Assuch, to fully understand how these drugs will interact and harness the immune response many tumor models such as those using zebra fish, drosophilia and c.elegans will not be appropriate for this project. Also, the small molecules we will be testing such as SnMP have a proven ability to cross the species barrier to mice, but it is unlikely will work as effectively in species that are more evolutionarily distant.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Where possible, pilot studies will be employed on a smaller number of animals, to gauge the effect of the therapies on tumour growth, based on which, group sizes will be refined to achieve the required level of statistical significance with the minimum number of mice. For our work with mice bearing spontaneous tumours, we have implemented a mouse welfare log which is kept in the animal room and is accessible to technicians that monitor our rooms. This log tracks the weight and welfare of all animals that have tumours. This log permits any deterioration of a mouse's welfare to be seen at the earliest possible time and decisions to be made quicker. We will continue to be mindful of ways to refine our implementation of the models we use and continue to select those that are the least toxic orharmful to address our preclinical research questions.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will continue to closely follow the NCRI guidelines as the current gold standard guidance for animalwelfare management in models of cancer.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

To ensure we are up to date with the most current advances in the 3Rs we will continue to closely follow new Home Office guidance and literature as well as remain in close communication with the BSU's NACWO and NVS. I have also registered an account at https://www.nc3rs.org.uk/ and receive the Newsletter updates. Also, myself and my lab, have copies of the NCRI guidelines https://www.nature.com/articles/6605642 and keep up to date with relevant literature, such as refiningprocedures for administration of substances to mice https://journals.sagepub.com/doi/10.1258/0023677011911345

INFLUENCE OF GENES ON THE DEVELOPMENT AND AGEING OF THE INNER EAR AND ITS SENSITIVITY TO OTOTOXINS

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, ortheir effects, in man, animals or plants

Key words

hearing, deafness, balance disorders, genes, ototoxicity

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The project aims to test how mutations in different genes affect the structure, function, developmentand ageing of the inner ear, and the sensitivity of this organ to medicines that are known to cause deafness as an unfortunate side effect.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 inner ear of humans and animals is essential for the senses of hearing and balance. It contains highly specialised organs: the cochlea and its sensory hair cells enable hearing, while the vestibular system allows us to balance and stay upright. However, the function of these essential organs can be compromised by many factors. These include mutations in

numerous genes, the ageing process, and unfortunate side effects of several commonly used medicines. For example, some broadly used antibiotics and cancer therapies may effectively save lives, while leaving a significant proportion of patients with hearing loss and/or balance disorders at the end of the treatment – such drugs are ototoxic. For this project, we will breed genetically altered mice which lack or have mutations in genes important for the development, function, drug response and/or long-term maintenance of the structuresin the inner ear over the lifetime of an organism.

What outputs do you think you will see at the end of this project?

Expected outputs include scientific publications describing the effects of mutations in genes that encode essential components of the inner ear, and an evaluation of a putative target for the preventionof deafness caused by the anti-cancer agent cisplatin and certain types of antibiotics.

Who or what will benefit from these outputs, and how?

Short term impacts on the scientific community could include an enhanced understanding of how the inner ear develops, how key structures in this organ are maintained for the lifetime of an organism, andhow medicines that can make one deaf as an unfortunate side effect gain access to and/or kill the cochlear sensory hair cells required for hearing. In the long-term, the outputs may improve the quality of life, both for the ageing population and for patients of all ages that are treated with the commonly used anti-cancer compound cisplatin and antibiotics like gentamicin.

How will you look to maximise the outputs of this work?

Outputs will be maximised by presenting our data at both national and international meetings, by collaborations with colleagues within and outside UK, and publications in relevant scientific journalsthat include both successful and unsuccessful approaches. Furthermore, we will share data with clinicians at our local county hospital with whom we have regular meetings on an annual basis.

Species and numbers of animals expected to be used

- Mice: 4200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using the mouse for several reasons. Firstly, the genome and the inner ear of the mouse are both very similar to those of humans. Secondly, there are numerous tools available for modifying the mouse genome, and many successful mouse models have been previously created for mutations that cause deafness and balance disorders in the human population. Finally, cochlear cultures - ears in a dish - prepared from newly born mice have proven to be a valuable system, both for studying the mechanisms of drug-induced hair-cell death and screening for potentially novel protective compounds.

As we are studying how mutations in genes affect both the development and ageing of specific structural components of the inner ear, we will be examining the ear at key stages in its initial development, from ~14 days of gestation to a postnatal age of 15 days, a point in time when hearing inmice begins, through to 15 months of advanced age.

Two parallel approaches will be used to determine if and how a deletion of a gene that renders cancercells resistant to the anti-cancer drug cisplatin protects sensory hair cells from damage by this drug.
Firstly, cochlear cultures prepared from newly born mice, and secondly, studies with mice between 3 and 6 months of age. The cochlear culture approach is advantageous as it will enable us to rapidly assess the effects of cisplatin on sensory hair cells. Furthermore, it is considered to be a mild procedure that uses material from an immature stage of life and is less sentient. However, as the properties and sensitivity of hair cells are known to change as they age, and as mature hair cells cannot be maintained in cultured cochlea, these findings will need to be confirmed with older animals.

Typically, what will be done to an animal used in your project?

Genetically-altered (GA) animals will be bred in the animal facility and biopsies will be collected between postnatal days 10 and 20 to allow for identification and genotyping. If the GA mice are carrying genes that can be turned on and off the gene will be activated or deleted, typically by the administration of an appropriate agent either by injection, or in the diet, either before or after the onsetof hearing.

For studies examining how mutations affect the structure, function, development and ageing of the inner ear, many of the mice will be humanely killed by a Schedule 1 method. Embryos and early postnatal mice will be humanely decapitated as cervical dislocation can cause damage to the inner ear.Tissues will be preserved for subsequent examination by microscopy.

When effects of the mutations on hearing are to be examined, auditory brain stem responses (ABRs) to sounds of different frequencies will be measured under general anaesthesia. For this purpose, fine needle sensors are attached to the scalp. These measurements will be followed by anaesthetic overdose and removal and preservation of the inner ear for further studies. In some instances, ABRs will be measured as function of age on up to four separate occasions followed by recovery at intervalsof at least 1 month before the animal is terminated and the inner ears are preserved.

When the effects of a given mutation on balance and vestibular function are to be examined, the mice will be subjected to one or more short behavioural tests on up to five occasions prior to humane killing,which may or may not be followed by preservation of the inner ear. To determine if early visual experience influences the effect of a mutation on the function of the vestibular system, litters of GA mice will reared in total darkness for up to 4 weeks of age before subjecting them to behavioural analysis. Additionally, and as mice are nocturnal and typically more active in the dark, we will monitor and compare the behaviour of mutants and control mice during an entire 24 hour light-dark cycle. Thiswill be done with infra-red cameras and will ensure we are not missing any unusual behaviours in mutants that only occur in the dark.

For our studies which aim to examine a potential route of entry for the anti-cancer drug cisplatin into sensory hair cells, toxicity assays will be performed on cochlear cultures prepared from new-born mice.Some genetically altered mice at 12 weeks of age will be injected with cisplatin (or saline as a control) once a day for 4 days followed by a 10-day

recovery period on 3 consecutive occasions (i.e., for a totaltreatment period of 42 days). Subsequently, hearing will be measured, and the inner ears will be preserved for assessment of numbers of cochlear hair cells.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected levels of severity range from mild to moderate. The methods for breeding and maintaining the mice, and the assessments of hearing and function of the vestibular system are not expected to have adverse effects. A small proportion of all genetically altered mice will exhibit abnormalbehaviour (circling and/or head bobbing), considered moderate. To study if a specific genetic mutation will protect the cochlear hair cells from the harmful effects of cisplatin, some mice will be exposed to aninjection protocol with this drug. Mimicking human patients undergoing cisplatin treatment, we expect this treatment protocol to cause progressive hearing loss in the normal mice, accompanied by slow weight loss over the 42-day treatment period.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Protocol 1 – mild – 100 % of mice will experience this severity.
Protocol 2 – moderate – ~35 % of all mice will experience this severity.
Protocol 3 – mild – 100 % of all mice will experience this severity.
Protocol 4 – moderate – ~25 % of all mice will experience this severity.
Protocol 5 – moderate – ~10 % of all mice will experience this severity.

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The ultimate aims of the project are to find ways of preventing hearing loss and balance problems caused by the unwanted side effects of certain medicines, mutations in various genes, and the ageingprocess. Whilst there are a small number of cell lines available that were derived from the inner ear and share some properties with sensory hair cells, and although inner-ear organoids (3-dimensional structures that bear a certain resemblance to the inner ear) can be generated from both mouse and human stem cells, none of these systems perform like the cells or structures found in ear of a living organism and cannot be used as a complete replacement for studying the questions we wish to address. We therefore need to use animals to fully achieve the aims of our project.

Which non-animal alternatives did you consider for use in this project?

We have considered the two ‘non-animal’ alternatives mentioned above, epithelial cell lines derivedfrom the inner ear of the immorto-mouse and inner ear organoids derived from human and mouse stem cells.

Why were they not suitable?

The systems described above are not suitable as the cells and structures produced lack any consistentuniformity in structure, do not produce the anatomical components we wish to study and are, relative tothose in the inner ears of living animals, insensitive to both sounds and ototoxins. Mouse cochlear cultures produced with tissues derived from the inner ears of newly born mice are the best less- sentient alternative.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are refined based on the usage on our current Project License for similar related projects. For the breeding and maintenance of mouse lines, and the mice generated for experiments, this equates to a likely maximum of 200 mice per year per line, or 1400 mice for the 7 mouse lines peryear, and a total of 4200 for the duration of the 3-year project license.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We considered the minimum acceptable numbers of animals that would have to be used for each typeof experiment to produce scientifically robust results. The experimental designs were derived in consultation with experts in statistics at this and other universities and will be further updated during the proposed work as necessary. We have also referred to sources such as the updated guidelines from NC3Rs and Norecopa

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We will use the most efficient breeding schedules possible, sharing tissues with other users and co- ordinating experiments with them whenever possible. We will collect inner ear tissues from ex-breedersfor our ageing studies, and preserve tissues for morphological studies from animals that have been used for testing hearing or balance for our morphometric studies. We will further optimise the numbers by always using animals that are on the same genetic background, that are of good health, and have been raised under standardised conditions. These practices will all help to minimise variability and ensure the number of animals is used is the minimum 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal models will be genetically altered mice carrying mutations in genes that are expressed in the inner ear. In some cases, these genes are only expressed at high levels in the inner ear and, as a result, any effect is likely to be restricted to the senses of hearing and/or balance and unlikely to causepain or suffering. Whilst social interactions may be compromised by a loss in auditory sensitivity, we have not noted any problems with wellbeing or breeding performance in mice that are severely deaf across the entire hearing range. Defects in the balance (vestibular) organs of the inner ear can lead to varying degrees of circling and/or head bobbing behaviour, but again this does not compromise breeding or feeding, nor does it lead to a weight loss or a deterioration in coat condition. Methods usedto monitor vestibular dysfunction are non-invasive, used for short periods of time at infrequent intervals, and are unlikely to cause pain, suffering, distress or any lasting harm. Hearing is monitored under general anaesthesia with fine needle electrodes attached to the scalp, a method expected to cause minimal pain.

Why can’t you use animals that are less sentient?

A proportion of the work proposed will be done using tissues derived from animals that have been terminally anaesthetised or with tissues derived from newly born animals. Mice do not respond to auditory signals before 14 days of age (due largely to immaturity of the middle ear) so potentially less sentient early postnatal animals cannot be used for testing hearing. Zebrafish are a species consideredto be less sentient and are, in some cases, a possible alternative. However, whilst they can hear, they do not have a cochlea akin to that in the human ear or a tectorial membrane, a structure of the mammalian inner ear that is essential for normal hearing and is one of our primary targets of interest.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Animals in our breeding colonies are monitored daily by the technicians in our animal facility and additionally at least once week by the licence holder and/or the research assistants running the projects. If hearing measurements are made over time in groups of animals, the animals will be additionally monitored daily by the research assistants involved for the first 3 days after recovery fromgeneral anaesthesia.

To minimise weight loss during cisplatin exposure mice will receive saline and dietary supplements twice daily and will be provided with free access to food pellets and a nutritionally fortified water gel.These mice will be inspected twice daily by the applicant and/or a research assistant who will weighthe animal and record their welfare and body condition, culling any animals that show an aversive response close to reaching the severity limits and humane end-points in the license.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

For all of our studies we will abide by published guidelines (NC3Rs, Workman et al, 2010 Br. J. Cancer), as well as local guidelines, and ensure best working practice. We will also adhere to theARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will stay informed about advances in the 3Rs via the regular monthly updates from the National Centre for the Replacement Refinement and Reduction of Animals in Research (NC3R) we receive viathe manager of our animal unit, via regular visits to the NC3R website, via attendance at relevant NC3R webinars, and via relevant 3Rs symposia held locally. We will implement any advances that arerelevant to our research following the guidelines provided.

MECHANISMS OF INFECTIOUS 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, ortheir 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 aimsmentioned in paragraph (b)

Key words

Respiratory infections, Invasive diseases, Vaccines, Therapeutics, Pathogens

Animal types	Life stages
Mice	adult, aged, pregnant, neonate, juvenile, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

To understand how pathogens such as bacteria, viruses and parasites cause disease. We aim to identify the host, environmental and microbial factors, as well as their associated mechanisms, whichcontribute to the onset and progression of disease. This will advance the current understanding of disease mechanisms, host-pathogen interactions, immune responses to infectious disease as well asthat of the impact of environmental influences such as pollutants. Altogether, our findings will be usedtowards the development of novel and more efficient approaches for the treatment or prevention of infectious disease.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The global burden of infectious disease surpasses those attributed to ischaemic heart disease or stroke. For instance, respiratory infections caused by bacteria, viruses or parasites remain amongst the top 5 most deadly infections, causing together 8.4 million annual deaths worldwide. In the UK, infectious diseases are a significant health and economic burden accounting for 8% of deaths and 10%of hospital bed days. Respiratory or gastro-intestinal infections represent the most common reasons for work absence, accounting for 27.4m days lost and an overall annual economic burden of £30bn in England alone. The relentless capacity of infectious pathogens to develop resistance to existing drugs
e.g. antibiotics, the emergence of new infections and re-emergence of infections linked to health inequalities or other environmental and societal changes, demands a renewed understanding of themechanisms of infectious disease.

A number of studies report that the inhalation of dust particles in hot dry weather may damage nose and throat mucosa creating favourable conditions for bacterial infection. Similarly, air pollution is the world's largest single environmental health risk, being responsible for an eighth of all global deaths peryear (WHO). Particulate matter, a key component of air pollution, is strongly associated with an increased incidence of infectious disease, including community acquired pneumonia, infective endocarditis, and cystic fibrosis. Our studies will increase our understanding of how environmental factors such as dust, heat and air pollution can cause increased risks of infectious disease and exacerbates chronic respiratory disease.

Multi-species co-infections pose one of the greatest challenges to world health, particularly in lower- income countries where major global pathogens such as HIV, malaria and soil-transmitted helminth (STH) infection are co-endemic. A pathogen's disease potential can be magnified by their relationship with other pathogens and co-infections involving intestinal helminths are of particular interest due to their ability to modulate systemic host immunity. This modulation ensures chronicity of infection from childhood through to adult life and influences immune homeostasis and inflammatory responses to microbial pathogens. Further understanding of the synergistic mechanisms underlying viral-helminth or bacterial-helminth infections would contribute towards designing low cost, efficient and coordinateddeworming programmes alongside vaccination, particular in settings where helminths infections areendemic.

Our growing and ageing population has become one of the most important global health challenges. The most recent reports suggest that in 2019, one out of every eleven people in the worldwide population was over the age of 65. By 2050, this proportion is expected to increase to one in every sixpeople over the age of 65. In some regions, such as in Europe and North America, one in four peoplewill beover 65 years old. Aging is a prominant risk factor for infectious disease. Our studies will also aim at dissecting the immune response, disease mechanisms and susceptibility in elderly animal models.

Our overaching aim is to identify key pathogen virulence factors e.g., gene or protein, environmental aswell as host immune factors which can be targeted for potential new vaccines and therapeutics.

What outputs do you think you will see at the end of this project?

Our work will provide;

[1] A detailed mechanistic understanding of how major bacterial and viral respiratory pathogens causedisease and the underlying molecular and cellular mechanisms, including

for example, pathogen interactions with host cells (e.g., nasopharyngeal, lung and olfactory epithelial cells, endothelial cells) and host immunity (e.g., macrophage signalling pathways, regulatory T cells responses, neutrophil influx, memory B cell and antibody production).

Development and testing of novel therapeutics against bacterial colonisation and/or invasive disease caused by significant human pathogens such as *Streptococcus pneumoniae, Pseudomonas aeruginosa, Streptococcus pyogenes (GAS), Streptococcus agalactiae (GBS), Staphylococcus aureus*and viruses such as Influenza A (IAV) and Respiratory Syncytial Virus (RSV).

Development of new vaccine candidates against major human pathogens such as those listedabove in [3].

Results that will be exploited in combination with *in vitro* studies, *in silico* computer modelling, andhuman organ-on-a-chip models - our findings will be highly translational and inform human clinical studies.

Multiple research publications in high quality peer-reviewed journals. We have a strong publicationrecord and we publish from all our funded studies (typically 6-10 publications per year).

Who or what will benefit from these outputs, and how?

In the short term, the primary beneficiaries will be the staff and students involved in the project, as wellas the research community more broadly, as they will benefit from access to research findings in the form of published research papers that will enhance knowledge in the field and stimulate further research. The research community will also benefit, in the short to medium term, from access to new experimental techniques that we will develop during this project, including those for the novel development of vaccines using reverse vaccinology techniques and unique models focusing on nasopharyngeal, lung, brain or blood infections. In the long term, the public will benefit from access tonew therapeutics or vaccines. In particular, children and the elderly who are highly susceptible to respiratory bacterial and viral infections people will benefit from the development of novel therapeutics and vaccines designed to protect them of infection.

How will you look to maximise the outputs of this work?

We aim to publish in open access journals to ensure maximum reach of our research and we also usepre-print servers (e.g. bioRxiv) to make our findings available at an early stage. All our high-impact publications are accompanied by press releases to make the wider public aware of significant developments.

We have a number of active national and international collaborations with leaders in their fields and these will enhance the impact and outputs of our work over the course of the project. For example, wehave contacts in the pharmaceutical and vaccine industry with whom we may wish to collaborate on the development of therapeutics and vaccines.

Species and numbers of animals expected to be used

- Mice: Estimated 23,250 mice over 5 years using 10 preclinical models of infectious disease,including therapeutics and vaccine studies.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We study bacteria and viruses that cause disease via entry to the respiratory tract and other mucosal surfaces, such as the vaginal tract. We are interested in understanding how the host immune response, co-infection with helminths, and environmental influences affect the upper airways (the noseand sinuses) and the lower airways (the lungs) e.g. inflammation, to cause dissemination and seeding of pathogens into the blood and tissues such as the brain. Of the animals with a respiratory tract and immunology sufficiently similar to that of humans, mice provide the best pre-clinical models to replicatemucosal tract, central nervous system (CNS) and other infection models and therefore the most appropriate for our purposes. For a majority of our experiments, we will use adult mice, as the diseasecourse of an adult mouse infected with the pathogens we use is very similar to that seen in humans. Inother studies, we will also use neonates, and juvenile mice. Neonates and juvenile mice are useful for studies aimed at developing new vaccines, as we typically aim to develop vaccine formulations targeting early life – when humans are the most vulnerable to infectious disease.

Typically, what will be done to an animal used in your project?

The protocols included in this licence aim to mimic the clinical features i.e., onset and disease progression of bacterial, viral or parasitic co-infection and range from asymptomatic carriage (Protocol 2) to invasive diseases such as pneumonia (Protocol 1), sepsis (infection of the bloodstream, Protocol3) and CNS infection (protocol 4). These experiments may range in length from 24-96 hours for pneumonia or sepsis experiments, 6-8 weeks for an upper airway carriage or chronic lung infection experiment, and 10-12 weeks for an immunisation study.

In the case of respiratory pathogens, we classically establish microbial infection by administering an inoculum of bacteria or virus suspended in saline solution to the nose of the animal and allow the liquidto be inhaled. In our sepsis or CNS infection model, we administer the pathogens directly into the bloodstream or the cisterna magna.

Infected mice are monitored for signs of disease based on our well-established mouse scoring systemwhich we have used for the past 20 years under Home Office Project Licence procedures. Mice may be subject to procedures, such as those designed to prevent or treat the infection - therefore, some mice undergo treatment with novel drugs e.g. antibiotic or novel drug, while some are given novel vaccine formulations to determine if they provide protection against subsequent infection, or mice mayalso be administered with substances designed to help us understand how microbes cause disease. An example of the latter would be administering substances that alter an aspect of host immunity, to see whether this alters the outcome of infection. Compounds are usually given in drinking water, are inhaled or are injected into the skin, muscles or blood.

We also sometimes use mice that lack a particular gene (knock-out mice) or that express a new gene(transgenics) in order to study particular processes that occur during infection.

What are the expected impacts and/or adverse effects for the animals during your project?

Once infected with a pathogen, mice typically either remain healthy e.g. asymptomatic carriage model,or slowly start to develop the characteristic signs of invasive disease e.g. pneumonia, CNS infection orsepsis. We have been using mouse models of respiratory and systemic disease for nearly 25 years and have very well established and reproducible models which obey consistent and predictable symptomatic patterns. These include reduced activity, mild transient weight loss and/or a starry coat with a hunched posture. Once clinical signs are observed, we see either a gradual deterioration over 24-72 hours depending on the pathogens, maintenance of mild symptoms for around 7-10 days, or else rapid recovery. Weight loss is very rare in bacterial infection models, but can occur in some of theviral infection models that we use.

Beyond the infection itself, the substances we administer to the animals and the routes and doses of administration do not typically cause harm. They may alleviate the clinical signs of the infection or mayhasten decline, but in either case, we have well defined humane endpoints and so terminate studies before animals suffer unduly.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

None of the protocols under this licence have a severity level higher than moderate. Our work on upperairway bacterial carriage (approximately 1/3 of our total animal usage) is typically mild in severity, although a small proportion of mice (<10%) may go on to develop symptomatic disease. Our work on pneumonia (1/3 of total animal usage) is classified as moderate, with mice developing symptomatic infection but our humane endpoints preventing excessive suffering. Of all our models, the viral lung infection model has the most adverse events, with mice experiencing moderate albeit transient weight loss in the days following infection. However, we use low numbers of animals under this protocol and published guidelines have refined the protocol to minimise adverse events. When performed correctly, mice under this protocol recover their weight and are alleviated of clinical signs within ~10 days of onset of infection.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mammalian physiology, anatomy and immune response impact the nature of host-pathogen interactions in ways that cannot be reproduced in a laboratory. This project licence is focused on understanding how pathogens cause disease and how the host immune system responds to infection.Although we routinely exploit in vitro lab-based

models to replicate in vivo settings (please see next section), for instance, the environment of the respiratory tract, the use of clinically relevant in vivo models is the only way to properly assess and elucidate complex pathogen-host interactions.

Which non-animal alternatives did you consider for use in this project?

In vitro systems (e.g., human organ-on-a-chip, cell culture, bacterial growth media) and *ex vivo* (e.g., human primary cells) offer some opportunities to replace animal use and we never use animals for early therapeutic screening. For instance, we classically use cell culture systems to study the effects ofindividual host factors on bacterial survival, phagocytosis, invasion and adhesion properties, etc.
Performing preliminary work and basic mechanistic studies in vitro ensures that animals are only usedwhen alternatives are unsuitable for the research question being addressed. Typically, we will use cellculture models for virulence screening and initial drug toxicity testing, before moving on to mouse models with selected bacterial isolates or promising drug/vaccine candidates.

Alternative infection models, that do not use rodents, include invertebrate species (for example, wax moth larvae and zebrafish models). However, they do not replicate the complexity of the immune responses seen in higher vertebrates, for instance, they lack the classical adaptive immunity and onlyrely on innate responses to protect themselves from pathogens.

Why were they not suitable?

Human experimental systems are not ethically appropriate for much of the work we undertake, such asdisease and pathogenicity studies. While non-animal systems can replicate many important aspects ofthe host, they cannot reproduce every feature. When studying the processes of bacterial pathogenesisor respiratory disease, for example, it is important to capture the full picture of the host environment aseven small changes in environmental conditions can have profound influences on the way in which themicrobes evolve. Similarly, if we aim to study key interactions between a pathogen and its host, we must consider that the interaction takes places in a host-specific context and that the outcome of the interaction may not be the same in an in vitro or ex vivo setup as it would be during an active infection.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

As a research group, we have a long standing experience on animal work, and continue to work closely with our biostatistics peers. All of our staff and students are required to attend the biostatitics workshops and courses offered on campus and are encouraged to use tools such as the NC3Rs Experimental Design Assistant (EDA). We have expertise to estimate the mouse numbers required to complete comparable projects with statistical significance. We are able to accurately determine expected effect sizes (with variance) of

interventions from previous work and we know the frequency atwhich adverse effects can occur in our protocols. The number of animals required has been determined based on our currently funded studies and the programme of work associated with each.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Our funded studies are peer reviewed, including our animal use statistical analysis plans, which was recently highlighted by the MRC as an exemplar of good practice and used in their animal models statistical courses. We use the NC3Rs experimental design assistant for study design, powering our experiments to achieve a defined primary outcome measure. Sample size calculations use estimatesof effect size from preliminary data or published work. Common experimental designs include time- point analyses and survival studies. In the latter, we monitor disease progression using a sensitive disease scoring system, recording physical/behavioural changes in the animal and using time to progress to humane practical endpoints as a proxy for survival time. Kaplan-Meier survival analysis compares virulence of bacterial strains or treatment and control groups.

In time-point analyses, we assess microbe and host responses over an infection time-course. We firstdetermine the key timeframe where the host-pathogen interaction under investigation can be studied, to reduce the number of time-points/mice needed in subsequent experiments. For acute infection, innate immune responses peak between 12 and 24 hours post-infection, and we avoid earlier or latertime points unless there is a compelling need to include them. In chronic lung infection with *Pseudomonas aeruginosa* or upper respiratory tract carriage with *Streptococcus pneumoniae*, key interactions occur later and alternative time-points are prioritised. Longitudinal data are analysed by two-way ANOVA with correction for multiple comparisons.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

The majority of animals used are purchased from external suppliers and we purchase only what is needed. When we breed mice, we use both sexes, where possible. Pilot studies are used to determineeffective treatment doses or to identify appropriate time points for analysis. Our team work closely with departmental colleagues to share resources and we regularly provide mouse tissue for others when wedo not require it ourselves.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use mouse models of infection with human bacterial, viral and parasitic pathogens. These are predominantly mucosal (respiratory and vaginal) infection models, although we occasionally use CNSor sepsis (blood infection) models when assessing therapeutic or

vaccine efficacy. The procedures forinduction of respiratory infection are generally minimally invasive, with bacteria or virus in suspension applied to the external surfaces of the nostrils with a pipette, before natural inhalation by the animal. Light anaesthesia is usually administered, to minimise stress.

In our model of intranasal infection, mice develop either upper respiratory tract (URT) infection, which is usually asymptomatic, or lower respiratory tract (LRT) infection, which induces pneumonia +/- sepsis. We can control this process by variation of dose, volume and bacterial strain. We never induceLRT infection unless necessary. If we want to study colonisation, for example, we induce URT infection, as this model causes the least pain and distress.

In our models of vaginal colonisation and vertical transmission, we have carried out titration studies todetermine the relationship between the inoculum dose and the rate of congenital transmission. The procedure that we use for induction of vaginal colonisation +/- transmission is minimally invasive, andconsists in gently applying a small volume of inoculum to the external surfaces of the vagina with a micropipette, before natural aspiration by the animal. In our models of helminths infection, oral administration (not injection) will be the preferred route of administration.

In all of the infection models described above, we may treat animals with antibiotics or a novel therapeutic, or else we might promote or inhibit certain host or pathogen signalling pathways, to studytheir function. When this is done, we first determine appropriate timing and dosing of the intervention,to minimise adverse outcomes for the animals whilst ensuring we achieve the desired effect of the intervention.

Why can’t you use animals that are less sentient?

The rationale for testing pathogen virulence, and drug/vaccine formulations in mouse models is well established and widely recognised as essential, when supported by appropriate prior in vitro studies.This project also investigates pathogen interactions with their host. Mice are the simplest preclinical *in vivo* model to use while also providing the most published information on their host immune responses. We use mostly adult mice for these experiments as the course of infection mimicshuman disease more closely than when juvenile animals are used. It is impossible to study microbial interactions over an infection time course in terminally anaesthetised animals.

While adult mice are the most appropriate choice for most of the work to be conducted under this licence, we will also use infant and aged mice to study immunity to infection in these age groups to reflect infection in human infant and the elderly, respectively. We will typically use infant or juvenileanimals in immunisation studies, as many vaccines are administered to humans in infancy.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

I have worked with mouse models of bacterial infection for 26 years, over which time we have developed robust infection models, whereby mice progress through well-characterised, reproducible disease signs following infection. In our pneumococcal pneumonia model, hunching and pilo-erection are typically the first signs to develop and mice are monitored with increasing frequency as they slowlyprogress to a disease stage where they present with weight loss or behavioural change (reduced activity, minor changes in respiratory rate). We have defined endpoints that allow us to collect valuable

information without undue suffering to the animal. The scoring system is sufficiently sensitive that the humane endpoint is rarely missed.

Most experiments are performed with outbred CD1 (*S. pneumoniae, S. pyogenes* and *S. aureus* infection), inbred C57Bl/6 (*S. pneumoniae* infection) or BALB/c (*S. agalactiae and P. aeruginosa* infection) mice and we can reliably predict time to reach humane endpoints, with some variation dependent on bacterial genotype or drug interventions. In our natural inhalation *P. aeruginosa* model,humane endpoints are rarely reached. In that model, a chronic long-lasting, low-density lung infectionis established and, after development of mild disease in early infection, mice are asymptomatic from ~48 hrs post-infection onwards.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Dosing and administration of agents, and withdrawal of blood, will be conducted according to LASAguidelines. Our disease scoring system was originally based on that of Morton (Morton DB, Nature, 1985, 317(6033):106) but has been updated to include more recent recommendations (Turner PV, Pang DSJ and Lofgren JLS, Comparative Medicine, 2019, 69(6):451). Animal monitoring will also takeaccount of the mouse grimace score (Miller AL and Leach MC, PLoS One 2015, 10(9):e0136000).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Our Biomedical Services team is very pro-active in advertising local and national 3Rs meetings, workshops and conferences. We have attended and presented talks at these meetings before and I always encourage my staff and students to attend. Important papers, posters and information leaflets are displayed in our animal unit, highlighting important work in 3Rs areas. I have an NC3Rs funded PhD studentship and have strong links with NC3Rs through my previously funded work with them. OurNC3Rs regional manager is in regular contact and on hand to provide support. We constantly aim to apply 3Rs principles to our research and work to implement refinements rapidly, as well as exploring alternative model systems in the laboratory.

DEVELOPMENT OF A NEW TREATMENT FOR THE INHERITED EYE DISORDER ANIRIDIA

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

therapy, aniridia, cornea, RNA therapeutics, limbal stem cells

Animal types	Life stages
Mice	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

To primary aim of this project is to develop new therapy options for the inherited eye disorder aniridia. Our work to date has identified a candidate that works in cells in a dish and now we wish to develop this new therapy so that it can be used in patients. This will involve improving the formulation to ensureit reaches the appropriate target cells in the eye and testing the new therapy's ability to prevent the disease from occurring or in slowing its progress.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 end goal is a therapy that can progress to clinical trials to treat human patients.

Patients with aniridia suffer from failure of the transparent front part of the eye, the cornea. This failure is painful andseverely limits vision. Current treatments for aniridia are supportive only, requiring complex surgical intervention and corneal transplantation. Even in the best situations, the relief for the patient is only temporary as the transplants ultimately fail as a consequence of the underlying genetic problem.

The therapy that we are developing treats the root cause of the disorder, and therefore we hope it willprevent the problems from occurring in the first place or will slow the development of the worst symptoms.

What outputs do you think you will see at the end of this project?

At the end of the 5-year period we anticipate having at least one new RNA-based therapy that is ready to progress into the final steps before human trials. We also anticipate having a panel of potential back-up molecules at earlier stage in development.

In addition, we will have information on the most effective modifications to RNA molecules to allow theirdelivery to cells of the surface of the eye including stem cells. This information will be valuable to the field, particularly for anyone attempting to develop a similar type of therapy for other inherited diseases.

We anticipate publishing at least one peer-reviewed primary research article specifically on aniridia andwill likely produce a second paper for the scientific field on the RNA modification strategy/delivery work.We will also share our work with patient involvement groups and at scientific conferences.

Who or what will benefit from these outputs, and how?

Short term, our delivery data will help other researchers in the RNA-therapy space in the developmentof their products. This should also reduce the number of animals used worldwide as a consequence ofresearchers progressing more rapidly to final formulations without requiring as many earlier trials.

Medium to Long term, the new therapy will benefit patients with aniridia, helping to treat their diseaseand improving their quality of life.

Long term, if our therapy works well for aniridia, it will provide proof of concept data to support the development of therapies for other similar diseases based on the same technology. We have identifiedup to 1000 disorders that could be addressed or aided using this approach.

How will you look to maximise the outputs of this work?

Outputs from this research will be shared through publication in peer-reviewed articles and throughconference presentations.

Aniridia specific work will also be shared with the EU-aniridia network; an international consortium of scientists working on this disorder, of which we are active participants. We will all share progress withpatients through presentations at Aniridia-UK meetings.

Species and numbers of animals expected to be used

- Mice: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have been selected for these studies for two reasons:

The RNA therapy we are developing is specific to the precise genetic code that our therapy targets.The target sequence for our lead candidate is perfectly shared between humans and mice but is not conserved with non-mammalian organisms.

There already exists an extremely well-characterised mouse genetic model of the aniridia diseasethat effectively recapitulates all aspects of the disorder. These animals have been used to test othertherapeutic interventions for this disease and, therefore, the results we obtain will be directly comparable to those prior studies. This ensures that the results will be interpretable and relevant.

Most early work to establish delivery modifications and routes of administration will be performed onyoung adult normal animals.

Treatment efficacy analyses being performed on juveniles of the aniridia model. The aniridia diseasecourse is progressive; starting therapy as early as possible is the best opportunity to impact the disease presentation and will minimised the harms to the animals.

Typically, what will be done to an animal used in your project?

The early studies will focus on delivery to normal mice. Topical delivery is highly desirable for long-termuptake by patients. Therefore formulations will be developed to either be delivered by eye drop or by injection into the white part of the eye (sub-conjunctival delivery). A third option of injection into the centre of the eye (intra-vitreal) delivery may also be developed to allow the therapy to reach the inner parts of the eye including the retina. This latter option will only be developed if we can achieve long- term stability of the therapeutic. Experimental duration will be short (under 3 days) for dosing/delivery studies, 28 days for duration studies. Analyses will be performed post mortem.

Studies in aniridia model animals will involve the optimised delivery approach from the options above.Again, primarily analysis of treatment efficacy will be via post-mortem examination of tissue. The Initialexperiments will assess our therapeutic intervention ability to increase expression of its target gene and will be measured at 3 days after injection.

Thereafter, we will conduct a similar series of experiments where we assess the therapeutic ability toslow the progression of the disease. This will use non-invasive imaging techniques including optical coherence tomography to measure the thickness of the cornea, measurement of intraocular pressure(the same imaging techniques as used in a standard human eye exam). These are painless procedures performed under sedation. Again, the primary measurements will be post-mortem. Theseexperiments will last up to 3 months. At the very end stages of the project we will perform overnight corneal wound

repair assay as a final assessment of treatment efficacy. These will only happen if the earlier experiments all show strong therapeutic effect.

What are the expected impacts and/or adverse effects for the animals during your project?

All the intervention approaches and the disease model itself are restricted to the eye tissue. The aniridia animals are otherwise healthy, breed normally and only suffer eye pain from their failing corneavery late in the disease course. This will not manifest in routine breeding. Our treatments will begin before this develops and the endpoints will be before even the untreated animals reach this stage of their disease.

The animals may experience mild pain at injection sites. There is a low risk of infection and inflammation; however, these will be rare events with short duration.

The imaging interventions including pressure assessment are non-invasive and should not cause pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Mild severity for 95%+ of the animals. Moderate severity in rare cases of inflammation or infection.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aniridia symptoms manifest as a consequence of defects in multiple different cell types which interact with one another in intact tissues. There are no effective in vitro models that can cover the whole disease course. Therefore, to determine if the treatment works, we need to use a genetic animalmodel.

Ex vivo models will be used for developmental purposes. However, the uptake by tissue, clearance rate and efficacy of the treatment are all likely to be substantively different in live tissue compared withdead, which is why live animal studies are also required.

Which non-animal alternatives did you consider for use in this project?

Prior to working with animals, we have used patient-derived cells-in-a-dish experiments to identify thatthe new therapy works in the right context. Before moving into the live animals, we will further test potential delivery options in these cells and also in post-mortem pig

eyes from animals used in the meat industry. These experiments will be used to identify the delivery agent and approach most likely to be effective.

Other organisms including drosophila and zebrafish were considered but are not suitable.

Why were they not suitable?

Gene structure and, specifically, the target sequence of our therapeutic agent are not conservedbetween mammals and insects or fish. Therefore they would not work in these contexts.

The ex vivo models are excellent surrogates for development of our therapeutic agents; however, they do not allow us to assess turnover and clearance. Moreover, in live animals the tear film and flow of thefluid within the eye are both relevant to whether and where the drug is delivered and also how long it remains in the tissue. These information are essential to allow progression into humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientificobjectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissueand reuse.

How have you estimated the numbers of animals you will use?

Animal numbers are based on a estimate of working through multiple therapeutic development cyclesbefore reaching our clinic-ready option. Most animals will be used in the early short term testing, with only the most promising candidate molecules progressing to the disease model.

We have used previously published work of the genetic model to identify the most effective experimental end-points that provide a clinically relevant assessment of efficacy but which are also easily measured with consistent differences between healthy and disease animals. This has informedour experimental design.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

In vitro (cells) and ex vivo (pig eye) experiments allow us to identify likely effect dose ranges andbest delivery options, thereby reducing the number of options requiring investigation in animals.

Using untreated eye as an internal control within an animal allows for stronger statistical tests(paired rather than independent analyses), further reducing numbers required per experiment.

We have no reason to anticipate a sex specific outcome, therefore all available animals can be usedin experiments, reducing wastage.

By carefully selecting our aniridia disease outcome measurements to maximise signal-to-

noise ratioand reduce between animal variability, we are able to design our experiments to use the fewest animals possible yet yield statistically robust and clinically valuable data.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

As mentioned above, only those putative therapeutics that pass our in vitro and ex vivo developmentpipeline will progress to animals.

Within animals studies, the approach will also be iterative with only those compounds that show promise in small scale, short term delivery studies progressing to dosing studies and only those that still show promise progressing to aniridia model and, again, only once satisfactory target modificationis achieved will the compounds be tested for their ability to rescue the disease.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emergingrefinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Eye drops, sub-conjunctival and intravitreal injection into mice eyes are all localised delivery options which cause minimal harm and accurately recapitulate human clinical scenarios. They are performedunder anaesthetic and pain relief will be provided. Indeed, most animals will not suffer any distress orsuffering beyond the period of anaesthesia.

The live imaging approaches are non-invasive and will performed on sedated animals. Again, no pain,suffering or distress is anticipated.

The aniridia genetic model effects are limited to the eye. The animals are otherwise healthy, do not display any symptoms of pain or distress and breed normally. Yet this model accurately reproduces thehuman disease course. Our intervention timing and assessment approaches ensure that animals will not suffer excess pain or distress. End points are terminal and most time courses are short to minimiseany pain or suffering.

Why can’t you use animals that are less sentient?

Our RNA therapy is based on the specific genetic sequence that it targets. Our lead RNA therapeutictarget sequence is identical in humans and mice and our cell-based experiments confirms cross- species activity. However, the sequence is not conserved in fish, fly, or other experimental organisms.

We plan to use post-mortem tissue in developmental stages.

Delivery of the therapeutic agents to the eye requires the eyes to be open, therefore treatment will bedifficult/impossible before 14 days. Ensuring the delivery is consistent

from animal to animal is essential to reduce variability. Adding extra variability will substantially increase the total number of animals required.

Most analysis will be post-mortem. The live animal analyses are non-invasive imaging approaches orshort-term wound repair assays. As the imaging approaches will not cause undue pain but provide a route to reduce animal numbers by providing time-course data, ensuring the we do not miss a key window of activity by relying solely on end-point assays.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Animals will be given pain killers and carefully monitored pre and post treatment for any signs of pain and distress. They will have enrichment available within their housing based. Surgical approaches willbe perfected and refined using post-mortem eyes to minimise potential for adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

For aniridia work, we will use published studies using the same animal model to estimate effect sizeand variability.

For efficacy studies, we will use a sample size calculation to work out the minimum number of animalsper group, for example:-

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2876926/
http://www.graphpad.com/scientific-software/statmate/#samplesize

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

In addition to staying abreast of the relevant scientific literature, we will regularly access the NC3R resource library detailing new and improved options to refine, reduce or replace animals in research.

MICROPOLLUTANT EFFECTS ON FISH HEALTH

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Micropollutants, Fish Protection, Ecotoxicology, Transgenics, Environment

Animal types	Life stages
Brown Trout (Salmo Trutta)	juvenile, adult, neonate, embryo
Redtailed splitfin (Xenotoca eiseni)	neonate, juvenile, adult, pregnant,
embryo Rainbow Trout (Oncorhynchus mykiss)	neonate, juvenile, adult, embryo
Medaka (Oryzias latipes) embryo,	neonate, juvenile, adult

Animal types	Life stages
Salmon (Salmo salar)	neonate, adult, juvenile, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to better understand the effects of exposure to pollutants on fish healthfor the protection of wild and managed populations. This will be achieved by exposing animals to various pollutants and measuring the effects on responses such as brain and liver function or behaviour. We will also develop and use fish in which we have altered their genetics to enable us to measure these effects more precisely and easily.

Potential benefits likely to derive from the project, for example how science might

be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 300 000 chemicals used in modern life for which we have toxicological data for less than 10 percent. Most of these chemicals end up in aquatic environments, and fish are amongst the most vulnerable wildlife populations to the effects of exposure. Often these exposures occur as chemical mixtures yet we know very little on their interactive effects on fish health (or in any other animal). Protection of fish, and other wildlife, is typically based on standardised basic tests that measure growth, development, and reproduction, yet adverse individual and potentially population- level impacts can manifest through more subtle effects on their physiology and behaviour, particularly after longer term exposure. Through the measurement of various markers of fish health such as brain and liver function or behaviour, along with the development and application of genetically modified fish models, we will better understand chemical effect mechanisms and develop more effective measurements of these health effects. Ultimately this work will contribute to better assessments of pollution exposure risk in fish for enhancing their health protection in natural and managed populations.

What outputs do you think you will see at the end of this project?

Under this project licence, the outputs we expect to deliver are several fold. Firstly, we will provide newknowledge to advance understanding of the health implications for fish exposed to specific classes of so called micropollutants (e.g. specific chemicals and particles which are found in many household products such as sunscreen, called nanoparticles) for which there is evidence for environmental concern. Secondly we will develop new methodology to improve our ability to protect wild and managed fish populations. Thirdly we will establish more refined procedures using less invasive approaches for measuring the impact of these micropollutants on fish. Finally we will generate publications, presentations and information for educational purposes.

The benefits will be delivered under three main headings, linked to the three objectives of thework:

How exposure to specific micropollutants affects individual fish health for application to the protection of fish populations. The main benefit will be to better understand the risks of exposure to selected micropollutants (and their mixtures) of concern in fish and thus the provision of information forsupporting the protection of fish populations, and development of mitigation strategies (e.g. switching to the use of alternative ‘greener’ chemicals). As an example, although there is worldwide concern on the impact of antimicrobial resistance (AMR) on animal and human health, almost nothing is known about how antibiotic pharmaceuticals impact the microbiomes on the skin and gill in fish, both of whichserve as primary barriers of protection for the prevention of disease.

Development and validation of new methods for contaminants of emerging concern, specifically for neuro-endocrine active chemicals (NEACs), pharmaceuticals, and nanomaterials. Current procedures for assessing the environmental impact of certain micropollutants are inadequate, as shown by the inability to effectively monitor the effects of chemicals that effect hormone levels (aka endocrine active chemicals) in fish entering the environment. The main benefit from this will be obtaining a greater understanding of micropollutant effects and their mechanisms of action. This is critical to enable us to

establish new testing procedures which ultimately, following validation, could be incorporated into environmental protection legislation. For nanoparticles, for example, there are still no validated test methods for hazard identification or environmental risk assessment. Our research will in turn contribute to providing the science needed to support legislation.We have been successful previously in this respect, for example data from our work in fish has been used to drive setting of a recommended discharge limit on steroidal oestrogens in the UK, and in developing new test systems for screening for NEACs by relevant environmental protection organisations. The work under Objectives 1 and 2 will provide a deeper understanding of how some micropollutants of the greatest emerging environmental concern affect health in fish, including their impact on organ-system physiology and behaviour, as well as better understanding the mechanisms through which these effects occur

Development of new models and sensitive biomarkers for monitoring and assessing the biological and mechanistic effects of micropollutants. Better approaches to micropollutant testing are needed to reduce the chances of unexpected adverse effects of these materials in the environment, and thereby provide better environmental protection. In this respect. We have previously developed novel genetically modified (transgenic) zebrafish models with fluorescent reporters that allow for the non-invasive visualisation of responses at molecular targets using imaging. These modelshave advanced our understanding of chemical effects, including those acting via endocrine disrupting and nervous system-mediated mechanisms. Furthermore, we have applied these models predominantly in non-protected embryo-larval life stages (with major 3Rs gains). The benefits this project will add will be the development and application of existing and new transgenic models to provide more informative data on the effects of micropollutants of particular concern in fish. This in turnwill allow us to identify more sensitive indicators of exposure and effect to provide earlier warning of environmental contamination than is currently possible.

Who or what will benefit from these outputs, and how?

The knowledge generated from work under this licence will allow for a greater appreciation on how the micropollutant substances we will study impact on fish and for identifying more sensitive and non- invasive biomarkers for environmental monitoring for the protection of wild and managed populations. As such, this work will benefit researchers studying animal physiology, developmental biology, ecotoxicology and animal health more generally. This understanding will also benefit regulators in the development of more effective monitoring, and in turn management, strategies for the conservation of natural environments in the interest of fish and fisheries. It will also support understanding for improving health standards for managed fish in aquaculture. Ecotoxicologists and regulatory bodies will benefit also from improved understanding on the potential of behaviour (i.e less invasive methods than conventional approaches) for assessing micropollutant exposure risks. We envisage that our transgenic fish models will help reduce the number of fish used in chemical and nanoparticle effects analysis in the future as each individual can provide a more informative and comprehensive analysis ofthe effects of micropollutants across whole body systems. These animals will also support refinement of chemical effects analysis through their application to non-protected life stages, thus reducing the useof protected animals. Society as a whole will benefit through the advancement of understanding on the effects of contaminants derived from man-made activities for supporting the protection of the natural environment, and in turn human health.

How will you look to maximise the outputs of this work?

We are very active in publishing our data in peer reviewed and open access journals (publishing around 15 research papers each year) and very regularly present (inter)nationally at scientific conferences, public events and stakeholder engagement groups. These will be our major routes for dissemination of our findings. We also have very extensive international academic research collaborations across Europe and Asia with whom will share in the new knowledge we generate from research under this Project Licence. We work closely with government organisations including nationaland international regulatory bodies, and with many major chemical, including pharmaceutical, industries. The applicant sits on various influential international committees for the development of fishtest guidelines and acts as a consultant for a number for major chemical industries for informing on fishtesting design. He is also co-director on Doctoral Training Programmes supporting in the training of PhD studentships for advancing next generation ecotoxicology working in partnership with industry, UKregulators and environmental protection groups. These partnerships provide further multiple routes for data dissemination and knowledge transfer that will maximise the benefit of developments made underthis project licence (for both successful and unsuccessful approaches).

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 30 000
- Other fish: No answer provided
- Brown Trout (Salmo Trutta): 250
- Rainbow Trout (Oncorhynchus mykiss): 1000
- Medaka (Oryzias latipes): 250
- Salmon (Salmo salar): 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

As the main aim of this project is to better understand the effects of micropollutants on fish, we will be undertaking micropollutant exposures of a range of fish species at life stages from eggs/embryos, neonates, juveniles, to adults depending on the specific aims of each exposure experiment. A range offish species are to be used as several aims and objectives require the need for different species. One aim is to better understand the impact of exposures to micropollutants on fish in UK rivers for the purposes of environmental protection. For this a range of UK indigenous fish species will be used: roach (*Rutilus rutilus*), carp (*Cyprinus carpio*), 3 spined stickleback (*Gasterosteous aucleatus*) and brown trout (*Salmo trutta*) and Salmon (Salmo salar). Rainbow trout (*Onchorhychus mykiss*) will also be used as it shows enhanced sensitivity to micropollutants compared with cyprinid fish species and itsuse will help ensure we protect the more sensitive fish species against the effects of micropollutants.

Tilapia species (*Oreochromis niloticus*, *Coptodon rendalli* and *Oreochromis shiranus*) are required to recreate exposure scenarios from tropical countries, for example for assessing the impact of antimicrobial pollution on skin microbiome composition. Where we wish to seek understanding on effects of contaminants for exposure periods over longer life periods and on breeding, more appropriate species to be used will be fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*)and medaka (*Oryzias latipes*) as these

species they develop quickly; it takes 4 months from embryo tofull sexual maturity compared with (for example) 3 years for the roach, and show clear sexual dimorphism and well characterised sexual development and courtship behaviours. We will use the viviparous fish, Redtailed splitfin (*Xenotoca eiseni*) to assess for maternal transfer of pollutants.

Although the zebrafish (*Danio rerio*) is not a native UK species, it represents the most recognised and highly studied model species for the generation of transgenic animals for the purposes of environmental (and human) health assessment. This includes it being the species in which the techniques used to generate such animals are most refined and understood. This is illustrated by our previous work in which we have developed zebrafish models for studying endocrine disrupting chemicals and micropollutants that induce toxicity via specific mechanisms. Specifically, zebrafish show development externally within an ovulated egg, are optically transparent in early development, show rapid development of major organ systems, are easily exposed to micropollutants on a small scale, and in which genetic modification is relatively easy and the techniques well established. All of these features combine to provide a model in which genetic modification is relatively simple, rapid, andeasily achieved.

Typically, what will be done to an animal used in your project?

The focus of the project is on the effects of micropollutants on fish health and as such procedures will revolve around the exposure of fish (any of the named species) to a range of micropollutants including,endocrine active substances, industrial chemicals, human pharmaceuticals and nanoparticles at environmentally-relevant sub-lethal concentrations. As the concentrations used are required to be of environmental relevance most of the effects are expected to be mild with those relatively few animals at the moderate level of severity resulting in few if any outward signs of toxicity. In some cases, to better understand the mechanisms behind these effects, it may be necessary to use concentrations that are slightly higher than those found in the environment (e.g. pharmacologically relevant in the caseof pharmaceuticals). Despite this, these exposures will be at most moderate in severity.

A typical chemical exposure experimental scenario (all named species) may be a 4 week exposure to asingle human drug or a mixture of drugs at ng/L to low ug/L concentrations, following which animals willhave terminal blood samples taken and then be schedule 1 killed for the harvesting of tissues for later analysis. Another experimental design may involve a 2 week exposure, at the end of which, animals are observed for abnormal behaviours associated with chemical exposure, for example after exposure to light or sound stimuli, and then blood samples taken and schedule killing undertaking with post-mortem analysis of tissues. In rare cases exposure may be undertaken for a longer period of time, for example, long enough for a species to reach sexual maturity or complete a full life cycle. This could, forexample involve micropollutant exposure at a low (environmentally-relevant) concentration for as long as a year for species such as the fathead minnow. When fish are used in which a fluorescence marker has been incorporated for the purposes of observing a specific molecular event (applies only to zebrafish in this licence), this would normally involve a short exposure period of 1-5 days in embryo- larval animals to take advantage of their optical transparency. Following exposure, larvae are typically embedded in a non-toxic viscous fluid or gel under general anaesthesia to hold them in position for subsequent fluorescent microscope observation, following which they will be schedule 1 killed.

To provide new genetically modified zebrafish for these exposure studies, the typical

process would beto inject very early embryos with DNA and non-toxic reagents that allow modification of the genetic makeup of the animal. Animals would then be assessed for changes associated with the modified geneat 2-4 days after injection, and then those showing the expected physical characteristics (phenotype) are then grown on to 10-14 days at which time a fin clip or skin swab is taken for genetic sequencing toensure the genetic modification has been successful (genotyping). Those animals found to be carrying the correct genetic modification will then be grown to adulthood, interbred multiple times to provide embryo-larvae for exposure to micropollutants, and when 18 months old, schedule 1 killed.

What are the expected impacts and/or adverse effects for the animals during your project?

As the overall aim of this project is to understand the impact of pollutants at environmentally relevant (i.e. low) concentrations, in most cases micropollutant exposure or specific procedures (e.g. the effectsof genetic modification) will be mild with little more than transient effects on the welfare of the animals used. For example, in our previous licence (PPL 30/3430) 87% of the animals used were classified as mild and just 13% were classified as moderate.

In the current licence, the only notable impacts/adverse effects are expected after exposure to micropollutants (e.g. chemicals and nanoparticles of environmental concern), and these are expected to be limited in number as the exposure concentrations used are likely to be relatively low in order to observe only sub lethal effects at environmentally relevant levels. Nevertheless, from our experience inour previous licence, the following impacts/adverse effects may occur as a result of exposure to a micropollutant:

Morphological defects (estimated duration in protected animals 8 hours until they are culled)Moderate water retention (estimated duration 1 day until they are culled)
Abnormal posture or loss of balance (estimated duration 2 days)

Any of these observed in adult animals would lead to those animals being humanely killed immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The expected severities range from mild through to moderate across all protocols. The numbers expected within each category can be estimated from previous figures (2016-2019) as an averageacross all protocols:

Mild ~87% Moderate ~ 13%
All animals used are fish and in many cases will be embryos and larvae of <14 days old.

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 haveconsidered 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 focused on understanding the impacts of exposure to micropollutants on fish for the protection and avoidance of harm to wild fish populations. We do use cell based approaches where appropriate (see below) and these can be highly useful in understanding basic function and interrogating mechanisms in detail. However, the complexity of a whole animal is vital to fully understand the impact on individuals and potentially the ecosystem more widely. For this we requireintact animal models to accurately recreate a full organismal response. For example, the transgenic zebrafish we have/will develop, that are responsive to specific classes of chemicals (e.g. environmental oestrogens) will allow us to understand the potential health effects of micropollutants ina more integrative manner, thus reducing the numbers of different whole animal tests required for hazard identification and environmental risk assessment. With increasing knowledge through the project, there is the potential for replacement of protected with non-protected animals (e.g. recent advances with transgenic zebrafish may allow detection of chemical effects in embryo-larval stages allowing for replacement of studies with intact fish).

Which non-animal alternatives did you consider for use in this project?

Wherever possible, relevant alternative methods are used to undertake this research. For example< we use *in vitro* approaches to screen for the toxicity of chemicals to fish using liver hepatocyte or gill cell culture systems, and/or fish embryo-larval stages (up to 96hpf) prior to undertaking *in vivo* experiments to refine and minimise animal usage in our whole animal studies. A significant part of our collaborative research programme has involved the development of alternative *in vitro* systems for assessing the biological activity of endocrine active chemicals (e.g. hormone receptor reporter assays),which we now adopt widely in our research and use a pre-screen prior to *in vivo* testing for chemicals suspected to interact with selected hormone receptors. This work will continue during the tenure of thislicence.

Why were they not suitable?

As stated above, the use of whole animals is essential to quantify the relative sensitivities/susceptibilities of different organ systems to micropollutant effects, to establish how theseeffects are integrated in the whole animal and to understand the complexities of toxicological processes more generally. It is also the case for some nanoparticles that target organs for potential adverse health outcomes have not been established and this can only be determined using whole animal approaches. No alternative methods are currently available to answer these questions.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot

studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers estimated reflect the undertaking of a large body of research across a range of micropollutants, and their mixtures for which we will investigate their health effects in fish. These numbers also reflect estimations from previous experience on the requirements for producing statistically validated data, and robust interpretations for informing the environmental protection of fish.

Predicted number of fish to be sued in the different protocols:

Protocol 1- Assessing the impact of micropollutant exposure on wild type fish physiology and behaviourin laboratory studies Fish (Teleosts): zebrafish, roach, carp, stickleback, zebrafish, fathead minnow, medaka, rainbow trout, brown trout, salmon, tilapia and redtailed splitfin (embryo-larval, juvenile and adult) Adults (400), juveniles (1000) and embryos (1000) per year

Protocol 2 -Assessing the impact of micropollutant exposure on genetically modified fish physiologyand behaviour in laboratory studies Fish (Teleosts)- Zebrafish Adults (200), juveniles (1000) and embryos (2000) per year

Protocol 3- Breeding and maintenance of genetically modified fish lines Fish (Teleosts)- Zebrafish,1500 per year

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

For every endpoint investigated, the numbers used will be minimised by employing careful experimental design. These include: utilising shared control groups and/or intra-individual controls; where possible power calculations; and previously generated datasets. Using these approaches ensures our methods obtain useable data but with the minimum number of fish. For micropollutant effects analyses, previous experience indicates that 6 to 16 animals are typically required in any one group. However, detection of small but biologically important changes in physiological parameters canrequire groups of up to 20 fish, particularly when physiological parameters show high natural variabilityin control fish. When this is the case, a paired experimental design and analysis will be used where possible, in which each fish acts as its own control for direct paired-wise comparison with an experimental manipulation. This approach dramatically increases the power of determining significant effects following a given treatment and therefore minimises the use of animals.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

In all cases the minimum number of fish will be employed. Wherever possible, multiple aspects of physiology and behaviour will be investigated within the same experiments/animals, to minimise the number of animals required. In this respect, the maximum amount of information will be extracted fromeach animal used by assessing multiple endpoints per individual, many of which are likely to be non- invasive (e.g. behaviour, imaging etc). The use of transgenic animals, often with multiple reporters that can be visualised non-invasively (e.g. via imaging) will enhance our ability assess interaction sites andbiological effects of specific classes of substances across many organ

systems in the same fish. This, therefore, will further enhance our ability to reduce the numbers of animals we require to gather meaningful data in the future.

Selecting appropriate treatment concentration ranges is also key: for most of the micropollutants we will study there are good data for their toxicity effects. The possible exceptions to this might be for a new emerging endocrine disrupting chemicals, pharmaceuticals or nanomaterials of possible concern. In this instance, we will use all available information for known effects in other animals (likely to be predominantly for mammals) and if there is good reason to expect the compound might induce adverse effects in fish, where available we would first apply *in vitro* tests prior to any protected fish exposure studies. We would also seek out any information on concentrations of the substances in the aquatic environment. This will be followed by embryo-larval tests (up to 96hpf) which we use extensively across several projects. For any juvenile or adult fish exposures a pilot (sighting) study will then be undertaken using a reduced number of animals (n=6 per treatment, 3 treatments) to establish an appropriate exposure regimen, prior to commencing the main study. The overall aim is to minimise anycompound-exposure related adverse effects in the definitive assessments.

The statistical analyses we apply will vary depending on the experimental design, but frequently includes the use of multivariate statistics. Consultation with very well qualified statisticians takes placeprior to all *in vivo* experiments with animals to ensure optimal experimental designs are employed throughout allowing for maximum power of analysis with minimal animal usage.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

When investigating the impacts of micropollutants, the most relevant species will vary according to theparticular environment at risk and the specifics of the chemical or particle, likely susceptibilities for pollutant impacts, and experimental convenience. We will only use fish in this work. Some of the fish species we will study are native to UK Rivers and indeed known to be affected by micropollutants suchas endocrine active chemicals, pharmaceuticals and nanomaterials (e.g. roach and sticklebacks).
Other species are laboratory models for which specific test guidelines have been established for chemical testing (e.g. rainbow trout, fathead minnow, zebrafish, medaka, and stickleback). For these species, much is known of their genetic makeup and as such our ability to better understand the precise effects of micropollutants and the mechanisms leading to their toxicity is greatly enhanced overspecies where less if known about their genetics and biology overall. Redtailed splifin (*Xenotoca esieni*) is adopted as a live bearing fish to study the hypothesis that micro pollutants (most notably nanomaterials) can be passed to developing embryos from exposed female fish during gestation.

The principal aims of our ecotoxicology work are to understand the effect of exposure to

real world concentrations of endocrine active chemicals, pharmaceuticals and nanomaterials and /or their mixtures contained in the aquatic environment. The duration of the experiments will vary depending onthe question addressed. In some instances, and most commonly, exposure will only for a few days to several weeks. However, in some cases the exposures will be for considerably longer periods of time, and include exposures over a full life cycle. These exposures however will mostly be mild in nature andaround concentrations around those that occur naturally in the environment. The purpose of this work is to understand what long term health outcomes can result from chronic exposures under environmentally realistic conditions. In some cases, to better understand the mechanisms behind these effects, it may be necessary to use concentrations that are slightly higher than those found in theenvironment (e.g. pharmacologically relevant in the case of pharmaceuticals). Despite this, these exposures will be at most moderate in severity.

Why can't you use animals that are less sentient?

As the main aim of this project is to better understand the impact of micropollutants on wild and managed fish population, fish are required in the procedures used. Fish are arguably the least complexvertebrates and, furthermore, we will use a range of life stages appropriate for the aims of the particular experiment, including non-protected embryonic and larval forms.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

A major aim is to investigate the effects of sub-lethal and environmentally relevant concentrations of chemicals, nanoparticles and their mixtures often at concentrations well below those causing below toxic levels. The choice of chemical for testing will be determined from the current available literature/intelligence on what micropollutants are of highest priority for investigation, and from our ownwork measuring levels in the environment. Indeed, it is through work of the latter nature that we have discovered contaminants that are high on the agenda for environmental impact assessment. The concentration ranges selected will, in all cases, be based on all available data (e.g. literature, pilot work, *in vitro* data etc), and as such treatment regimens should induce the minimum degree of suffering in the exposed animals. All fish will be inspected frequently and experiments terminated as soon as the necessary data are collected and at the lowest level of severity possible. Many of the proposed techniques merely involve non-invasive observation of behavioural or physiological function in free swimming or anaesthetised animals. The only invasive procedures under this project licence in intact animals include the removal of peripheral blood samples, introduction of tags using a hypodermic needle into an internal body cavity or muscle block, or the removal of small sections of a fishes fin or small number of scales (for genotyping and sequencing of fish). The use of fin clipping is widespread for genotyping fish, but a growing number of publications are proposing skin swabbing as a more refined alternative. There is, however, still some debate around which method is less stressful for the fish and if skin swabbing is appropriate in earlier life stages, so as part of this project we will further investigate the appropriateness of skin swabbing for genotyping fish. Provided adequate comparability in terms of DNA quality, low contamination and sample size between skin swabbing andfin clipping are achieved, we will move to skin swabbing for all genotyping work under this licence.
These data will also inform other projects within our facility that still routinely use fin clipping.

The fish will be held in tanks appropriate for their size and where they can move freely. As

a rule of thumb the biomass of fish will less than 5g/litre of tank water. Exposure conditions will vary depending on the test substance, but principally we will adopt flow–through with a continuous movement of water through the tanks. For all exposures dissolved oxygen saturation will exceed 90%. Adult fish maintained in groups in tanks in the laboratory will develop social hierarchies and this may result in aggressive behaviour between different ranks of fish within the social hierarchy. This is a normal aspect of life in fish species that form social hierarchies and fish normally settle into their appropriate social rank very quickly after being put together (e.g. a few minutes to a few hours). Thereafter aggression is reduced and behaviour of the subordinate and dominant fish act to avoid direct contact and the potential for physical damage. Aggression is most pronounced when fish are held for extendedperiods in pairs and occasionally 2 fish will be relatively equally matched in terms of dominance attributes which can result in excessive and continued aggression with significant physical damage to both fish. Consequently, in procedures of >12 hours, we will hold fish individually where necessary, or in groups of 4-20 and thus reduce the likelihood of any cases of excessive aggression. Shorter term pairing of animals may be undertaken for breeding purposes, for example where there needs to be an accurate record of parentage for geno/phenotyping. In all cases, expert advice will be sought from the animal care staff to establish the appropriate density to maintain each species, and also where mitigation against any potential aggression problems is needed due to an experimental requirement forshorter term suboptimal housing densities. Such mitigation could include reduced duration of exposure, use of environmental enrichment, careful size matching, the avoiding sex bias for each species etc. In embryo-larval exposures, where individual animals are used as our experimental replicate this necessitates the housing of animals in individual wells of microwell plates during compound exposure. Prior to commencing the experiment, embryo-larval stocks are held in groups of around 50 embryo-larvae in Petri dishes (circa. 35 ml volume) until experimental use where they may be transferred to microwell plates. Typically, 24 (3 ml) or 48 well (1.6 ml) plates are used for animals upto 8 dpf, and larger volume wells used for older animals (12 well (6ml) or Petri dishes up to 14 dpf).

Daily water changes are undertaken on Petri dishes, and the size of microwell plate well is selected toensure that animals have enough room to move freely and oxygen levels are maintained during experimental durations.

For genetic modification, animals will be killed as soon as the observed phenotype is sufficient to yieldthe desired data. For GM animals used, in the vast majority of cases, these are merely gene-reporter animals showing no adverse effects under normal conditions.

In experiments injecting RNA or DNA into embryos may lead to altered development of the embryo or significant adverse effects on the growing fish. A wide range of early developmental changes could occur and are most likely to involve changes in body shape (e.g. spinal curvature, jaw malformations, changes in heart shape). All embryos with altered development causing significant adverse effects such as clear heart oedema or widespread tissue damage will be killed by Schedule 1 procedures priorto the stage at which they are capable of independent feeding.

In the vast majority of cases for our work on genetically modified fish, embryo larval fish will be used (>70% expected to be <14dpf). Only under very specific circumstances will older animals be used, forexample for the investigation of a specific endpoint that requires analysis of an older animal (e.g. complex social behaviour) and when genetically modified fish are maintained and bred to provide larvae for subsequent contaminant effects analysis.

In addition to the use of generic indicators of adverse effects as humane endpoints, information will begathered to provide more specific humane endpoints for use on both existing and future models.

Post-operative analgesia is not widely used for fish, as there is currently no definitive data to confirm the efficacy of any drugs on all species or the recommended doses to use. However, we have recentlybeen awarded a grant to investigate the efficacy and tolerability of anaesthetics and analgesics in embryo-larval zebrafish, and we will apply any learning from this project to the appropriate animals used in this licence. As is required, we will discuss the need for analgesia following fin clipping (whereused) with the NVS on a case by case basis, although as stated above it is our intention to move towards skin swabbing as a less invasive method for genotyping provided adequate comparability in terms of DNA quality, low contamination and sample size is demonstrated.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We adhere to the Home Office, UKRC and NC3Rs guidance on the application of the 3Rs to ourresearch. We also adhere to the PREPARE guidelines for planning animal experimentation and ARRIVE guidelines for the publication of our *in vivo* experimental findings.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

As a laboratory we are highly active in the promotion and application of the 3Rs to our work as evidenced by some of our recent publications and presentations including at fora promoting the use ofalternative models in toxicology and pharmacology. Moreover, some of work we undertake is directly funded by the NC3Rs and we have been active in the development of refined techniques throughout the tenure of previous licences, such as the development of the non-invasive neural imaging approachin non-protected larvae.

We will continue this approach throughout the tenure of this licence by keeping up to date throughpublications, conference attendance, collaboration and funding body interactions.

DECIPHERING THE CROSS-TALK BETWEEN THE NON-IMMUNE AND IMMUNE SYSTEM IN TYPE 1 DIABETES.

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

autoimmunity, therapy, thymus, ageing, B cells

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to determine how developmental and immunological changes in lymphoid andnon-lymphoid tissues may lead to autoimmune responses against insulin-producing cells.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 chronic condition characterized by the inability of the body to produce, or respond to, thehormone insulin. As a consequence blood sugar (glucose) is not metabolised efficiently leading to life threatening complications e.g. heart attack, stroke and complications that decrease the quality of life
e.g. amputation of limbs and depression. Currently 415 million people live with diabetes in the world, and the World Health Organization predicts this will rise to 642 million by 2040. In the UK, 400 thousand people live with diabetes, and treating the complications of the condition utilizes 10% of theNHS budget.

Type 1 Diabetes (T1D) occurs when the immune system -predominantly T and B cells- attack and destroys the insulin producing beta cells in the pancreas, termed autoimmunity. Approximately 10% ofall diabetes incidences are attributed to T1D. There is no cure due to an incomplete understanding of the complex mechanism that leads to immune cells attacking insulin-producing cells.

Normally, T and B cells with autoimmune potential are destroyed during their development. In people and animals that develop autoimmunity, this quality control step to remove B and T cells reactive to selftissues is impaired and we need to know why it is impaired.

Studies of the mechanisms contributing to human T1D development utilize cadaver tissue donations ofdeceased T1D patients. Unfortunately, for several reasons it is not possible to analyse mechanisms that precipitate T1D development in live humans. As such, animal models of T1D have been invaluable at deciphering the complexities in the immune response associated with T1D development, particularly before the manifestation of T1D. The importance of animal models in developing treatments for T1D is exemplified by the recent announcement that the US Food and Drugs Administration has approved the world's first immunotherapy for T1D, teplizumab; an immunotherapy borne from studies in the non-obese diabetic (NOD) mouse, a spontaneous model of T1D.

This project will provide new information about the autoimmune mechanism in T1D, and through dissemination of our research findings to scientists and public, drive new therapies to ameliorate T1Dand potentially, other autoimmune immune conditions.

What outputs do you think you will see at the end of this project?

There are a number of predicted outputs from our proposal:

- advancement in our knowledge of the complexity of the immune system in the thymus andpancreas, both in health, autoimmunity and as we age.

- new peer reviewed publications describing novel immune processes that characterize progression to type 1 diabetes, and other autoimmune conditions where the thymus is a keytarget e.g. lupus, myasthenia gravis.

- invitations to present our research at national and international conferences, enabling newcollaborations with scientists and clinicians.

- participation of research students in a cutting edge research programme, inspiring the nextgeneration of biomedical scientists

- developing patient participation networks with stakeholders, enabling sharing of information about our research in an accessible format to people who live with autoimmune conditions, andencourage their input in direction of the research programme

- maintain a strong reputation within my discipline leading to invitations to shape funding policies ofmajor Charities and Research Councils.

Who or what will benefit from these outputs, and how?

The benefits and beneficiaries include:

Research community (Timescale of 1-5 years):

- Researchers in fields of autoimmunity: B cells are known to be important for mouse/human T1D, and there is a need for better therapeutic strategies that target B cells. Our data documenting thymic B cells -a type of B cell- as important mediators of T1D adds a new dimension to the relationship between B cells and T1D development. Furthermore, that the detrimental role of thymic B cells occurs at the late, preclinical stage where we currently have limited biomarkers or therapeutic interventions, our single cell gene profiling and potential translation to human T1D willbe of particular interest of T1D researchers. The autoimmune conditions myasthenia gravis and lupus are also characterized by inappropriate thymic B cell activity, and there is a desire to generate therapies that target shared mechanisms in immune-mediated inflammatory diseases. In this regard, our studies will be of interest to researchers in the field of autoimmune conditions.

- Researchers in the field of regenerative medicine and developmental biology: Shrinkage of our thymus- a tissue important for producing T cells- is a well known age-associated phenomenon, and contributes to the elderly's susceptibility to infections. Thymic shrinkage in humans (and mice) is characterized by increased thymic B cell numbers, and the risk of developing autoimmunity increases with age. Furthermore,radiotherapy impairs thymic function impeding thesuccess of bone marrow transplantation. Our studies will be of interest to scientists in the fields ofregenerative medicine and developmental biology interested in the ageing thymus, as our studies could yield potential avenues to restore thymic function following radiotherapy or in an ageing population.

People living with type 1 diabetes and their family/carers (Timescale of 1 to +5 years):

- Dissemination of our research through outreach events and patient public initiatives will enable open dialogue of animal experimentation and how it can address key questions for the condition the people live with. Our studies will bring insight of the complex thymic microenvironment to stakeholders delivered in an open way to facilitate both understanding and feedback on experimental questions. Our endeavours to bring our research to the public has increased uptakein children into clinical trials for new T1D therapies, and led to increased donations to Charities that fund our work.

How will you look to maximise the outputs of this work?

The project is multidisciplinary and international. The animal studies are conducted in parallel with clinical research. The applicant has collaborations in place with researchers in the UK and abroad. These researchers have archival biobanks of human tissue, including the thymus, from donors who were type 1 diabetic at the time of their passing. The applicant also has access to repositories e.g. Juvenile Diabetes Research Foundation (JDRF) network of pancreatic organ donors with diabetes (JDRF nPOD), that contain many types of tissues from donors. The applicant's team publish in open access journals and deposit manuscripts prior to publication in pre-print servers to facilitate rapid data exchange. Social media, and our laboratory website, is used to rapidly disseminate key information.
We have a Patient and Public Involvement and Engagement plan in place with our local Diabetes UKSupport Network, and meet four times per year to discuss our research discoveries and direction.

Species and numbers of animals expected to be used

- Mice: 4150 mice

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The non-obese diabetic (NOD) mouse is the most robust animal model of T1D development, recapitulating the genetic and immunological events that are characteristic of human T1D development. Studies in NOD mice have determined non-immune cell and immune cell changes occurin the lymphoid tissues several weeks prior to T1D manifestation. Similarly in humans, investigations ofserum or peripheral blood lymphocytes demonstrated that antibody responses, and T cell responses, to human beta cells occur many years prior to T1D development. Recent availability of donor tissues from T1D cadavers have documented similar changes in the pancreas to that seen in diabetic NOD mice- a prevalence of B cells that correlate with rapid T1D development in children. The NOD mouse isinvaluable for assessing the age-specific (neonate to adult) changes in the non-immune and immune cell compartments of relevant tissues as the animals progress to T1D. In addition, there is greater availability of immunological tools compared to other model systems of human conditions, and there isextensive conservation between mouse and human immune systems.

Typically, what will be done to an animal used in your project?

Experiments will follow the PREPARE guidelines (https://norecopa.no/prepare).

Typically this project aims to understand the non immune cell and immune cell changes that take placein the thymus, or other tissues, that lead to T1D development. In this regard, the majority of experimental (NOD) animals (>70%) will be culled for tissue retrieval without undergoing an experimental procedure.

Experimental mice may undergo procedures where substances/cells are injected to determine their effect on stromal, or immune, cells. A typical animal undergoing this procedure may receive five or sixinjections, although the majority of such experimental mice normally receive one or two injections.

A small proportion of experimental animals (<5%) will be given one radiotherapy treatment and will bereconstituted with appropriate cells to examine the role the cells play in T1D progression.

To maintain our animal lines, animals will be bred using best practice husbandry techniques. For T1D susceptible strains female breeders will deliver a maximum of two litters to minimise risk of developingT1D whilst pregnant or their young are suckling.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the animals in this project show no outwards signs of distress or suffering. Injections produce mild discomfort and there may be some minor transient stress associated with handling. Female NODmice develop T1D between 12-23 weeks of age: ~2% develop the condition at 12 weeks, the majority80% develop the condition between 15-18 weeks. By 23 weeks of age 95% of females will be type 1 diabetic. Our studies focus on the prediabetic phases of the condition, and animals used for experimental purposes are humanely killed prior to the development of type 1 diabetes.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Animals that do develop type 1 diabetes (2%) or undergo radiotherapy treatment are termed"moderate" and constitute <5% of total number of mice used in the entire project.

All other procedures are "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 haveconsidered 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 is complex. Autoimmune diseases, i.e. diseases where the immune system inappropriately attacks our own tissues, always involve a multitude of cells, molecules and pathways whose function can be altered depending on the inflamed status of the environment in which they reside. Many of these changes will inevitably involve the contribution of molecules that are as yet undiscovered. As such, it is impossible to recapitulate in a test tube the multicellular, multistep conditions that contribute to disease. In addition, human thymic tissue is not easily accessible from people living with T1D, and donors are diabetic negating studies of the pre-diabetic phase that we areinterested in.

Which non-animal alternatives did you consider for use in this project?

Research in other laboratories is underway to develop in the laboratory a 3-D organ model of thehuman thymus. If successful this will be an important resource for our research.

We are currently developing a computational model of the thymus that we aim to use to inform someaspects of our research.

We use existing open access datasets of the murine and human donors to supplement, compare withand add value to our data, minimising repetition of experiments conducted by others.

We use human donor tissue- pancreas and thymus- from repositories to translate our

research tohumans.

Why were they not suitable?

Both the laboratory 3-D model of the human thymus, and the *in silico* murine/human thymus are notyet established.

Existing open data sets of the thymus use healthy donors, and minimum information is provided ontheir risk of developing autoimmunity, particularly T1D. Datasets that relate to T1D, tend to focus onpancreatic tissue, lymph nodes or peripheral blood lymphocytes.

Tissue, including thymic tissue, that is available from depositories are derived from deceased diabeticdonors, negating the ability to look at the thymic events that precede T1D development.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our estimates of animal usage are based on consideration of the scope and duration of funded work,staff workload, balance of experimental and analysis time and the need to ensure adequate training and research planning.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We use the NC3Rs Experimental Design Assistant (EDA), coupled with statistical analysis using appropriate software and local statistical advice. These combined approaches ensure the minimumnumbers of mice are used to produce statistically robust data.

We use our own bio-bank of archived tissue for optimization of new protocols, and to generatepreliminary data to validate new research avenues.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Unnecessary production, or import, of genetically altered animals will be avoided by searchingcryobanks and databases.

State of the art technology will be used to allow analysis of multiple parameters of immune cellphenotype from minimum numbers of animals.

We cryopreserve excess immune cells from extracted tissue and use these cells for future optimizationof experimental conditions and/or new *in vitro/ex-vivo* immunological investigations.

We always use the most up to date best practice in experimental design and implementation of techniques. To determine the minimum number of animals required per experiment, we perform pilotstudies.

In future, we aim to use our computational model of the thymus 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Strong similarities between mouse and human genome/immune system coupled to ease of manipulating animal models genetically make the mouse an ideal model to decipher the complexities of autoimmune diseases. Studies in non-obese diabetic (NOD) mice- a robust animal model of spontaneous T1D development- have generated major discoveries on the immunopathology of T1D inman, leading to the identification of several key cells/molecules that are potential targets for therapeutic intervention. More recently, the availability of humanised mice as a model system of a human immune system enables investigations into the dysfunctions of the human immune system thatdestabilises effective control of immune responses to human tissue.

By keeping ourselves up to date with advances in biomedical techniques, we have been able to refine an experimental approach from a moderate to a mild severity limit. Thus, we initially wished to look at movement of cells in the body, and this required cells from a donor mouse to be labelled and injected into recipient animals (moderate severity). However, with the availability of reporter mice where the cells of interest carry a fluorescent tag, we no longer need to inject labelled cells into recipient animals.

Although 95% of female NOD mice in our colony develop T1D, this tends to occur between 18-23 weeks of age, and we focus on deciphering immunological and stromal events that occur prior to T1Dmanifestation. As a consequence, experimental endpoints in most animals occur before any adverse event, and the studies are characterised as sub-threshold. For animals that undergo procedures requiring injection of substances/cells, we restrict the total number of injections (and if appropriate, local anaesthesia) per animal as advised by the NVS.

Why can’t you use animals that are less sentient?

The mouse is the lowest sentient mammalian species for the study of type 1 diabetes. The complexityof the immune response in T1D cannot be recapitulated in less sentient species, and as the condition develops in adults, immature life stages are not appropriate for detailed analysis.

How will you refine the procedures you're using to minimise the welfare costs

(harms) for theanimals?

We adhere to the rolling program of advances in husbandry introduced by the animal facility e.g. implementation of environmental enrichment, changes in handling methods (for example using tubesto pick animals up, or cupped hands) and changes in the policy regarding re-use of needles, i.e. we always use single-use needles.

Animals will be closely monitored for signs relating to the development of T1D (excessive waterconsumption/bed wetting/loss of weight).

Body score conditioning sheet and grimace scoring will be used alongside increased monitoring ofanimals undergoing procedures to minimise any adverse events.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

The applicant uses the NC3Rs Experimental Design Assistant and associated webinars to inform therobust experimental design. All experimental data is reported using the ARRIVE2 guidelines.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

The applicant keeps abreast of new technological advancements and approaches in experimental design that may reduce animal usage whilst maintaining robustness in data output. Open dialogue withinvestigators and review of the NC3Rs website enables optimal animal welfare and experimental design. If necessary, amendments to this project will be sought to pilot new techniques that benefit the ethos of the 3Rs. We have regular animal facility meetings where internal and external speakers sharenew findings and discuss best practice. We also keep up to date with the latest NC3Rs developments through their newsletter.

IDENTIFICATION AND CHARACTERISATION OF THERAPEUTIC TARGETS FOR CARDIAC DISEASE

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Heart, Heart Failure, Cardiovascular, Therapy, Cardiac remodelling

Animal types	Life stages
Mice	adult, neonate, embryo, juvenile, pregnant
Rats	adult, neonate, embryo, juvenile, pregnant
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required,and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The project aims to define the functions of selected components of mechanisms/processes involved incardiac remodelling across a broad range of animal models of heart disease.

A retrospective assessment of these aims will be due by 16 September 2028

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cardiovascular disease causes 160,000 deaths each year in the UK which equates to 435 people perday. There are around 7 million people living with cardiovascular disease in the UK with a healthcare cost of ~£9 billion and a cost to the UK economy of £19 billion a year.

A common end point of many cardiovascular diseases is heart failure (HF); a complex clinical syndrome caused by structural and/or functional cardiac abnormality. Over 500,000 people in the UK have been diagnosed with HF and despite optimised medical therapy mortality rates remain unacceptably high (5-year mortality rates are ~50%). HF has a major adverse effect on quality of life commonly leading to fluid retention in the lungs causing undue breathlessness even at rest, swelling ofthe legs/ankles and depression.

There are multiple cardiac diseases that lead to HF including; heart attacks (myocardial infarction; MI), high blood pressure (hypertension) and structural changes to the heart (cardiomyopathy). These diseases result in cardiac remodelling; a complex array of molecular and cellular changes within the heart that clinically manifest as altered heart architecture and contractile function. Initially, patients withcardiac remodelling may demonstrate compensated contractile function. However, a significant proportion of these patients will undergo progressive deterioration of cardiac remodelling leading to HF.

Novel therapeutic strategies to preserve heart function and limit cardiac remodelling are therefore urgently required to treat patients with cardiac disease and limit progression to HF thereby improvingsurvival rates and quality of life. Identification of new therapeutic targets can be achieved by using animal models to enhance our understanding of the mechanisms/processes involved in adverse cardiac remodelling and HF.

What outputs do you think you will see at the end of this project?

Through increasing our understanding of the science underlying the functional and structural changesto the heart that occur after a range of cardiac diseases we can inform the development of therapeuticstrategies to prevent heart failure. Such treatments would reduce the socioeconomic burden of heart failure in the UK and improve the quality of life of patients living with heart disease.

Short Term Outputs:

The primary expected benefit is the generation of **new knowledge, defining and characterising themechanisms/processes important for cardiac remodelling** in a broad range of cardiac diseases. This work will lead to publications in scientific journals and presentation of research findings at local, national and international conferences.

Medium Term Outputs:

Components of identified mechanisms/processes (which can be at any level e.g. molecular, organelle, cellular, extracellular, organ etc.) will then be manipulated to **generate new data on the importance of selected components for cardiac function in the whole animal**. Importantly, this new data will inform preclinical studies aimed at determining the therapeutic potential of using these components as targets to limit progression of HF in patients with cardiac disease, for which there remains no cure. The

data generated will be used to support future grant applications.

Long Term Outputs:

In the longer term, the potential benefits of this study are that data generated may have far reaching implications for treatment of a range of cardiac diseases benefitting patients and clinicians by contributing to the **development of effective therapy** which will ultimately reduce the economic andhealth burden of HF. Knowledge gained on cardiac remodelling may also **contribute to clinical guidelines** for the treatment of patients with HF.

Who or what will benefit from these outputs, and how?

The expected benefits arising from this project are multi-fold:

Short Term Benefits: The main beneficiaries will be scientists in the field through are advancementsin knowledge and understanding of heart failure.

Medium to Longer Term Benefits: Again, scientists in the field will be the main beneficiaries but this aspect of the project will also be beneficial to pharmaceutical companies looking to prioritise targets fordrug development; a huge unmet clinical need. Securing industrial interest promotes two-way flow of knowledge and guidance to help expedite and focus not only the mechanistic research itself but also the future potential for translational development. The ultimate aim is for any positive findings to lead tochanges in clinical practice and in turn enhance the quality of life of patients with cardiovascular disease.

An additional benefit over the duration of the programme of work will be staff and students working on this programme becoming independent scientists trained in the implementation of 3Rs. This new cadreof skills trained people strengthen the research communities required to meet future scientific challenges.

How will you look to maximise the outputs of this work?

All of the approaches we intend to use have been described in the literature and we have built up considerable expertise in assessing cardiac function (phenotyping) and complex microsurgical procedures over the last 10 years. We have also published in high profile publications the use of studies using gene therapy to treat hearts with MI and genetically modified animals. I have establishedcollaborations to learn their methodology for induction of heart disease due to increased blood pressure. Moreover, my close collaboration with international colleagues who are actively developing new small molecule compounds means we can be the first to test these compounds in our rodent models, addressing our key objectives and achieving many of the above benefits quicker (e.g., development of a new gene therapy and drugs for cardiac disease).

The data will be presented at national and international conferences, public engagement outreachactivities, academic journals and press releases by those involved in the programme of work. We publish our studies in peer reviewed scientific journals and will also aim to use journals that permitnegative studies where appropriate.

Species and numbers of animals expected to be used

- Mice: 27500

- Rats: 15000
- Rabbits: 1800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice and rats will be used because of our ability to mimic clinically-relevant cardiovascular disease in these species. Furthermore, they provide an effective way of enabling genetic modification thereby enhancing our understanding of the role of genes and development of cardiac disease. Adult rabbits will be used for some experiments as they provide an intermediate sized heart to mimic humandisease but also because the heart muscle cell from this species more closely resembles some aspects of human heart muscle cell function compared to rodents. We will also utilise heart tissue from rodent neonates where we require to culture cardiac cells for prolonged periods of time; a difficult technique achieved with adult cardiac cells. The most appropriate models will be chosen based on ourongoing studies or from the published scientific literature.

Typically, what will be done to an animal used in your project?

During this project we will typically induce cardiac disease in animals purchased in from a recognisedbreeder or animals bred in-house that are genetically manipulated. These models of cardiac disease can be induced surgically or non-surgically. In separate experiments, we take mice without cardiac disease and assess cardiac function and can test interventions.

We will then assess the characteristics of the cardiovascular system in our models at baseline and post-induction of disease. This typically includes measurement of the electrical activity of the heart (ECG), cardiac contractile function, blood pressure, cannulation of blood vessels for monitoring, blood sampling, administration of substances and microsampling. Some animals will undergo acute/chronic administration of substances/components to modulate physiological function, induce cardiac remodelling and/or required for imaging (all with appropriate controls). These substances/components include potential therapeutics. Additionally, some animals will undergo delivery of components that canalter gene function.

Upon terminal anaesthesia some animals will undergo more in-depth assessment of the cardiovascularsystem. Animals will then be humanely killed and tissue taken for examination. Where minimally compromised tissue is required this will be done under anaesthesia.

The duration of model can be as little as one day or as long as 52 weeks. A typical animal would undergo one surgical/nonsurgical procedure to induce cardiac dysfunction, cardiac imaging (echocardiography), blood pressure measurements at weekly intervals, and more in-depth assessmentof the cardiovascular system under terminal anaesthesia and be on procedure for between 1 day and 8weeks.

The duration that the animals will be exposed to cardiac disease will be the minimum required to obtainsufficient data about the acute and chronic changes in cardiac structure

and function. At the end of the procedures the animals will be killed humanely.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected impact/adverse effects for the animals on the project are dependent on the model of cardiovascular disease. Any animal that develops clinical signs of heart failure arising from cardiovascular disease will be humanely killed with an option to assess cardiac function under terminalanaesthesia beforehand, with discussion if necessary, with the NVS and named animal care and welfare officer (NACWO).

Surgical induced cardiac disease: e.g. myocardial infarction and transverse aortic constriction.

Deaths from animals undergoing complex microsurgery predominately occur during the operationunder anaesthesia and therefore pose limited cost to welfare. From this point, we have a post- operative mortality rate of up to approximately 25% on a rolling (typically quartely) basis.

Non-surgical induced cardiac disease: e.g. myocarditis

Although strain specific the mortality is approximately 10%. Infected animals (e.g. with Coxsackievirus B3) are closely monitored during the acute phase of the infection, looking for signs indicative of heart failure. An intestinal inflammatory reaction could develop with this model and in certain mouse strains. Mice that show this response (e.g. weight loss, hunching) rather than cardiac specific, will be assisted with placement of gelatinous food or treats inside the cage and will also be monitored closely. The non-cardiac signs are expected to be transient and improve over time but if any animal develops “severe” signs such as marked and persistent (>48 hours) it will be humanely killed.

Non-surgical induced cardiac disease: e.g. heart failure with preserved ejection fraction (HFpEF).

Most of the manipulations to induce HFpEF by themselves are not expected to produce significant adverse effects. However, some manipulations that induce high blood pressure can sometimes lead tofatalities, usually associated with dramatic cardiovascular events such as ruptured blood vessels (aneurysms), abnormal heart electrical activity (arrhythmias), sudden cardiac failure or massive stroke.Animals used in studies involving such manipulations will be monitored at regular and frequent intervals and any whose welfare gives rise to concern at any point will receive prompt veterinary attention or will be humanely killed. Except in the case of small group sizes (e.g. <10 animals) where e.g. 1 death out of 6 animals may represent a >15% mortality rate, we do not typically expect overallmortality on a rolling basis (typically measured quaterly) to increase above 15%.

The mouse models detailed do not show any changes to their behaviour or health as a result of anychange to their genes.

Pain as a result of surgical procedures: all animals undergoing surgery may experience some pain. Pain relief (analgesics) will be given in consultation with the named veterinary surgeon (NVS) and foras long as necessary.

Models on procedure and phenotyping:

Mortality risks due to anaesthetic for imaging/ECG - risks are minimal and are the same for anaesthesia (<1%). The NVS will be consulted should this increase above 10%. In all cases, the number of anaesthetic/imaging sessions will be kept to the minimum possible and will never exceed 32sessions. The imaging sessions represent a non-invasive way to monitor progression of disease and inform determination of humane endpoints. Gaps between sessions will be as long as possible and typically no less than 24 hours.

More in-depth cardiac phenotyping (e.g. pressure-volume catheter measurements) - transient discomfort from induction of anaesthesia. Intra-operative mortality rates due to these measurementsduring terminal anaesthesia are <15%. Pressure drops will be monitored in order to mitigate animal deaths intra-operatively. Administration of substances are not expected to have adverse effects but ifthese occur they will be discussed with NVS.

Blood loss is minimal during cannulation - risk of major blood loss is rare, but if it occurs, the animalwill be killed by a Schedule 1 method.

Injections & blood sampling - Intravenous and subcutaneous injections carry the risk of blood clots, bruising and blood loss. These adverse effects will be minimised by good technique. Blood sampling will not exceed 15% of total blood volume in any 28 day period and hence should not result in anaemiaor (reduced blood volume) hypovolaemia.

For implantation of substance delivery devices (e.g. mini-pumps) or devices enabling continuous phenotyping (e.g. telemetry devices) - Post-operative pain will be controlled by analgesic drugs givenon the advice of the NVS.

The substances which are administered to modulate physiological function/cardiac remodelling. Most of the substances which are administered to modulate physiological function are not expected to produce significant adverse effects. However, some substances do cause adverse effects. Although they are used according to published literature, they can sometimes lead to fatalities usually associated with dramatic cardiovascular events such as ruptured blood vessels (aneurysms), abnormalelectrical activity (arrhythmias), sudden cardiac failure or massive stroke. Animals used in studies involving such drugs will be monitored at regular and frequent intervals and any whose welfare gives rise to concern at any point will receive prompt veterinary attention or will be humanely killed. When some substances are applied to animals models in which there is already organ/tissue pathology a mortality rate of between 20-25% has been reported. Except in the case of small group sizes (e.g. <10 animals) where e.g. 2 deaths out of 8 animals may represent a 25% mortality rate, we do not typically expect overall mortality on a rolling basis (typically quaterly) to increase above 15%.

Any animal that develops clinical signs of heart failure will be humanely killed with an option to assess cardiac function under terminal anaesthesia beforehand, with discussion if necessary, with theNVS and named animal care and welfare officer (NACWO). For slightly longer periods of weight loss
e.g. >20% of pre-operative body weight over a 3-4 day period, advice will be sought from the NVS andacted on promptly or animal humanely killed.

Any animals kept singly-housed will be handled with due care and empathy and will be provided withsuitable enrichment.

Expected severity categories and the proportion of animals in each category, per

species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

All animals experiencing surgical and non-surgical procedures to induce cardiac disease are denotedas severe (~100% severe) (e.g. myocardial infarction, transverse aortic constriction, myocarditis and heart failure with preserved ejection fraction (HFpEF)). All animals undergoing these procedures are given analgesia.

All breeding and maintenance of genetically modified animals is mild (~100% mild) as is obtainingminimally compromised organ/tissue/blood.

The cumulative effect of those animals not undergoing induction of cardiac disease but who will experience repeated monitoring measures, ECG, echocardiography, drug, gene manipulation, is notexpected to exceed moderate severity (~100% moderate) as the least harmful route and lowest volume of administration will be chosen for delivery of substances, the nature of the monitoring methods are broadly non-invasive and animals will be acclimatised or familiarised with these within thestudy design.

In our previous experience of similar studies involving multiple assessment/intervention steps in combination with genetic modifications, we have identified no lasting harm as a result of cumulativeadverse effects. Animals on these studies maintain weight, show normal behaviour.

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 16 September 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animalswere affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is difficult to obtain viable human heart muscle including suitable non-disease human heart muscle.There is considerable variation in age, medication and underlying pathology of any obtainable humantissue and there is the likelihood of progressive disease being present. It is also not possible to investigate the processes at well-defined time points after a single incidence of damage. Substantial prior and continuing organ/tissue/cell experiments will inform and limit the number of animal experiments required and where possible as much information from one animal will be obtained.

Which non-animal alternatives did you consider for use in this project?

Access to human tissue to study cardiovascular diseases is limited due to various factors including: (a) the inability of heart muscle cells to replicate to any great extent which limits acquirement of human heart tissue for experimentation and (b) the plethora of drugs which patients with cardiac disease are on which can confound data. Both (a) and (b) limit access to appropriate control cardiac tissue from healthy patients. These factors lead to the crucial need for the development and characterisation of animal models of cardiac disease which mimic human heart disease for which no alternatives exist. Weare aware of the limitations of alternative preparations in cardiac research but where possible we will utilise data collected from organs and tissue experiments (including stem cells, cardiac slices) to replace the requirement for animals. However, when positive data are generated in cell systems, the next step is to progress to animal models, before final translation to clinical trials in humans.

Why were they not suitable?

For some of the early studies, the cell culture models are suitable. But there reaches a point where there are limitations associated with interpretation of results in these models; cells are of immature status and therefore results may not be predictive of the cellular response in the adult heart. The immature status of stem cells and use of culture for cardiac slices precludes these preparations beingof sufficient similarity to adult cardiac tissue to inform accurately their translational potential relative tolive animal models. Furthermore, the complexity of the 3D beating heart and its inter-relationship withthe circulation and other organs cannot be replicated yet in sufficient detail using alternative models.

A retrospective assessment of replacement will be due by 16 September 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 for the full 5 years duration of the project licence and are based on ongoing projects within the group and with collaborators. We have also been awarded a 5-year programme grant and additional project grants and so the animal numbers also incorporate the experiments outlined in these proposals. Pilot studies performed on previous licence provide information on the lowest group sizes required for each experiment and we have a good understanding of the degree of variation in our models from extensive studies and the literature. The estimated numbers are based onthese group sizes for the appropriate comparison of intervention and control groups.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We have considerable expertise in minimising the number of animals required whilst ensuring generation of robust data as evidence by publication track record. We will use statistician advice whererequired. Sample sizes will be set from our knowledge of the literature, pilot experiments, previously performed experiments. For the majority of measurements this leads to a typical group size of 8-15 animals. We will use serial measures where possible to gain as much information as possible without the need to increase animal numbers. Where appropriate, we will randomly assign animals to experimental groups and blind studies and utilise PREPARE and NC3Rs' ARRIVE guidelines to guide best practice. In terms of genetically altered mice, where suitable lines already exist, animals will beobtained from the relevant supplier. Otherwise, we will make/obtain the required lines with help fromBiological Services and collaborators. We will measure production and breeding performance and ensure the minimum numbers of animals are used in the programme. We will aim to minimise shamsurgeries as neccesary but cannot remove their need due to the requirement of control data in publications.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Pilot studies will be used to optimise dose and efficiency where appropriate. We will share tissue withinour group across experiments and with collaborators and other groups within our institution where it does not compromise the experiment. Efficient breeding will be employed where possible including efficient breeding strategies, replacing breeders before reproductive performance declines, and replacing non-productive breeders as soon as possible.

The flexibility of being able to use mouse/rat/rabbits is aimed at reducing and refining the number of animals used rather than increasing them. Experiments will not be repeated in both species where unnecessary. The decision as to what species is to be used for a particular set of experiments will depend upon a clear decision at that time as to whether the use of the species tissue with the particular technique maximises the ability to detect a difference between experimental groups for eachmeasure and hence decreases the numbers of animals used.

A retrospective assessment of reduction will be due by 16 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse, rat and rabbit animal models will be used as determined by the scientific question and the relevance of the species for human translation. The models used within the licence represent key cardiovascular diseases of humans and have been optimised to cause the least pain, suffering and distress to the animals. We have developed clear humane end points in order to limit potential sufferingand these will be implemented as necessary. Our protocols have been chosen based on the literature to provide maximum detailed information necessary to understand the process of adverse cardiac remodelling whilst at the same time ensure that the animals under investigation experience the least pain, suffering, distress or lasting harm. Phenotyping of our models (and respective controls) and administration of drugs/interventions are performed by the least severe/painful method available e.g.often administering via food/drink, use of non-invasive assessment of cardiac function e.g. echocardiography or under terminal anaesthesia.

Myocardial Infarction: This model typically involves surgically-induced permanent or temporary ligation of the coronary artery to cause myocardial heart damage. Animal can recover within hours to afew days due to compensatory heart function. This type of injury falls within the severe band. Appropriate analgesia is given pre- and post-operation to reduce pain.

Transverse Aortic Constriction: This model typically involves surgically-induced ligation of the aorta to induce and increase in cardiac muscle size. Animal can recover within hours to a few days dueto compensatory heart function. This type of injury falls within the severe band. Appropriate analgesia is given pre- and post-operation to reduce pain.

Myocarditis: This model typically involves injection of a pathogen to induce inflammation of the heart. This type of injury falls within the severe band. We have developed clear humane end points inorder to limit potential suffering and these will be implemented as necessary.

Heart Failure with Preserved Ejection Fraction: This model typically involves administration of substances to induce high blood pressure (hypertension)/metabolic challenge with or without a diet that leads to increased weight gain. This type of injury falls within the severe band. We have developedclear humane end points in order to limit potential suffering and these will be implemented as necessary.

We will utilise ARRIVE guidelines to report data and use blinding and randomisation where appropriateto reduce unconscious bias.

The ultimate benefits arising from the project licence will be to improve diagnosis and treatment ofcardiac disease.

Why can’t you use animals that are less sentient?

There are no alternatives to using an animal model to examine the consequences of MI and other cardiac diseases on cardiac function at the level of the whole heart and single cell. Many international groups have established rat, mouse and rabbit models of cardiac disease and these represent the lowest mammalian vertebrate group in which you can fully characterise human relevant adverse cardiac remodelling. We will continue to utilise our current laboratory data to inform us whether severeprocedures are required or can be

obtained using protocols of lower severity.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We will utilise in all models post-operative monitoring and care (including e.g. analgesia where appropriate, maintenance of body temperature, softened diet to encourage eating etc) to minimise anysuffering. Animals will be regularly checked to determine if any deterioration in their condition or the adverse effects of therapy. Should this occur we have defined limits and end points that determine ouraction which can include advice from the veterinary surgeon and humane killing.

Animals are regularly monitored and pain can be assessed by using scoring sheets and will inform theabove post-operative care provided to the animals. Acclimatisation and regular handling of animals reduces stress as does training in certain functional assessment procedures e.g. exercise testing and blood pressure measurement.

The decision as to what species to use for a particular set of experiments will depend upon: (a) obtaining the maximum signal to noise ratio, e.g. some antibodies work best in a particular species, (b)physiological mechanisms under investigation e.g. calcium handling function in rabbit tissue is more similar to humans and (c) use of transgenic breeding. The appropriate anaesthesia and pain relief measurements will be undertaken in all animal experiments as will aseptic techniques. We will constantly review the literature and N3CRs website for ways to refine the severe disease models and other routine techniques (e.g. serial blood microsampling).

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Percie du Sert et al - The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research(2020).

PLOS Biology https://doi.org/10.1371/journal.pbio.3000410Local guidelines and NC3Rs website.
Zacchigna S, Paldino A, Falcão-Pires I, Daskalopoulos EP, Dal Ferro M, Vodret S, Lesizza P, Cannatà A, Miranda-Silva D, Lourenço AP, Pinamonti B, Sinagra G, Weinberger F, Eschenhagen T, Carrier L, Kehat I, Tocchetti CG, Russo M, Ghigo A, Cimino J, Hirsch E, Dawson D, Ciccarelli M, Oliveti M, LinkeWA, Cuijpers I, Heymans S, Hamdani N, de Boer M, Duncker DJ, Kuster D, van der Velden J, Beauloye C, Bertrand L, Mayr M, Giacca M, Leuschner F, Backs J, Thum T. Towards standardization ofechocardiography for the evaluation of left ventricular function in adult rodents: a position paper of the ESC Working Group on Myocardial Function. Cardiovasc Res. 2021 Jan 1;117(1):43-59. doi: 10.1093/cvr/cvaa110. PMID: 32365197.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will continue to utilise the scientific literature for the latest refinement to our models and phenotyping to ensure the lowest possible level of suffering. Scientific conferences together with locallyrun seminars/workshops/training will be used. The NC3R webpage also will provide a forum for best practice and expertise in functional assessment.

A retrospective assessment of refinement will be due by 16 September 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved forfuture work of this kind? During the project, how did you minimise harm to the animals?

REAGENT PRODUCTION AND SCREENING: IMMUNOLOGICAL TOOLBOX

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Immunity, Tools, Vaccines, Antibodies

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The primary focus of this project is to address the acute shortage of experimental tools (called reagents) that are available to study veterinary immunology. By developing new tools, we aim to improve immunological knowledge which will benefit the development of vaccines and diagnostic testsfor animal diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The control of infectious diseases relies on vaccines and accurate diagnostic tests. Alongside this, immunotherapy is being increasingly used in treatment of chronic conditions such as cancer and autoimmunity. Each of these requires significant knowledge of the immune system of animals (and humans) in health and disease. A major constraint to the understanding of the immune response in veterinary species (including livestock

and companion animals) is a lack of tools (reagents) to study the immune response in detail. This lack of tools is a major barrier to progress in vaccine development,and for immunotherapeutics.

An important type of tool that we use to measure the immune response are antibodies: these recognise and bind to specific parts of cells or their products (targets). We can measure whether theseantibodies bind to their targets as a way to determine whether certain cells or responses are present inanimals that have disease or have been vaccinated.

The major output will be the generation of these highly specific antibodies. This will be of value to researchers within the veterinary immunology community. The requirement for, and impact of, these reagents will be assessed and prioritised by a steering committee overseeing this Immunological Toolbox activity. This will ensure that the reagents made in animals will be of impact to the wider community. Alongside this we will engage with industrial partners for distribution and commercialisationso that the outputs are widely available.

What outputs do you think you will see at the end of this project?

The major output will be a series of new tools enabling researchers worldwide to measure the immuneresponse of animals. This will influence the development of vaccines, diagnostic tests and other methods to control diseases. We will publish our data in peer-reviewed journals and through a dedicated website.

Who or what will benefit from these outputs, and how?

The primary focus of this project is to address the acute shortage of veterinary immunology reagents currently available. By generating new tools and reagents we will increase the capability to understand specialised cell populations, their products and functions. In the medium term (3-5y) this will allow us todetermine how these cells and their products influence the interaction with, and control of disease- causing microorganisms or the response to vaccines. These reagents may also be used to define immunological correlates of vaccine induced protection which can then be used to identify candidate vaccines for disease protection studies. Longer term (up to 10y), we expect the data from the short andmedium-term goals to feed directly into the development of vaccines or diagnostic tests for important diseases of livestock and companion animals. The reagents developed may also be utilised for immunotherapetic purposes to treat chronic illnesses including cancer and autoimmune conditions.

How will you look to maximise the outputs of this work?

The majority of work carried out under this project licence will be done in consultation and collaborationwith our network of veterinary immunology colleagues worldwide. This will allow maximum dissemination of knowledge and distribution of tools across the community enabling significant output. We recognise the very high value of publishing 'negative' data and the importance of shared experience to minimise duplication of effort. We will publish data in peer-reviewed journals, and we willmake all information available through a dedicated website.

Species and numbers of animals expected to be used

- Mice: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will utilise adult mice. Mice are routinely used to generate monoclonal antibodies and the procedures to be used are very well established. This results in robust, reliable outputs. We will useadult animals as they have fully developed immune systems.

Typically, what will be done to an animal used in your project?

Typically each animal will be blood sampled prior to, and after each immunisation. A maximum of fourimmunisations will take place and following a final immunisation animals will be humanely culled for tissue retrieval.

What are the expected impacts and/or adverse effects for the animals during your project?

The procedures carried out under this project licence are of mild severity with no expected adverse effects as each animal will receive a small number of injections and blood samples will be taken. Weexpect no more than momentary discomfort from these procedures. At the end of each carefully planned study animals will be humanely culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

All animals will experience no more than mild severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The generation of highly specific antibodies cannot be achieved without the use of mice as the cells and processes required for their induction require whole body systems. We have processes in place toidentify alternative methods by screening existing reagents or non-animal tools that may work in somecircumstances. We will always investigate alternatives to the use of animals as our first step before progressing to experiments in animals.

Which non-animal alternatives did you consider for use in this project?

Before we initiate animal experiments we will always consider non-animal alternatives. Wherever possible we will use existing reagents tested for specificity, or cross-reactivity to the target molecule ofchoice. We will also investigate alternative techniques such as antibody phage display and recombinatorial libraries, or DNA aptamers. However, these require further investigation before we canbe confident they are suitable alternatives. The generation of monoclonal antibodies for use in a rangeof immunological assays requires the use of mice, it cannot be done in vitro with cell lines or other in vitro systems at present although we will continue to monitor the literature to identify alternatives and investigate these if they become available.

Why were they not suitable?

Only where we have examined current resource and attempted alternative methods (resource permitting) will we proceed to animal studies. The de novo generation of monoclonal antibodies cannotbe done without the use of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have extensive (>20 years) experience of the experiments described in this application. We willuse this knowledge to estimate the number of experiments to be undertaken, and the numbers of animals to be used in each study.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The design of each experiment will be assessed by a statistician as part of our study approval process.We have refined our techniques for monoclonal antibody generation through extensive experience. Wewill always design each experiment involving the lowest number of animals possible and this will only be increased if the desired immune response is not seen.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

In some cases, we may co-immunise the same animal with different antigens in order to increase the likelihood of antibodies that work across species or can be used for multiple purposes. This will reducethe number of animals used where antibodies are required that display broad reactivity.

All experiments will be appropriately controlled. For the generation of monoclonal antibodies this willinclude comparison of responses in small blood (serum) samples taken

pre-immunisation to those taken post-immunisation and post-boosting. Care will be taken when testing these sera that the antibodies detected are specific for the antigen used to immunise, and to rule out non-specific responses.

Wherever possible we will archive material (sera, tissue samples/cells taken post-mortem) to allow additional analyses in future. For example, we may store spare spleen cells to enable additional fusions for monoclonal antibody production to be carried out. As part of good laboratory practice all ofour experiments are officially reviewed by a named vet, animal care technicians and a statistician before they can be undertaken. These study protocols include aims, numbers of animals, treatments,assessment of adverse effects, end points and our data analysis methods which are carefully scrutinised.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice in this project. We will use protocols that are well established, and which have beendesigned to allow us to induce immune responses with the minimum of pain, suffering and lasting harm. We will use doses of immunogens and timescales that do not cause overt disease or suffering,and we will continually assess our methodology and refine where necessary.

The majority of animals will be subject to procedures that we only expect to cause minimal, transientdiscomfort. However, we will carefully monitor the wellbeing of the animals at regular intervals to monitor pain and to minimise harm.

Why can’t you use animals that are less sentient?

The outputs from this work require the induction of an effective immune response. In mice, immune responses are not fully developed until the animal reaches maturity. Hence, we need to use adult animals for our studies. The use of less sentient animals is not possible due to potential differences inthe immune systems that would result in a failure to generate suitable responses that are needed for our reagent generation. The animals need to remain alive throughout the procedures as generation ofimmune responses takes several days or weeks.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

By measuring the immune response in the sera of immunised animals we can potentially reduce thenumber of immunisations required to induce an effective response. This would reduce the number ofprocedures and thereby minimise the potential adverse effects.

What published best practice guidance will you follow to ensure experiments are

conducted inthe most refined way?

We will refer to guidelines published by the UK National Centre for the Replacement, Refinement, andReduction of Animals in Research (NC3Rs).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will monitor correspondence and guidelines from the NC3Rs on a regular basis and implement relevant changes in a timely manner. The project licence holder is in frequent contact with the namedveterinary surgeon and the named animal care workers: this will enable any changes to be made where appropriate.

Home Office

UNDERSTANDING MECHANISMS OF TISSUE DEGENERATION AND REGENERATION

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Drugs, Chemicals, Diet, Degeneration, Regeneration

Animal types Life stages

Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

This project aims to better understand how substances such as drugs, chemicals and dietary agentscause tissue degeneration and/or stimulate tissue regeneration.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work has the potential to advance our understanding of how substances such as drugs, chemicalsand dietary agents cause tissue damage and recovery. This could support the development of new medicines, protect the public against adverse reactions and ultimately contribute to the evolution of non-animal experimental approaches that could replace such standard animal tests.

What outputs do you think you will see at the end of this project?

The main outputs are likely to be publications and other communications with relevant audiences (e.g.presentations at scientific conferences). It is possible that the work will directly or indirectly support thedevelopment of certain new medicines by the pharmaceutical industry, or lead to the development of kits for measuring molecules known as 'biomarkers' in blood and/or tissues to detect tissue degeneration and/or regeneration.

Who or what will benefit from these outputs, and how?

This work has the potential to benefit patients, clinicians, academic researchers and industry by (a) advancing our understanding of how substances such as drugs, chemicals and dietary agents cause tissue damage and recovery, which are features of many adverse reactions seen in patients; (b) allowing new drug candidates with the potential to cause an adverse reaction to be identified and rejected at an earlier stage of development, rather than progress to patient trials and or wider clinical use; (c) developing novel approaches to identify patients that are predisposed to a particular adverse reaction, and characterizing biomarkers that enable the earlier identification of patients experiencing such a reaction, thus limiting their exposure to the substance and informing clinical diagnosis and prognosis; (d) identifying novel strategies for mitigating the adverse effects of otherwise effective drugs, and for preventing tissue damage and/or promoting recovery and regeneration in other medical contexts such as natural disease; (e) ensuring that computational models and non-animal experimentalsystems (which could ultimately replace some standard animal toxicology tests performed e.g. during drug development) are informed by high quality data generated in relevant settings.

How will you look to maximise the outputs of this work?

We will disseminate the findings of our work by publishing in open access (no fee) scientific journals,through presentations at scientific conferences, via social media and through press releases.

Species and numbers of animals expected to be used

- Mice: 2900
- Rats: 850

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.
We will primarily use mice, and less frequently rats, as they share many anatomical and physiologicalsimilarities with humans and several useful experimental models of tissue damage and regeneration have been established in these species. Adults will be used in all cases.

Typically, what will be done to an animal used in your project?

Typically, animals will be exposed to a substance (such as a drug, chemical or dietary component) viainjection, oral gavage, topical administration or supplementation of their food/drinking water, for a period of days or weeks. The substance will cause tissue degeneration or promote regeneration.
Alternatively, a surgical procedure may be used to cause direct tissue degeneration.
Where necessary,animals will undergo anaesthesia during some procedures.
Measurements will be taken from the animals during (e.g. using non-invasive imaging) or after the procedure, e.g. using collected blood or tissues.

What are the expected impacts and/or adverse effects for the animals during your project?

Manageable adverse effects will include short-term pain (minutes/hours), moderate weight loss (hours/days), tumour formation (days/weeks) and surgical wound infection (hours/days). Animals willbe monitored for signs of more severe adverse effects (e.g. failure to respond to gentle stimuli, laboured respiration, prolonged diarrhoea) and humanely culled to minimise distress.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

We estimate that 100 % of animals in our studies will fall within or below the moderate severity category. Animals will be monitored for signs of ill-health and we will use defined endpoints to determine when studies must be stopped (using approved, humane culling techniques) to minimiseanimal distress and the potential for any animals to fall into the severe category in this project.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The goal of our work is to investigate mechanisms by which drugs, chemicals and dietary componentscause tissue degeneration and/or stimulation regeneration. It is necessary to include some animal research in our overall programme of work, to facilitate analysis of whole organ degeneration/regeneration, and consider unanticipated, off-target effects of novel therapeutic interventions prior to their use in patients.

Which non-animal alternatives did you consider for use in this project?

Cell/tissue culture techniques including the use of immortalised cell lines or primary cells cultured in 2Dor 3D, as well as more complex models such a combinations of cells cultured in 3D and precision cut tissue slices.

Why were they not suitable?

The above cell/tissue culture techniques are informative (and will be used whenever possible/appropriate) but these are currently unable to mimic the complex environment and interactions between different cell types that underpin most forms of tissue degeneration/regenerationin a living body over a suitable period of time.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 species, we have estimated the group size and number of experimental groups within a typical study. We have multiplied these numbers by the estimated number of studies to be conductedover the course of this project.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We will ensure that a minimum but sufficient number of animals will be used in any studies by using statistical power calculations to determine group sizes. Where necessary, this will be done in collaboration with a local biostatistician and informed by our own historical data or data obtained fromthe literature. We will make use of the NC3R's Experimental Design Assistant tool to plan studies.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Whenever possible, we will use cell/tissue culture techniques and human tissue biopsies to address our research questions. Where possible, animal usage will be minimised by making use of our ability tomonitor tissue degeneration/regeneration and associated events using minimally-invasive imaging techniques and measurements of circulating biomarkers. These techniques allow repeated measurements to be taken over time in the same animal, limiting the need for large group sizes. We will also ensure that any tissues collected during/after animal studies are archived and stored appropriately, avoiding unnecessary repetition of experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will administer non-lethal doses of relevant substances to the animals, using routes associated withminimal pain, or perform surgery under general anaesthesia. Where possible, we will make use of non-invasive imaging under general anaesthesia for monitoring animals. Humane endpoints will be used to minimise suffering.

Why can't you use animals that are less sentient?

Whilst tissue/cells may be obtained from some animals under terminal anaesthesia, to provide materialfor ex vivo experiments, studies performed under other protocols in this licence will typically last for longer than it is acceptable to anaesthetise animals for (e.g. several days/weeks) hence most studies will be conducted using conscious animals. Animals at a more immature life stage, or species that areless sentient, are not considered to mimic the human responses to substance exposure as closely asadult rodents.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

If deemed necessary, we will increase the frequency of animal monitoring during a procedure, or at certain points (e.g. immediately following surgery) to ensure timely intervention or humane culling of any animals displaying significant clinical signs. We will use peri- and post-operative pain relief to minimise suffering during/after surgery. We will make use of non-invasive imaging to monitor animalsduring procedures, when appropriate.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Whilst no formal published guidance exists for the models we will use in this project, we will follow general guidance from the Animals in Science Regulation Unit (ARSU) and UK Research and Innovation (UKRI) to ensure our studies follow best practice. Each study will be preceded by the writingof an experimental study protocol which will outline our objectives, methodology and endpoints.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Throughout this project we will communicate regularly with veterinary, NACWOs and husbandry staff atour Establishment, receive updates from the NC3Rs through receipt of electronic and printed media, and attend NC3Rs symposium and scientific conferences. These approaches will allow us to be awareof the most recent advances in the field and will enable us to implement these into our research in a timely manner.

TRACKING TWAITE SHAD TO EVALUATE SEASONAL DISTRIBUTION IN BRISTOL CHANNEL MARINE PROTECTED AREAS

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 oranimals
  - Research aimed at preserving the species of animal subjected to regulated procedures aspart of the programme of work

Key words

Twaite shad, Marine Protected Area, Acoustic tags, Marine distribution, Survival

Animal types	Life stages
Other fish species	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

To compare the marine seasonal distribution, freshwater and marine survival rates of twaite shad (*Alosa fallax*) spawning in multiple rivers draining into the Bristol Channel, in order to extend basicscientific knowledge and inform management and regulatory decision making.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Fish which migrate into freshwater to spawn, such as Twaite shad may have to migrate past coastaland in-river developments to complete their life cycle. Developments such as weirs, barrages, tidal lagoons or major abstractions have the potential to impact on

survival of both juvenile and adult stages.

This is particularly important for species such as shad (*Alosa fallax*), which spawn multiple times and may therefore be subject to cumulative impacts. The lack of data on marine migration and distribution,and hence potential impact, has impaired the ability of developers to assess impacts and propose suitable mitigation, compromising marine licence applications and potentially putting the fish populations at risk. The abstraction being built for Hinkley point C, and proposed tidal lagoons in Swansea Bay and elsewhere in the Bristol Channel are major developments where this work has significant current application.

This project will tag and track up to 450 Twaite shad from the Rivers Tywi, Usk and Wye, and comparethe results with existing movement and survival data from fish tagged in the River Severn. The tags regularly emit a coded acoustic pulse which can be detected and decoded by fixed passive receivers, enabling individual movements to be followed. Adult fish will be tagged in freshwater, and their migration to sea followed, to develop quantitative survival, migration and availability data for a specificcoastal location.

What outputs do you think you will see at the end of this project?

Quantitative data on distribution and residence times of twaite shad in the Carmarthen Bay MarineProtected Area.

Quantitative data from strategic development areas such as Swansea bay (Tidal Lagoon) andBridgwater Bay (Hinkley point c abstraction).

Qualitative data on coastal distribution and migration paths in the wider Bristol Channel area.

River and sea survival rates.

Comparison of similarities and differences in migration patterns with twaite shad tagged in the RiverSevern.

Who or what will benefit from these outputs, and how?

Support for Marine Renewable development is an important element of Welsh Government policy. However lack of data on key marine species, particularly anadromous fish, is recognised as a strategicinformation gap for Marine Renewable energy and other developments such as major abstractions for power generation, by both regulators and industry (see for example ref 1 below.).

Developers and regulators will be provided with valuable data as the study progresses. Information specific to the area will benefit local regulation (Natural Resources Wales) and inform evaluation of current and future development proposals as well as aiding understanding of the effectiveness of the MPA. The information will therefore provide both short and long term value. Results describing migration and behaviour patterns in inshore areas will have wider utility and will benefit assessments by regulators elsewhere UK regulators (Environment agency, Marine Management Organisation, Natural England and Marine Scotland). The value regulators place on this data is reflected in financial commitments (tag and receiver purchase) to help support the work.

Local angling associations are keen that evidence is developed to ensure that both the fish

population and fishery are protected through the regulatory process for marine developments. They will be directlyinvolved in providing practical assistance with our fieldwork, including fish capture.

A final project report will be published and made publicly available. The more important results will bepublished in peer reviewed journals. We would also expect to provide information regularly during thecourse of the project to the wider scientific community through conference papers and publications.

1. Marine Energy Wales. Tidal range: Critical Evidence gaps and how to address them. Workshop report, March 2022. http://www.orjip.org.uk/sites/default/files/u53/ORJIP%20OE%20MEW%20Tidal%20Range%20Worksh op%202022%20V1.pdf

How will you look to maximise the outputs of this work?

We expect to produce reports, conference papers and peer reviewed publication.

We are already working closely and collaborating with Natural Resources Wales, Natural England,Environment Agency, and the Devon and Severn IFCA, as well as local angling interests.

Species and numbers of animals expected to be used

- Up to 450 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.

Twaite shad (Alosa fallax) are a highly protected anadromous species which are found in four UK rivers, all draining into the Bristol Channel. They normally spawn a number of times. They are highly protected, being primary species features of Special Areas of Conservation (SAC) in the Rivers Usk, Wye and Tywi, and primary species features of the Severn, Carmarthen Bay and Pembrokeshire Marine Protected Areas (MPA). Consequently they are particularly important for regulation of marine developments, including the cooling water abstraction for Hinkley Point C, Marine Renewable Energy (MRE) developments (tidal range, tidal stream and offshore wind), as well as management of the impact of activities such as gravel abstraction and fishing.

We have previously undertaken marine tracking of twaite shad tagged by the 'Unlocking the Severn' project team in the river Severn under a separate Home Office licence. This has been a highly successful project and has enabled a good picture of marine migrations of shad spawning in the Severn to be developed. Early data from the work have already been used in a public inquiry looking atproposed modifications to the Hinkley point C abstraction, and were referenced in the inspectors conclusions.

We do not, however, know if Twaite shad spawning in the other rivers follow similar migration paths to those spawning in the Severn. This project aims to track fish, initially

from the River Tywi and subsequently from the Rivers Usk and Wye, in order to compare movements with the extensive Severndataset to help understand the extent to which conclusions from Severn data may be transferable.

Typically, what will be done to an animal used in your project?

Fish will be captured using rod and line. They will be anaesthetised and tagged with an acoustic tagthrough an incision approximately 1cm long (or less). The incision will be closed with a dissolvable suture and covered with a suitable covering to initially prevent the wound from infection during initialhealing. Analgesia will be applied as appropriate.

After tagging adult fish will be held facing into the water stream until they are able to hold position andactively swim upstream. They will then be released to continue normal lives.

What are the expected impacts and/or adverse effects for the animals during your project?

Experience has shown that fish rapidly recover from anaesthesia and surgery and are not expected tosuffer any lasting long term harm as a result of the procedures under this protocol being carried out.

The procedures carried out in these protocols will be done under general anaesthetic and therefore fish will be subjected to no more than mild stress as a result of capture and handling. There may be some mild post-operative discomfort, but experience of staff carrying out the work will ensure that fishare only released when they are recovered and able to swim upstream against the flow.

A literature review has suggested there is a small risk that the fish could hear the tag. They are thoughtto be capable of hearing at 69kHz (the tag frequency), but the literature evidence suggests that the hearing threshold for these species is higher than the tag output, so it unlikely that they actually hear the tag. The same tags have been used extensively since 2018 with the same species in the River Severn (320 tagged), and in studies in the Netherlands and Ireland without any obvious problems or unusual behaviour. We therefore believe that any risks are low, and outweighed by the project benefits.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

We expect the severity to be moderate for all fish tagged. The tagging approach is intended tominimise discomfort or damage to the fish.

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you

have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project aims are to look at the behaviour and distribution of twaite shad (*Alosa fallax*) in the wild inorder to gain information to manage and protect the species in the context of specific development areas. There are no practical alternatives to generate this data.

Which non-animal alternatives did you consider for use in this project?

Theoretical modelling has already been utilised to look at potential distribution and movements in relation to developments in the Bristol channel. This work has identified the species most at risk from developments, which include twaite shad. However all such work has to be grounded in empirical datato validate assumptions and parameterisation of models.

Why were they not suitable?

Work with fish tagged on the Severn has helped to inform the validation of modelling and predictions.However without these comparative studies we do not know whether these assumptions can be applied to fish spawning in other catchments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We need to use sufficient fish to provide robust estimates of survival and marine distribution to informmodels which can then be used to predict impacts without the requirement for further experiments using live fish. We have drawn on survival and movement data from our recent collaboration with the 'Unlocking the Severn Project' to inform our sample sizes, and will begin with pilot studies of 50 fish ineach of the Rivers Tywi, Wye and Usk to validate both capture methods and survival rates. A key partof our aim is to compare results with those already obtained using larger numbers of tagged fish fromthe Severn and we have therefore reduced numbers tagged in the each river in this study to numbersthat we anticipate, based on existing data, will be sufficient to evaluate survival and determine the extent to which the existing Severn dataset are transferable.

In practice the key factor in determining the number of fish required will be survival at different life stages. The numbers given above and design and actual numbers will be set on a 'pilot study' basisand informed by past results as the project evolves.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

See above. We have used empirical data available from our collaboration on the River Severn and areplanning to use an adaptive experimental design which will be updated on

an annual basis to determine numbers deployed in each year enabling the number actually tagged to be the minimumnecessary to achieve the objectives of the study.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Ongoing review of data, developing computer models which can be used in subsequent studies toreduce requirements for similar work.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The capture and tagging methods we are using with acoustic tags are well established, and have beenrefined for twaite shad during the work undertaken on the Severn. The methods we are using are designed to allow the fish to return as rapidly as possible to normal behaviour with minimal long term effects.

Why can’t you use animals that are less sentient?

Our objective is to understand observe behaviour and distribution of Twaite shad under naturalconditions, including at sea. This cannot be achieved by other means.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Capture method

Rod and line is our preferred method and has been used in the Severn study with success. This minimises the likelihood of scale damage while the fish is held in traps and the subsequent risk ofdisease.

Choice of tags

We are using the smallest tags available consistent with the objectives of the project, including tag life and tracking in the marine environment.The tags we are using are specifically designed by the supplier (Innovasea) for work with the species and life stages we are using. They are tough and smooth tominimise any issues if ingested by a predator.

Tagging and recovery procedures

The anaesthesia technique we are using is well established. If tagging during dark hours light will bekept to a minimum to reduce stress. Aseptic surgery techniques and single use scalpel blades and suture needles will minimise risk of infections.

Each incision will be covered with a suitable temporary wound barrier to provide a temporary barrier,reducing discomfort and providing protection from infection.

Sutures will be checked prior to transferinto recovery and holding tanks. Analgesia will be applied via subcutaneous injection.

Fish will be recovered and released by holding them facing into the current to maximise flow over thegills until they are capable of holding station and swimming against the current.

All procedures will only be performed by suitable trained and qualified individuals (ie PIL holder;training and competency record kept by NTCO)

Humane end-points and limits of severity

If internal damage to organs were to occur during surgery, the fish would not be allowed to recover andwould be euthanized by a schedule 1 method.

If fish fail to recover from anaesthesia they will be euthanized by a schedule 1 method

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

There are a number of published studies using these tags and techniques. However methods evolvecontinuously and we continue to share experience with others to develop best practice through conferences and direct conversations with other groups, including NRW (via our NVS), from Hull International Fisheries Institute, from the Atlantic Salmon Trust, and from the Game Conservancy Trust. All the above are undertaking current licenced work with these species and our approach and protocols seek to take the best from each, consistent with our objectives.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will continually review the literature. We will attend conferences, such as the recent SAMARCH workshop, which brought together salmonid tracking researchers, and the 'Unlocking the Severn' Shadconference workshop, which brought together species specialists. We will continue to network with others to share and learn from further developments, both as research understanding of the field develops and to improve our tagging methods to minimise any potential adverse effects. Where appropriate we will update our protocols and methods.

MYELOID CELLS IN LUNG INJURY AND REPAIR

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Immune Cells, Lung, Infection, Fibrosis, Regeneration

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

This project will examine the role of group a of immune cells called 'myeloid cells' in the processes ofinjury, scarring, repair and regeneration in the lungs that result from infection and/or environmental damage.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Lung scarring or fibrosis in the form of the disease- idiopathic pulmonary fibrosis (or IPF) is a devastating disease with a median survival of 3-5 years from diagnosis, a prognosis worse than many cancers. Every year around 5000 people are diagnosed with IPF in UK

each year, and there is no curefor the disease. Current treatments only slow the progression of the disease. This project examines therole of a group of immune cells called myeloid cells in the causation of IPF, specifically, which subtype of myeloid cells is involved in progression and stopping lung fibrosis.

People with IPF also suffer periods of accelerated fibrosis called acute exacerbation or AE-IPF which herald a worse outcome – 80% of patients who suffer an AE-IPF episodes die within 3 months of the episode. We will also investigate whether lung infection (using influenza virus) can cause AE-IPF and how. From our previous studies, we know that influenza infection has a major impact on myeloid cells,and we want to test the possibility that a rush of myeloid cells to the lungs, caused by infection could be a cause of accelerated fibrosis in the lungs.

We are also working on the role of myeloid cells in influenza virus infection, with the aim of generating better vaccines. Influenza still has the potential to cause huge pandemics. Influenza can spread easily,mutate, reassort, cross species barriers and inflict injury and death upon its host. Influenza viruses lead to regular winter epidemics and intermittently to widespread pandemics related to the development of new strains of the virus. Although vaccines are available for influenza, every year nearly 0.5M people still die world wide from influenza. Amongst various reasons for this high death rate, two are (i) limited understanding of how the immune system remembers its encounter with any one flu strain and (ii) the inability to control lung injury associated with severe influenza. In this project, we question if high levels of myeloid cells help the immune system remembers its encounter with a flu strain and improves the recall of immune response in its subsequent encounter; and how injurious myeloid cells can be controlled in the lungs.

What outputs do you think you will see at the end of this project?

Outputs will include peer reviewed published papers, presentations of our data at conferences andsymposia and a greater understanding of the contribution of myeloid cells to both lung fibrosis andinfection within the scientific and clinical community.

This new understanding of the roles of myeloid cells in fibrotic lung disease allows the development of therapeutic strategies to inhibit pro-fibrotic myeloid cells and promote cells which encourage regeneration of healthy tissue. As current drugs can only slow progression of disease, finding potentialcell targets which can stop or reverse lung fibrosis would substantially improve clinical outcomes for patients.

In influenza, the key potential benefit of this work relates to new knowledge in the area of immunology and immune memory to influenza. Our findings may allow us to develop new ways of boosting immuneresponses to more effectively combat influenza viruses. This is particularly important in elderly patientswhose immune systems function differently than in youth. If we are better able to understand how to effectively activate immune responses then we can develop more advanced vaccination strategies.

Who or what will benefit from these outputs, and how?

Short term benefits of this project are increased understanding of the role of myeloid cells in lung infection and fibrosis. The data generated could potentially be used quickly to advance understandingin mechanisms of disease in lung fibrosis and infection eg within 3 years if publications can be generated within 2-5years.
Longer term benefits for this work include the potential development of new drugs for both lung fibrosistargeting the myeloid cell pathway. The patient burden of these diseases is

considerable and the current therapies can only slow disease progression and not offer a cure. Therefore, if we were to identify specific types of myeloid cells in the lungs which contribute to disease, confirmed in both animals and human studies, and determine the point of their action and the dominant lung conditions required for their generation; then the path to ‘drugging’ this pathway become possible.

These outputs are expected to benefit

Our own group by increasing our knowledge of myeloid cell biology in lung fibrosis and influenzainfection, and completion of relevant scientific publications

The scientific community working in fibrosis and influenza

Our industrial partners interested in using myeloid cells as cellular targets for therapy

Patients with lung fibrosis and/or severe influenza infection

How will you look to maximise the outputs of this work?

Outputs will include peer reviewed published papers, presentations of our data at conferences and symposia and a greater understanding of the contribution of myeloid cells to both lung fibrosis and infection. The studies will generate papers that will be published in high impact factor journals. They will also be communicated and disseminated at national and international conferences. These data willbe translated to human studies to establish their role of myeloid cells in human lung fibrosis and influenza, and eventually to determine how they may be used in collaboration with our industrial partners to design or reposition drugs for patients with lung fibrosis and to inform better vaccine designfor influenza infection.

Species and numbers of animals expected to be used

- Mice: 20,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We chose mice because their immune system and lungs are sufficiently similar to humans for us to draw conclusions on processes like scarring and lung injury. We are unable to use less sentient animals because we need to have the ability to model the lungs rather than disease processes only, since the complex lung environment greatly impacts on the differentiation and behavior of myeloid cells. For example, zebra fish do not have lungs. Generally mice will be used as adults to better mimicdisease in humans. Mice may also be used as embryos and pups to help understand how myeloid cells affect the developing lung.

Mice can be genetically modified to allow alteration of genes and proteins which are important [or hypothesized to be important] in immune cells, IPF and influenza. For example, genes which alter thefunction of key immune cells or genes which promote repair

in the lungs. We will also use mice with genetic modifications which allow specific populations of immune cells to be tracked allowing us to understand when immune cells play their role in the disease process. The huge variety of genetically modified mice means that we would be able to use mice which are currently available.

Typically, what will be done to an animal used in your project?

Mice will be bred and maintained that have genetic modifications which allowing us to manipulate theirimmune systems or track immune cells.

Mice will be anesthetized and agents which cause lung scarring and/or infection will be injected into the lungs. The process of anaesthesia and administration into the lungs takes a few minutes. For influenza, the mice are anesthetized and influenza virus in liquid is administered through the nose. When lung scarring agents are given the liquid is administered directly into the lungs via the trachea, and an injection of painkilling drugs is given to the mice to reduce any discomfort they may experience.

For both influenza infection and administration of lung scarring agents the mice will typically lose weight as the disease progresses about 7-9 days after the initial dose they will reach the most weightloss, then they will recover the weight loss around 10-14 days after dose.

Immediately after administration of lung scarring agents the mice may have an altered breathing pattern which will resolve after a few minutes due to the liquid entering the lungs. At the peak of disease around days 7-9, depending on the amount of agent used to treat the mice, they may have anincreased respiratory rate which will return to normal within a few days.Mice may also be given drugs and/or treated with radiation to alter their immune system.

The infection or fibrosis disease course will then be followed, with duration of experiments ranging froma few days up to 12months.

Mice will be treated with agents to cause genetic modification at different points of their development totarget immune cells. Mice may be given additional immune cells to alter their immune populations.

Mice may have the lungs imaged by microscopy or MRI lung imaging.

What are the expected impacts and/or adverse effects for the animals during your project?

During the administration of lung scarring agents an injection of painkilling drugs is also given to themice to reduce any short-term discomfort they may experience.

The mice may suffer some discomfort during the injury and inflammation phase of our lung scarring, they are also likely to experience lack of appetite and weight loss as well as altered respiratory rate, with these effects are likely to last around a week. We will provide low level wet food to encourage eating. Mice infected with influenza have similar clinical symptoms to humans and experience lack of appetite leading to weight loss, mice typically regain their starting weight about 10 days after they wereinfected. Some of the substances we administer to alter gene expression can cause transient diarrhoea, typically less than 48 hours, after which the weight loss caused by this will be resolved.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

- Mice
- 10% subthreshold
- 25% Mild Severity
- 65% 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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The lung is a very complex environment and we cannot accurately replicate the intricate networks of both resident and incoming cells as well as molecule interactions within a non-animal model cell culture system. We are unable to collect samples to adequately reflect disease severity and progression in patients as they are often severely ill as the sampling process is both invasive and highrisk and not clinically required. Understanding disease progression is a key aspect of our research andunfortunately, we cannot sample human lung from the same patient on multiple occasions. Therefore, only in an animal model system can we examine the sequential changes in lung physiology as progression from injury to inflammation and then repair and fibrosis occurs.

Which non-animal alternatives did you consider for use in this project?

We have investigated and considered a number of established and cutting-edge non-animal models todetermine if they could be utilized in our program of work to answer our aims. These include human tissue culture models which mimic aspects of the lung and immune systems such as: air-liquid interface epithelial cultures, organoid models and lung-on-a-chip.

Why were they not suitable?

Currently, these non animal culture systems can only use one or two immune and structural cell subtypes simultaneously rather than the dozens of structural and immune cell types that contribute to disease pathogenesis and repair. Within our group we have developed and made use of these systems to look at specific interactions between cells but unfortunately, these models cannot yet recapitulate the complex nature of the lung environment. Additionally, acquiring the human tissue needed for these culture models would require invasive procedures in very sick patients without clinicaljustification. Therefore we need to use animal models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used both statistical models, published data and experience gained from previous studies toestablish the minimum number of animals required to obtain outcomes in a given study.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We use our own experience of these models, published data and online tools eg NC3R's ExperimentalDesign Assistant to carefully design our experimental projects to ensure we use the lowest number of mice but still meet sufficient numbers to ensure statistical power to our data outputs.

Our experience of both lung scaring and infection models has allowed us to develop models which reduce harm to the mice we use by giving the lowest dose of scarring drugs or infectious agent whilestill producing the necessary effects needed for the experimental program.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Animals are only purchased or bred when a defined experimental plan with clear hypothesis, numbersand objectives has been established. Mouse numbers for individual experiments are refined based onprevious work within the license as well as published data. Breeding colonies will be managed in line with the best practice guidelines. Particular attention will be paid to genetic stability and good breedingperformance. Data from breeding animals are readily available from the in-house database and will beused to make decisions on future breeding animals and also to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

We maximise the data outputs that can be achieved from each mouse i.e. storing organs, blood and tissues for later analysis as well as on the day analysis e.g. flow cytometry. Where appropriate ‘control’or ‘mock-treated’ and ‘treated’ mice are shared between researchers and studies. We are pursuing high dimensional studies i.e. single cell sequencing or mass cytometry which have lower input numbers of mice than traditional flow cytometry or molecular biology 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 welfarecosts (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use the least invasive method of administering the agent which is by non-surgical intratracheal instillation under general anesthesia. There is no injury to any tissue during this procedure and the mouse recovers and is able to eat and drink after the brief anesthesia. We also treat the mice with painkilling drugs locally to the neck so that any discomfort is alleviated. This is a significant improvement over intra-tracheal administration via surgical incision of the skin and trachea. Each timewe administer lung scarring agents we also treat the mice with fast acting painkilling drugs injected under the skin of the neck so that any discomfort is alleviated.

In order to better mimic the multiple small inflammatory events that are hypothesised to drive humanfibrotic lung diseases can we opt to give lower amount of bleomycin more frequently to the mice – to effectively split the dose. By administering a lower amount of Bleomycin albeit more frequently, inflammation in the lung is lessened and the associated adverse events for example weight loss are lessened. The infection model uses very brief general anesthesia and the virus is given by inhalation of dropletsthrough the nose. We have substantial experience in working with influenza in mice and are able to give the lowest possible dose of virus to achieve the experimental outcomes, therefore the disease burden in mice is as low as possible. We also developed a symptom scoring system to monitor the disease course and allow us to intervene rapidly with additional care like easily reached and palatablefood.

Why can’t you use animals that are less sentient?

It is not appropriate to use a less sentient animal model because we need to have the ability to modelthe lungs rather than disease processes only, since the complex lung environment greatly impacts onthe differentiation and behavior of myeloid cells.

The mouse and human immune systems share major similarities, and mouse and human lungs have similar anatomy and components for example type 1 and 2 alveolar epithelial cells and bronchial epithelial cells lining a similar division of trachea and bronchi and the full complement of innate and adaptive immune cells and response allowing us to extrapolate findings. This would be impossible using lower protected animals (such as zebrafish) or non-protected animals (such as fruit fly), becauseboth their immune and respiratory systems are highly divergent from the one in humans. Mice need tobe used as adults to reflect the process of disease seen in humans where fibrotic lung diseases affectsolder adults.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

In consultation with trainers and vets we refined the anaesthetic regimen from injectable to inhalation for our lung fibrosis model. This offers the benefit of minimal animal handing, higher predictability, morerapid recovery and the flexibility to adjust anaesthetic depth. We also administer a painkiller under the skin of the neck to alleviate any discomfort each time we treat the mice with lung scarring agents. In addition, we provide soft diet at floor level after each procedure to improve access to food and water and have collaboration with

animal care staff to find the best method of delivering soft food post- procedure, examining options like gel packs and low-hanging soft food dispensers.

We also use the lowest severity of treatment required to achieve scientific outcome. By working collaboratively, during procedure we ensure constant monitoring of animals throughout the procedure and that the procedure is completed as quickly as possible to prevent potential adverse effects resulting from prolonged use of specialised equipment, as well as minimise the duration of anaesthesiaand its related side-effects.

We have developed a scoring system for daily monitoring of animals based on welfare assessment and weight which allows early intervention and rapid elimination of avoidable pain, suffering, distress and lasting harm for any animals whose behaviour deviates from the norm. Animals are killed immediately where there are clear indications of distress so that a humane endpoint of a procedure isapplied as early as possible.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will make use of the following published and online resourceswww.nc3rs.org.uk https://www.lasa.co.uk/ https://science.rspca.org.uk https://www.biologie.uni-konstanz.de/leist/caat-europe/http://altweb.jhsph.edu/

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

The establishment where we will conduct this work has a excellent programme to ensure researchers have an up-to-date knowledge of 3Rs best practice. A local dedicated 3Rs information officer maintainsa regular newsletter as well as holding yearly virtual and in-person 3Rs Research Days and Symposia.At least one member of our group will attend and disseminate key points from this symposium in our weekly lab meeting and all licence users have access to the 3Rs newsletter. The local Animal Welfare and Ethical Review Committee also examines both licences and activity at set points during the term ofthe licence and makes recommendations to the licence holders to improve adherence to 3R principles.We will also make use of online resources to ensure we follow 3Rs best practice including

www.nc3rs.org.uk https://science.rspca.org.uk https://www.biologie.uni-konstanz.de/leist/caat-europe/

IDENTIFICATION OF NEW THERAPEUTIC TARGETS AND INVESTIGATION OF BIOLOGICAL AND SYNTHETIC THERAPIES FOR ISCHAEMIC DISEASE

Identification of New Therapeutic Targets andInvestigation of Biological and Synthetic Therapies for Ischaemic 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, ortheir effects, in man, animals or plants

Key words

Cardiovascular Disease, Ischaemic Heart Disease, Lower Extremities Damage, Diabetes Mellitus andHypercholesterolemia, Therapeutic Angiogenesis

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 required,and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

This project is designed to better understand the causes of ischaemic disease, a reduction

in blood flow caused by the occlusion of major arteries by “atherosclerotic plaques” (e.g., the build-up of fats, cholesterol and other substances) and the death or misfunction of small blood vessels (also called “microvessels”): capillaries and arterioles. These conditions are particularly frequent in people affectedby diabetes mellitus and/or high cholesterol level in their blood (a condition which is called “hypercholesterolemia”). The ultimate goal of this research is to develop new therapies that are able tomeet the clinical needs of the patients who suffer from ischaemia in their heart or legs.

A retrospective assessment of these aims will be due by 21 September 2028

The PPL holder will be required to disclose**:**

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Arteries thickening or hardening by fat in the blood (this is called “atherosclerosis”) results in insufficient blood supply to tissue and organs. Current treatment for poor blood supply to the heart andlegs often consists of surgical interventions used to remove fat and material obstructing the vessel.

However, there are limitations because these treatments are not always possible and can often leave the patient with side effects and disabilities requiring additional interventions. Moreover, oxygen and blood deprivation to the heart and limb is not exclusively caused by an obstruction of one or more arteries. In fact, small vessel disease (called “INOCA”) can cause a type of oxygen and blood starvation in the heart that cannot be treated by surgeons or interventional cardiologist, and it can even cause a heart attack (this type of heart attack is called “MINOCA”). It is widely accepted that no other optimal cure is currently available for small vessel disease in the heart. In the lower leg, particularly in diabetic patients, small vessel disease causes ulcers which are difficult to heal and prone to infections and gangrene, often leading to the need for foot or leg amputation. These ulcers represent a very severe treat to the patients’ capacity to walk and overall wellbeing. Moreover, both the presence of leg ulcers and the foot or leg amputation are associated with increased mortality. It is estimated that almosthalf of the patients will die in the 5 years following a first-time leg, and the risk of death highly increases in patients with diabetes.

Treating insufficient blood supply in diabetic patients represents a big and unresolved challenge. Diabetes Mellitus represents one of the major threats to human health in the 21st century1, 2. DiabetesUK has recently reported that there are 3.9 million people living with diabetes in the UK. Around 700 people a day are diagnosed with diabetes. That's the equivalent of one person every two minutes.
According to the International Diabetes Federation, the number of patients with DM will further increase by 35% in the next 30 years. Cardiovascular disease is the leading cause of death in the diabetic population, where the prevalence of heart disease is responsible for two-thirds of deaths. The Framingham Heart Study, developed in the USA in the late ‘70s, was the first to show the increase of cardiovascular disease across all age groups for

individuals with diabetes compared with those withoutdiabetes. Diabetes promotes oxygen and blood deprivation to the heart muscle and limb by worsening arteries thickening and promoting small vessel disease. Moreover, diabetes disrupts the mechanism normally taking place after sudden blood deprivation to a tissue (heart, limb) and which consist of the formation of new small vessels in the attempt to restore blood flow.

Unfortunately, currently available anti-diabetic drugs are not able to avoid cardiovascular complicationsin diabetic patients. It is also worrying that diabetic patients have more adverse events and complications after the surgical interventions used to unblock the arteries to re-establish the circulationin the heart or legs.

Our studies will increase the option to treat blood and oxygen deprivation in the heart and lowerextremities by enhancing the formation of new vessels around the affected area. We will also investigate therapies to cure poor blood/oxygen supply in patients with diabetes and high bloodcholesterol level.

What outputs do you think you will see at the end of this project?

This project licence will contribute to global efforts aiming to provide a definitive treatment of ischaemicdisease in the heart and limb muscles. It will identify new therapeutic targets and test therapeutic approaches.

Our work will allow to:

Improve the understanding of the process that causes tissue ischaemia (i.e., insufficient blood perfusion). This will allow identification of new “therapeutic targets” (i.e., disease-causing molecules,whose activity can be modified by a therapeutic intervention).

Identify new therapeutic strategies to protect the blood vessels and regenerate them once theyare lost to ischaemic disease.

The expected outputs include: 1) publications of scientific articles in highly respected journals; 2) presentations at national and international conferences and workshops); 3) Knowledge gained from thetherapeutic experiments on animals described in this project have the potential to lay foundations for the treatment of diabetic patients suffering from blood vessel disease in the heart and legs; 4) Use of new methods and techniques to promote the 3Rs principle (replacement, refinement, reduction) in various areas of this project. For example, where possible we will test potential therapeutic strategies using cultured cell lines in the laboratory instead of whole animals (replacement), we will employ a technique recently described to experimentally induce heart attacks which unlike traditional methods does not require traumatic tissue incisions with potential blood loss (refinement), we have powerful statistical methods and assistance at our disposal to determine the minimum number of animals necessary to obtain meaningful results (reduction).

Who or what will benefit from these outputs, and how?
Progress in the areas described above will not only be of significant interest to the wider cardiovascularbasic science research community but could also provide animal data to initiate a “clinical translational pathway” and underpin part of the regulatory requirements for setting up clinical studies or trials.
Longer term benefits would be to render therapies developed in the laboratory accessible to patients with diabetes and heart or vascular complications, to improve their health-related quality of life, and tohave positive impacts on their long-term morbidity and

mortality.

How will you look to maximise the outputs of this work?

Several factors will ensure the maximal output of this project:

Collaboration within our multidisciplinary team, comprising highly skilled staff working with expertisein animal models, molecular and cellular biology, data analyses and clinical studies.

Collaboration between Institutions. Our Institution is a leading organisation nationally and internationally in medical research. We have optimal support and access to top-class facilities andexpertise which attract multiple collaborators.

Our capacity to integrate animal work with analyses on clinical samples, cell models and bioinformatic analyses will maximise the value of the animal data.

Being part of national and international consortia, which favour the exchange of information and thecontinuous training of staff and exposure to new concepts and discoveries, will enhance the dissemination of our work.

Dissemination of our research results via publications of scientific articles in highly respectedjournals and presentations at national and international conferences and workshops.

Species and numbers of animals expected to be used

- Mice: 14625
- 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.

Preliminary studies of cell functions will be carried out before engaging with animal studies. We will beusing adult mice and rats for these studies to reflect the average age of the human population usually affected by ischaemic disease in their heart and legs. Multiple studies on rats and mice have reproduced key features of ischaemic disease, diabetes and high levels of cholesterol in the blood.
Mice are particularly valuable because of the range of genetically modified animals available for this project. However, rats are larger in size and hence are preferable for studies where therapeutic substances engineered in the laboratory are implanted in the animal’s heart. Moreover, an individual rat produces about 10 time more material, which we can use for cell and molecular biology studies tocomplement the in vivo investigation.

Typically, what will be done to an animal used in your project?

The animals in our project are typically used as models of the human conditions of poor circulation (ischaemia) in the leg or heart. Working with these ischaemia models, we will

investigate the mechanisms underpinning the disease and test the therapeutic potential of substances, especially theircapacity to promote angiogenesis (the growth of new blood vessels) and improve the circulation.
Animals will develop ischaemia as consequence of diabetes and/or high blood cholesterol and/or willbe induced by a surgical intervention to occlude a major blood vessel which deliver the blood flow to the heart or a leg. We also have a separate protocol used to test the proangiogenic activity of substances in healthy mice.

Typical scenario 1:

A mouse will be microchipped and will progessively develop diabetes and/or high cholesterol blood level (caused by GAA, high fat diet feeding, 5 IP injections of low dose streptozotocin or one single IV injection with a Pcsk9). Before and after the onset of diabetes and/or high blood cholesterol level, bodyweight will be measured once per week and blood will be taken from a tail vein for blood tests (with minimal rest intervals of 7 days). After at least 1 month from onset of diabetes and/or high blood cholesterol, the mouse will receive superficial skin wounds (one per leg) and, at the same occasion, it will also receive a therapeutic proangiogenic treatment (typically by gene therapy or exosome therapy),via injection in the limb adductor and gastrocnemius muscle and/or by topical application on the woundarea. Next, the mouse will be submitted to weekly non-invasive colour laser analyses of the blood flow in the limb muscles and wound area. At the same occasion, when the mouse is still anaesthetised, the wound area will be measure with a caliper. The mouse will be finally perfusion/fixed for histology.
During the whole protocol, the mouse will be regularly checked for immediately recognisable adverseevents.

Typical scenario 2:

A mouse will be microchipped. Next, it will receive (at the same occasion) unilateral limb ischaemia surgery, superficial skin wounding (one per leg), a therapeutic proangiogenic treatment (typically by gene therapy or exosome therapy), via injection in the limb adductor and gastrocnemius muscle and/orby topical application on the wound area. Next, the mouse will be submitted to weekly non-invasive colour laser analyses of the blood flow in the limb muscles and wound area. At the same occasion, when the mouse is still anaesthetised, the wound area will be measure with a caliper. The mouse will be finally perfusion/fixed for histology. During the whole protocol, the mouse will be regularly checked for immediately recognisable adverse events.

Typical scenario 3:

A mouse will be microchipped and will progessively develop diabetes and/or high cholesterol blood level (caused by GAA, high fat diet feeding, 5 IP injections of low dose streptozotocin or one single IV injection with a Pcsk9). Before and after the onset of diabetes and/or high blood cholesterol level, bodyweight will be measured once per week and blood will be taken from a tail vein for blood tests (with minimal rest intervals of 7 days). After at least 1 month from onset of diabetes and/or high blood cholesterol, the mouse will receive (at the same occasion) unilateral limb ischaemia surgery, superficialskin wounding (one per leg), a therapeutic proangiogenic treatment (typically by gene therapy or exosome therapy), via injection in the limb adductor and gastrocnemius muscle and/or by topical application on the wound area. Next, the mouse will be submitted to weekly non-invasive colour laser analyses of the blood flow in the limb muscles and wound area. At the same occasion, when the mouse is still anaesthetised, the wound area will be measure with a caliper. The mouse will be finally perfusion/fixed for histology. During the whole

protocol, the mouse will be regularly checked for immediately recognisable adverse events.

Typical scenario 4:

The animal (either mouse or rat) will be microchipped and will progessively develop diabetes and/or high cholesterol blood level (caused by GAA, high fat diet feeding, 5 IP injections of low dose streptozotocin or one single IV injection with a Pcsk9). Before and after the onset of diabetes and/or high blood cholesterol level, body weight will be measured once per week and blood will be taken froma tail vein for blood tests (with minimal rest intervals of 7 days). After at least 1 month from onset of diabetes and/or high blood cholesterol, the animal will receive a therapeutic proangiogenic treatment (typically by gene therapy or exosome therapy), via IV injection. Next, the animal will be submitted to bi-weekly non-invasive echocardiographic analyses. The animal will be finally perfusion/fixed for histology. During the whole protocol, the animal will be regularly checked for immediately recognisableadverse events.

Typical scenario 5:

The animal (either mouse or rat) will be microchipped. The animal will experience a heart attack (eithertype 1 or type 2 myocardial infarct) and at the same occasion, it will receive a therapeutic proangiogenic treatment (typically by gene therapy or exosome therapy), via cardiac delivery. Next, the animal will be submitted to bi-weekly non-invasive echocardiographic analyses. The animal will be finally perfusion/fixed for histology. During the whole protocol, the animal will be regularly checked for immediately recognisable adverse events.

Typical scenario 6:

The animal (either mouse or rat) will be microchipped and will progessively develop diabetes and/or high cholesterol blood level (caused by GAA, high fat die feeding, 5 IP injections of low dose streptozotocin or one single IV injection with a Pcsk9). Before and after the onset of diabetes and/or high blood cholesterol level, body weight will be measured once per week and blood will be taken froma tail vein for blood tests (with minimal rest intervals of 7 days). After at least 1 month from onset of diabetes and/or high blood cholesterol, at the same occasion, the animal will experience a heart attack(either type 1 or type 2 myocardial infarct) and receive a therapeutic proangiogenic treatment (typicallyby gene therapy or exosome therapy), via cardiac delivery. Next, the animal will be submitted to bi- weekly non-invasive echocardiographic analyses. The animal will be finally perfusion/fixed for histology. During the whole protocol, the animal will be regularly checked for immediately recognisableadverse events.

Typical scenario 7:

A mouse will be microchipped and receive an sc injection of (cooled liquid) Matrigel containing a testsubstance or placebo. The mouse will then be monitored for adverse effects, but otherwise left undisturbedfor the remainder of the protocol (up to 4 weeks), when it will be perfusion-fixed for histological analyses.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects during the protocols to be used in the current programme may occur as

consequenceof the procedures employed to induce diabetes, heart attacks or damage to blood vessels in the lowerextremities, similar to those observed in patients with ischaemic disease.

We expect the diabetic animals will experience adverse effects after surgically induced heart or limbischaemia more often than the non-diabetic animals. We do expect mice and rats to have similar frequency and severity of adverse effects.

The most likely adverse effects to occur relate to changes in eating habits and body weight in diabeticanimals.

Animals will have poor circulation in the leg in the first week after induction of limb ischaemia (up to 80% animals) and temporary difficulty in ambulation in the first day thereafter. Some animals will have to be humanely killed in the first week after the operation to prevent development of foot necrosis following limb ischaemia surgery (up to 3% in non-diabetic mice and up to 10% in diabetic mice). Afterthis critical time window, the animals with limb ischaemia will progressively recover blood perfusion andmostly remain symptom-free until the end of the protocol.

Up to 30% of animals (both mice and rats) that will be induced with a severe heart attack (technically called “myocardial infarction” or “MI”) are expected to die during the duration of the protocols, due to the sum of the acute consequence of the injury and post-MI heart failure. The expected cumulative death is higher (up to 40%) when an heart attack is induced in animals with pre-existing diabetes and/or high cholesterol level. The animals can experience acute mortality in the first days after the MI operation. Moreover, symptoms of heart failure (difficulty breathing, reduced mobility, fatigue) might appear after 2 weeks, leading to prompt humane killing. Some animals will die when still anaesthetised and will therefore not experience any pain or stress. In our research program, the mortality rate after severe MI is high for mice and rats. The death rate in the animals is comparable to the human patients,where the severe heart attacks are frequent. In people, acute MI is associated with death in circa 1 outof 3 people and death often occur prior to arrival at the hospital. Approximately half of all patients are re-hospitalized within 1 year of their heart attack. Unfortunately, these numbers increase for the diabetic people.

Relevant foreseeable stresses also include the need for repeated anaesthesia maximum three times inthe week of the operation to induce heart or limb ischaemia and then at the maximal frequency of onceper week) for in vivo imaging and repeated intravenous blood drawing, up to the limits authorised and at weekly or longer intervals. Rarely, diabetic animals will develop severe forms of diabetes.

Unexpected adverse effects could also arise from the test substances and their delivery.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Mild or subthreshold: 54% mice, 5% ratsNon-recovery: 0.5% mice, 1% rats
Moderate: 40% mice, 68.5% rats

Severe: 5.5% mice, 25.5% rats

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 21 September 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animalswere affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our project integrates *in silico*, *in vitro* and *ex vivo* work with animal work.

The use of animals is an essential component for our research programme. Studying disease progression consecutive to heart attack or poor circulation of the lower extremities involves examining the intact heart and limbs and vasculature system in the whole animal, as both respond to the complexinterplay between mechanical stress, autonomic nervous system, vascular, endocrine and inflammatory systems.

Small Mammals are also essential to screen therapeutic candidates before proceeding to preclinicalwork in large animal models and clinical validation in first-in-human studies. Both the UK Medicines and Healthcare products Regulatory Agency (MHRA) and the USA Food and Drug Agency (FDA) require proof of efficacy in animal species for clinical trial approval.

Which non-animal alternatives did you consider for use in this project?

In our work we use different type of experiments and replacement has been possible in part of our work using cells cultured in dishes or using human tissue samples derived from biopsies from volunteering patients undergoing surgery. We also make use of publicly available data deposited byother scientists, which we process using computational algorithms.

Why were they not suitable?

Ischaemic disease develops progressively and is influenced by the environment, where metabolisms, hormones, immune and blood cells, the nervous system and the gut bacterial to name just a few all communicate with the blood vessels of the heart and limbs. Therapeutic angiogenesis is also dependent of the environment. It is not yet possible to recreate the complexity of this environment in adish or *in silico*. It is possible that our research could contribute to develop new knowledge progressively supporting the advancement of *in vitro* and mathematical modelling alternatives to work on living animals.

A retrospective assessment of replacement will be due by 21 September 2028

The PPL holder will be required to disclose**:**

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The projected number of animals reflects the number necessary to achieve the scientific objectives outlined in the programme of work described in this research programme and this has been informedby our previous experience and that of others.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We have made an effort in limiting the numbers of animals in each our research programmes by providing adequate statistical advice (i.e., power calculations to establish the minimal number of animals required per group and per experiment), and assistance in experimental design to all our personal licensees. We are continuously undertaking literature and web searches (Google, pubmed.com, nc3rs.org.uk) in an effort to find alternatives to any procedure that causes more thanmomentary pain or distress.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Use of pilot studies involving a small number of animals is an integral part of our experimental designand may be used for example to identify the safest, reasonable dosage of a novel compound (small scale dose/response study) to be used in subsequent larger studies. This approach will help reducingnumbers of animals used in this project.

Refining skills is a continuous process required to reduce variability from data collected in animals, thus skills precision help reducing number of animals in a study. With this aim we have actively engaged in several joint sessions with other experienced scientists from other groups in the department to optimise surgical techniques and imaging methods and share experience. Wheneverpossible, we will engage in visits to other Labs in the UK and overseas with excellent records on mouse/rat work.

Using the latest imaging technologies available to assess rodent cardiac and vasculature function (echocardiography, MRI techniques), by means of which multiple new key parameters can be measured, we have striven to maximize the information obtained from each animal during the same imaging study. These novel parameters show promise in the detection of subtle cardiovascular changes in rodents treated with new compounds, which would potentially be missed by conventionalimaging, this allows us to use fewer animals

than before to identify differences between treated and control groups.
From each animal, we will typically obtain more than one type of quantitative data. As example capillary density (by histology) will be obtained on heart and limb specimens collected from animalswhich have already undergone in vivo imaging

At the end of a study, we seek to acquire as many organ samples as possible from euthanisedanimals.

A retrospective assessment of reduction will be due by 21 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Everything will be done to attenuate pain, discomfort, infections and stress. We will adopt refined microsurgical and aseptic techniques to minimise the adverse effects of surgery. Precautions include pain killer administration, supplementary heat and mashed food at floor level post-operatively, and if post-operative recovery is slower than expected supplemented oxygen is provided. Animals displayingany of the following clinical signs — breathlessness, discolouration of the skin, reduced mobility, eyes or nose discharge, diarrhoea, darkening and ulceration spreading beyond the toes, difficulty walking —will be monitored closely and supportive measures may be implemented in conjunction with the local veterinary surgeon, and if there is no improvement within 24 hours, then the animal will be humanely killed. All animals will be carefully and individually monitored after surgery.

With the ambition to further refine our models and methods, we are updating our knowledge via interactions with other scientists based in the UK and overseas. We also reviewing the literature andthe nc3rs.org.uk website.

The Matrigel plug angiogesis model allows us to study the in vivo angiogenic effects of substances in a mild severity context. It consists of implanting in the animal flank a plug containing proteins or non-cancerous cells modified before the implantation. The Matrigel, which will solidify as a small plug following injection into the animals. The plug is not expected to grow over time. The plug will be colonised by blood vessels, which grow more or less as responses to the tested substances and will be explanted after the death of the mice to quantify the blood vessels growth. The study typically last 2-4 weeks. The animals are not expected to experience major adverse events. We do not expect the mice will experience pain, discomfort or limited mobility.

Refinement: as a difference from some other groups, we do not use cancer cells to stimulate angiogenesis. Immunocompromised mice (used when human cells are implanted in the Matrigel) willbe housed in a barrier environment, thereby minimising the likelihood of infection.

High blood cholesterol and diabetes models are created by feeding animals (mice and rats) with high fat diets, and/or using a drug (streptozotocin, STZ), which damages the pancreas cells responsible for insulin production, and/or by using animals with genetic alterations that predisposes them to the development of these diseases. High blood cholesterol and/or diabetes will progressively cause small vessel disease (microangiopathy) in several organs (heart, limb, kidneys) and reduce thecardiac function (cardiomyopathy).

Refinement: The studies have been designed to end before animals develop severe diabetes and became symptomatic for cardiac complications. After diabetes onset, animals will be checked daily forsign of sufferance, dermatitis, or obesity-induced impaired mobility in animals with type-2 diabetes mellitus (T2DM) and regularly screened to assess urine and blood glucose levels and to monitor the evolution of cardiac changes (by echocardiography). To model type-1 diabetes mellitus (T1DM), othergroups have used STZ, compound which at high doses immediately kills the insulin producing β cells in the pancreas. We will use a refined protocol based on repeated injections of STZ at a low dose to induce T1DM in mice and rats. Used according to this refined protocol, STZ elicits an immune and inflammatory reaction in the pancreas, which more progressively damage the β cells. This is better tolerated by the animal and importantly mimics better the insurgence of human T1DM.

Acute Limb ischaemia (LI) model consists of the surgical occlusion of the femoral artery in one leg and it can be associated with the induction of a wound on the calf of the same leg (to mimic the humancondition of critical LI). The procedures are developed in under aseptic conditions, keeping the mouse under general anaesthesia. Our plan is to surgically induce limb ischaemia (LI) in one leg by ligating the femoral artery while the other leg, which remains well perfused, is used as an internal control. Afterthat we will inflict one circular skin laceration (4-5 mm diameter) in both the left and the right lower leg at the calf muscle level, to subsequently assess and compare wound healing capacity in hindlimbs withLI in comparison with “internal controls”. Blood flow reduction observed in the immediate post- operative hours/days in the ischaemic leg will naturally improve and normalise within 5 weeks after surgery. The calf skin laceration in both legs is expected to completely heal within the first 5 weeks following injury. The LI and wound healing models will be developed in healthy animals (both wild type and genetically altered, GA) and in animals with high cholesterol level and diabetes (both wild type andGA). Animals with diabetes and/or high cholesterol blood level are expected to require longer to reachblood flow recovery and closure of the skin wound. To accelerate the angiogenesis (new blood vessel formation) responses and improve tissue perfusion, test substances might be delivered locally (injections in the ischaemic leg muscles and topical application to the wounds), or systemically, either during the operation or at different times points before or after it.

Refinement: We induce limb ischaemia in one leg, only. The other leg is used as internal control whenmeasuring the post-surgery blood flow recovery to the animal foot and the local angiogenesis responses. The operations takes course under the most refined protocols for anaesthesia and analgesia. Animals are inspected daily and will be promptly humanely killed if they have foot necrosis.Animals are also killed if the wound is infected

and does not respond to antibiotic treatment. When thelimb ischaemia model is developed on hypercholesterolemia and diabetic animals, the specific refinement strategies described above will be also apply.

The **Myocardial Ischaemia** with Coronary Occlusion model The 'heart attack model', also named myocardial infarction (MI) model is based on the ligation of one major coronary artery, which delivers the blood to the heart. This intervention results in tissue death (infarction) due to inadequate blood supply to the affected area, after a couple of weeks the infarcted zone is progressively replaced by a fibrotic scar. The surviving myocardium is starved of blood flow and is therefore ischaemic. Substancesmight be delivered locally to the infarct border (typically by direct injection with a needle and syringe, orby application of medicated patches/scaffolds on the heart surface). New blood vessel formation can be triggered this way to improve tissue perfusion. Animals can die early (usually within 3 days) after theoperation (by cardiac rupture or fatal arrhythmia) and develop heart failure thereafter (typically, after 2 weeks post-MI). The MI model will be developed in healthy animals (both wild type and GA) and in animals with high cholesterol level and diabetes (both wild type and GA). Animals with diabetes areexpected to experience more severe adverse events.

Refinement: The MI model of coronary ligation is well validated, refined and already used by many experts in the field. During the course of this project, we aim to further refine the model by importing anew advanced technique recently published by another group. This new approach allows to occlude the coronary artery in mice without resorting to traumatic tissue incisions and open chest surgery, hence it proved to be less invasive and deadly.

Alternatively, we will induce MI by giving the animal a high dose of a drug (β-adrenoceptor agonist (such as isoproterenol – ISO or isoprenalin), which increases the heart rate. is also Upon ISO administration, ischaemia occurs due to the imbalance between cardiac stimulation and decreased coronary blood flow. This model, which is also called "type 2 MI" or "MINOCA" (Myocardial Ischaemiawith Nonobstructive Coronary Arteries).

Refinement: This model has been used for decades by many groups and is currently already used inour department. We are not planning to further refine it during the course of this project.

The animal models that will be used in studies carried over for over several weeks during which we willuse imaging methods at regular intervals to measure how the blood vessels in the heart and extremities function and grow, in response to the tested substances and the overall impact on the heartfunction and wound healing. Imaging will be done under anaesthesia from which the animal will recover.

The Applicant of this animal licence is already experienced with the animal models and methods included in this project, has used them in published scientific paper, and has been awarded research grants employing these models. The establishment staff are also experienced with the models (with exception of acute limb ischaemia). The models and methods proposed in this project have been usedfor many years by many groups, which have contributed to their progressive refinement. This has supported the capacity to prevent adverse effect and reduce the pain, suffering and distress caused byunavoidable adverse effects on the animals.

Why can't you use animals that are less sentient?

We propose to use mice since they are considered the least sentient mammalian species

with genes that can be readily modified to alter expressions of key molecules, allowing for proof-of-principle studies of the heart and blood vessels. In addition, they represent the lowest group of mammals with acardiovascular system similar to man, and for which there are good models of heart attack and lower extremity arterial disease. In a few experiments, we will use rats because their larger size facilitates some surgical procedures and allows for the extraction or more biological material to be used for ex- vivo and in vitro studies. For example, in the 'living myocardial slice' ex-vivo assay rats are humanely killed and their heart collected and placed in a cold solution and sliced using a vibratome, to producedmyocardial slices. These tissue preparations are cultured up to several days and used to investigate fundamental biology questions and to conduct pharmacological studies.

Similar to recuperating patients in hospitals, animals recovering from surgery will be closely monitoredusing individual daily health sheets for signs of distress and heat therapy. Animals receiving injections will be monitored daily by the operator (who holds a personal HO licence -PIL- to perform the work onanimal approved in this research programme). Where problems arise, we shall consult the named animal care and welfare officer (NACWO) and veterinary surgeon and offer pain relief, treatment, or cull animals as appropriate.

Most animals used in this project will die at the end of a terminal procedure without suffering since killing takes place whilst the animal is under full anaesthesia. A small proportion of animals that underwent surgery with recovery will be immediately killed if they show signs of uncontrolled pain, or signs of suffering that are greater than minor and transient or in any way compromises normal behaviour. As with man, deaths is expected in some of the animals subjected to experimental heart attack. All animals that have not undergone a terminal procedure will be humanely killed at the end ofthe protocol.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Surgical procedures on animals are performed on Mondays–Thursdays to allow for adequate monitoring in the subsequent 24-48 hr. We only perform surgery before 3pm to allow sufficient time foranimals to recover and be monitored and assessed before being returned to their holding room. Goodaseptic methods are in place to prevent bacterial infection of animals during and after surgery. Proper anaesthetic (Isoflurane) is used to avoid potential pain and distress during surgery. Mice are lying on aheated pad throughout surgery to prevent heat loss from being anaesthetised. Mice receive appropriate painkiller before surgery and for 24hr or longer if needed to reduce pain. Saline is given atthe end of the operation (through one injection under the skin) to counter risk of dehydration. Mice canrecover in a heated chamber following procedures under general anaesthesia (surgery), and their health/well-being is periodically checked after recovery. Mashed food is placed in the cage at floor level; food pellets and water are provided. Heat therapy is provided whenever necessary to improve recovery. Mice are checked daily during key periods, such as the first week post-operatively

Diabetic, hypercholesteraemic, obese mice used in this project are closely monitored through their lifetime. They are weighed once a week and require frequent monitoring for movements, behaviour, water and food consumption. At some point they will require more frequent cage change than normalmice and water addition, since moderate diabetes causes frequent urination and increases water consumption. Skin condition will be inspected regularly since obesity causes skin problems (rashes, infection, etc.).

Some animals may have an altered immune system making them more susceptible to infection. Animals with altered immune status will be housed in a barrier environment thereby minimising thelikelihood of compromising health.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

The conduct of animal experiments is for the most part guided by the LASA, PREPARE, and 2019ARRIVE guidelines, and information found on the NC3Rs website.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will constantly review our protocols and experimental design to reduce, refine and replace the use of animals in conjunction with the support of experienced NACWOs and the named veterinary surgeon (NVS). Additional support from the facility consists of dissemination/organisation of relevant event and conferences (e.g., 3R Advisory group, LASA or NC3Rs events, etc.). Training/assessments on the newest most refined techniques will be provided by the named training and competency officer (NTCO)and NVS. We will attend our quarterly animal facility's operation committee meeting which provides a forum for discussion on all matters related to animal welfare, care and use on the campus. We will keep informed about any advances by reading scientific publications and speaking with colleagues within the College and elsewhere.

A retrospective assessment of refinement will be due by 21 September 2028

The PPL holder will be required to disclose**:**

- With the knowledge you have now, could the choice of animals or model(s) used be improved forfuture work of this kind? During the project, how did you minimise harm to the animals?

DEVELOPMENT OF BRAIN TUMOUR MODELS IN MICE FOR TUMORIGENICITY SCREENS AND PRE-CLINICAL THERAPEUTIC TRIALS.

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, ortheir 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 aimsmentioned in paragraph (b)

Key words

Medulloblastoma, Cancer, Therapy, Orthotopic

Animal types	Life stages
Mice	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required,and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

Develop, investigate, and treat brain tumours (cancer) in mice.

A retrospective assessment of these aims will be due by 21 September 2028

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Medulloblastoma is the most common malignant brain tumour of childhood with approximately 80 newcases diagnosed every year in the UK. Over the last 50 years, advances in standard treatments (i.e., surgical removal of tumour, radiotherapy, and chemotherapy) have led to long-term survival rates of approximately 70%. However, for survivors there is a burden of tumour and/or treatment related late- effects, with ~40% of long-term survivors suffering from ≥1 severe chronic medical problem.

Furthermore, we have identified biologically-defined groups of the disease which do not respond tocurrent therapies (e.g., MYC-driven medulloblastoma; < 10% survival). Moreover, medulloblastomacomes back (disease relapse) in 30% of patients and is usually fatal. Overall, medulloblastoma therefore accounts for a disproportionately high amount (~10%) of childhood cancer deaths.

This project will initially focus on developing new brain tumour mouse models of medulloblastoma. Wewill i) develop mouse models which mimic some of the known biological drivers of aggressive disease(e.g., MYC-driven medulloblastoma), ii) use a repertoire of brain tumour models to investigate additional candidate biological drivers of disease progression and relapse which we call tumorigenicity screens, and iii) use these findings to identify new treatments/combinations of treatments to be tested in our medulloblastoma mouse models. New treatment approaches will need to be tested in appropriate mouse models to understand whether they firstly cause harm (are toxic), and secondly arebeneficial i.e., are better at treating the tumour than our current therapies. These trials will deliver the essential evidence to progress these treatments into human clinical trials.

What outputs do you think you will see at the end of this project?

Development of a team at the establishment with a new suite of technical skills in animal work that willbe supportive of collaborations and can be shared globally with partners.

Development of the first medulloblastoma mouse models at the establishment, including potentiallynovel models and tumorigenicity screening systems.

Identification and/or evaluation of specific targeted treatments in these novel models.

Dissemination of pre-clinical information through national and international working groups, conferencepresentations and open publications.

Who or what will benefit from these outputs, and how?

In the short-medium term, the outputs will initially benefit the local scientific community at

the establishment, followed by collaborators and the wider childhood cancer community. Ultimately, the outputs will provide key information for the development of future early-phase clinical trials in children, which will be supported through the already established national and international networks of the applicant. Longer-term this project will provide the platform for routine development of brain tumour mouse models at the establishment including novel models from patient-derived tissue (human cancer cells which are taken directly from the patient tumour). This in turn will support the international effort todevelop suitable, workable mouse models for all childhood brain tumours.

How will you look to maximise the outputs of this work?

I will continue to expand my collaborative networks in the field of both childhood brain tumour researchand mouse model development. Where appropriate early resources and findings will be made available to the wider research team and establishment through local group and institute research-in- progress meetings. Similarly, findings and resources will be shared with collaborators actively engaged with the project. This early sharing will maximise the impact of outputs, and aid in experimental refinement and validation.

Validated outputs (including negative findings) from this project will be disseminated to the scientific community through national and international working groups, conference presentations and publication in open access journals. Publication of novel discoveries will be announced, supported by the establishment's press office, to disseminate findings more widely and broadcast via social media. Activities to entice a varied audience will also be undertaken. This will include providing written piecesfor non-academic audiences, and I will also utilise additional outreach platforms to maximise the impact of this research.

Species and numbers of animals expected to be used

- Mice: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have been chosen for this project as the least sentient being that these models can be developedin. Mice importantly model the supporting tissue surrounding the tumour (tumour microenvironment), blood vessel development, and metastatic disease spread, all of which is observed and important in understanding the human disease. In treatment trials, mice will also display treatment side-effects which will provide vital information for selecting new treatments. Furthermore, mice can be genetically modified as appropriate and immunocompromised strains (mice without a working immune system) willbe used as they will be more likely to accept human cancer cells which are taken directly from the patient tumour (known as patient-derived samples). The age of the species has been selected to mostrepresent the age at which childhood tumours typically develop in humans and is also in line with collaborative experience and expertise within the field.

Typically, what will be done to an animal used in your project?

A mouse undergoing this study will be obtained from collaborative breeding programmes,

breeding programmes at the establishment, or purchased commercially. Mice will be group housed in individually ventilated cages, unless needing to be singly housed for welfare reasons, and maintainedin appropriate biosecurity conditions depending on the mouse strain. Their housing conditions will match those within the code of practice (e.g., enriched environment). The animals will be handled using a low-stress techniques (i.e., tunnel handling).

Subcutaneous model development

We will develop subcutaneous (tumour cells injected into the fat under the skin) mouse models as an adjunct to our orthotopic animal models (tumour cells injected into the relevant part of the body i.e., thebrain). Subcutaneous models will be particularly useful for testing new treatments that may not initiallyget into the brain and thus orthotopic models will not be helpful to understand whether these new treatments are beneficial and worth developing further so that they can ultimately be delivered to the brain.

Animals will be shaved and experience tumour cell injection into the flank into the fat under the skin (subcutaneously). Animals may require a general anaesthetic for this procedure. Animals will make afull recovery following this procedure and will be closely monitored using appropriate injection site inspection, tumour measurements with callipers, and where required non-invasive imaging under general anaesthetic. Animals will only experience tumour cell injection once. The animals will beterminally euthanised at an appropriate time point using humane methods.

Orthotopic model development

Animals will experience a surgical procedure under general anaesthetic, whereby they will have a small part of their head shaved, positioned in a surgical frame, a small hole made in their skull, andtumour cells injected through this hole into the relevant part of the body i.e., the brain (orthotopic). Animals will make a full recovery following this procedure and will be closely monitored using appropriate non-invasive imaging under general anaesthetic and scoring techniques for tumour development. Animals will only experience this surgical procedure once. The animals will be terminally euthanised at an appropriate time point using humane methods.

Treatment trials in tumour bearing mice (subcutaneous)

We will develop subcutaneous (tumour cells injected into the fat under the skin) mouse models as anadjunct to our orthotopic animal models (tumour cells injected into the relevant part of the brain).

Subcutaneous models will be particularly useful for testing new treatments that may not initially get intothe brain and thus orthotopic models will not be helpful to understand whether these new treatments are beneficial and worth developing further so that they can ultimately be delivered to the brain.

Animals will be shaved and experience tumour cell injection into the flank into the fat under the skin (subcutaneously). Animals may require a general anaesthetic for this procedure. Animals will make afull recovery following this procedure and will be closely monitored using appropriate injection site inspection, tumour measurements with callipers, and where required non-invasive imaging under general anaesthetic. Animals will only experience tumour cell injection once. Upon tumour development, animals will be entered into treatment trials. These will include, for example, the administration of a compound(s) or

control by intravenous (into the vein) injection, intraperitoneal (thespace around the abdominal organs) injection, subcutaneous (into the fat under the skin) injection, intramuscular (into the muscle and requiring a general anaesthetic) injection, gavage (direct administration into the gut using a tube) or via fluid/food alternation until an appropriate humane endpoint or a scientific purpose has been obtained. The administration method and frequency may vary depending on the compound. Animals will be terminally euthanised at an appropriate time pointusing humane methods.

Treatment trials in tumour bearing mice (orthotopic)

Animals will experience a surgical procedure under general anaesthetic, whereby they will have a small part of their head shaved, positioned in a surgical frame, a small hole made in their skull, and tumour cells injected through this hole into the relevant part of the body i.e., the brain (orthotopic). Animals will make a full recovery following this procedure and will be closely monitored using appropriate non-invasive imaging under general anaesthetic and scoring techniques for tumour development. Animals will only experience this surgical procedure once. Upon tumour development,animals will be entered into treatment trials. These will include, for example, the administration of a compound(s) or control by intravenous injection, intraperitoneal injection, subcutaneous injection, intramuscular injection (under general anaesthetic), gavage or via fluid/food alternation until an appropriate humane endpoint or a scientific purpose has been obtained. The administration method and frequency may vary depending on the compound. Animals will be terminally euthanised at anappropriate time point using humane methods.

Dose finding and toxicity studies in non-tumour bearing mice

Animals will be entered into dose finding and toxicity studies of novel compounds. Dose setting at a low dose in initially no more than three mice will be undertaken. If no toxicities are observed, a furtherthree mice may be tested at a higher dose and so on until an effective dose level is reached. If the initial dose produces evident toxicity, doses will be reduced. Compounds will be administered using intravenous injection, intraperitoneal injection, subcutaneous injection, intramuscular injection (under general anaesthetic), gavage or via fluid/food alternation. The administration method and frequency may vary depending on the compound. All animals will be monitored for weight, skin colouration, abdominal distention, hydration status and stool consistency a minimum of twice weekly. Animals willbe terminally euthanised at an appropriate time point using humane methods.

What are the expected impacts and/or adverse effects for the animals during your project?

Subcutaneous model development and treatment trials

Animals will be assessed for pain both before and after tumour cell injection and where appropriate given pain relief. Where appropriate animals will be anaesthetised, shaved and tumour cells injected. Following tumour cell injection, animals will be closely monitored using appropriate injection site inspection, tumour measurements using callipers, and where required imaging (under general anaesthetic) for tumour development and tumour response to treatment. During this we anticipate animals may show signs of weight loss, poor grooming, poor intake of food and water. Animals will be scored against a local scoresheet which will define the humane endpoints and guide appropriate welfare interventions such as soaked diet or a heated environment. Veterinary care will be called uponwhere appropriate.

Orthotopic model development and treatment trials

Animals will be assessed for pain both before and after surgery, given pain relief during surgery beforetumour cell injection (perioperatively), and where appropriate given pain relief following surgery for as long as deemed necessary. Following recovery from surgery, animals will be monitored using imagingtechniques (under general anaesthetic) and scoring for tumour development and tumour response to treatment. During this we anticipate animals may show signs of weight loss, poor grooming, loss of balance, poor intake of food and water. Animals will be scored against a local scoresheet which will define the humane endpoints and guide appropriate welfare interventions such as soaked diet or a heated environment. Veterinary care will be called upon where appropriate.

Dose finding and toxicity studies in non-tumour bearing mice

All animals will be monitored for toxicities associated with novel compound(s). This will include monitoring for weight, skin colouration, abdominal distention, hydration status, and stool consistency aminimum of twice weekly. Animals will be scored against a local scoresheet which will define the humane endpoints and guide appropriate welfare interventions such as soaked diet or a heated environment. Veterinary care will be called upon where appropriate.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Subcutaneous injections, tumorigenicity screens and treatment trials
100% of the animals are going to experience a moderate severity category.Orthotopic injections, tumorigenicity screens and treatment trials
100% of the animals are going to experience a moderate severity category. An unknown percentage ofanimals may experience a severe severity category.

Dose finding and toxicity studies in non-tumour bearing mice

Up to 30% of the animals may experience a moderate severity category.

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 21 September 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animalswere affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you

have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals (mice) support the development of these brain tumour models and subsequent treatment trialsbecause they have an appropriate tumour microenvironment (the tissue surrounding and supporting tumour development), model blood vessels development, metastatic disease spread, and exhibit similar treatment side-effects to what we would observe in humans.

Which non-animal alternatives did you consider for use in this project?

2D cell lines (tumour cells that grow indefinitely as two-dimensional cells on a flat surface in a petri dishor flask under laboratory conditions).

3D cell lines (tumour cells that grow indefinitely as three-dimensional clusters of cells in a petri dish orflask under laboratory conditions).

Why were they not suitable?

2D cell lines have been and will continue to be utilised to shortlist appropriate compounds for testing inanimals. Animal tests are required to assess compound side-effects and tumour response. 2D cell lines do not grow in a representative tumour microenvironment, they do not model blood vessel development, metastatic disease spread, or exhibit treatment side-effects when compared to tumours in a mouse model. Therefore, cell line responses to treatments are not always reliable or reproducible in human clinical trials. 3D cell lines in medulloblastoma are yet to be developed in the establishment but are an aspiration of the applicant and wider research team. Internationally, efforts to develop 3D medulloblastoma cell cultures which more reliably represent the tumour microenvironment remain challenging. Furthermore,3D cultures still do not model blood vessel development, metastatic disease spread, or treatment side-effects in the way that mice models do.

A retrospective assessment of replacement will be due by 21 September 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For the establishment of new medulloblastoma brain tumour models (orthotopic or subcutaneous),three mice will be injected per medulloblastoma tumour type.

Upon establishment and characterisation of each model, appropriate models will be selected for use, for example in our pre-clinical treatment trials. We have defined that for most treatment trial groups wewill require five animals per treatment arm with appropriate controls for both drug and animal. This is based on previously published data and our local experience and will provide results that are scientifically significant. Upon calculating the number of animals required a total number of animals to complete our pre-clinical treatment trials will be injected with tumour cells. For our dose findings and toxicity studies, three animals per dosing level will be required and we anticipate that no more than five dosing levels will be required per novel compound. This is again based on previously published data, our local experience, and will provide results that are scientificallyrelevant.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

For model development, we will predominantly be using immunocompromised mice (animals without aworking immune system). These strains of mice have been selected as they are most likely to accept tumour tissue from another species, e.g., human, and therefore this will increase our chances of success and reduce the number of mice required for model development.

All treatment will first be tested in cell lines before being short-listed for our mouse treatment trials. Where possible treatment doses for mice will be obtained from previously published studies or workundertaken by our collaborators to reduce the number of dose finding studies required in mice.

Both sexes of mice will be used. Results for both sexes will be combined and analysed together, butsex will be assessed as a confounding factor. Only significant differences attributable to sex will be accounted for in future study designs.

Longer term I aspire to develop 3D cell cultures to better represent the tumour microenvironment, andthus reduce the number of animals required in future experiments.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We will obtain mice from either approved suppliers or internal/external collaborators. This will reduce the number of animals required as we will be using efficient breeding programmes and will only requestthe exact number of animals required. This will also support reproducibility and reduce any effects of genetic drift (small genetic changes that get passed on to subsequent generations of mice).

Our initial medulloblastoma model development will be undertaken in small numbers of mice to characterise each model and aid future selection of the most appropriate model(s) to take forward, for example, into treatment trials. Furthermore, our initial work with patient-derived tumours (i.e., cells taken directly from the human tumour and grown in mice) will be first undertaken in established patient-derived samples that already reliably grow in mice. This will reduce the initial number of mice required to develop these models in our hands.

Where possible treatment doses for mice will be obtained from previously published studies or work undertaken by our collaborators. Where this data is not available, we will use the minimal number ofmice possible (e.g., three mice per dosing level with an estimated maximum of five dosing levels) to establish the maximal tolerated dose of any

novel compound.

Upon completion of our treatment trials, within the childhood brain tumour research community, it is now best practice when investigating new treatment approaches, to validate any findings in a secondappropriate mouse model which may be undertaken in a separate institution. We will follow this guidance where appropriate and work with our collaborators and international networks to prioritiseresults, and future translation into human clinical trials.

A retrospective assessment of reduction will be due by 21 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animals transported to the establishment will have a period of habituation (7-10 days). Animals will be handled using low-stress techniques (i.e., tunnel handling). We will have appropriate scoring and behavioural assessments in place to detect treatment toxicities, pain and response to pain relief whererequired. We will have tight humane endpoints in place to minimise suffering for the mice, for examplein those mice that develop orthotopic brain tumours, this includes regular imaging of mice to accuratelyassess both the size and growth rate of the tumours. Furthermore, those animals undergoing treatment are likely to experience a prolonged life and tumour reduction.

We will use regular inspection (subcutaneous tumours) and imaging techniques (subcutaneous and orthotopic tumours) to monitor for early tumour development alongside scoresheets to monitor animalwelfare. These scoresheets will be regularly reviewed and where appropriate refined to provide the most accurate assessment of animal welfare.

Previous in-house and collaborative experience has shown that for the injection of tumour cells subcutaneously, a 1:1 mixture of matrigel:tumour cell prevents tumour cells from dissipating in the flank, enabling more reliable tumour development and a reduction in engraftment failure. We will observe other refinements such as avoiding needle reuse, and those acquired from our collaborators. These will include the use of a surgical needle as opposed to a microdrill to create burr holes in the skull, this will minimise the damage to normal brain tissue. Tumour cell injection sites will be shaved which will improve asepsis and reduce the risk of infection. We will use dissolvable sutures for surgicalsites which will reduce the need for animal handling and further procedures to remove the sutures.

Why can't you use animals that are less sentient?

Less sentient beings such as zebra fish and nematodes (roundworm or threadworm), are not suitablealternatives to mice. They are not routinely established for use in the field of childhood brain tumour research. Furthermore, they do not mimic the tumour microenvironment, blood vessel development,metastatic disease spread, or treatment side-effects in the same way that mice models do.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

All animals will receive appropriate peri-operative pain management. Animals will be housed in an enriched environment and handled using low-stress techniques (i.e., tunnel handling). Any animal thatdemonstrates vocalisation or sensitivity during treatment will be assessed by a NACWO/vet before continuing treatment. Where appropriate we will use the most refined/least invasive method of compound administration while gaining scientific results. We will refine our surgical techniques and models by working with the named veterinary surgeon (NVS) to improve asepsis and use the most up-to-date anaesthesia and pain management protocols.

During the development of our medulloblastoma brain tumour models all new PILs to the technique willundergo a period of cadaver training supervised by local and/or external experts. We have already refined our surgical technique through the use of a surgical needle, as opposed to a microdrill to make our burr holes. Use of imaging is also a refinement for better monitoring of tumour establishment and growth rate. Use of scoresheets has further refined our techniques for the monitoring of mice.

Where required we will include pilot studies to refine, establish and select appropriate tumour models to take forward into tumorigenicity screens and treatment trials. Longer term when we are looking to develop new in-house patient-derived models, where appropriate, we will undertake pilot studies to determine the minimal number tumour cells required to develop a brain tumour, thus reducing the post-surgical pain and/or inflammation experienced by the mice. During the length of this project, we will continue to work closely with our established collaborators to introduce new refinements where appropriate.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Alongside the guidelines listed below, I will also adhere to local AWERB standards for research animals, and where appropriate support the development of new standards for refinements discoveredduring the project licence.

Code of practice for housing and care of animals bred, supplied or used for scientific purposesLASA guidelines
RSPCA Animals in science guidelinesUFAW publications
NC3Rs and procedures with care website

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

The local Animal Welfare Ethical Review Body (AWERB) named information officer, named animal careand welfare officer (NACWO), named training and competency officer (NTCO)

and veterinary team regularly inform and disseminate improvements and recent studies involving replacement, reduction, and refinement (3Rs). During the 1-, 3- and 5-year review of the project licence, I will update on implementation and/or the consideration of the 3Rs that has occurred during the previous period, alongside a review of the linked training plan, scoresheets etc. with a particular focus on refinement.

Alongside this review process I will also encourage my team to explore new ways to observe the 3Rs,supporting small grant applications for example to advance this work.

A retrospective assessment of refinement will be due by 21 September 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved forfuture work of this kind? During the project, how did you minimise harm to the animals?

Home Office

SAFETY TESTING OF MEDICINAL PRODUCTS USING NON-HUMAN PRIMATES

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 aimsmentioned in paragraph (b)

Key words

Non Human Primate, Regulatory, Safety Assessment

Animal types	Life stages
Cynomolgus macaques	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required,and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Uses non-human primates

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

This project licence authorises the conduct of studies in laboratory non-human primates (NHP) toevaluate the safety, quality and effectiveness of medicinal products for the avoidance, prevention,diagnosis or treatment of debilitating or potentially life-threatening conditions in man, in terms of general toxicity and whole body system exposure.

A retrospective assessment of these aims will be due by 22 September 2028

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 medicines have the potential to benefit in new or improved disease treatments. Before potential new medicines are administered to humans their safety must be evaluated. This testing is a mandatory legal requirement and provides information on risks to people taking new medicines. Often,the new medicines we test on this programme will be highly specific for a molecular target or receptor, which often make them less likely to have side effects than traditional medicines.

The test substances examined under this licence may include: chemical pharmaceuticals, biological pharmaceutical products derived from biotechnology (such as; gene therapies and monoclonal antibodies, stem cells and other therapeutic cell lines, cellular fractions, vaccines, serums and allergens). Substances with potential medical utility other than therapy (e.g. diagnostic imaging agents,liposomal encapsulation, nanoparticle carriers) and substances associated with drug candidates (e.g. metabolites, impurities, excipients, degradation products, placebos and/or novel vehicles for clinical trials) may also be investigated under this project licence.

The primary aims of this project are to support the development of these medicinal products through acquisition of data to 1) Support selection of new candidate molecules for further evaluation and development. 2) Demonstrate the safety-hazard profile of a new medicinal product prior to the initiationof clinical trials involving humans 3) Demonstrate the hazard profile of a medicinal product, in order to meet the regulatory requirements for marketing authorisation. Further aims include validation of new experimental conditions including the collection of blood/tissues to support drug development and thevalidation of non-animal alternative methodology.

These studies are run to satisfy the requirements of UK/EU (and sometimes international regulatory authorities) who are independent of governments) which require the testing of pharmaceuticals in a non-rodent species. At present there are no alternatives that don’t use animals that are scientifically, ethically or legally acceptable as replacements for systemic toxicity assessment. In addition, we onlyuse NHPs when no other species is suitable based on the nature of the drug (so if a drug can be tested in say a rodent, dog or a pig, and we would expect an equal outcome to the study then we would use them instead of a primate). These studies are performed prior to potential new drugs advancing into human clinical trials.

Primates will only be used where scientific justification shows that the purpose of the programme ofwork cannot be achieved by the use of animals that are not primates.

What outputs do you think you will see at the end of this project?

The overall benefit of this project is that it supports the development of safe, new medicines to improvethe health and quality of life of human patients by generating high quality data that is acceptable to regulatory authorities and enables internal decision making within our client's organisations.
Achievement of the objectives of this licence will enable safe development candidates to progress andwill also help to remove unsuitable candidates from the development pipeline

at an early stage, thus saving animals and resources.

Study reports will be included in regulatory submissions to allow regulatory authorities to make judgements on whether to permit clinical studies or to licence a drug. Global guidelines recognise thatthe justification for animal-based regulatory toxicology and safety testing is the need for regulatory authorities to have sufficient information to assess the risks to which humans are exposed to by new 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 drugsthat help alleviate human conditions. These new drugs may work better in the clinic, relieve or curediseases 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 developfurther new drugs.

One of the key benefits is the production of data that is required by regulatory authorities, to ensuremedicines can be dosed safely to humans. These drugs that will be tested are for debilitating or lifethreatening human 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 anew drug, and find a dose that causes no 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 drugsinto 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 usedfor, or to support, regulatory purposes (e.g. to show that a certain chemical is safe for human exposure).

Where appropriate, we collaborate with our customers to share data we have produced in the form ofScientific publications that are in the public domain.

We are able to advise our customers on which studies are required in their development programme and on suitable study designs, based on our experience and on knowledge gained from previous post-registration feedback from customers and/or regulators, leading to focussed 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 toconfidentiality issues. However, this work will contribute to the safety of chemicals that the public and animals are exposed to.

Species and numbers of animals expected to be used

- Cynomolgus macaques: 5300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using adult non-human primates on these studies. We only use non-human primates as a non-rodent species, when other non-rodent species (like dogs and/or pigs) are unsuitable to get the answers we need from the studies.

This is often because for the type of pharmaceuticals we are testing (for example 'biologics', peptidesor antibodies) we can only see any toxic effects if we use primates, maybe because the biological target of the drug is only present in a primate, or maybe because the requirements of the study meanwe can only use primates to get the results we need that will satisfy global regulatory bodies.

We are not allowed to use primates by law unless there is no other animal we can use that will give usthe results we need to satisfy the regulatory authorities.

Typically, what will be done to an animal used in your project?

Animals are dosed by the intended/likely route of human or animal exposure (for example oral administration, injection, infusion or inhalation), and observed regularly to monitor appearance,behaviour and clinical health.

Some animals may undergo a surgical procedure under general anaesthesia, eg placement of a deep vein catheter for intravenous infusion, or implantation of a monitoring device or minipump. Investigativeprocedures carried out in these studies are similar to diagnostic procedures that might be used medically to monitor progress of a human patient and include, for example, collection of blood and urine samples for laboratory investigations, or ECG monitoring to assess heart rate/function, or examination of the eyes using an instrument similar to those used by opticians. Animals undergoing surgery receive the same sort of care as a patient would in hospital. We discuss their pain relief and use of antibiotics with a veterinary surgeon before we start. We administer drugs as necessary and give them plenty of time to recover from surgery before we use them in experiments. These surgical procedures are carried out only for essential purposes.

Typically, on this project, animals are dosed over a period of time with test substances, and usually sampled (e.g. blood or urine) before having tissues taken after they have been humanely killed for extensive toxicology analysis. Studies would range from a single dose, to repeat-dose studies whichcan last up to 1, 3 or rarely 12 months. Study durations are dependent on the specific regulatory testbeing performed. Some animals are left dose free for a few weeks after dosing is complete to see if any effects of the test substances can be reversed.

Dosing of animals is commonly done orally using a flexible tube or by capsule. Other common routesinclude by injection using a syringe and needle, maybe directly into a vein or under the skin.

Blood samples are usually taken from easily accessible veins. We are limited to how much

blood wecan take at once or, cumulatively, over a month. If we need a large blood sample, we would do this when the animal is anaesthetised and we would not let them recover consciousness.

Where possible, we try to take as many of the tissues and samples we need after the animals havebeen humanely killed after all dosing had been completed.

If we need to take a urine sample for analysis, we would put an animal into a special collection cagewhich is smaller than their normal cage. The animal can still move around.

Other more unusual tests might include assessment of retinal function, assessment of neural function, taking small samples of tissue under general anaesthesia, collection/examination of body fluids such as tear fluid or semen, collection under general anaesthesia and examination of lung washings or spinal fluid, body temperature by rectal thermometer. A minimal degree of restraint or confinement maybe required for some procedures. Where appropriate, positive reinforcement training (using treat rewards) is used to encourage co-operation in (and minimise any stress of) handling/procedures.

Some animals may be used on procedure on more than one occasion (re-use); such re-use is limitedand strict criteria are applied, eg veterinary examination indicates that it is appropriate to do so. Mostanimals are humanely killed at the end of the study to allow detailed examination of the organs, something required by the regulatory authorities to evaluate if there have been any toxic effects on organs and tissues.

What are the expected impacts and/or adverse effects for the animals during your project?

When dosing an animal by injection or taking blood, the amount of pain an animal feels is similar to what a patient would feel having an injection done by a doctor. If we have to repeatedly inject animalsusing a needle and syringe, we would choose different sites to do this where possible. If we can take blood samples when an animal is deeply unconscious, then we do. If we need to take repeated bloodsamples or need to dose repeatedly then we try and use different sites. Of course, everyone who performs these procedures are trained to a high standard and hold a UK personal licence outlining their competency in the procedure.

Typically we can do this in the animal’s home cage though . Occasionally we may need to take a urinesample for analysis, so we would then put an animal into a special cage which is smaller than their normal cage. The animal can still move around however, and we'd normally introduce an animal to thiscage to acclimatise them to it. . Virtually every animal will get used to their new cage within about 15 minutes and are fine.

Generally, if we have to use any equipment to help us get the results we need, we acclimatise our animals to it so they get used to it and tolerate the procedure when we start dosing them. So, we carefully introduce them to things like restraint gradually, for short periods at first, and usually they accept it after a while. And if they don't acclimatise, we take them off the studies, to stop causing anyharm.

Dosing with pharmaceuticals may cause adverse effects in some studies. A percentage (~46% based on last project) may show transient subtle to mild clinical signs. Moderate signs of adverse effects maybe seen in some animals (~53% based on last project), usually in the higher dose groups. Lethality and/or severe effects are not study objectives in any of the protocols within this licence, and are not expected.

We observe our animals at least twice a day, and the people who do this know the signs when an animal is ill. If an animal is ill, we would check it more frequently, and consult vets and other senioranimal care staff for advice and guidance in its care.

Most animals are expected to experience no, or only mild, adverse effects during the course of the study such as slight weight loss. A small percentage of animals may show more significant adverse effects, such as more marked weight loss, reduced activity, vomiting or tremors. No animals would beexpected to die or to suffer prolonged adverse effects as a result of the procedures, and where necessary early humane end-points are applied, under veterinary guidance as necessary, to prevent this; such end-points might include interventions to discontinue dosing, or to provide supportive treatments, or if necessary to humanely kill the animal.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

On the last project, about 46% of animals experienced mild severity, and around 53% of animals wereclassified as having experienced moderate severity. The moderate severities in the last project were either due to treatment-related signs of moderate severity (mostly in preliminary studies) or because asurgical procedure, e.g. cannulation, was involved.

It's impossible to predict the proportion of severities expected on a service licence like this, as this will be dependent on what study types we are asked to perform, however, a distribution between ‘mild’ and‘moderate’ severities similar to those in the last project are anticipated.

All protocols on this licence are classified Mild or Moderate only, there is no intention to perform anyprocedures that are Severe in nature.

What will happen to animals at the end of this project?

- Killed
- Kept alive

A retrospective assessment of these predicted harms will be due by 22 September 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animalswere affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Pharmaceutical testing is a mandatory regulatory and legal requirement and provides information on risks to people taking new medicines. At present there are no alternatives that don't use animals that are scientifically, ethically or legally acceptable as replacements for systemic toxicity assessment. TheNHP (e.g. macaques) is a recognised non-rodent species suitable for toxicity studies, and is only usedwhen the dog or pig is shown to be unsuitable.

In vitro and in silico methods (test tube work not using animals and computational methods) are usedin combination with animal studies to inform study designs and assist in understanding of potential toxicity but cannot yet replace in vivo (animal) studies (except on some rare occasions) and only withapproval from global regulatory agencies.

We maintain a constant awareness of regulatory guidance and ensure that where non-invasive methods exist which fulfil the regulatory requirement, they are used in preference to animal studies.

The regulatory requirements for testing in the NHP are mainly for UK/EU regulators, but occasionallyother regulators in other countries like the US for example. If the requirements for these non-UK/EU tests are over and above the requirements for a UK/EU regulators, or the test required is more severe,then we consult the Home Office to ask for prospective authority to run such tests.

Which non-animal alternatives did you consider for use in this project?

There are currently no scientific and legally acceptable evaluations of systemic toxicity which will satisfy regulatory requirements and provide sufficient safety data other than use of animals, though validated in vitro tests for specific organs are used wherever possible. As new in vitro methods becomeavailable and achieve regulatory acceptance during the course of this project they will be validated andused to replace in vivo procedures. Where available, review of scientific articles, non-animal methods and other animal data such as metabolism and pharmacology information will be utilised to reduce animal use.

As a specially protected species, the non-human primate is selected for safety assessment studiesonly after careful determination that it is the most biologically appropriate species with relevance toman, and that there is no other acceptable candidate species that is not a primate.

Why were they not suitable?

Although there are test tube tests that can model some parts of 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 test tube tests that brings all these complex happenings together, like we see in animals and humans.

That's why we need to test the new drugs in animals, as they have similar physiology and processesas humans, and that testing gives us a good idea what may happen if they were ever tested in, or exposed to humans.

However, in all cases we will assess whether data already exists or can be generated in other ways other than the use of animals, and that we will ensure that animal reduction, replacement or refinementstrategies and alternatives provided in the regulatory guidance will be considered, and animal use avoided where possible.

A retrospective assessment of replacement will be due by 22 September 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received from customers in the past. It is, however, impossible to accurately predict the number of studies that may be performed, inthe circumstances.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Studies are designed to provide maximal data and statistical power (where appropriate) from the minimum number of animals considering that it is better to increase the number of animals used toachieve the objective than to use too few animals and risk having to repeat the study.

For regulatory studies, guidelines require the number of groups and animals per group to be adequateto clearly demonstrate the presence or absence of an effect of the test substance; core study designs are based on international guidelines where these exist. Otherwise reference is made to standard study designs with input from the Department of Statistics, where appropriate, to identify the optimum number balancing the need to achieve study objectives while avoiding excessive animal use. These internal designs are reviewed and updated in line with changing external guidelines and internal refinements that either minimise numbers or reduce severity.

Whenever possible, common species of animals are used such that a large amount of controlbackground data is available. This reduces the need for large control groups.

What measures, apart from good experimental design, will you use to optimise the number ofanimals 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 thatin the same animal, rather than using separate ones, when possible.

Before our main studies, we use smaller groups of animals in exploratory studies to get an

idea of the doses we need to use for the main studies. These preliminary studies are important as they give us confidence that the doses we are using are correct prior to testing them in bigger groups of animals asrequired by global regulators.

A retrospective assessment of reduction will be due by 22 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use adult non-human primates (macaques). We only use non-human primates whenother species (rats, mice and other large animals like dogs and/or pigs) are unsuitable for scientific reasons.

The models we use are the least invasive procedures, for the least amount of time necessary to get the information we need. They are carried out using standard and recognised techniques by fully trained staff. We also have veterinary clinicians on hand for advice and on the occasions we have toanaesthetise the animals and for general advice on animal welfare.

If we have to repeatedly inject animals or withdraw blood using a needle and syringe, we would choosedifferent sites to do this where possible to minimise local adverse effects. Where appropriate we placetemporary 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.

For all surgical procedures pain relief will always be provided. Surgical procedures will be carried outaseptically and to at least the Home Office minimum standards for aseptic surgery, and in accordancewith the principles set out in the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017) (LASA is the Laboratory Animal Science Association). Basically any animals who undego surgery will get the same standard of care as a patient who needed surgery in hospital.

For situations involving restraint procedures (e.g. in a restraint device or in a metabolism cage) the animals are habituated to this equipment starting with short periods, then building up. Most animalshabituate fine to this equipment, but if they don't (rare) we remove them from the study.

Why can’t you use animals that are less sentient?

Non-human primates are only used when no other species is suitable to get the information we need.In fact, we have to prove that the primate is the only species that will give us the answer we need (instead of rodents or dogs or pigs) that will translate to the effect we would see in man.

Most of these studies require repeat dosing for days, weeks or months, to assess potential adverseeffects in man, so it is not practical to perform them under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Animal welfare is of utmost importance and Good Surgical Practice will be observed for any animal undergoing surgical procedures. Surgery will be conducted using aseptic techniques (to prevent infection) which meet at least the standards set out in the Home Office Minimum Standards for Aseptic Surgery. Before we start surgery, we agree with a veterinary surgeon what pain killers or antibiotics the animals need both before and after the surgery. When recovering from surgery, we give the animals extra heat and monitor them closely until they start behaving normally again. We then checkthem at least twice daily before they go on study.

During dosing and restraint, animals are constantly and closely watched for signs of distress.

All procedures are subject to ongoing assessment and technique improvement and we participate incross-company working parties on best practice. Animals are regularly reviewed for general health and a veterinary surgeon are on call at all times to assess and relieve any adverse events.

Refinements to improve the animals experience include but are not limited to group housing, environmental enrichment, including novel toys and foods, human interaction, acclimatisation and training to procedures, to move around the cage and to leave the cage voluntarily as required, forageopportunity and calming measures such as stroking/gentle talking are used to help animals have a better experience of restraint.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Regulatory guidelines (ICH)

Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies S3A(1994)

Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies (S3B) (1994)

Duration of Chronic Toxicity Testing in Animals (Rodent and Non Rodent Toxicity Testing) S4 (1998)Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals S6(R1) (2011)
Safety Pharmacology Studies For Human Pharmaceuticals S7A (2000)Immunotoxicity Studies for Human Pharmaceuticals S8 (2005) Nonclinical Evaluation for Anticancer Pharmaceuticals S9 (2009)
Guidance on Non-clinical Safety Studies for the Conduct of Human Clinical Trials and MarketingAuthorisation for Pharmaceuticals M3(R2) (2009)

OECD Guidelines

OECD 409 – Repeated Dose 90-Day Oral Toxicity in Non-Rodents (1998)OECD 417 – Toxicokinetics (2010)
D 452 – Chronic Toxicity Studies (2009)

Summary of Considerations in the Report from the OECD Expert Groups on Short Term and LongTerm Toxicology (2006)

Other guidelines

Notes for guidance on repeated dose toxicity. Committee for Proprietary Medicinal Products (CPMP),2010.

Guideline on the evaluation of control samples in non-clinical safety studies: checking for contamination with a test substance. Committee for Medicinal Products for Human Use (CHMP), 2005.

LASA/NC3Rs: Guidance on dose level selection for regulatory general toxicology studies forpharmaceuticals.

Notes for guidance on non-clinical local tolerance testing of medicinal products. CPMP, 2001.LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017) Diehl et al. A good practice guide to the administration of substances and removal of blood, includingroutes and volumes. Journal of Applied Toxicology: 21, 15-23 (2001)

Non-Rodent Selections in Pharmaceutical Toxicology (Smith & Trennery, ABPI ‘Points to consider’,2002)

Gad et al. Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routes inmultiple species with notes on methods to improve utility. International Journal of Toxicology: 1-84 (2016)

NC3Rs: Overview of 3Rs opportunities in drug discovery and development using non-human primates(Drug Discovery Today, 2017)

NC3Rs: Recommendations from a global cross-company data sharing initiative on the incorporation ofrecovery phase animals in safety assessment studies to support first-in-human clinical trials (Regulatory Toxicology & Pharmacology, 2014)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This will be achieved by regular discussions with our Named Information Officer, colleagues in AnimalTechnology, and by attending appropriate training courses and conferences, or getting feedback fromsuch events.

A retrospective assessment of refinement will be due by 22 September 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved forfuture work of this kind? During the project, how did you minimise harm to the animals?

Home Office

DYNAMICS IN TISSUE MECHANICS, GENE TRANSCRIPTION AND SIGNALLING DURING TISSUE FORMATION AND REGENERATION

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Tissue formation, Tissue dynamics, Tissue regeneration, Tissue mechanics, Regenerative medicine

Animal types	Life stages
Xenopus laevis	adult, embryo, neonate, juvenile
Xenopus tropicalis	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to uncover key mechanisms that drive the formation, regeneration and repairof complex tissues.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 primary goal in regenerative medicine is to identify and implement novel treatments aimed at improving our ability to regenerate injured, diseased, or aged tissues and organs. Because mammals exhibit poor regenerative capacities, there is an interest in understanding the regenerative mechanisms employed by organisms with higher regenerative capacity, such as planarians, Hydra, fishand amphibians, to help inform new

possible regenerative therapies in humans.

The work undertaken in this project will advance our understanding of the molecular and cellular processes that underpin regeneration and repair. In turn, this work will provide critical informationnecessary for the design and eventual implementation of novel therapies aimed at promoting regenerative healing in humans.

What outputs do you think you will see at the end of this project?

The primary output of this project will be a greater understanding of the cellular and molecular mechanisms of tissue formation, repair and regeneration. More specifically, how dynamics in tissue mechanics, gene transcription and signalling drives the formation and repair of tissues in vertebrate organisms. Ultimately these outputs of new knowledge will be published in high quality, peer reviewedand open access journals.

Who or what will benefit from these outputs, and how?

Short term: the research community, in particular scientists interested in tissue mechanics and gene transcription dynamics during development and regeneration will benefit from the scientific publicationsgenerated during this project.

Medium term: the tools and techniques developed during this project (for example mutant and transgenic lines, imaging techniques) will be available to the scientific community to be applied to newresearch questions

Long term: this work will generate new hypotheses to improve repair and regenerative capabilities thatcan be tested first in mammalian models such as mice and then be translated to humans.

How will you look to maximise the outputs of this work?

We will openly share our data with our collaborators and the scientific community through participation to meetings and conferences. All scientific results will be published in high quality, open access and peer reviewed journals in a timely manner. To avoid publication bias, we are committed to publishing allfindings from this project, both positive and negative.

Transcriptomics datasets generated during this project will be made publicly available by being deposited at the European Molecular Biology Laboratory - European Bioinformatics Institute (EMBL- EBI) repository. Software and scripts implementing workflows, algorithms, and macros will be availableon GitHub. Mutant Xenopus lines will be deposited at the European Xenopus Research Centre (EXRC,https://xenopusresource.org/).

Our research aims to understand the mechanisms that underpins regeneration in vertebrates with theview to translate these findings in species with poor regenerative abilities (i.e. mammals), it is critical that we communicate our findings to clinicians and the public in general. We will continue existing collaborations with clinicians and participate in public engagement events.

Species and numbers of animals expected to be used

- Xenopus laevis: 5000

- Xenopus tropicalis: 14500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The African clawed frog Xenopus was chosen for this study because of its low sentience (neuronalcomplexity) and because as a vertebrate, it shares many of the same genes and developmental pathways with humans. As tadpoles, Xenopus display great regenerative capabilities, being able toheal a wound without scarring or to regenerate a whole appendage like its tail.

Most of the experiments proposed in this project will be performed in early embryos or parts of embryos that can be cultured in vitro (explants) prior to the protected stage. These approaches allow us to investigate cellular/tissue mechanisms in the context of complex 3 Dimensional (3D) tissues while providing an excellent non-protected animal alternative to most in vivo studies. However, for thestudy of the formation and regeneration of complex tissues such as the spinal cord, it is necessary to perform some work in vivo in post-embryonic larval stage as it is not possible to recreate the full complexity of the tissue and its environment in culture.

Finally, we need to raise transgenic and mutant lines to adulthood to generate mutant embryos.

Typically, what will be done to an animal used in your project?

Most of the frogs used in this project will be used for breeding purposes. Typically, frogs will be injectedwith reproductive hormones that induce the maturation of oocytes and subsequent laying of eggs. Thisprocedure can be repeated multiple times over the adult lifespan, with appropriate rest between procedures, as adult frogs replenish their stock of gametes over time.

Embryos obtained from these crosses will often be injected with genetic material that can express afluorescent protein or compounds that prevent the expression of a particular genes. These embryoswill mainly be used at pre-larval stage. At post-larval stages, tadpoles will mainly be subjected to tailamputation or spinal cord transection. This is done under anaesthetic and whilst it will cause discomfort, tadpoles regrow a functional tail, or repair their spinal cord within 7 days without long- lasting consequences.

Occasionally, we will raise embryos with modifications in their genomes (inactivation of a gene orexpressing a transgene) to adulthood to generate genetically modified embryos. In this case, all animals will be carefully monitored to ensure that they are developing normally. Adult frogs will begenotyped by isolating genomic DNA from toe clippings or skin swabbing whichever is the most effective and humane. Frogs will then be identified either by their natural skin pigment pattern or microchipping.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of our protected animals lead healthy lives. It is anticipated that only transient minordiscomfort should occur in the adult animals during injection procedures but adverse reaction to the hormones is not expected.

Injected constructs may cause death or developmental abnormalities before tadpoles reach the protected stage. Harmful genetic alterations in embryos may be evident as altered morphology before free-feeding stage but some genetic alterations may result in a harmful phenotype later during development. Particular attention will be given to the morphology of tadpoles as well as their behaviour(for example ability to swim). Any larval tadpole showing signs deviating from normal development or behaviour will be euthanised immediately at pre-independent-feeding stage.

Any animal that will be raised to adulthood will be monitored at least 3x a week throughout their development to ensure that no deviation from normal development occurs. In particular, we will assesstheir ability to swim, their growth rate and any sign of ill-health (bloating, changes in activity level, skin discoloration). Any adult showing these signs will be euthanised immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Adult frogs will experience only mild procedures (injection of hormones, generation of transgenic lines).Post-feeding stage tadpoles subjected to spinal cord transection or tail amputation will experience a moderate level of pain. This will be the case for about 50% of the animals used under this projectlicence.

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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The production of frog embryos require that adult animals are induced to ovulate or mate via the injection of reproductive hormones, and this is a regulated procedure. However, to study the formationand regeneration of complex tissues and organs, it is necessary to perform some work in vivo, as it is not possible to recreate fully the complex environment of tissues in culture. Therefore, experiments where we are investigating the multi-tissue events responsible for tissue repair and regeneration will necessitate working with post-embryonic larvae stages. However, we have chosen to pursue this workon a “lower” vertebrate (i.e. Xenopus frogs) with lower neurophysiological sentience.

Which non-animal alternatives did you consider for use in this project?

We perform experiments using cell culture systems (for example neuronal or epithelial cell lines) for very specific purposes and we use them when appropriate. We also use cell lines for preliminary experiments (for example to test imaging probes) prior to using them in the Xenopus embryo, makingour animal experiments more focused and reducing animal usage.

Why were they not suitable?

We are investigating the cellular mechanisms taking place in complex 3D tissue environments, that include multiple cell types and interactions between various tissues. Currently, this complexity cannotbe accurately replicated using in vitro models. Furthermore, in vitro studies do not allow us to identifynew signalling events from unexpected sources and surrounding tissues.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissueand reuse.

How have you estimated the numbers of animals you will use?

The number of animals has been estimated based on previous licences covering our work. We havebased our assumption that we will use a similar number of animals as in the previous 5 years.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

In all our studies we aim at reducing animal numbers to a minimum by using the NC3R's ExperimentalDesign Assistant (https://www.nc3rs.org.uk/experimental-design-assistant-eda) web application and the PREPARE guidelines (https://norecopa.no/PREPARE). We will also consult with our in-house statistical service for advice during the course of this project. More specifically, we have applied to all our experiments very stringent methods to obtain statistically meaningful results with the minimum number of animals possible.

Where possible, we calculate the precise number of embryos per time point per experimental conditionbased on previous experience or published data. If these data are not available, we will perform low number pilot experiment to estimate the magnitude of change and the intrinsic variability of the data to determine sample size. These numbers will be updated as more recent and relevant data becomes available.

Data analysis will be conducted according to a pre-specified statistical analysis plan drawn up in conjunction with establishment-based statisticians. Important experimental results will be designed withbiological replicates and repeated and validated via an alternative follow-up experiment to minimise thelikelihood of spurious non-replicable results.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

To reduce the number of matings, we ensure that a maximum number of embryos are used. A singlemating produces 1000s of eggs, generating multiple experimental units (i.e. a single frog embryo thathas been subjected to a procedure) per conditions per mating. Experimental units not immediately used are banked (for example by fixing or freezing). We also co-ordinate between the different members of the group to share clutches of embryos to reduce the number of mating.

Recently, we have introduced means to identify individual frogs by microchipping (for Xenopus tropicalis) and by identification of pigment pattern via stored photographs for each individual (for Xenopus laevis). This allow us to ensure that animals are kept at the ideal density in each tank, for example by combining frogs from different genotypes. Furthermore, this allows us to monitor the healthand reproductive capabilities of each frog to prevent keeping animals longer than necessary.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use Xenopus laevis and tropicalis in our research. These are related species with distinct advantages: laevis embryos are bigger (about 1mm in diameter) making explant and grafting experiments much easier. However, the genome of Xenopus laevis is more complex and the generation time much longer that Xenopus tropicalis. Therefore, Xenopus tropicalis is much more suitable for genetic experiments such as generation of knockout or transgenic lines. Whilst zebrafish also has regenerative abilities), Xenopus has important advantages for our work: it is a tetrapod and therefore evolutionary closer to mammals, its genome (for tropicalis) is much closer to the mammalian genome, it is much easier to obtain explants, to perform graft experiments and Xenopus larvae are much more efficient at regenerating their tails. Amphibian embryos in general, and frog embryos more specifically, have been used to investigate the mechanisms responsible for tissue development for overa century. Indeed, much of what we currently know about how the vertebrate embryo develops has come from experiments initiated in frog embryos. Using an established experimental species reduces the use of animals, as one does not have to replicate accrued knowledge from other species.

Adult animals are used in this project for the purpose of generating embryos. The production of frog embryos require that adult animals are induced to ovulate or mate via the injection of reproductive hormones, and this is a regulated procedure. It is anticipated that only transient minor discomfort should occur in the adult animals during injection procedures but adverse reaction to the hormones isnot expected.

As the embryos develop ex utero, experiments and harvesting embryos can be done without harming the parent. Xenopus is an extremely tractable system and techniques such as transplantation, injectionof genetic material and imaging of fluorescent proteins are standard in the laboratory. The transparency of Xenopus tadpoles and the ease of

culturing explants allows one to follow the behaviour of labelled cells (for example green fluorescent protein) over long periods of time. Chemical modifiers are easily applied by addition to the media. Some of our study involves analysis of regeneration. In our tail amputation assay, tadpoles are anaesthetised and the tail tips are removed (<50%, not exceeding 2mm width). Although the amputation damage is visible, it is still a very small amount when compared to the entire tadpole, the tissue is rapidly regenerated. Furthermore, these types of injuries occur regularly in nature.

Why can't you use animals that are less sentient?

Xenopus is a low-complexity vertebrate model that permits reliable and robust translation of findings tomammalian biology (including humans). Most obviously, the study of spinal column development and regeneration requires a vertebrate model.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Although extensive experience informs us that most of our regulated procedures (injection of reproductive hormones into adult frogs) are minimally-invasive, mild in severity and well-tolerated, we monitor frogs carefully by recording biological metrics like weight during procedures, and by constantlyinteracting with NACWOs and NVS to maximise welfare. Unusual events are flagged via our institutional recording system and we routinely review and refine our protocols. Procedures that may cause distress to the tadpoles or frogs are performed under deep anaesthesia. After procedures tadpoles will be closely observed and monitored for any signs of distress. During a procedure, any protected animal showing signs of distress will trigger the cessation of an experiment and subsequent protocol review.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

There are many useful resources on the NC3Rs website (e.g. NC3Rs experimental design assistant).We are constantly reviewing and improving Xenopus husbandry by implementing best practice from the community (Slack channel with more than 120 participants, close contact with the European Xenopus Research Centre in Portsmouth and the National Xenopus Resource in Woods Hole, USA).Publications include the Xenopus book (Cold Spring Harbwour) and Xenopus protocols (Humana Press).

The new PREPARE Guidelines will also be strictly adhered to (https://norecopa.no/PREPARE.)

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We have previously held funding from the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs), and we maintain strong links with local and national advisors. NC3Rs regularly holds online workshops and institutional events aimed to improve experimental procedures with a focus on the 3Rs. We continually review and refine our protocols withdiscussion between our researchers, Named Animal Care & Welfare Officers (NACWOs) and NVS.

USING LUNG FUNCTION ANALYSIS TO DEFINE IMMUNE MEDIATED MECHANISMS IN THE INDUCTION OF ASTHMA

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Asthma, Immunology, Mechanism, Therapy, Pathway

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to use murine models of allergic asthma combined with immune cell analysisand lung function testing to determine the molecular pathways of asthma induction and modulation.
The goal is to identify targets and test preventive and therapeutic interventions against asthma

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Asthma is a major respiratory disease, affecting both adults and children. The World Health Organisation (WHO) estimates that a staggering 262 million people were affected

by the disease in 2019, with 455,000 people dying from asthma episodes (https://www.who.int/news-room/fact- sheets/detail/asthma). With such a high global burden and death rate it is important to continue to determine the underlying processes that lead to asthma episodes and to determine how new therapiescan interrupt these processes and thus have potential to prevent or treat disease. The success of this approach has been seen with effectiveness of new biologic compounds in those individuals who suffer from specific types of asthma that involve a specific cell type that is affected by the biologic compound.Unfortunately, there are a range of asthma types which do not respond to the new biologic compounds.For these patients broad acting steroid inhalers are critical in reducing symptoms. These steroids have other impacts and are hard to deliver during asthma attacks. To continue recent success, we are working to link key clinical understanding in humans to defined and flexible models of inflammation in mice. By linking the clinical work with the mouse model, we can target candidate processes identified from clinical work and define the role of one specific compound in the steps from immune sensitization to inflammation-induced changes in lung physiology and ultimately in lung function. The mouse provides a model where the unknown unknowns are accounted for by flexing only one compound at a time.

What outputs do you think you will see at the end of this project?

Expected outputs are an understanding of immunological processes leading to inflammation and reduced lung function. The target outputs will be understanding of preventive and/or therapeutic interventions that limit inflammation and lung function reduction and thereby increase our ability to reduce induction of asthma and episodes of asthma. We expect to identify new targets for interventionand to test known stimulators/inhibitors of already identified targets with the aim of having better understanding of the processes involved in asthma. Targets may be identified that can then contributeto development of products that can be delivered in the clinic.

Who or what will benefit from these outputs, and how?

In the short-term we will be able to assess the potential for known stimulators/inhibitors of specified targets to change the inflammatory and lung function outcomes in an asthma model. Medium term outcomes will be determination of the specific processes involved in asthma development. In the long term and due to the role of animal models in testing pharmaceutical interventions, we expect that someinterventions may be progressed through to clinical trials, which could lead to new drugs and treatments for asthma.

How will you look to maximise the outputs of this work?

The work will be developed through discussions with clinical experts, animal modellers and immunologists. These discussions will identify hypotheses, which when tested using the procedures outlined in this application will result in data sets that will inform the scientific and clinical asthma and lung function fields. The data sets will be disseminated through publication in peer-reviewed journals and through presentations at scientific and clinical meetings. There will be direct outreach to pharmaceutical companies (some are already aligned with the clinical groups) and to groups for whomthis model will be useful.

Species and numbers of animals expected to be used

- Mice: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice provide a model wherein single molecules can be targeted within a pathway, and they allow hypotheses regarding the role of these molecules in specific pathways to be tested. They provide a model wherein the unknown unknowns are accounted for and where simple interventions can be undertaken leading to defined and interpretable outcomes. They are an established model for antigen- induced lung inflammation that mimics some elements of the initiation and expression of asthma. We are also able to measure lung function using established tools that have been verified within the field of preclinical asthma work. The key to effective use of mice in the asthma model is to be constrained in the interpretation of the data and to place it within the context of experimental medicine and clinical data. Mice have been used in allergic models of asthma as they provide a model wherein, we can control almost all aspects of their lifestyle, they are genetically identical and there are extensive tools that we can use to measure, intervene and manipulate their molecular and cellular responses. We will use adult mice as their immune response and their lungs are developed to a stable state and their lungfunction can be measured.

Typically, what will be done to an animal used in your project?

Animals will receive some sensitizing agent through their noses. This agent is the kind of thing that might drive an allergic response like hay fever (house dust mite antigen is one example). The mouse will be asleep while the agent is put on its nose and will inhale the agent as a cloud into its lungs. The volume of agent is very small, and the agent goes into very small droplets during the inhalation process. The mice will undergo this delivery of agent through the nose either daily for a couple of weeks or three times a week for three weeks. This sensitization would not last more than 5 weeks. Wedo not expect the mice to show outward signs of discomfort during this process but there will be a change in their immune response. Some mice will be killed at the end and the immune response in the lung measured alongside any changes in the lung structure. Other mice will be anaesthetised, and a tube will be surgically placed in their lungs and an agent that constricts the airway muscles in the lungs. The ability of the lung to respond to the agent is measured by a machine (FlexiVent) connected to the tube in the mouse lungs and determines how healthy the lung is (lung function).

To investigate how the sensitization activity changes the lung function an intervention will be given to some mice. This intervention can be delivered via different routes at the same time as the sensitizationprocess. Interventions will be any acceptable delivery route based on the UK wide guidelines and will be overseen by the local animal welfare committee.

The anaesthetic given prior to the use of the FlexiVent machine is strong and the mice will not be brought out of the anaesthetic; this is called terminal anaesthesia. Once the experiments have beencompleted the mice will be humanely killed without coming out to the anaesthetic.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect the mice to show any adverse effects from either the sensitisation process or theirconnection to the machine that measures their lung function. We think this because the mice will be lightly anaesthetised during the delivery of agents through the nose and they will be heavily anaesthetised during the lung function test. The delivery of the anaesthetic in both cases will be a transient effect. Things that could change the adverse effect on the mice are the nature of the sensitizing and/or intervention agent. To avoid undue adverse effects, we will mostly be using agentsand procedures that have been shown to be without adverse effect. If we need to use a new sensitizing or intervention agent, we will use what we know about how it works and discuss this with the experts within the facility and monitor mice during the treatments more frequently. If limited information is available, we will start with a low dose and work upwards to ensure no adverse effectsare observed at the same dose where the agent is having an effect.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Although any one event will have little to no impact, the repeated nature of the sensitization andintervention will result in the cumulative severity reaching a moderate severity level.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered 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 a need to determine whether the interventions identified in vitro have the capacity to alter the behaviour of immune cells and lung function in vivo in the context of a complex tissue such as the lung.

Which non-animal alternatives did you consider for use in this project?

Tissue culture models will be used, including the use of ex vivo models using primary human cells derived from airways of asthmatic individuals, such as the primary bronchial epithelial cell (PBEC) model. These models are critical to narrowing the choice of interventions and determining those allergens and interventions that require the use of the in vivo model to demonstrate efficacy within thecomplex tissue.

Why were they not suitable?

While in vitro systems are useful for intracellular pathway determination they do not allow for integration of blood flow, air flow, altered inflammation and airway smooth muscle behaviour over time.The in vitro and ex vivo (human) models are limited due to low numbers of cells that can be collected and grown from each patient. Cell lines loose

characteristics after long term culturing and are limited inour ability to interpret the data. Cell line models investigate only one type of cell response, they don't give a complete picture showing full immune response, the effect of the disease on lung function or on inflammation within the lung.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Good experimental design will be used. Group sizes will initially be of 5 animals and will be adjusted asdetermined by statistical analysis we will use previous data reported in the literature to these models.
Control animals (in receipt of sham intervention – or mice that have had sham sensitization) will be used to demonstrate the outcomes are dependent on the intervention. Expected effect size will be used to guide animal numbers to ensure sufficient numbers are used to provide robust data but that noextra mice than are required are used.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Statistical analysis of data from the published literature and experience have informed initial animal numbers. Ongoing statistical analysis and definition of the measurable effect of any intervention will beused to refine animal numbers as the studies develop.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Animals will be utilised to analyse as much data from tissue as possible. Animals will be used for lung function data or for generation of lavage fluid both assays cannot be undertaken on the same mouse. Blood will also be taken from each animal to measure immune response. Some mice will be used for immunological studies and some for lung function as required by the nature of the procedures. Efficientbreeding strategies will be used, and mice will be bred from a core colony with animals being available to other users to ensure animals are utilised.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm

to the animals.

We will use the mouse model of immunological sensitization. We will largely use the intranasal deliveryof antigen for sensitization, this is to allow mucosal delivery of antigen. To facilitate inhalation in a robust manner mice are anaesthetised to avoid movement and to assure regular breathing. The anaesthetic also serves to reduce the mouses ability to feel stress or pain during the intervention. We have the capacity to deliver antigens and interventions via other approved LASA routes and these will be without anaesthetic as they are transient in nature and delivery of the anaesthetic increase handlingand stress. The terminal anaesthetic prior to the tracheotomy will reduced the pain and stress felt by the animal and will allow the collection of critical data sets for the objectives with the least pain and distress. The mice will be killed by schedule 1 (Option 2, 4) or cardiac bleed (option 3, 4) before wakingfrom the anaesthetic. We are not expecting animals to suffer more than transient discomfort from the procedures.

Why can’t you use animals that are less sentient?

Mice are the least sentient animals that can be used that will allow us to take lung function measurements as well as all the tissue needed for analysis of inflammation in the lungs.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The procedures listed include repeated delivery of antigens to sensitize animals. The literature supports the need to undertake repeated delivery, but the known standard is 3 intranasal procedures per week for three week using house dust mite antigen. These procedures will mostly be undertaken on Monday Wednesday and Friday with no activity on Saturday and Sunday providing a break from handling. The use of anaesthetic while a stressful procedure, reduces the overall stress from repeatedintranasal instillations. We will use the minimum number of sensitizations to induce the inflammatory phenotype in the mice and continue to read the literature to ensure we are using the most refined protocol. Delivery of the interventions will also be limited to the most refined protocol based on literature and known dose response data. We will acclimatise animals to procedures and general handling throughout. Animals will be monitored daily for any adverse effects.

We do not expect adverse effects based on the literature and our use of known sensitizing or intervention agents. If we use novel agents, we will undertake pilot studies with escalating dose andfrequency to obtain the required phenotype with the least interventions.

If mice start to show signs of stress sensitization or intervention will cease.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

LASA guidelines for dosing.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will stay informed by reading of scientific literature, from staying up to date with information fromthe NC3Rs, and from information circulated from NIO. We will consult with our local NC3Rs representative.

Home Office

NEW THERAPEUTICS AND DIAGNOSTICS FOR FILARIAL 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, ortheir 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 aimsmentioned in paragraph (b)

Key words

Filariasis, River blindness, Elephantiasis, Therapeutics, Diagnostics

Animal types	Life stages
Mice	adult
Gerbils	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

- This project will use infections of laboratory rodents (gerbils & mice) with human & veterinary filarialworm parasites. Using rodent infections, we aim to:
- determine the mechanisms by which novel drug therapies may target filarial parasites or modify filarialdisease,
- identify new biomarkers of filarial disease and
- evaluate novel filariasis therapeutics and diagnostics.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Filariae are thread-like parasitic worms that infect humans and animals. Filarial infections are spread by the bites of mosquitoes or flies. Filariae cause the disabling diseases, river blindness and elephantiasis in humans. These infections are targeted for global elimination by The World Health Organisation. In cats and dogs, filarial infection causes heartworm disease, which is potentially fatal. Veterinary filariasis can also cause disease in humans. Filariae induce disease when the worms die inthe body, provoking an inflammatory reaction and collateral damage to the eyes, skin, lymphatic system or heart and lungs. Over 90 million of the world's poorest populations suffer with filarial disease. Veterinary filariasis is spreading in America and Europe due to the effects of climate change and emergence of drug resistance.

Whilst there is no vaccine to protect humans or animals against filariasis, there are drugs that work to block the spread of infection between people or prevent infection in dogs. For elimination of human filariasis, donations of standard anti-filarial drugs by pharmaceutical companies are distributed to at risk populations. These drugs need to be taken every year by the majority of affected communities forup to 15 years to achieve elimination. This approach has failed to eliminate filarial infection in certain situations. Three important reasons for the failure of this strategy using standard anti-filarial drugs are:

It is difficult to distribute drugs sustainably and at scale in resource poor countries especially wherethere may be disruptions caused by conflict there are signs that filarial parasites have developed drug resistance and people can suffer a range of inflammatory adverse reactions following treatment causing reducedadherence.

An additional drawback of current treatments used to eliminate human filariasis is that they do notimprove pre-existing disease (an estimated 40 million elephantiasis patients worldwide).

In areas where veterinary filariasis is a problem, there is only one type of drug in use which is effectiveat stopping infections from developing. Pet owners need to give their animals this treatment routinely, as frequently as every month. There are signs that the prevention of heartworm is failing due to the development of drug resistance.

To determine where to scale up elimination with drug delivery, when to stop an elimination program andto confirm that there is no re-emergence of disease after elimination, there needs to be accurate and affordable methods of detecting filarial parasites. Unfortunately, current diagnostics are not ideal for these uses meaning that it is problematic to know where and for how long to treat at risk populations.

Our aims are to develop new short-course drugs for curing human filariasis or preventing veterinary filariasis. We aim to develop new ways to diagnose filariasis which improve on current methods. We will also research the events in the body that trigger elephantiasis and test drugs that may block these disease-causing pathways. Further, we will research the mechanisms of adverse reactions to standardanti-filarial drugs and test whether co-treatment with certain anti-inflammatory drugs may prevent adverse event occurrance.

What outputs do you think you will see at the end of this project?

- supported by preclinical evidence gained in this project, our outputs may encompass:
- registration a new curative drug for human filariasis

- the clinical development of a new veterinary heartworm preventative & curative drug
- the adoption of a mouse model of heartworm by industry to reduce the number of severe proceduresundertaken in cats and dogs
- start of proof-of-concept clinical trials to test the benefit of an adjunctive therapeutic for filarialdisease
- the clinical evaluation of a new electromagnetic diagnostic sensor of lymphatic filariasis
- the identification of circulating biomarkers of lymphatic filariasis for diagnostics development

Who or what will benefit from these outputs, and how?

Registration of a new curative filariasis drug by end of the project is feasible based on the current position of two anti-*Wolbachia* candidates in phase I and II clinical trials. The data derived from this project will provide evidence of the molecular target and/or mode-of-action of these candidates when given in combination with anthelmintics which in turn could support phase II/III and registrationdossiers submitted to The Food and Drug Administration regulator during the project lifetime.

The availability of a new short-course cure will dramatically curtail human filariasis elimination time- frames and may accelerate global elimination targets in 90% or greater of affected countries between2030-2040. Thus, new cure(s) will benefit current vulnerable populations (over 1 billion at risk of infection) and endemic countries scaling up or implementing existing elimination programmes (51 and34 countries affected with lymphatic filariasis and onchocerciasis, respectively). The knock on socio- economic impacts will be to reduce a drain on the limited resources of local and national health systems and improve the economic productivity of millions of currently affected populations.

Proof of concept that a new class of drug is effective in the prevention of veterinary heartworm diseasewill be of immediate benefit to the animal healthcare industry who may wish to take up clinical development and commercialisation. Realisation of a new class of heartworm drug within five years from project ending will safeguard protection of client-owned cats and dogs resident in heartworm endemic areas where resistance to standard treatments are emerging. The new treatment may also beadopted as a safer, more acceptable cure for heartworm in dogs compared with current standard treatment and offer a hiterto unavailable curative treatment for cats.

We continue to work with major commerical end-users to encourage uptake of a mouse model alternative to cats and dogs for early stage drug screening of heartworm preventative compounds. We project that one major contract research organisation and one industry client will have fully adopted themodel by the end of the project. More widespread adoption of this model by heartworm preventative research and development companies and academia, feasibly within 5-10 years beyond the end of theproject has the potential to reduce the total number of long-term cat and dog procedures which my cause severe harm by as much as 50%.

Our preclinical research into repurposing registered anti-inflammatory drugs as a lymphoedema disease alleviating treatment may translate into proof-of-concept repurposing human trials in human filariasis patients. Beyond the end of the project, large scale trial evaluations may result in recommendations to use these treatments in up to 51 countries remaining with 36 million lymphoedema suffers who presently do not have access to any affordable medicines to treat their condition. We also aim to prove whether it is possible to reduce adverse reactions to standard drugs used in filariasis elimination

programmes if co-treated with anti-inflammatory adjunct treatments. This may impact on the long term success of the control and elimination of river blindness using ivermectin, with an estimated 220 million individuals still requiring annual mass drug administrations.

The establishment of a wearable biosensor that works to detect filariasis infections in mice will supportearly proof-of-concept human trials within the project life time. If sufficiently accelerated through large scale field testing, this has the potential to be adopted by endemic countries and their implementation partners to support LF elimination both as a way of surveying for stopping mass drug administrations and to monitor for any resurgence of infection. Identifying protein or lipid filarial parasite-produced biomarkers using experimental animals in this project could similarly be translated through to a point-of-care diagnostic in a 5-10 year time frame.

How will you look to maximise the outputs of this work?

Findings from the project will be reported at the earliest stage in national and international conferencesand at invited speaker seminars to academic and global health professional practitioners such as The International Congress for Tropical Medicine & Malaria, The American Society for Tropical Medicine & Hygiene and The American Heartworm Society Triannual Symposium.

The project investigatory team is networked with The UK Lymphatics Science Meeting and the US- based Lymphatic Seminar Series which together comprise a prominent cadre of ~200 international lymphatic research experts including clinical investigators. We will present findings at both fora duringthe project life-span to emphasise translational opportunities in the pharmacological treatment of lymphoedema to a broader scientific community.

Data and methodological advancements will be made available at the earliest opportunity via open access biomedical and veterinary journals with online repositories for primary data. Clinical journals willbe selected where suitable to inform a wider medical audience of the potential clinical applications of the project discoveries. Preclinical lipidomics data will also be made freely available on the BBSRC MetaboLights data repository. Major outputs will be highlighted in social media and institute research centre websites. The investigatory team will also target selected review articles to disseminate cross- cutting therapeutic or technological opportunities to a wider biomedical community at the early stage ofthe project. We will make use of the NC3Rs Gateway Journal and the Filariasis Reagent Resource Repository (FR3) website to also detail methodological details and/or negative data sets.

The investigatory team and partners are experienced international experts in their fields, with substantial complementary professional networks extending from academia to clinical practitioners, industry, global health funders, NGDOs and filariasis public health policy makers. Findings of the thispreclinical project will be communicated to expert committee groups such as WHO NTD DiagnosticsTechnical Advisory Group, Mectizan Donation Programme, Bill & Melinda Gates Foundation Macrofilaricides Expert Group, Drugs for Neglected Diseases initiative and national filariasis elimination programme Health Ministry links we have prior supported to implement mass drug elimination programmes.

We will chair workshops embedded into The Coalition of Operational Research (COR)-NTD to posit clinical applications of our preclincial research findings and articulate a case for onward clinical testing.

Species and numbers of animals expected to be used

- Mice: 1657
- Gerbils: 246

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will be using adult mice, including genetically modified strains and gerbils to test our hypotheses and realise our objectives. We are using these species because they are susceptible to infections with filarial parasites that cause human and animal disease and we can easily maintain them within specialist laboratories. Filariae cannot be grown outside the body. A major use of rodents in this projectwill be to grow the different life-cycle stages of the parasites for onward testing in the laboratory. In thenext phase of our project, we will be able to test drugs for efficacy, understand how drugs work to kill parasites and investigate new diagnostic approaches against these target human and animal filarial parasites in the context of a mammalian host. A further advantage of using mice is that their immune system is very well studied and is closely related to humans. We can therefore study in detail how filarial parasites invoke host inflammation to cause disease and identify host immune responses that we may be able to target to improve or prevent the worst aspects of filariasis pathology.

Typically, what will be done to an animal used in your project?

We will typically experimentally infect gerbils and mice under the skin or into the peritoneal cavity and allow the parasites to mature. Where the parasites grow is related to each individual species of filarial parasite, the life cycle stage of the parasite and also the infection route. For instance, lymphatic filarial parasites will infect the lymphatic system if inoculated under the skin. After an incubation period, we will remove various larval or adult stages of parasites for drug testing outside of the body or treat the infected mice or gerbils with drugs to determine the effects inside the body. In some experiments our goal will be to understand the host immune responses which cause disease after infection. In this case, we may use genetically modified mice with loss of specific inflammatory processes or inject micewith drugs or antibodies to modify a specific facet of the host immune response and measure the effects on disease. We will typically measure disease by recording temperature, measuring changes ininflammatory mediators in circulation or using bio-imaging to visualise changes to the lymphatic system. We may also use mice and gerbils to evaluate new devices placed on the skin to diagnose filarial infections inside the body and to take serial blood samples to identify circulating molecules outside of the body which might be in future developed into new diagnostics. Infections in mice which cause pronounced disease will typically only be run for one to four weeks, whereas infections in mice or gerbils which typically cause more mild, sub-clinical disease may be run for up to 4 or 18 months, respectively. We estimate that in total we will use up to a maximum of 1657 mice and 246 gerbils over the course of the five year project.

What are the expected impacts and/or adverse effects for the animals during your project?

The experience of pain and suffering caused by the filarial infection in mice and gerbils varies dependent on the route, species, life-cycle stage and quantity of filarial parasite used. Through experience in running experiments for many years, we can set maximum tolerated inoculation levelsand we normally infect below this level.

Infections that are contained in the peritoneal cavity do not trigger clinical symptoms and only a minor inflammatory response is evident localised to the peritoneal cavity, even when infections aremaintained for many months. For this reason, where this approach is applicable for our scientific outcome, we always use this approach.

Infections into the blood with blood-stage microfilarial larvae of parasites may cause a degree oftransient ill health due to change in blood pressure in the heart and lungs which will affect the breathing and mobility of the animal before the numbers of parasites distributes more evenly aroundthe body. This ill health is normally temporary, over the first few hours after inoculation. We will treatthese animals with drugs which rapidly kill the microfilarial stage but which co-incidentally can causesystemic febrile inflammation. The mice may show loss of activity, loss of appetite and some weight loss over one to two days before recovering.

Infections of microfilariae into the skin cause some mild, localised inflammation and irritation as the parasites accumulate in the cooler regions of the skin, especially the ears, which dissipates within twoto three weeks.

infections with dog heartworm larvae under the skin do not cause any clinical ill health in mice overthe first 4 weeks of infection. The larvae at this stage establish in the subcutaneous tissues and muscle.

infections with lymphatic filarial parasites under the skin rapidly infect the lymphatic system. In micethis initiates a pronounced immune-mediated inflammatory response which effectively kills the larvae within one to two weeks but simultaneously provokes immunopathology (damaged and dysfunctional lymphatic system). The mice might suffer some swelling, pain and irritation localised to one hind limb. The condition is normally stable or very gradually progressive and so we limit the time course of theseinfections typically for 2-4 weeks. In gerbils, infections inside the lymphatics occurs with less pronounced inflammation which allows for long-term infections. Gerbils may occasionally suffer from parasites infecting the heart and lungs which, as the filariae gradually grow, may cause interference ofnormal blood pressure and consequently the animals may begin to show reduced mobility. The incidence of this is low and usually is slow to develop after the first 6 months of infection, which is regularly monitored for.

Apart from the infection procedures, In most cases, other treatments and sampling procedures might cause temporary minor pain and tissue damage due to for instance, an inoculation and short-term inflammation at the site of infection or due to scratching a tail vein to sample blood. This may occur onseveral different occasions in the life of the animal and last for one to two days before healing. Some procedures run the risk of more pronounced ill effects in a proportion of animals used, where for instance, higher level doses of drugs may cause some toxic side effects, or where the treatments to suppress or modify the immune response make the animals feel ill. These effects will typically last for between one to seven days and such treatments may be stopped prematurely based on the extent of ill health exhibited.

Long-term procedures in gerbils may cause gradual ill health due to risk of co-infections or age-relatedloss of welfare and partly due to the cumulative effects of sampling procedures.

We will attempt to treatthese problems when they first become apparent e.g. skin infections but if the welfare issue persists orworsens after one to two days we will humanly cull the animal. For these reasons we have a maximumage and duration of infection before gerbils must be euthanised.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

We have estimated that 62/236 gerbils (25%) and 828/1657 mice (50%) may experience up to moderately severe adverse effects. The remainder of animals used will experience mild severity effectsof procedures undertaken.

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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Filarial parasites have a complex life cycle and require a mammalian and insect host to develop and reproduce. Filariae do not survive long-term and are not reproductively active in culture outside of thebody. With NC3R funding we have attempted to modify culture techniques to grow infectious larvae derived from mosquitoes outside of the body. Our attempts using co-cultures with various mammaliancells and three-dimensional cultures has failed to support the growth of these parasites to a reproductive adult stage.

Drugs are absorbed, distributed, metabolised and excreted by the body in a complex dynamic whichcannot be precisely emulated by replacement techniques. This necessitates using animals to determine how drugs mediate pharmacological effects on filarial parasites.

Assessment of filarial disease requires a whole organism approach to appropriately model the complexeffects of multiple cell types and pathological tissue changes at the site of inflammation or infection.

Prospective diagnostic biomarkers produced by filariae may behave differently when in circulation of aliving animal. For example they may be rapidly broken down or, conversely, be bound to plasma proteins and antibodies and persist for variable periods after death of parasites in the body. Biomarkerdetection therefore requires evaluation after infection and drug mediated cure in animals.

microwave signals that are characterised to be specific to detect the presence of filarial parasites in theblood or skin may be altered by the dialectric properties (insulation) of living tissues the parasites are found within and surrounded by. For this reason, the microwave

patterns produced by filarial parasires need to be evaluated in a living animal.

Which non-animal alternatives did you consider for use in this project?

Advanced 2D, 3D and organoid in vitro cultures to propagate *B. malayi* or *D. immitis* from infectiousstage to mature reproductively active adult worms.'Organ on a chip' *in vitro* models to link liver organoids to parasite growth systems via microfluidicssystems are a conceivable solution to model drug exposure and liver metabolism *in vitro.*

Why were they not suitable?

We have in the past trialled several methods to grow the mammalian life cycle stages of *B. malayi* or
D. immitis in complex co-culture systems. These attempts have proven unsuccessful. Whilst we have developed methods to prolong adult *B. malayi* worm survival *ex vivo*, this still requires an animal host to provide a source of parasites. It might be possible in the future, with advancements in *in vitro* organoid models of the lymphatic system (the natural parasitic niche of this parasite), to establish an environment to propogate reproductively active adult *B. malayi.* However, such studies have not been placed in the public domain and we are not currently funded to undertake such expensive and high-risk experimentation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals estimated is a maximum based on our research objectives. We have built incheck-points where we may decide to stop further testing (ie in the event of a drug, anti-inflammatoryor diagnostic approach not working at an early stage of evaluation). Therefore the actual numbers used may be less than currently estimated.

For the production of different life-cycle stages of filarial worms to use in onward experiments outside of the body we have extensively considered how many of each stage and species of filarial parasite wemay require over the five years of the project to achieve our objectives. We have based this on prior experimental designs and knowledge of, for instance, yields of DNA or protein from individual worms orpools of larval stages. We have then applied empirical estimates of the typical range and average yields produced in mice and gerbils based on meta-analysis of multiple prior infection studies. In the production of certain life cycle stages, gerbils can be repeatedly sampled and maintained for long periods, reducing overall animal use. Further, we have established that certain immunodeficient mice produce higher yields of larval and juvenile adult parasites and so we will employ these strains and life-cycle stage in testing wherever applicable to reduce overall animal use.

We have used existing data to assess the minimum group sizes needed to test with a degree of statistical rigour whether the drug compounds work against filariae or filarial

disease. For drug testing, we have considered the primary outcome measurement of the study (the efficacy effect) and the typicalrange of values of this measurement in untreated mice. From reference drugs, we know the likely maximum effects and have applied statistics to ascertain a group sample size to determine a minimally desired effect of the experimental drug treatment. This minimum effect is aligned to 'target candidate profiles' which are used to decide whether to take a drug forward into veterinary or human testing (e.g.≥70% efficacy following ≤7 days treatment).

Similarly, for testing how the immune response to filarial infection induces disease, we will be using mice where specific molecules and cells of the host response will be modified or impaired. We have determined, through prior research, a way of reproducibly quantifying disease e.g. inflammatory mediators in circulation or changes to lymphatic system. We also know the expected range of these measures in infected diseased mice. Thus, we have designed our group sizes to test whether we canmodify the disease or inflammation by at least 50% as a meaningful effect which would justify onwardtesting in humans.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We followed NC3Rs' experimental design guidance to plan experimental design including advice andsupport for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We used a free to use software (G-Power) to calculate group sizes based on the minimumbiological effects we have set as threshold for 'go-no:go' decisions justifying further preclinical testingor as dossiers / paper reports advocating clinical testing.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Diagnostic testing will require a preliminary round of pilot evaluations in mice and gerbils. Only if we determine a robust and reproducible signal of parasite detection (diagnostic sensitivity) will we procedeinto further animal testing.

At the end of each experiment, we will harvest as many tissues as possible at post-mortem for cyro- preservation or fixation. This is because there are many additional readouts that we can measure if ourprimary outcome measures are positive. This will help further refine our research questions and objectives and may further reduce use of total animal use.

In addition we will always use surplus filarial parasites harvested from untreated or sham treated control animals for onward experiments. This will reduce overall animals required for the propagation offilarial life-cycle 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm

to the animals.

Rodents are the lowest sentient mammalian group that are susceptible to filarial infections.

Gerbils are the lowest outbred vertebrate susceptible to *Brugia* filarial worms. Gerbils tolerate theseinfections within the peritoneal cavity without clinical signs of disease or ill health for protracted periods. Length of infections are limited to 18 months to avoid decline in welfare due to age or cumulative effects of sampling procedures.

Gerbils can also tolerate long-term active lymphatic infections. They are the lowest sentient species that are fully susceptible to intra-lymphatic infections and generally, single infections do not cause overtpathology or only very gradually does overt lymphoedema swelling start to occur. Infections in the heart and lungs can occur which may gradually cause pathology. For this reason we will limit infectionsto 18 months and monitor carefully for signs of ill health.

Mice, including immunodeficient strains, will be used in shorter term procedures for *Brugia* and *Dirofilaria* infections as they either produce a higher level of pathology or produce more yields of parasites and are more ethically acceptable alternatives to using natural host species (cats and dogs).Where pathology is the expected outcome of the study, the extent of pathology is sometimes localisedand does not progress beyond a moderate level. In other instances the pathology may be systemic butis normally transient and self-resolving.

Why can’t you use animals that are less sentient?

The long periods of development of filarial parasites and their host-restriction to mammals and the chronic nature of pathology development precludes any less sentient or immature alternatives than therodent models we have selected.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

To minimize development of any welfare issues due to ageing, gerbils will be maintained for a maximum period of +18 months post infection / +24 months of age (whichever is sooner). Through tenyears experience, not exceeding these time frames safeguards against the majority of age-related lossof welfare issues which arise. However, we will put in place more vigourous monitoring when gerbils reach 12 months of age including weekly weight monitoring. We will always attempt remedial treatments if applicable when loss of welfare is suspected or identified. When loss of welfare does arise we will not allow an animal to exceed a moderate level of suffering before humanely culling, judged by appearance, behaviour any specific clinical symptoms and/or weight loss.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We follow best practice guidance for handling, housing and husbandry of mice and gerbils: https://nc3rs.org.uk/3rs-resources/housing-and-husbandry.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

I am an NC3R grantee, an NC3R expert panel member and chair of the institutional

AWERB. Throughthese activities I and receive regular communications from stakeholders such as LASA, NC3Rs and RSCPA. Our AWERB members are proactive in attending off-site seminars on aspects of 3Rs and report back to practicioners. Similarly I receive information and advice from the secondary establishment biological services unit on best practice.

UNDERSTANDING PROTEINS THAT INTERPRET GENOME CONTEXT 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, ortheir effects, in man, animals or plants

Key words

Rett syndrome, Brain, Human disease models, Intellectual disability, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

We aim to investigate the function of a number of proteins involved in chromatin regulation which are associated with human neurological disease, including autism and intellectual disability. We would liketo find out how some of these proteins interact with each other, and whether the effects of mutating them are reversible.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 hope to discover more about how mutation of these proteins leads to malfunction in the brain and nervous system. The proteins we wish to study have been found to be mutated in human disease andunderstanding what they do in health will help develop new

treatments for these diseases. Treatmentscould include gene therapy or pharmaceutical approaches.

MECP2 is mutated in the severe neurological disease, Rett syndrome (RTT). We have previously shown that you can cure neurological symptoms in a mouse model of RTT by adding back functionalMeCP2, indicating that it should be possible to cure RTT patients using, for example, gene therapy. Ifwe can show that a similar reversal is also possible for other conditions, it will increase interest in developing therapies to cure these conditions, as was seen with RTT.

Gene therapy strategies to treat RTT are advancing towards clinical trials in patients. Gene replacement potentially has some disadvantages due to the importance of getting just the right level ofMeCP2 in each brain cell. Our work to develop a gene editing therapy for some RTT mutations could solve this problem by correcting the mutated gene itself rather than adding back more copies.

What outputs do you think you will see at the end of this project?

This work should lead to a greater understanding of the function of a number of proteins implicated inintellectual disability and neurological disease in humans. It will involve the production of a number ofmouse lines modelling human disease which could be made available to the wider scientific community. Work on genome editing could lead to constructs that can be advanced towards clinical use.

We plan to publish the work at the earliest opportunity.

Who or what will benefit from these outputs, and how?

The expansion of basic understanding of how certain mutations affect brain function, in the form of published studies, will benefit the scientific research community working in this area, and may also influence work to develop potential therapies. Mouse models of human disease, once validated and characterised, will benefit those working on those particular proteins or diseases, and may also be ofuse for preclinical studies, as has been the case with our RTT mouse models. This would potentiallybenefit clinicians and patient communities in the longer term.

How will you look to maximise the outputs of this work?

We would hope to publish as much of our work as possible. We have a long-track record of sharing ourmouse lines, tissues, cell lines derived from mutant mice and advice on management of our established lines. We will continue to do this. We also have a history of collaboration, through which weshare expertise, mouse models and reagents.

Species and numbers of animals expected to be used

- Mice: 16,000 (3,200 animals per year)

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 for our studies. Up to this point we have heavily concentrated on the gene mutated in RTT, *Mecp2*. We judge the mouse to be the least sentient species in which it is possible to model human *MECP2*-deficiency well, as it is a disease which affects the function of the network of cells in the brain as a whole, rather than individual cells. MeCP2 protein levels in the brainincrease greatly after birth, and the neurological effects are seen only from around 6 weeks of age, atthe earliest. We are now extending our work to a number of other proteins involved in neurological disease which may function during embryonic development and/or postnatally. In a similar way to *Mecp2*, these need to be studied in the whole organism. The life stage that is studied may vary for different gene mutations.

Typically, what will be done to an animal used in your project?

The vast majority of animals covered by this project licence will be used in breeding protocols. Fortissue harvest for *ex vivo* studies, animals will be euthanised by an authorised method and tissuescollected.

A small number of animals will receive prospective treatments, administered via the least invasive appropriate route possible, after which they may be allowed to develop neurological symptoms notexceeding moderate severity.

Animals being monitored for neurological symptoms will be weighed, scored for various symptoms while being handled, and may be put through non-invasive behavioural tests such as measuring theiractivity in an open arena.

At the end of procedures (or if the defined humane endpoint is reached) animals will be euthanised byan authorised method and tissues collected for further study.

What are the expected impacts and/or adverse effects for the animals during your project?

Most animals will experience sub-threshold severity. Animals with *Mecp2* mutations may be allowed todevelop neurological symptoms (altered gait, reduced activity, tremor, breathing abnormalities) which will not be allowed to progress beyond moderate severity. They may lose weight but they will not be allowed to go below 80% of their previous maximum weight. Animals with mutations in other genes involved in intellectual disability may experience behavioural abnormalities and possibly mild skeletal abnormalities.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

It is expected that the vast majority of animals (approximately 90-95%) will experience sub-thresholdseverity. Based on past projects, about 5% of animals will experience mild severity and less than 3%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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although some abnormalities can be detected in *Mecp2*-deficient cells grown in a dish, or in biochemical assays, the effect of MeCP2 mutations can only be truly assessed in the context of a whole animal, as they affect the entire network of cells in the brain and nervous system. These in turnaffect the functioning of the rest of the body. This will likely be the same for other genes mutated in neurological disease.

When testing potential therapies, it will be necessary to see that the treatment removes or reduces thesymptoms seen in the whole animal.

Which non-animal alternatives did you consider for use in this project?

As we are primarily interested in the brain, we have used cultured neurons (nerve cells) for some of our studies, including looking at mutant protein levels. We are able to make targeted alterations to genes in both mouse embryonic stem cells and human LUHMES cells, which can both be differentiatedinto neurons in a dish. Some studies, for example looking at how proteins bind to each other, can be done by simple transfection of cell lines. We will continue to use these methods whenever possible.

Why were they not suitable?

As explained above, they are suitable for some aspects of our work. They can't be used to study theeffects of mutation on the organism as a whole, where functional defects lead to atypical behaviours,neurological or structural abnormalities.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 past usage for our group (over the past 5-10 years), comparing the number of lines we have maintained at various time in the past with the number we propose to hold tocarry out this project. Our colony management strategy means that we maintain fixed stock levels for most of our lines, making it possible to estimate the number of animals that will be used fairly accurately.

What steps did you take during the experimental design phase to reduce the

number of animalsbeing used in this project?

We use isogenic mouse lines, where appropriate, to reduce variability and therefore the number ofanimals required. When assembling experimental cohorts, we use timed matings to generate an appropriate number of pregnancies to produce the animals we will need. Careful planning of experimental design and statistical analysis will be carried out in advance to ensure an appropriatenumber of animals are used.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We have a written breeding and maintenance strategy for each mouse line we hold, after the initial development period. This details the number of breeding pairs and stock animals for each line, as well as instructions for culling of animals that will not be required or will develop harmful phenotypes. These instructions are carried out by our experienced animal technician who is in daily contact with our animals. Where excess animals are produced, or at the end of a study, we collect relevant tissues andstore them in an indexed "Tissue Bank" for *ex vivo* studies. All our lines are cryopreserved at the earliest opportunity so that they do not need to be kept as live animals when not required for experimental use.

Before an experiment where, for example, a therapeutic agent is to be administered to treat neurological symptoms, we will perform a smaller pilot study to assess whether the agent is having aneffect before going on to conduct a larger 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Many of our experimental procedures will be performed on mouse models of Rett syndrome (RTT), which we have created and characterised. We have many years of experience working with these lines. We are also planning to create new mouse lines which either model human disease or help us understand the function of proteins involved in the function of the brain. These genes include *Tbl1x, Tbl1r, Setd5, Ankrd11* and *Nipbl*. Whilst the RTT-like phenotype can be severe in rodents, our experience and knowledge will allow us to monitor the animals closely and will take the following stepsto ensure that pain, suffering and distress are kept to a minimum.

We have devised a scoring system which we use at least weekly to assess the onset and progressionof RTT-like symptoms in mice. This scoring system has been published and is widely used by researchers using RTT mouse models. It has clear endpoints which determine when an animal shouldbe humanely killed to prevent prolonged suffering.

Male *Mecp2* mutant mice start to show symptoms from about 4 weeks of age, which progressively worsen. They are humanely killed before 3 weeks if they are not needed for

an experiment. They are not kept alive long enough to become severely affected through use of the phenotypic scoring scheme.Mutant mice used to provide tissue for cell culture and other experiments are euthanised while still presymptomatic.

Why can’t you use animals that are less sentient?

Based on our experience with MeCP2 and RTT, we judge the mouse to be the least sentient species inwhich it is possible to model human *MECP2*-deficiency well. Mutations in MeCP2 in non-mammalian vertebrates affect embryonic development and do not accurately reflect the symptoms seen in RTT. The effects of these mutations in mice are only seen post-weaning (from 4 weeks in males and six months in females). Many of the symptoms they cause can only been seen in a conscious animalperforming normal behaviours.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Our detailed phenotypic scoring system has been applied to our RTT mouse models for many years. Itwas originally developed after using a much longer scoring procedure and picking out the particular way in which the mice were affected. If we see that any of our new mouse models are affected in wayswhich aren't covered by our current system, we will adapt and tailor a scoring scheme to that particularmutant. In the early stages of development of a new line, animals will be closely monitored to pick up on any unexpected consequences of a mutation.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Guidance on the Operation of the Animals (Scientific Procedures) Act (1986) - https://www.gov.uk/guidance/guidance-on-the-operation-of-the-animals-scientific-procedures-act-1986

Various publications from the Laboratory Animal Science Association (LASA) - https://www.lasa.co.uk/current_publications/

Resources on the website and in newsletters from the National Centre for the Replacement,Refinement and Reduction of Animals in Research (NC3Rs) - https://www.nc3rs.org.uk/

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Our technical staff are members of professional bodies (Institute of Animal Technology, Royal Societyof Biology) and take part in continuing professional development to ensure their knowledge is as current as possible. Updates from bodies such as the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and Understanding Animal Research (UAR) come through their newsletters and are also passed on to technicians and researchers throughyearly 'roadshows' run by our establishment. In the interim, updated advice is passed on through animal unit meetings and e-mails. Regular communication with our veterinary staff will ensure techniques are reviewed and refined where necessary and appropriate.

INFECTIOUS DISEASE IN WILD BIRD POPULATIONS

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 oranimals
  - Research aimed at preserving the species of animal subjected to regulated procedures aspart of the programme of work

Key words

Disease, birds, pathogen, parasite, virus

Animal types	Life stages
Wild bird species	embryo, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate the impact of disease on wildlife populations. It aims to considerhow hosts may differ in their behaviour to influence both their likely exposure and ability to deal with infection and how, in turn, this may affect the prevalence, transmission potential and virulence of different pathogens that are naturally encountered.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Wildlife populations experience a range of diseases that may not only impact on their own health and welfare but may transfer to humans or domesticated animal populations. The pathogens that cause these diseases often exist in different forms that vary in their level of harm. However, we know little about the ecology of these pathogens and the route from

harmless to harmful disease. Understandinghow wild animals contract, tolerate and transfer particular pathogens, including those related to commercially important diseases or that have zoonotic potential (i.e. can transfer to humans), is therefore key to both conserving natural populations of animals in our environment and controlling anyrisk to ourselves and our livestock. Birds have been identified as one of the main groups that warrant further study as they move freely around our environment and harbour a range of pathogens that can cross species boundaries. We will explore whether particular individuals are more likely to be exposedto infection (e.g. males versus females, juveniles versus adults, migrants versus residents) and whether individuals differ in how they are affected. This will allow us to build up a picture of the likely impact of the disease on different populations and is a first step in establishing the most likely patternsof disease transmission between species of concern.

What outputs do you think you will see at the end of this project?

Our expected outputs include data on pathogen prevalence across different populations of birds and seroprevalence (level of antibodies measured in blood serum) indicating levels of previous exposure indifferent hosts. This will be shared with partners and co-published in scientific journals and policy reports. The data will be shared through open access data repositories so it can be used more widely by the wider research and policy communities.

Who or what will benefit from these outputs, and how?

The direct outputs from this proposal will benefit the research community interested in avian ecology,conservation biology, host-pathogen relationships, virology, disease ecology and epidemiology.

The outputs will benefit stakeholder organisations that own or manage parts of the natural environmentin the UK, for example our national nature reserves, to help inform management decisions that affect staff, researchers, citizen scientists, tour operators, land managers and the general public when populations are experiencing disease outbreaks.

The outputs will be helpful to policy advisors responsible for the overall licencing framework fordifferent activities in the natural environment.

More broadly, our work will help to inform the general public about the ongoing disease outbreaks, theimpact it is having on our wild animal populations, and the value of UK science in addressing these issues of public concern.

More generally, the data we generate can be used more widely to inform on human health risk fromzoonotic disease.

All data will be open-access and follow FAIR guiding principles.

How will you look to maximise the outputs of this work?

The work is part of a major collaborative project and funding has been secured both to publish resultsand present widely at relevant conferences and workshops. We are working closely with key partnersand with relevant public bodies to ensure our data can be utilised to inform current policy and be shared more widely with all relevant stakeholders.

Species and numbers of animals expected to be used

- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Wild animal populations suffer a range of diseases in common with human and domesticated animal populations. Understanding the group of pathogens responsible is therefore critical to both human andanimal health as well as understanding their likely impact on wildlife. Birds have been identified as one of the main groups that warrant further study as they move freely around the environment and harbour a range of pathogens that can cross species boundaries. Establishing levels of disease prevalence and levels of immunity to disease at different life stages and different components of the population (e.g. males vs females, juveniles vs adults, migrants vs residents) is important as they may differ in how much they are affected and the role they may play in onward transmission both within andbetween species. We also look at eggs in the nest that may have been deserted or fail to hatch as these can tell us about the infection status of their parents.

Typically, what will be done to an animal used in your project?

Birds will be caught and have a ring with a unique number attached to the leg so it can be identified after it's release back into the wild. All birds are caught by people fully trained in catching in the focal species. In some species, e.g. seabirds, birds are caught individually. In other cases, catching methodssuch as mistnests may be used. These are set in areas designed to catch the target species. Any birdsthat are caught that do not require sampling are immediately released. No endangered species are currently within the group of birds to be targeted and all personnel can distinguish species listed in Annex A of Council Regulation 338/97 that lists these species. While caught it will be weighed and have morphometric measurements taken (usually tarsus or bill/beak depending on species), have a small blood sample taken and swabs will be taken from the back of the mouth and from the cloaca.
This will enable us to test for presence or absence of specific pathogens of interest and for antibodiesto pathogens to show if individuals have previously been infected and recovered (or in the case of chicks that receive maternal antibodies to protect them when they first hatch) whether their mothers have been previously infected and recovered. Generally we can gain all this information from a single individual or nest in less than 10 minutes in a single procedure. In some cases, birds may also have alogger attached as part of ongoing monitoring work to track movement related to planning requirements for renewable energy projects. This may be attached either to a leg ring, or attached to feathers on their back or tail (depending on what is most appropriate for the species as specified and licenced by The British Trust for Ornithology ‘Special Methods’ licences). These will either be removed after 3 weeks or are naturally moulted off after 1-8 weeks in the case of those attached to feathers.
Geolocation loggers weigh <0.5% of body mass and GPS data loggers weigh <3% of body mass and there is no evidence of any negative effects of these loggers on fitness in the populations in which theyare used. This provides additional opportunity to explore the relationship between movement and infection around the natural environment.

What are the expected impacts and/or adverse effects for the animals during your project?

The likely impact on the birds being caught and handled includes elevated stress levels from beingcaught (up to 10 minutes in the hand), temporary discomfort while blood is withdrawn (30 seconds)and while swabs are taken (5 seconds per swab).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Mild

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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Levels of infection in natural populations can only be measured by directly testing in vivo for infection inthe field. This is needed to establish basic levels of disease prevalence and to understand which components of the population are most affected. Levels of immunity can also only be detected by measuring this directly from blood samples and is important in establishing levels of immune protectionthat may exist in a population, how effective this is in preventing further infection and therefore the likely future impact of further exposure to infection in the future. Although levels of infection can sometimes can be obtained from dead birds, these are often not representative of the wider populationand therefore results would be biased. In addition, our work requires following individuals that have been infected, recovered and survived to assess levels of immunity. We also need to follow individuals as they undertake seasonal migrations to track how disease may move around the environment and toidentify key transmission routes. There is no method of capturing the same information *in vitro*.

Which non-animal alternatives did you consider for use in this project?

We are not using animals as a model system for study but are interested in measuring levels of infection and immunity in wild animals as they are important in themselves. We are exploring the possibility of using faecal samples to test for both levels of infection and antibodies where i) faecal samples can be collected and assigned to individual birds and ii) the pathogen of interest can be detected in faeces. This is part of our experimental work to refine procedures for use by the wider community but needs verification using standard approaches to assess suitability. There is currently noindirect methodology for detecting other diseases we are interested in (e.g. respiratory viruses where no replication occurs within the gut) or the immune responses generated to them using any other means. Some information can potentially be obtained about particular pathogens from dead birds and

from environmental sampling.

Why were they not suitable?

While sampling dead birds for specific pathogens can tell us about one component of the population (iethose that are susceptible and have died) - a full understanding of the future impact of infection requires an understanding of the proportion of individuals that have been exposed to infection and survived. This will also be the component of the population that can potentially play a major role in disease in transmission and therefore this is the key component of the population requiring study.

Environmental samples can tell us if disease is present in an area but cannot tell us about individualresponses to infection that is needed for modelling future disease scenarios and mitigations.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissueand reuse.

How have you estimated the numbers of animals you will use?

Prevalence levels can change from year to year and differ between populations and species therefore numbers proposed are maximal and are continually adjusted based on minimum likely estimates required based on current prevalence in any population. Assessing numbers required in a responsive rather than pre-determined mode is one of our continual refinements to minimise the number of birds that require to be sampled. It is also essential due to the unpredicatable nature of disease progressionin the wild. For example, the current avian influenza outbreak is one key pathogen of interest which until recently has mainly affected wildfowl but has now moved widely into seabird species. A number ofpasserine species have now also been reported to be affected though in lower numbers. We have the expertise and statistical support to assess minimal numbers required to address key questions in this responsive mode.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Sample sizes proposed are based on our previous data that give indicative levels of prevalence in particular species. Sample sizes will be continually revised if prevalence levels in the wild change substantially. We are working as part of team that includes professional statisticians that can assist inundertaking these assessments.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

The power to detect relevant effects is increased if random variation between individuals can be accounted for. We therefore utilise a mixed modelling approach to analyse our data. In particular, typical of pathogen distributions in host populations, the deviations from group means are typically non-normally distributed – our statistical framework allow us to

specify other error structures, most commonly binomial, and thus to maximise the information that can be gained for the minimal number of samples. Several of our study populations have been monitored for over twenty five years so thereis considerable background biological data that can be utilised in the analysis to explain background variation in other traits to increase the power of analyses allowing minimum numbers to be utilised.
Where we can, we are sampling birds that are already being caught for other projects, such as trackingwork being done to enable marine renewable developments to be sited with minimal impact. This both limits the number of birds being caught and maximises the information gained from a single catch and there is synergy by combining this work as tracking information is also useful in understanding how infection may be moved around the environment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emergingrefinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our work is carried out on wild birds to test the impact of pathogens that affect them directly. The methods we use are the least invasive available for detecting levels of infection and immunity. The majority of populations we are working with have been monitored over many years so there is considerable background biological data that can be utilised in the analysis to explain background variation in traits we will be measuring allowing greater explanatory power in our models. The populations we are working with are also the same populations where the findings from our results could be used to establish disease mitigations. The methods of capture and handling of the birds andall the listed procedures have already been utilised successfully over a number of years without any detectable adverse effects in direct comparisons with individuals in the same population that have notbeen part of these procedures.

Why can’t you use animals that are less sentient?

Although some information can be gained from the examination of carcasses, fully understanding the level of infection in the population, levels of immunity and routes of transmission, we need to sample the live bird population. Furthermore, while some pathogens have high levels of mortality (e.g. highly pathogenic avian influenza), other important pathogens (eg low, pathogenic avian influenza, avian paramyxoviruses) may have sub-lethal effects in wild birds but still confer a substantial risk of spilloverinto other more susceptible species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We are continually monitoring the impact of our field work and associated procedures and exploring ways to reduce handling times. Post handling observation is a standard part of

our procedures. We usethe data we collect in the field to continually review our proposed sample sizes to ensure sampling the appropriate number of birds for given levels of infection prevalence in the field.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

All catching and handling of birds to conduct the procedures is carried out according to British Trust forOrnithology (BTO) guidelines by handlers who have been deemed competent by a BTO licence holder.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Our establishment regularly shares information from NC3Rs.org including their newsletters and eventswhich are useful in keeping up to date with general developments. More specifically attending scientificmeetings and talking with colleagues in the field keeps us up to date about advances that could enable us to refine our fieldwork procedures. Our new grant application specifically aims to explore alternativeways to measure immune responses less invasively but these require validation before fully adopting them going forward.

GENETICS OF CARDIOVASCULAR DEVELOPMENT AND FUNCTION

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Heart development, Heart disease, Genetics, Congenital heart defects

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

Mutations in genes are known to play a significant role in the development of both heart defects in new-born babies and in heart disease at all stages of life. Therefore, the overall aim of the project is touse genetically modified mouse models to investigate different genes which are involved in heart development and to understand how the loss of function of these genes affects cellular and signalling pathways within the heart leading to heart defects and disease, and how this can be prevented with potential supplements.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Babies born with heart defects account for one-third of all abnormalities present at birth (~0.8% of live births). Additionally, one quarter of all deaths worldwide are due to heart disease and, therefore, from birth to old age, these conditions are a significant burden on

the health care system due to the costs oftreatment and care required. Experiments undertaken on this project will provide valuable data which will allow us to understand why errors, known as mutations in certain genes, or disruption of signalling pathways which are required for a cell to function, can alter the normal structure or function of the heart, leading to ill health and life-threatening symptoms. This could lead to the identification of new disease-causing genes for heart abnormalities. These new genes could then be searched for errors in unborn babies, patients or asymptomatic patients, allowing affected individuals to be identified.

Furthermore, one focus of the project is to look at whether medication such as vitamin supplementationcould be a possible straightforward treatment for heart disease.

What outputs do you think you will see at the end of this project?

This project will generate new data to contribute to understanding why babies and adults develop heartdefects and disease. The most likely output for the project will be disseminating the data collected in the form of new scientific peer-reviewed publications and presentations at national and international conferences.

Who or what will benefit from these outputs, and how?

Our data will contribute to understanding the basic science behind how the heart develops and what goes wrong in heart disease. The studies, which focus on understanding the role of different genes orsignalling pathways in heart development and function, will reveal new disease-causing genes. This will be beneficial to families whose babies are born with heart defects allowing determination of their genetic risk of having a child with a heart defect.

Therefore, the data generated from this program of work will provide short-term benefit to the scientificcommunity in the field of heart and great blood vessel research who will benefit from the sharing of ourdata in the form of publications or presentations. Medium-term, the benefits would be that any new disease-causing genes identified could be included in a list of genes which are searched for mutationswhen a patient is assessed by a clinician. Moreover, long-term, the early identification of mutations in disease-causing genes will allow constant monitoring of these patients before any abnormalities in heart function are evident and hence by giving them early treatment it could stop the progression to heart failure. We hope that giving nonharmful medication such as vitamin supplementation, will help the patients with abnormal hearts, and improve their quality of life.

How will you look to maximise the outputs of this work?

We will maximise the outputs of our work through national and international collaborations during theproject and aim to publish the data collected in peer-reviewed journals and present at national and international meetings. Publications and successful grant applications based on this work will be publicised on social media platforms such as Twitter.

Species and numbers of animals expected to be used

- Mice: 15200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 as the mouse is the most appropriate and simplest model to use asthe different stages of heart development and how the blood flows through it, including the genes and cellular processes involved, and the final structure of the heart and blood vessels closely mirror those in humans. Therefore, using the mouse we can study the role of different genes which have similar functions in mice and humans.

To do this we will look at all stages of the life course of the mouse. To look in detail at the developmentof the heart we will start examining embryos from embryonic day 8.5 through to birth and the postnatal stages after birth. The function of the heart will be monitored from juveniles to adulthood, allowing us tomonitor the impact of the loss of a gene.

Typically, what will be done to an animal used in your project?

The majority of our planned studies focus on investigating the effect of changing the function of a geneon the heart. The main procedure we will do is simple breeding experiments through natural mating of different genetically altered mice. Embryos will be collected at specific time points from the pregnant mother who will be humanely killed. In some cases, genetically altered mice will be born and the function of their hearts may be measured at specific time points. We will also collect tissues from thesejuvenile and adult mice. The mice will be genotyped using an ear notch. In some experiments, mice may be given vitamin supplements in their diet to see if this makes a defect in their heart better. In some experiments the pregnant mother or juvenile/adult mouse may be injected with substances that do not cause harm but can later be detected in the developing embryo or different tissues, respectively.

What are the expected impacts and/or adverse effects for the animals during your project?

In some cases, juvenile or adult GA mice may develop a form of heart disease which may affect the heart function but not the overall health of the mouse. These mice and the corresponding control micemay receive vitamin supplementation in food and water and may undergo analysis of their heart function. For the analysis of heart function, the mice are anaesthetised. There is a small risk of hypothermia where the body temperature of the mouse drops, but this is very rare. The mice are placed on a heated mat during the procedure and placed in a warm incubator following the procedureto ensure the body temperature is maintained.

The mice will be monitored for adverse effects such as changes in their activity, appearance and bodyweight.

At the end of all the experiments the animals are killed by a humane 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 animaltype)?

The expected severity level for animals used for breeding and maintenance of the lines will be subthreshold to mild as they will experience only standard breeding practices and genotyping and thiswill account for approximately half of the mice used in this PPL.

The other half of the mice may experience moderate severity (although the majority will be mild) as they may develop heart disease with age and may undergo procedures including supplemented diets,injections, and cardiac function analysis.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Heart development is a very complex process which involves many different genes, cell types and cellular processes to allow the correct formation of the 3D structure of the four chambered heart whichis correctly linked to blood vessels in order for the heart to function properly. Therefore, to study these processes and to understand what happens in development or disease progression, we need to use mouse models as unlike cell culture-based experiments, they have a fully functional heart which is verysimilar to the human heart in how it develops and functions. Due to the accessibility and knowledge of mouse genetics which allows for genetic manipulation, the mouse can be used to study the role of genes in heart development and disease.

Which non-animal alternatives did you consider for use in this project?

There are limited cell lines which will be used to complement the studies in the mice. For example, there are cardiomyocyte (heart muscle cells) - like derived cell lines, which can be used to study gene function and cellular expression. Additionally, stem cells have the ability to be turned into different cell types including cardiomyocytes. Both of these cell lines can be cultured and maintained relatively easily and can be used to look at cell growth and behaviour in a controlled manner in a dish. However,neither type of cell line mimics the features of a mature cardiomyocyte. We considered using computermodelling in this project but found that this was not appropriate due to the complex interaction of cells and tissues within the developing embryo.

Why were they not suitable?

Although cell lines are cheaper and are relatively easy to culture, they have limited experimentalcapacity in terms of looking at heart development and function which include:

Cells grown in petri-dishes sit on plastic, which is much stiffer than where they reside in the body. The increased stiffness can change their behaviour and they become "super

activated" or fail to do thejob they would in the body. These abnormal behaviours could lead to the identification of non-relevant pathways or fail to predict drugs which are likely to be ineffective in the disease.

Organ disease development and resolution is regulated by many different types of cells communicating with each other within the damaged organ as well as through communication with white blood cells and receiving signals from other organs (delivered by circulating blood). Recreatingall of these internal and external organ damage signals is currently impossible to model in culture.

Maintaining cultures or organs alive and sterile for weeks, months or years is very challenging andwill not mimic all the natural events cells or organs deal with and so will lack context that is very important in most diseases or normal processes.

Heart development and disease are regulated by many different cell types which communicate witheach other, and also are exposed to signals from the circulating blood. Therefore, with the current technology this is impossible to recreate in culture as the cell lines are restricted to a single cell type and lack all the different signals.

The cell lines previously mentioned, are limited as importantly they lack the part of the cell which isneeded to make a heart cell beat (contract) which is a key process in investigating heart function.

Therefore, there are no 3D cell culture systems available that could replace mouse models. Using genetic engineered mice will help advance our understanding of both the natural processes in heartdevelopment and those in disease to help identify new targets for drug discovery.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 extensive experience of using mouse models and understanding what the minimal numbers required are for each experiment to produce statistical outcomes. Our work is based on using GA mice carrying either a mutation (an error) or a deletion (where part or all of a gene is missing) for a specific gene to study the effect on the developing and adult heart. Using biological inheritance, where we can work out which genetic information is passed onto each mouse embryo/pup born, we can estimate the number of mice carrying one copy of the affected gene (these mice are required for colony maintenance and for experiments) and the number of mutant embryos and mice which carry 2 copies of the affected gene we will generate. We can workout which embryos/mice carry no, one or two copies of the affected gene by looking at their genotype.For experiments which require the collection of heart tissue to study, for example, gene and protein expression patterns, and for those involving heart function analysis, we aim to collect 3-6 different embryos/mice of each genotype. We can then do statistical analysis to look for differences which are caused by the different genotypes and

not by normal biological variability.
We use animals of both sexes in our experiments.

We have consulted with the Animal Welfare Ethical Review Body (AWERB) statistician and colonymanager when estimating the numbers for this project.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The experimental design was planned using our prior knowledge and with advice from experienced investigators, animal unit staff, a statistician, and using the NC3Rs Experimental Design Assistant. Experiments are carefully designed, for example taking into consideration, group sizes, genotypes andare age matched for control and experimental groups. To avoid any variability in our studies all our mice are maintained on the same genetic background which means the same strain (C57Bl/6) of miceis used for breeding.

Throughout the project, group sizes will be constantly re-evaluated and updated as required to ensurethe minimal number of mice are used and there is very little wastage. As a general rule, only mouse lines which are currently being used will be expanded and then reduced following the completion of a set of experiments. When using new strains, or combinations of currently used strains, small numbersof animals will initially be analysed to check for any abnormalities in the heart. This will ensure that large numbers of mice which do not have the expected heart defects will not be produced.

We have planned to utilise cell culture as an alternative model to ask specific scientific questions, which will complement the mouse studies and may lead to a reduction in animal numbers in futureexperiments.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We maintain a high level of colony management to ensure through our breeding protocols and experiments we always reduce the number of mice with the incorrect genotype. To do this, we always ensure we use the best genotypes for each line. We only breed mice during their maximum breeding age and not beyond and carefully monitor the frequency and number of litters obtained and replace breeding cages when required. Breeding colonies that are not required in the medium/long term will bestored as frozen embryos or frozen sperm, to minimise the continued production of GA animals,

When generating new strains, we will carry out pilot studies using small numbers of animals which arecollected at specified time points to establish if these mice have any heart defects of interest prior to breeding more mice.

We will conserve tissues from individual animals by storing as frozen samples from mutants and extracontrol embryos/mice, so they can be preserved and used over long periods without needing to produce new animals for experiments. This will maximise outputs from animal procedures and minimise the number of animals used.

An important part of our project is cardiac imaging to assess the heart function of the mutant comparedto the control mice. By using non-invasive imaging techniques (such as electrocardiogram (ECG), magnetic resonance imaging (MRI) or ultrasound) means the mouse is not harmed in any way, and thisallows the same mice to be used at a number of

different time points, hence reducing the number of animals required.
Hence, we always ensure we maximise outputs from each animal while minimising the number ofanimals 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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use genetically altered (GA) mouse models in which genes of interest can be altered or deletedand we will study the effect of this on the heart and blood vessels during development and in adult mice.

All mice are handled using low stress handling methods to reduce any stressors to the animals.

We generally maintain our mice to carry one altered copy of a gene and this has no effect on the healthof the mouse. We will breed these mice together to generate embryos, foetal forms, new born, juveniles and adults for tissue collection for analysis. Prior to collecting the embryos, the pregnant female may be injected with a substance. The substances will cause no effect on the mother and the injection is a mild, transient pain. Animals will be humanely killed.

In some cases, the breeding trio may be given supplements in their diet (food and water) which has noimpact or cause any distress to the animals as the mice continue eating and drinking as normal. This isless stressful than for example giving the supplements by a more stressful invasive method like injections.

In some mouse lines, the mice which carry two faulty copies of a gene are able to survive birth and livenormal lives. In these cases, the mice will be closely monitored and the heart function may be predominantly be measured by ECG, and occasionally by MRI or ultrasound. The mice are closely monitored and allowed to recover in a warm incubator.

Why can’t you use animals that are less sentient?

Due to the complexity of the processes involved in mammalian heart development the use of other animal models such as chick, zebrafish, or insects (for example, flies) are not appropriate. Zebrafish for example have a different heart structure as they have two chambers, compared to the four chambers in humans and mice. Mice are being used in this project because this is the simplest organism that has a similar heart and blood vessels to humans and therefore can be used to investigate the roles of different genes in heart development. We are able to modify gene function easily in the mouse and have the necessary tools. Moreover, there are no suitable cell culture systemswhich would replace the complex 3D structure of the heart, especially for discovering disease mechanisms as these would be different in culture.

For the study of heart development, we will be using immature embryos and foetuses. However, for thestudy of the progression of heart disease it will be necessary to study the mice post birth and into adulthood to mimic what happens in humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

All mouse lines, regardless of genotype are checked daily by the animal staff and if required supportivecare will be provided to minimise distress or suffering and to improve welfare. We ensure early socialisation to human contact of mice in our care to reduce the stress of being handled to an absolute minimum and we use tunnel handling to pick the mice up. A large majority of our experiments will involve the collection of embryos and foetuses so the animals involved in breeding will have no observable adverse effects.

Following any procedure using anaesthesia (cardiac function monitoring by MRI, ECG and ultrasound),the mice will be allowed to recover in warm incubators. In some situations, it may be required to transport the mice for imaging to a different facility and in this case the mice will have 5 days ofrecovery prior to imaging.

For mouse lines that may present with heart-related issues, health monitoring will be carried out regularly with the aid of a local establishment or specifically defined score sheet, and data recorded, for example, weight, activity, appearance, and body condition. If a welfare risk is identified, the impactwill be minimised by working with the Veterinary team to administer appropriate treatment.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Alongside the guidelines listed below, I will also adhere to local AWERB standards for research animals, and where appropriate, support the development of new local standards for refinementsdiscovered during the project licence.

Code of Practice for Housing and Care of Animals Bred, Supplied or used for scientific purposes

LASA Guidelines

RSPCA Animals in Science guidelines

UFAW Guidelines and Publications

NC3R’s and Procedures with Care

I will consult with the Colony Manager to review genetic health, breeding practices and overall colonyhealth and management at regular intervals.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

The local Animal Welfare Ethical Review Board (AWERB), Named Animal Care and Welfare Officer (NACWO), Named Training and Competency Officer (NTCO) and Veterinary team regularly inform, anddisseminate improvements and recent studies

involving reduction, replacement and refinement.

Alongside external resources including (but not limited to); collaborators, peers, conferences and labanimal and animal welfare bodies.

During the 1, 3 and 5 year review of the project licence, I will update on the implementation or consideration of the 3Rs that have occurred during the previous period, alongside a review of the linked training plan, score sheets etc. in collaboration with the NACWO, NTCO, and Veterinary teamwith a particular focus on refinements.

NEUROTRANSMITTER ACTION, ION CHANNELS AND RECEPTOR MECHANISMS

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Glutamate receptors, NMDA receptors, Parkinson's Disease, Alzheimer's Disease, Dopaminergicneurons

Animal types	Life stages
Mice	neonate, juvenile, adult
Rats	neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to improve knowledge and understanding of neurotransmitter action in the brain and in particular of the neurotransmitter glutamate and its action at one type of receptor for glutamate, the NMDA (N-methyl-D-aspartate) subtype of glutamate receptors. We aim to enhance understanding of the action of drugs and medicines at this receptor and of the functional properties ofthese NMDA receptors in order to contribute to understanding disease processes in the brain such asin Schizophrenia, Parkinson's and Alzheimer's diseases.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Aberrant glutamate receptor signalling is now widely recognised as a key contributor to diseases of thenervous system including stroke and epilepsy, and neurodegenerative

diseases such as Parkinson's Disease and Alzheimer's Disease. In addition, NMDA receptors and their contribution to synaptic plasticity are a key feature of mental health problems such as depression and schizophrenia. The experiments described in this project will provide new information relevant to understanding drug action at NMDA receptors and so help with understanding drug action at these receptors.

Parkinson's disease and Alzheimer's disease are two example conditions that illustrate well why basicresearch generating new knowledge about NMDA receptors in the brain is important. Parkinson's Disease affects approximately 1% of the population over the age of 65yrs. Although symptoms are initially mild, this is a relentless neurodegenerative disease that eventually impairs all movement. The primary feature of the condition is the slow degeneration of dopaminergic neurons in a region of the brain known as the substantia nigra (one of the types of neuron which are the subject of this study).

The cause of this degeneration is unknown and current treatments are symptomatic only. Current treatments do not slow the underlying disease progression. One current theory of the disease progression is that NMDA receptors contribute to dopaminergic neuron cell death and so knowledge ofNMDA receptor properties in these neurons may benefit future research in this area while knowledge of drug action at these receptors may aid with development of improved drug therapies in the future.

One current treatment for Parkinson's Disease, amantadine, acts as a weak blocker of NMDA receptor.

Alzheimer's Disease accounts for about 60% of all dementia in the UK and has huge economic and social impact on our society with an annual cost of more than £26 billion in the UK. Alzheimer's disease is a progressive neurodegenerative condition that results in severe cognitive decline, characterised by failing memory, compromised decision making and impaired ability to form and retainnew memories. In many brain circuits, the formation of memories is dependent on activation of NMDAreceptors at synapses (points of communication between neurons) and the study of synaptic transmission is one of the key areas of current research into Alzheimer's Disease. Overactivation of NMDA receptors is recognised as one potential contributor to neuronal cell death and so the study of NMDA receptor pharmacology may lead to new 'neuroprotective' drugs that could be useful in neurodegenerative disease. One current treatment of Alzheimer's Disease is the drug, memantine which acts as a blocker of NMDA receptors and can help to improve cognitive function in AD patients.

What outputs do you think you will see at the end of this project?

The research in this project is largely about studying NMDA receptors. The main output is knowledge of NMDA receptor function and pharmacology. In the short-term and by the end of this project the mainoutputs will be knowledge that will be used by other researchers in academia and industry engaged in further brain research, as evidenced by our previous research publications in highly regarded basic research physiology, pharmacology and neuroscience journals.
Specific benefits of this research include advances in understanding drug action. Benefits of this work include enhanced understanding of the mechanisms of action of recently developed NMDA receptor 'allosteric potentiator' drugs. One of our current areas of research and one of the first areas likely to benefit from work under this project is in understanding the action of recently developed potentiator drugs. Drugs like this could be used in the future to enhance dopamine release from dopaminergic neurons in Parkinson's disease and hence help to relieve movement problems for Parkinson's Diseasepatients

which are caused by loss of dopamine-producing neurons in the brain. The investigation of NMDA receptor potentiator drugs will be the subject of the next experiments we will make and will be amajor part of our next publication.

Who or what will benefit from these outputs, and how?

In the medium term, knowledge created by this project will benefit other researchers engaged in pursuing understanding and treatment of brain diseases such as Alzheimer's or Parkinson's. One aspect of this is the pursuit in the pharmaceutical industry of better 'neuroprotective' drugs which couldhelp to slow or even stop neurodegeneration in the future. Thus, the long-term potential beneficiaries of this project extend to patients with neurological disorders. The severe unmet clinical needs of thesepatients mean that further research is urgently needed in these areas. The results of experiments conducted under this project may also aid understanding of disturbances of mental health such as depression or schizophrenia that are now known to have an NMDA receptor component, although the experiments are not directed specifically at these conditions.

We currently collaborate with academic research groups in the US, where several promising NMDA receptor modulator drugs have been developed. By collaboration we can share our latest knowledge with drug developers who are working on related problems while our research benefits from knowledgefrom them about the latest drugs under development.

How will you look to maximise the outputs of this work?

In order to maximise the outputs of this work, strict attention is paid to creating high quality open access publications that via peer review and subsequent dissemination among researchers can optimise the benefits that can be achieved from this knowledge. In addition to published output, I regularly give invited lectures, conference communications at national and international physiology andpharmacology scientific meetings, and presentation of research outputs as part of public engagement.

Examples of this include lectures during postgraduate training courses given to PhD students and members of the pharmaceutical industry, invited lectures at advanced neuroscience courses in developing countries, and High School public engagement lectures. In addition, my international collaborations in Europe and in the United States facilitate knowledge exchange and enhance efficientuse of new knowledge in further research. In these ways the outputs from this work can be communicated at multiple levels of the scientific research community with the goal to make new knowledge and understanding as widely available as possible.

Species and numbers of animals expected to be used

- Mice: 60
- Rats: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project does not require any in vivo work. The experimental approach we use prepares acute slices of brain tissue that preserve closely the physiology and synaptic connections of intact nervous system. In this way we can study neurotransmitter receptors and synaptic transmission with most relevance to normal physiology and thus achieve more precise understanding of the actions of drugs inthe nervous system. However, the preparation of brain tissue from animals of defined age and developmental stage means that animals need to be killed using methods appropriate to the optimum preservation of the brain tissue for physiological and pharmacological measurements that are made in vitro. The animals most useful and best suited for these experiments are small rodents: rats or mice mainly between 7 days old and young adult stages. For the experiments in this project, the rat or mouse nervous system very closely matches that of the human nervous system and so provides a verygood model system in which to develop our understanding of physiology and drug action. Animals of different developmental stages are used to optimise relevance of experimental results to normal physiology or pathophysiology as appropriate. Young animals often provide the most consistent experimental preparation and also the opportunity to investigate mechanisms at defined developmentalstages. In contrast, older animals are sometimes needed to precisely study mechanisms relevant to diseases of the ageing nervous system. The benefits of these approaches are to provide high-quality experimental data at defined developmental stages and, where appropriate, at ages relevant to specific disease processes.

Typically, what will be done to an animal used in your project?

In order to achieve the above benefits, acute slices of brain tissue are used to make high-resolution,patch-clamp electrophysiological measurements. For 7 days old animals, the animal is killed by decapitation as this provides a rapid (seconds) end to life by exsanguination without damage to the brain tissue to be used for experiment. For older animals, the animal is killed by rapid anaesthetic overdose (~ 1 min) followed by decapitation which provides a painless and stress-free end to life by exsanguination. Subsequently the prepared brain slices are washed with a large volume of physiological salt solution which effectively dilutes the anaesthetic to negligible concentrations in the brain tissue before beginning the experiment. In this way a potential confound of anaesthetic effect onmeasured physiological parameters or drug effects is avoided. Prepared brain slices are maintained inconstantly oxygenated physiological salt solution and are viable for > 8 hours and since multiple brain slices (~10) can be prepared from a single animal, this provides tissue for multiple experimental observations to be achieved from a single animal.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are anticipated using this procedure. Mild transient stress during handling of an animal is to be expected and this will typically last no more than a few minutes between removal of theanimal from its home cage to end of life. Decapitation in the neonate will create transient pain before the almost instantaneous drop in blood pressure to the brain will result in unconsciousness. In older animals mild stress during anaesthetic inhalation is possible, but transient (< 30 seconds) before the animal loses consciousness which is checked by loss of reflexes. The procedure will be carried out under qualified supervision or by fully trained and competent individuals. Should an animal display anyadverse effect at any stage they will be immediately killed by a suitable humane 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 animaltype)?

The expected severity of the decapitation procedure is mild (100%). The procedure will be carried outunder qualified supervision or by fully trained and competent individuals using a suitable dedicated apparatus. Animals undergoing terminal anaesthesia are continuously monitored and checked for endpoint (absence of reflexes). Rarely (<1% of animals), anaesthesia could cause adverse effects such as agitation in which case the animal will be killed by a suitable procedure. Adverse effects duringterminal anaesthesia are minimised by using a maximal anaesthetic concentration.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The questions I aim to address specifically relate to the function and dysfunction of the mammalian nervous system, and to understanding the physiology of the synaptic transmission. These questions can only be addressed ultimately through the use of living, functioning mammalian brain tissue with relatively intact neuronal circuits that enable empirical measurements of neuronal physiology to be made. Such tissue can only be obtained from animals. The in vitro brain slice preparation will be usedfor the majority of the proposed experiments, using brain tissue removed from rats or mice. Rodents are the most appropriate species for these experiments because of the wealth of knowledge currently available on rodent glutamate receptors and rodent models of human diseases. Rodents are the lowest vertebrate species suitable for the preparation of brain tissue for physiological measurements. Key pharmacological systems share similar characteristics in both rodents and humans. Compared with using *in vivo* models relevant to the study of synaptic transmission, the brain slice model reduces the number of animals required, and also minimises the severity of the procedures applied to the animal.

Which non-animal alternatives did you consider for use in this project?

Experiments in tissue-cultured cell lines expressing receptors of interest can be used for some studiesof drug action at neurotransmitter receptors. However, receptors expressed in these cells lack direct relevance to receptors in neurons.

Human stem cells grown and differentiated in tissue culture display some of the properties of neurons(including expression of some neurotransmitter receptors), particularly of neurons early in development.

Why were they not suitable?

Tissue-cultured cell lines (e.g. HEK or CHO cells) lack many of the relevant biochemical

systems relevant to understanding of the nervous system (e.g. the precise receptor protein composition in specific neuron types) and crucially, tissue-cultured cell lines do not make synapses and so cannot beused to investigate drug action at synapses. Because of this cell lines tend to be useful for studies of the receptors in isolation, but lack the crucial relevance to intact nervous system that is needed to understand receptor function at synapses and drug action in the brain.

Human stem cells grown and differentiated in tissue culture do not display consistent cell-type specificproperties (e.g. comparing excitatory or inhibitory neurons with dopaminergic neurons) and so do not reflect the properties of neurons *in vivo* and cannot be used as model systems to understand the actions of drugs at receptors and ion channels in neurons.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on the type of experimental recordings needed for this project (electrophysiological recordings from single neurons) and the likely success of individual experiments combined with the precision of this type of measurement, the number of animals that will be used in order to reach a clear conclusionis estimated based on past experience and knowledge of the variation in experimental measurementsobserved and the size of drug effects being measured.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The key to reducing the number of animals being used in experiments like this is experimental design and efficient use of the experimental tissue. Each brain slice is used for only one drug application, so each new recording is made in a fresh tissue sample avoiding potential confounds due to cumulative drug effects. Thus use of brain slices allows multiple experimental observations of drug action to be made using tissue from a single animal (technical replicates) which reduce the variation of the average effect measured across independent animals, therefore minimising the number of animals used for each experiment. A single neuron recorded in the whole-cell patch-clamp configuration can provide its own ‘internal’ control recording before application of a test drug. In some experiments, the effects of multiple concentrations of a single drug can be tested on a single brain slice. Randomisation of the sequence of drug concentrations applied is used to avoid systematic effects of the sequence of changes in drug concentration that could bias measurements. These approaches reduce measurementvariation and so allow smaller drug effects to be more reliably detected, or allow less brain tissue (and so reduced animal numbers) to be used when measuring a drug effect.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

In silico methods of modelling drug action are used in order to optimise experimental design and data analysis and to minimise the number of animals used. We have precise models of drug action (see e.g.Gibb, 2022) and receptor activation allowing prediction of effect size and so optimum choice of experimental conditions such as experimental drug concentrations. Each experimental animal provides several brain slices and where possible, brain slices are shared among laboratory personnel making these measurements, so reducing animal usage. Because the brain has two substantia nigra regions, each coronal brain slice can be cut into two equal pieces providing up to 12 tissue samples foruse in each experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rodents are the most appropriate species to provide brain slice tissue for these experiments because of the wealth of knowledge currently available about rodent glutamate receptors and rodent models of human diseases. Compared with using *in vivo* and behavioural models to study drug action, use of electrophysiological recordings from neurons in brain slices minimises the severity and number of the procedures carried out on animals in order to achieve a statistically viable set of measurements of synaptic transmission or of drug action at neurotransmitter receptors. Because multiple brain slices areobtained from each animal, these provide technical replicates so that brain slices as a model system reduce the number of animals required while increasing the level of detail and consistency of observations. Suffering is minimised by handling the animal carefully to avoid stress and by rapid end of life. Variability of experimental tissue is minimised by good training to precisely apply the procedurein preparing the brain slices, and by ensuring all equipment is well maintained.

Why can’t you use animals that are less sentient?

Rodents are the lowest vertebrate species suitable for the preparation of brain tissue for physiological measurements of this kind where the results need to be of direct relevance to drug action in other mammals and humans. Key physiological and pharmacological systems in the brain share similar characteristics in both rodents and humans and the properties of synapses in rats and mice are directlycomparable to what is known from limited studies of human brain tissue (e.g. surgically removed tissuebiopsies).

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

By updating and refreshing knowledge, by studying advances (eg in anaesthetic use) and by observingprocedures by other trained individuals, the procedures used in this project for the preparation of acutebrain slices will be continuously examined for possible refinements that can both optimise the quality ofexperimental results or reduce further the impact on each animal.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Several published guides to optimising electrophysiological recordings from acute brain slices (e.g. Bischofberger et al. (2006) Patch-clamp recording from mossy fiber terminals in hippocampal slices.Nature Protocols, Vol. 1(4), 2075. doi:10.1038/nprot.2006.312

Experimental design and data analysis guides (e.g. The Experimental Design Assistant: Percie du SertN, Bamsey I, Bate ST, Berdoy M, Clark A, Cuthill I, et al. (2017) The Experimental Design Assistant.
PLoS Biol 15(9): e2003779. https://doi.org/10.1371/journal.pbio.2003779)

Animal Research, Technical Advice published by UK.GOV
(https://www.gov.uk/guidance/animal-research-technical-advice)

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Avenues to stay informed about advances in the 3Rs include i) discussions with colleagues, ii) regularupdate discussions at scientific meetings such as those of The Physiological Society and British Pharmacological Society, iii) I subscribe to the NC3Rs e-newsletter which comes via e-mail and includes updates on funding opportunities to investigate alternatives to animal experiments, information about 3Rs events and publications iv) seeking advice where appropriate from local NACWO and staff of our Biological Services Unit, v) regular discussions at editorial board meetings ofBritish Journal of Pharmacology. Implementing advances effectively may require undertaking training or refresher courses such as provided by Jackson labs or observation of colleagues' procedures.
Discussion of advances in tissue preparation techniques (which can help to minimise experimental variation and so reduce animal usage) happens regularly by attendance at specialist electrophysiologycourses such as the annual Plymouth Microelectrode Techniques Workshop.

REGULATING GLIAL FUNCTION TO PROMOTE HEALTH AND REPAIR IN THE CENTRAL NERVOUS SYSTEM

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain, glia, Neurodegenerative disease, Healthy aging, Brain repair

Animal types	Life stages
Mice	neonate, juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to examine extracellular and intracellular signalling pathways that regulateglial cell functions that are critical for maintaining a healthy brain and promoting its repair.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 neurodegenerative diseases are often inadequate. Glial cells are critical for brain function and largely determine the pathogenesis and outcome of neurodegenerative diseases.However, we do not fully understand the complex cell biology of glial cells in health or disease. This project aims to provide this fundamental information that will lay the foundation for potential new therapies.

What outputs do you think you will see at the end of this project?

This work is primarily fundamental research that will generate new knowledge on the phenotypic, biochemical and physiological processes that underpin glial function and regulate brain health andrepair.

The primary outputs of the project will be publications, as well as training of the next generation ofyoung scientists. Key outputs of the project will be the identification and/or exclusion of potential targets for the future development of therapies aimed at the regulation of neurodegenerative processes.

Who or what will benefit from these outputs, and how?

This fundamental research will be of interest to the thousands of neuroscience researchers andclinicians worldwide working in this area.

In the medium term the project will be of interest to the commercial and public sectors.

It is likely that with the approaches used in this project will rule in or out novel drug targets that havethe potential to promote brain regeneration, which in the longer term may have therapeutic potential.

How will you look to maximise the outputs of this work?

Findings will be made available to other scientists and potential end-users through publication in peer-reviewed journals and presentations at scientific conferences and meetings. All data will be published in full in open access journals, including unsuccessful approaches and 'negative' findings. In addition, the data will be presented at national and international conferences, at stake-holder meetings of the Spinal Research Trust, Alzheimer’s Research UK and Multiple Sclerosis Society, as well aspresentations to the public and communication with local and national media.

The databases generated in this project will be made freely available to other scientists andcommercial sectors interested in neurogenesis in the brain.

Species and numbers of animals expected to be used

- Mice: 10000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 neonatal, juvenile, adult and aged (up to 15 months old) mice. The overall aim of the project is to study how glial cells maintain the health and repair the nervous system at different periodsof life, so we need to use animals at different ages.

Mice will be used as the neuroanatomy and neurophysiology of this species is well understood. Most ofour knowledge of glial cells in health and disease is based on studies in mice, which provide the best compromise in terms of correlating with human biology whilst exhibiting the lowest level of sentience.

In contrast, glial cells do not replicate the complex anatomy and physiology or neurone-glial interrelationships of humans in in silico or in vitro systems or in animals with lower levels of sentience,such as frogs, fish, or invertebrates.

Typically, what will be done to an animal used in your project?

We will use genetically altered mice in which glial specific genes drive cellular expression of fluorescent proteins, which refines unequivocal identification of glial cells and reduces the number of mice that would otherwise be used for cellular identification by other anatomical techniques. In the majority of experiments, mice will be killed by a humane method to enable us to harvest tissue for in vitro and ex vivo experiments, which reduces suffering and the number of mice used overall. In these experiments, we will determine the effects of small molecules on glial signalling pathways and determine how they regulate glial neuroprotective functions. Following on from these studies, the mostpromising agents may be tested in vivo; mice will be anaesthetised for the administration of agents by the most suitable route (into the body fluids of the animal, nasally, or directly into the fluid compartmentof the brain (ventricles) by injection), after which they will be allowed to recover and return to their cages and after a suitable period of time (1 day to several weeks), the mice will be killed humanely to harvest the tissue and determine the outcome on glial cells. In some cases, mice may be treated with the most efficacious agents following treatment of a gliotoxin that is injected focally into a very small area of the brain of anaesthetised mice to reproduce localised pathological changes observed in neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease.

What are the expected impacts and/or adverse effects for the animals during your project?

Most animals experience no more than mild discomfort from anaesthesia. Occasionally, administrationof agents directly into the brain may cause transient mild problems, but we will design the experimentsto minimise these possibilities, by using agents with known pharmacology and toxicology, testing theseagents in a test tube, and starting with low doses in animals to induce partial effects that are sufficient for us to determine their functional importance, whilst minimising harm to the animals. Animals that do not recover full functionality quickly after drug administration 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 animaltype)?

Subthreshold: 60%

Mild: 20%

Moderate: 20%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered 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 determine the glial signalling pathways that regulate healthy brainfunctioning and repair in neuropathology.

The research questions necessitate the use of animals since the anatomy, physiology and neurochemistry of glial-neuronal interactions can only be elucidated by studying in tissue or the animalitself.

Which non-animal alternatives did you consider for use in this project?

We have considered in silico techniques, tissue culture and brain organoids, using cell lines and human cells, and we will incorporate and develop these models during the course of our project. Wewill develop these models for studying aspects of fundamental glial cell biology.

Why were they not suitable?

In silico and in vitro models will be used to achieve important objectives of this project, such as identifying key regulatory pathways that promote glial repair mechanisms. However, brain function anddisorders arise from the interactions of a multitude of individual cell types, and it is not currently possible to fully replicate the molecular, morphological and functional complexity and interactions of these different brain cells, in basic in vitro cell systems.

In order to develop robust computer models, we first need to have accumulated a sufficiently detailedset of data to feed into the algorithms. The current lack of available primary data relating to the parameters under investigation in this project limits the use of such technology at this stage.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This estimate is for the number of mice required for the breeding and maintenance of the transgenic mouse colonies to provide tissue and animals for the proposed experiments based on pilot studies andour published data. To minimise animal use for breeding, we use breeding regimes that minimise production of animals of the wrong genotype, as well as using multiple tissues from the mice (such as the cerebrum, hippocampus, optic nerves, cerebellum, brainstem and spinal cord, as well as peripheralnerves, internal organs and gastrointestinal tract), and sharing any spare animals with other researchers. We have extensive experience in using these models and can therefore accurately estimate the

number of animals we will need per group to obtain statistically valid results. As we generate more results, we will continually assess group sizes to see whether the number of animals can be further reduced from those currently proposed. We will also use the latest statistical methods for data analysis to reduce sample size.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

I have made use of all local support including our statistician and other resources such as the NCRsexperimental Design Assistant.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Our use of tissue culture systems from mice will reduce the number of experiments that need to use live animals. We conduct pilot experiments where necessary to ensure that our experimental systemsare optimised before conducting full studies. We will carry out studies in a stepwise manner to minimise the number of animals used. We and our collaborators work on body systems other than thebrain, and we will share tissues with them to reduce the total number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

In the majority of experiments, mice will be killed by a humane method to enable us to harvest tissue for in vitro and ex vivo experiments to identify neuroprotective agents. The most promising agents maybe tested in vivo in anaesthetised mice and, to determine their neuroprotective efficacy, in some casesa small volume of gliotoxin may be administered focally into a small area of the brain of anaesthetised mice to reproduce pathological changes observed in neurodegenerative diseases, such as multiple sclerosis and Alzheimer's disease. This experimental model has known and minimal risks to the animal and has reduced suffering compared to models that cause more severe neurodegeneration, such as genetic models of multiple sclerosis and Alzheimer's disease. In cases where there are known harmful effects that are part of the experiment, we have selected methods where the effects are highly localised and transient and should not cause clinical harm to the animal. In the case where we are developing pharmacological modulation of glial therapeutic targets, we have designed the experimentsso that the function of these molecules can be altered incrementally, to cause effects on the cells without clinical harm to the animal.

Why can’t you use animals that are less sentient?

Our aim is to understand how modifying signalling pathways in glial cells can maintain a

healthy brain and promote repair. We will use transgenic mice because they enable us to specifically affect potentialdrug targets in specific cells. Mice are the primary preclinical model for the development of novel therapies and wherever possible we use in vitro methods. A number of lower vertebrate species, for example larval stages of Xenopus and Zebrafish, are available for screening novel compounds in vivoand, where possible, we will seek to collaborate with experts in these species to refine our experiments. However, the detailed background knowledge on glial cells is missing in these species and the little that is known has demonstrated glial physiology and anatomy does not comply with that of the human nervous system. We plan to develop the use of less sentient species during this project, but currently this will not preclude the use of mice for preclinical experiments and we will screen compounds in silico and in vitro, then test the most promising ex vivo, and only those proven to be effective will be examined in vivo.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

At all stages of the project, we will conduct welfare assessments on the animals to evaluate the impactof procedures and will review our procedures to determine whether harmful procedures can be minimised without adversely affecting scientific outcomes. We will refine our delivery of therapeutic agents to the brain by improving the efficacy of the non-invasive technique of intranasal delivery, with the aim of achieving accurate delivery of effective doses to the desired site/s of action, which currentlyrequires administration of these agents by local and controlled injection via ultrathin needles directly into the brain or spinal cord to by-pass the blood-brain barrier; the latter is the current route of administration of agents into the brain and spinal cord in humans, such as epidural anaesthetic in childbirth and intracerebral injections in neurological diseases. We will also seek advice from the named people and other scientists working in the discipline.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

I will keep up to date with best practice through published guidelines issued by LASA, NC3Rs, RSPCA,the HO and any other relevant sources.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

I will keep up to date by reading scientific articles and attending conferences. I will also liaise frequentlywith the named veterinary surgeon, named animal care and welfare officer, and named information officer.

Home Office

STEM CELLS AND ADAPTIVE MOLECULAR PHENOTYPE IN COLORECTAL 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, ortheir effects, in man, animals or plants

Key words

Stem cells, therapy, cancer, cell plasticity, tumour microenvironment

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

Cancer cells are capable of adaption to change. This adaption is mediated through changes in the chemical messages that control cancer cell fate and is responsible for the ability of some cancer cells to resist drug treatments. These altered chemical messages can result from the combination of mutation in the cancer cells DNA themselves and from variation in the cells that surround and support cancer cells. We believe these adaptive changes can be assessed and measured in cancer cells usinga new tool we have developed called the stem cell index, and we wish to use this in advanced cancer models to see if we can assess a tumours' response to a drug treatment and improve therapeutic killing of cancer cells.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 cells are subject to the forces of natural selection. When cancer cells are exposed to chemotherapies, some cells will be killed, but we believe that other cells within the tumour survive andare capable of responding to these evolutionary forces (selective pressures) by changing their behaviour and appearance - so called cellular plasticity. This plasticity is responsible for some tumourseventually developing resistance to chemotherapy agents, by adapting to their changing circumstances. This adaptive cancer cell evolution depends not only upon the genetic makeup of the tumour cells themselves but also on the environment within which those cells reside. This is made of up of a variety of supporting stromal cells and a constellation of different immune cells. The cancer cells and these surrounding microenvironmental cells are engaged in a two-way conversation, communicating with each other through the secretion of chemical messages. These chemical messages ultimately control the activity, appearance and the behaviour (fate) of the cancer cells.

We believe that tumour cell plasticity is controlled by the crosstalk between a cancer cell and its environment, and that we can measure this key tumour cell attribute, and how it responds to drug therapies by looking at some molecular markers of cancer stem cells. Using a combination of advanced biological models and contrasting these with measurements taken from human tumours, wewill explore these concepts and test the impact of established and novel drugs on tumour adaption andcell plasticity. We believe that if we can prevent a tumour from adapting to a selective pressure, like that imposed by a chemotherapy drug treatment, then this will improve the cancer cell killing effect of new combinations of anti-cancer drugs.

Specific aims include:

Increase our understanding of the chemical messages that mediate communication between cancercells and their surrounding microenvironment, and the impact of this signalling on the cellular architecture of intestinal tumours. Use existing and novel drugs to interrupt the tumour-microenvironmental crosstalk, reduce the cellular plasticity response of cancer cells to a chemotherapy drug and see if this can improve cancercell killing

Assess whether cellular plasticity is required for cancer cells to spread to other organs (metastasise)and assess which chemical messages are responsible for the successful colonisation of a different organ by intestinal cancer cells.

Ultimately, we hope this work will lead to an improved ability to measure tumour response to drug treatments, test the impact of existing and novel therapies at inhibiting the chemical messages that allow cell plasticity and assess whether we can reduce or inhibit the ability of cancer cells to spread toother organs.

What outputs do you think you will see at the end of this project?

The work has a clear line of sight to patient benefit. We expect this work to provide insight into thefollowing translational outputs:
Identifying morphogen signalling targets to reduce stem cell plasticity and adaption in cancer

Preclinical testing of the stem cell index as a molecular tool to assess tumour response to therapy,and guide drug administration.

Preclinical testing of new or repurposed agents in mouse models to demonstrate favourable efficacyand toxicity profiles and expedite human clinical trials

Our previous preclinical work with novel drug targets or agents has led to a patent application andresulted in clinical trials, although these can be hard to foresee at work outset as drug activity andcontext is rarely predictable.

We anticipate that the work programme will generate >5 primary publications in the 5-year period withmore stemming from collaborative work. It will also underpin a further program of work.

Who or what will benefit from these outputs, and how?

Mouse work is undertaken based upon observations made on human tissue and all of these fundamental observations have an impact on human disease. Specific beneficiaries from this researchwould include:

The investigating group.

Collaborators. The international collaborators involved in this project are all experienced colorectal cancer researchers. The project will generate multimodal animal and human data which will be sharedacross the collaborative network.

Other cancer researchers. Cell plasticity is not confined to colorectal tumours but is relevant acrosssolid and liquid tumours. Researchers working on other diseases. The tools/algorithms we will develop for assessing cellular neighbourhoods could be used in other organs and diseases to assess cell-to-cell interactions (such asinflammation, infection and autoimmune disease)

The data, functional models and tools that we will develop in this proposal (e.g. animal models)would be available to the wider scientific community following publication and/or subject to data sharing/material transfer agreements.

Pharma. We will undertake preclinical testing of a novel drugs and if successful will work with thedrug companies on drug development.

Clinical triallists. We will preclinically test the application of measurement of the stem cell molecularphenotype in guiding therapy scheduling

Clinicians and patients. This work has a clear translational focus as we seek to address thetranslational questions above.

How will you look to maximise the outputs of this work?

The outputs of this work will be maximised by collaborations with scientific and clinical colleagues. New knowledge will be communicated through publication in open access journals. Any acquired datawill be shared on data access platforms and new analysis tools will be made freely available through Github.

Species and numbers of animals expected to be used

- Mice: 41000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The animals used in these experiments will be mice, which are the most appropriate and least sentientmodel for studying cancer. These animals will be genetically modified strains, carrying targeted mutations or transgenes. We will mainly using adult mice in which relevant genes have been altered and these mice may be aged (up to 2 years) to allow time for the formation of cancers.

Typically, what will be done to an animal used in your project?

Mice will be bred and tissue taken for both identification and to determine their genetic status. Mice of the appropriate genetic status may be dosed with a gene deleting agent typically by the oral gavage route. Mice may undergo injections to test of novel drug treatments. Some mice may undergo surgical procedures to introduce cancerous cells into the body to allow the study of the spread of these cells to other tissues (metastasis). Some mice may be monitored for signs of cancer and disease progression,up to 2 years of age. After these procedures' mice will be humanely killed and multiple tissues harvested for further analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Some experiments will only cause mild adverse effects such as mild anaemia. Other mice will experience moderate harmful side effects such as weight loss or tumour development. Animals will bemonitored regularly as the disease progresses and the animals killed when defined limits are met (for example when a certain tumour size has been reached). It may take time for the cancers to develop so some mice may be kept up until 2 years of age. It is expected that the duration of the experiment will be no longer than 3 months once the phenotype is visible.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Mice 33% Subthreshold, 40% Mild 27% 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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our work is based on observations made in human disease, such as the increased prevalence of bowel cancer in inflammatory bowel disease. Human tissue and cell line work is useful to generate hypotheses and constituent about 5-10% of our work. However, functional mechanisms need to be tested in biological models as data from cell line work are often unconvincing in the absence of in vivodata.

In order to explore epithelial-mesenchymal pathways in vivo work is required, the use of mouse modelsis an essential and complementary approach for both the understanding of the initiation and progression of tumours, and for the development and testing of therapies. Moreover, since our aim is to take some of the interventions forward into clinical use, animal testing is essential such that humans are not given interventions for which the evidence of efficacy is low, or toxicity high.Lower species can share some cancer-causing pathways with humans but many do not live long enough to allow tumour growth. Their organs and immune systems can also differ significantly, thus are not suitable models for cancer research. Mice however do develop tumours in as a result of mutations in genes known to cause cancer in humans, and powerful new technologies now allow us to recreate precise mutations, and combinations of mutations in mice. Using these models, we can followcancer progression from the appearance of the first few tumour cells and investigate how, when and where these develop into cancers. We can also treat these mice with existing or new therapies to investigate which tumours respond well and poorly to each therapy.

Which non-animal alternatives did you consider for use in this project?

Over recent years, significant advances in culture techniques have allowed generation of established murine intestinal organoid, and tumour derived spheroid cultures. Similarly, the advent of CRISPR- cas9 based gene editing approaches have allowed these established culture systems to be modified genetically. These approaches are now applied regularly as an alternative to animal usage in vivo, andhave a number of advantages including increased scalability and flexibility.

Why were they not suitable?

Unfortunately, these approaches are as yet unable to address the majority of questions related tometastatic dissemination and immune/stromal co-option in the primary tumour.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is based on experience over years of working with transgenic mice carrying some of the alleles tobe used in these experiments.

What steps did you take during the experimental design phase to reduce the

number of animalsbeing used in this project?

We will always use the minimum number of animals required to achieve of scientific objectives. Wewill optimise breeding strategies as efficiently as possible to achieve the greatest number of desiredgenotypes. Advice is sought and we use various tools to inform of experimental design (online tool: NC3R's Experimental Design Assistant, www.nc3rs.org.uk/experimental-design-assistant-eda).

We will be conducting our experiments so that we comply with the PREPARE guidelines – (https://norecopa.no/prepare) and the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines).

We try to reduce variation in data by housing the mice together where possible and ensuring that theyare genetically identical.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Existing mutant mice will be procured from collaborators or commercial sources, thus reducing the breeding needed to set up a new mutant stock. New mutant models will be generated by commercialproviders or by the local transgenics facility and transferred to this licence only once established.

Breeding strategies will be followed to generate the mice of necessary genotypes. Complex genotypesare sometimes required, with multiple alleles/transgenes necessary to replicate the human situation of multiple genetic alterations being present in cancer. Breeding strategies will be optimised to result in asmany useful offspring as possible.

Gestation times, weaning age and litter sizes will be used to calculate the numbers of mice required tooptimise the size of the colony. If appropriate, embryos will be frozen to minimise breeding numbers.
Where appropriate pilot experiments will be performed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority of our experiments require us to breed and then compare tissues from mice that are normal (wildtype) or genetically altered to recapitulate human disease (mainly cancers). The study of the genes and pathways may potentially lead to their targeting in novel therapies. The breeding protocols cause minimal distress and those breeding animals that develop any symptoms will be killedbefore these progress.

Some of our experimental animals will develop cancers and in some cases these cancers

will spread to distant sites in the body. By allowing these cancers to progress to this advanced stage will allow asto study the important biological processes involved, which may in turn allow us to develop ways to exploit them to develop new treatments.

We will continually seek ways to improve all our animal work and reduce the level of suffering to theanimals.

Why can't you use animals that are less sentient?

We need to use adult rather than embryo stages as cancer develops in adult mice.

Lower species can share some cancer-causing pathways with humans but many do not live long enough to allow tumour growth. Their organs and immune systems can also differ significantly, thusare not suitable models for cancer research.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Potential refinements to reduce welfare costs to the animals include:

We activate mutations in experimental mice only. This means that breeding, stock and control animals will not carry the active mutation and will not experience any adverse effects. In many cases the mutation will only be activated in a subset of adult mice and in specific tissues thereby reducing thetime the mouse carries tumour burden and any unwanted or unexpected effects in other tissues.

We will always deliver drugs by the most humane methods, for example by gavage where possible. We will aim to use the fewest number of administrations to achieve a biological effect.

Enhanced monitoring in the timeframe when mice expected to have a phenotype, e.g. dailyweighing

Providing mash at cage floor level to all animals after oral gavage

Providing extra enrichment and housing to animals if they have to be singly housed

Focussing irradiation to the specific tissue of interest (in contrast to whole body irradiation)

peri-operative pain relief will be administered and continued after surgery for as long as requiredto alleviate pain.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Some of the resources we use are:

www.nc3rs.org.uk https://norecopa.no https://www.lasa.co.uk
We consult any newly published guidance of the establishment Named Information Officer and NamedAnimal Care and Welfare Officer. we attend local events discussing the 3Rs.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will attend local "Animal Welfare" meetings in which we share best practice, data and refined methods. We will attend relevant meetings organised by our local NC3R regional manager. We willfollow activity at www.nc3rs.org.uk and https://science.rspca.org.uk. We will keep our training up-to-date and adopt new methods when they will improve animal care.

T CELL REGULATION AND CONTROL OF AUTOIMMUNITY

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir 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 aimsmentioned in paragraph (b)

Key words

Diabetes, Autoimmune disease, Immune regulation, T cells

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

This project aims to study how the immune system is regulated to prevent it from causing damage toour own body.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 is a powerful weapon and essential for our health; without it we would quickly succumb to infection. However, the immune system can also cause damage to our own tissues in some circumstances. For example, the autoimmune disease type 1

diabetes occurs when the immune system attacks the insulin producing islet cells in the pancreas. Insulin production controls the uptake of sugar from the blood and so must be replaced by injections in people with type 1 diabetes. Excess sugar in the blood leads to serious long term complications such as nerve and small blood vessel damage. There are approximately 400,000 people in the UK living with type 1 diabetes, including nearly 30,000 children. Incidence is increasing year on year for reasons that are not clear and there is currently no cure. This project aims to understand how the immune system is normally regulated, to stop it making aberrant responses. We know that a protein called CTLA-4 is involved, and people with mutations in their CTLA-4 gene can develop an immune dysregulation syndrome. This project will use mouse models, in combination with patient data, to better understand how CTLA-4 operates to keep ushealthy. To specifically study the immune response associated with diabetes, we have developed a mouse model in which we have altered the immune system so that it attacks the pancreas. These mice develop diabetes in a manner that is similar to diabetes in humans. For example, the same types of immune cells appear to enter the pancreas in these mice as in people with diabetes. In addition, antibodies specific for pancreas proteins can be found in the blood of both mice and humans during diabetes development. Because we have an animal model to study diabetes onset, we can manipulateit to ask which genetic pathways are important for causing or preventing disease. We wish to use our mouse models to better understand the cellular and molecular pathways that cause type 1 diabetes so we can develop and test new immunotherapy approaches.

What outputs do you think you will see at the end of this project?

By the end of this project we will have obtained new information about how the immune system is regulated, particularly in regard to how the regulatory protein CTLA-4 works and how autoimmune diseases like type 1 diabetes develop. We will have conceived (and performed early testing with) newimmunotherapies for controlling type 1 diabetes. Our findings will be published in academic journals and presented at international conferences so that our insights can be used by others.

Who or what will benefit from these outputs, and how?

In the short term, we envisage our data may help to guide clinical decisions about which individuals may benefit from particular treatments. These individuals may be people with mutations in CTLA-4 pathway genes, people with autoimmune diseases like type 1 diabetes, or people being treated withCTLA-4 antibodies as "checkpoint inhibitors". In the longer term, we envisage that new therapeutic strategies arising from our work may be used to provide novel ways of treating immune-mediated disease.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work by presenting our research findings at international conferences and in academic publications, ensuring to report our methods in sufficient detail for the work to be reproduced. To reach a broader audience we will write reviews discussing our key findingsto make them more accessible to a range of scientists, including those working in different disciplines.We will also engage with relevant patient groups, particularly where patient samples have been donated for our research, to feedback the insights we have generated from both the clinical material and the associated animal work. Where appropriate, we will seek industry-based collaborators to takethe work forward.

Species and numbers of animals expected to be used

- Mice: 20000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice in this study since we have generated or accumulated several relevant strains that allow us to address precise research questions in a controlled manner. For example, we have generated a mouse model of type 1 diabetes that has already yielded useful information about diseasemechanisms in human diabetes. We analyse patient samples alongside our mouse work wherever possible to ensure our research is relevant to people. Mice will be used at life stages that are required to achieve the scientific aims of this project (typically adult).

Typically, what will be done to an animal used in your project?

Mice used in this project may receive injections in the tail vein, or at other sites, in order to introduceimmune cells and/or antigens. Many of the animals will experience blood sampling to assess their immune status and some mice will be given a low dose of irradiation. Some mice will develop autoimmune diabetes or systemic autoimmunity. A small number of mice will undergo a surgical procedure (lasting approximately 30 minutes) that mimics a procedure performed in humans (pancreatic islet transplant).

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of the mice used during this study will not experience adverse effects and many of the procedures will result in no more than transient discomfort. However, some mice will develop formsof autoimmune disease (where the immune system targets the body's own tissues) so that we can study how this occurs and how we can interrupt the process. We will minimise the discomfort experienced by these animals by monitoring them closely (e.g. using blood glucose readings in the same way as for people with type 1 diabetes) and ending the experiment at ethical timepoints (typicallyno later than 3 weeks after the classification of a mouse as diabetic). Some mice will undergo a short surgical procedure (~30 minutes). They are expected to recover quickly and will be given appropriate pain relief 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 animaltype)?

Severities expected in mice used in this project will be sub-threshold or mild (approximately 85-90%)and moderate (approximately 10-15%).

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered 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 understand how the immune response is normally regulated to prevent tissue damage. Our programme of work includes both animal and non-animal work. Tissue-damaging immune responses are complex, involving many different cell types that interact and migrate around the body. While we can study selected aspects of this process *in vitro*, it is not possible to understand how they all fit together culminating in tissue damage without *in vivo* work. We use our mouse model ofdiabetes to reveal novel findings that we can then study in humans with diabetes; for example, the animal model pointed us towards a particular type of immune cell (Tfh cells) that we were subsequently able to identify in patients with diabetes. Likewise, our work on CTLA-4 biology spans both mouse and human, and incorporates many *in vitro* approaches. For example, we have developed novel *in vitro* assays to study the ability of CTLA-4 to capture its ligands from adjacent cells. Thus, there are many aspects of our research that can be pursued without the use of animals. However, to study the ability ofimmune cells to become activated, migrate to distinct anatomical sites and ultimately cause an autoimmune disease, it is necessary to perform experiments in animals.

Which non-animal alternatives did you consider for use in this project?

Human data, *in vitro* cell culture, and *in silico* modelling are regularly considered for aspects of our work. These approaches are all used to some extent in the project, but they cannot completely replacethe use of animals.

Why were they not suitable?

In order to explore what causes harmful immune responses, we need to access cells from tissues as well as the blood, and to block or genetically manipulate pathways under defined experimental conditions to understand how they regulate disease. These approaches are not possible in humans, although we use human data as much as we can to inform our experiments. Cell culture experiments are used to pinpoint selected elements of our research questions (e.g. understanding how 2 cell typesinteract) however we are not able to recapitulate complex autoimmune conditions *in vitro*. Likewise, *insilico* modelling of patient or animal data can be used as a component of our research, but it is limitedby the information that we input.

These approaches can provide predictions, but ultimately experimentation is required to test those predictions and understand what they mean for the development of disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals required for this programme of work based on our previousexperience, taking into account the scientific goals and the nature of the experiments proposed.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Multiple individual experiments will be designed during the course of this project, and where appropriate these will use the NC3Rs experimental design assistant (EDA). Throughout this process, EDA is consulted to validate the chosen randomisation strategy and handling of variables (including nuisance variables), and the EDA analysis suggestion tool is used to choose the most appropriate method of analysis for each experiment. Statistical assumptions of the chosen analysis methods (e.g. normal distribution and homogeneity of variance for parametric tests) are assessed as outlined in Bateand Clark (2014). The number of animals used in experiments will be dictated by the magnitude of difference between the control and experimental groups in the pilot study and the spread of the experimental data. Where appropriate, the EDA power calculation tool will be used for group size calculations. Alternatively, for studies with more than two groups, group sizes will be calculated using other appropriate statistics calculators. This will ensure that we use an appropriate number of animalsto achieve statistically valid results. Experimental variability will be further controlled by assigning animals to experimental groups using an unbiased computer-generated randomisation procedure.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

To minimise the use of animals in experiments that do not turn out to be informative, pilot experiments are performed, typically involving 3-4 mice per group. This ensures that large group sizes are not used in experiments that are unlikely to yield useful information. Whenever possible members of the group share tissue samples, thus eliminating the need to take separate animals for their respective experiments. Furthermore, we have optimised methods used to retrieve cells from organs thereby reducing the number of animals required to obtain a certain cell count. Animal numbers will also be minimised by keeping records of the number of cells typically obtained from donor mice bearing different genotypes. This allows accurate planning of the number of donor mice of each type required in order to provide cells for a given number of recipients, ensuring that the minimum number of animalsare used. In certain experiments, we are able to minimise the number of recipient mice used by transferring both normal and gene-deficient cells into the same recipient. In these experiments the cellsfrom the normal mice can be identified because they express a particular surface marker (Thy1.1) that is naturally found on cells from certain mouse strains. By breeding this marker onto some of our wildtype mice (Protocol 1) we are able to distinguish wildtype cells from gene-deficient ones. We will use this approach wherever possible to allow both wildtype and gene-deficient cells to be studied in a single host animal, thereby reducing the number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

In some experiments, mice will receive one or more injection (similar to a vaccination) and we expect only minor and transient discomfort in these animals. In other experiments, mice will experience autoimmune responses, where the immune system reacts to parts of the body. In our diabetes model, for example, the immune system reacts to the pancreas and destroys the insulin-producing cells. By choosing this tissue to target, we can test the degree of pancreas destruction without killing the animal.This is because as the insulin-producing cells become destroyed, the animal loses its ability to control its blood glucose levels. By monitoring blood glucose levels (using the same type of glucometer used by people with type 1 diabetes) we can obtain accurate kinetic data from a single animal showing pancreas destruction over time. Such an accurate measure of tissue damage enables close monitoringof disease progression and thus prevents unnecessary suffering to the animal.

Why can’t you use animals that are less sentient?

The scientific aims of this project could not be achieved in more immature, less sentient or terminally anaesthetised animals because to investigate the pathways that regulate the development of immune-mediated diseases it is necessary to study how immune cells become activated, migrate to distinct anatomical sites and ultimately cause disease over time. The complexity of autoimmune diseases - involving multiple cell types and multiple anatomical sites - and the time periods required for the response to develop, preclude the use of less sentient animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We will minimise suffering in our experiments by use of humane endpoints and refining our experiments (in terms of the nature and number of interventions) as much as possible. Furthermore, allprocedural animals will be closely monitored, and where appropriate, will also receive post-operative care and pain management treatments.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow guidance on procedural refinement published by the NC3Rs and LASA.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Since the last PPL submission, we have attended several 3Rs-focused seminars by

NC3Rs as well asengaged with our regional NC3Rs programme manager. During this project, we will continue to follow the latest updates from the NC3Rs to keep up with advances in the 3Rs and study the appropriate resources to ensure new 3Rs practices are implemented correctly and efficiently. Within our group, wekeep records of 3Rs improvements and discuss them in our lab meetings. All new starters in the groupare introduced to 3Rs concepts and best practice and directed to the NC3Rs website.

Home Office

PHYSIOLOGICAL SIGNIFICANCE OF ORGAN PLASTICITY

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Intestine, sex, reproduction, obesity, diabetes

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand the impact of the intestine on other organs and on whole-body(patho)physiology, with a view to identifying novel, clinically relevant mechanisms that could be leveraged to treat diseases such as obesity and diabetes.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 shape, size and function of our internal organs can differ dramatically across our life and between individuals, and we call this phenomenon the organ plasticity. Organ plasticity, especially gastrointestinal plasticity (remodelling of our gut system), is closely related to our body’s energy metabolism. Abnormal organ plasticity will lead to imbalances in energy metabolism, and in some cases, this will result in diseases such as obesity and diabetes. For example, the gut becomes bigger during pregnancy and nursing which may allow mothers to absorb extra nutrients (energy) to feed the developing baby. But work in other animal model leads us to believe that, if the enlarged gut fails to shrink to its normal

size after pregnancy or lactation (milk production), this can result in weight retention due to excessive nutrient intake. Although the above example is set in females and during the period of pregnancy and nursing, other factors such as diet, age or sex also contribute to organ plasticity and energy metabolism. We believe that it is important to undertake our work because it aimsto thoroughly investigate all factors which influence the organ plasticity and energy metabolism, and therefore will provide the effective personalised treatment to the two most prevalent diseases worldwide, namely obesity and diabetes.

What outputs do you think you will see at the end of this project?

The outputs of the work will include:

Increased understanding of the relationship between organ plasticity and energy metabolism.

Increased understanding of how various factors (e.g. environment, diet, sex) influence organplasticity and metabolism and how they contribute to diseases such as obesity and diabetes.

Identification of new targets for drugs aimed at the prevention and/or treatment of these diseases.

Development of new methods to diagnose and evaluate disease progression and/or monitor theefficiency of treatment.

Written information such as publications available to other researchers and the public to increasetheir understanding of organ plasticity and diseases.

Presentations at scientific meetings and public engagement events.

Who or what will benefit from these outputs, and how?

In the short-term (0-5 years), our work will benefit:

Developmental biologists: they will increase their understanding of how adult organs change andadapt and how this plasticity can result in abnormal growth and tumour formation;

Researchers: they will increase their knowledge in how organ plasticity is involved in diseases suchas diabetes and obesity;

Our current research: outputs from the work such as publications and public engagement events willhelp us to get further funding to continue our research, inspire and train early-stage scientists in research, and convey the importance of fundamental research to the general public.

In the long-term (> 5years), our work will benefit:
Opportunities to work with other research groups: our work will identify genes and processes in thebody that contribute to organ plasticity and energy metabolism. Future work with pharmaceutical companies may lead to the design and testing of new drugs based on our findings.

Clinicians: clinical trials using new drugs can be undertaken. Our research will also enrich

the knowledge of doctors in treatment of obesity and diabetes. In turn, this will help us in recruiting patientsfor future clinical studies.

Patients: if clinical trials are successful and new drugs are approved for clinical treatment, patientssuffering these diseases will benefit. Importantly, their families will receive indirect benefit from our work that will help improve their quality of life.

The Economy: effective treatment of obesity and diabetes will reduce the financial burden of the UKhealth service and/or worldwide.

How will you look to maximise the outputs of this work?

We will use a number of approaches to maximise our outputs:

Working with other groups to bring new expertise, access to new reagents and/or new technologiesthat we do not currently have ourselves.

Working with industry to develop treatments.

We will use a variety of approaches to share our findings, including the traditional routes of journalpublications and conference presentations, but also including social media threads, podcasts and interviews.

For approaches that report null results, we will deposit our findings on preprint serves, as well asdiscussing them at scientific meetings to ensure that the information is accessible as widely as possible.

We will also publish our methods in addition to papers describing our scientific findings, in order tomove the whole field forward and increase the rate of progress.

Species and numbers of animals expected to be used:

Mice: Mice: Over the 5 year licensing period, we expect to use a total of approximately 14000mice. This includes breeding and maintenance of colonies plus experimental work on virgin, pregnant and lactating mice (I note that the expected number does include the pups of pregnant/lactating dam). This projected number of animals is our best estimate of the numbernecessary to achieve the scientific objectives of the programme and has been guided by our previous experience.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

We will use mice, primarily adult in age, for our studies.

We have successfully used flies as research models to study organ plasticity and energy metabolism.It is important to validate our findings made in flies by using mammalian models before they can be translated to humans. Mice are the lowest mammalian species available for studying human diseasessuch as obesity and diabetes. In addition, the mouse is the most suitable mammalian species for genetic manipulation, for this study.

The techniques required for generating genetically modified miceare well established, so we will be able to use them to establish how specific genes contibute to intestinal (patho)physiology.

In some cases, we will use pregnant mice to study pregnancy and lactation on organ plasticity andenergy metabolism, therefore ‘pregnant adult’ will be also a life stage for this project.

Typically, what will be done to an animal used in your project?

What will be done on an animal in our project depends on the objectives we plan to achieve. We haveidentified several likely scenarios:

When studying diet and organ plasticity/energy metabolism, we will typically give (male and female) mice an altered diet (e.g. a high fat diet, a calorically restricted diet or a diet lacking or supplemented with a specific nutrient) for a maximum 30 weeks. Fasting (removal of food) may be applied on occasion. During fasting, mice will continue to have access to water, but may not have access to food for up to 20 hours. Most animals will be humanely killed after these dietary interventions, and the tissues/organs of interest will be collected for laboratory studies. Our main interest is the intestine but we may collect additional organs from the same mouse (e.g. liver, kidney,adipose tissue) if we have reasons to suspect that the intestine may be impacting these organs. Lessthan 10% of animals will undergo further experiments to check their metabolic state before being humanely killed for tissue/organ harvesting.

When studying the effect of sex hormones on organ plasticity/energy metabolism, we will havetwo approaches:

We will surgically remove sex organs (ovaries or testes) from mice after sexual maturity and check theorgan changes by comparing to mice that underwent sham surgery. Alternatively, we will inject hormones to mice.

We will then either humanely kill the animals and remove their tissues/organs for laboratory studies, orcontinue with metabolic tests before humanely killing the mice and harvesting their organ(s). Animals that undergo this procedure will be kept for a maximum of 12 weeks following surgery.

When studying the impact of certain genes or diets on gut activity, we will disturb the normal gut structure by adding a chemical called dextran sulphate sodium (DSS) to animals’ drinking water, whichdamages the lining of their gut. These mice will not undergo any further metabolic tests. They will be killed shortly after DSS treatment or within 7 days after DSS. The tissues/organs of interest will be harvested for laboratory studies.

When studying the impact of pregnancy and lactation on organ plasticity and energy metabolism,the female may receive an altered diet before mating, and then undergo different experimental routes:

They may be killed during pregnancy for tissue/organ harvesting. If it is important to understand the effect of intestinal sex or plasticity on reproductive output, dams will need to be singly housedduring pregnancy and lactation.

They may be killed during lactation and the tissues/organs of interest will be collected; the pupswill be killed at the same time but their tissues will not be used in our studies.

Some metabolic tests such as tolerance tests and imaging tests may be applied on pregnantmice once before they are killed for tissue/organ harvesting.

Dams will receive an altered diet for a maximum of 30 weeks. The type of tests carried out involve injection of substances such as insulin into animals before taking blood samples (to measure glucose levels) from the tail at different times. Mice will be fasted for a maximum of 20h prior to these tests. Thetests themselves will take no longer than 3 hours. A maximum of 8 blood samplings will be performed in each test. The blood sampling procedure is similar to the ones on humans.

Whilst the majority of our pregnancy experiments will involve looking at a single pregnancy, a subset(<5%) will make use of mice that have experienced up to three pregnancies. The total duration of thethree pregnancies will be in the region of 18-20 weeks.

Any single female may be pregnant up to 3 times before being killed so we can study the impact of thenumber of pregnancies on organ plasticity. In these cases, these females will not undergo repeated procedures; if there is a need to repeat a test in different pregnancies, different animals will be used forthis purpose.

Some tests involve animals being singly housed in specially designed cages which can measure a mouse’s energy/calorie burning or feeding behaviour. Animals will typically be singly housed for a maximum of 7 days (normally 2-3 days) following a 7 day-period of acclimatisation, and the test may be repeated once. For experiments in which food intake is monitored in special cages over the course of pregnancy/lactation, single housing will last for a maximum of 6 weeks - equivalent to the degree ofsingle housing of a standard lactation experiment.

Some tests involve the mouse undergoing surgery to insert a tube into a blood vessel in the neck, afterwhich the mouse is injected with insulin through the tube, and the blood glucose level measured at timed intervals. Animals will only have the test once and will be killed immediately after the test.

Some mice will be injected with a dye and have images taken afterwards to track the dye but will be imaged whilst anaesthetized to avoid stress and discomfort. Each imaging session will last a maximumof 30 minutes. This process will be repeated a maximum of 5 times over the animal's lifetime. Other animals will be constrained in a tube for less than 5 minutes and undergo specialist imaging to monitortheir body fat composition. An animal will be imaged by one of the two methods and will never undergoboth processes.

What are the expected impacts and/or adverse effects for the animals during your project?

In general, for the majority of animals we use, we do not anticipate any significant adverse effects.Animals undergoing procedures such as surgery are expected to make a quick and full recovery.

In some mice, the expected impacts and/or adverse effects might be:

Hight fat diet may cause a greasy coat to mice, but this will not irritate them. In rare cases, thismay lead to slight skin damage which is treatable.

Long periods of high fat diet may lead to diabetes to mice. They may present polyuria (urinate alot) and polydipsic (overly consume water), but we will frequently change their beddings and top up their water bottles.

Fasting and restricted food intake may cause temporary weight loss. We will monitor weight lossto ensure it is not higher that 15% of their starting weight at any point during the procedure. A 20% weight loss limit will exceptionally be allowed for up to 72 hours following a dietary alteration to allow for initial fluctuations in weight following a switch in diet, which tend to stabilise after 2-3 days.

In some of our studies, we may administer drugs that may result in local inflammation around the injection site and/or cause reduced food or water consumption leading to weight loss or dehydration. We will monitor mice for these effects and if they cannot be treated and persist for more than 72 hours,we would humanely kill the animal.

Administration of DSS could lead to internal organ damage and the clinical signs could be bleeding from the back passage and diarrhoea; these mice will be monitored twice daily and will behumanely killed before showing these symptoms.

Administration of insulin in some tests may lead to hypoglycaemia (low blood sugar level). Animals may be distressed, struggle to breathe, or may fall unconscious. We will closely monitor thesesigns during and after injection and they will be humanely killed if the symptoms last more than 3 minutes. Some studies require the mice to be singly-housed (e.g. to monitor food intake, measurement of breathing rate), and in these cases the animals will be given objects in their cages, such as cardboardtunnels, to encourage normal behaviour. For all studies requiring single housing, we will plan to do these at towards the end of the study so that we can keep this to a minimum.

Some mice will undergo surgical procedures such as ovary removal or implantation of tubes. Pain, distress, unhealed wound, wound infection or open wound could occur, but these situations willbe monitored daily and appropriate actions such as pain management, antibiotic treatment or restitching wound will be taken accordingly.

Treatment of pregnant mice with diet changes or substances could theoretically cause miscarriages, or abnormal maternal behaviour after birth. Care will be taken to avoid these side effectsby using appropriate doses that have been shown not to interfere with pregnancy and normal mothering behaviour.

It is important to note that in all the instances described above, we will closely monitor the expectedadverse effects and take suitable actions to either control the adverse effect within their limits or humanely kill the mice, therefore the duration of the adverse effects will be minimum.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Based on past experience, we would expect the following distributions of severity to apply across ourtotal animal usage.

Sub threshold: 60-75%

Mild: 20-30%%

Moderate: 5-10%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our inital discoveries that the intestine differs between the sexes, and that the sex of the intestine is important to sustain reproduction were initally made in fruit flies. Fruit flies do not have all the cell types present in human GI tract (intestines). They also do not have organs that are purpose-built for reproduction such as the mammalian placenta, raising the question of whether in mammals intestinal changes are also required to support reproduction. The GI tract of Mus musculus is much more similar to ours, and the methods we have will allow us to investigate both the genetic mechanisms underlying intestinal sex differences and reproductive intestinal changes, as well as understanding why these may be important.

Which non-animal alternatives did you consider for use in this project?

We have considered using cell-based approaches such as organoids (mini-organs). We also successfully used flies as our experimental model and will continue to use both organoids and flies inthe future whenever they allow us to answer certain scientific questions.

Why were they not suitable?

Cell-based approaches are useful for some studies. However, it is not possible to simulate interactionsbetween different cell types, and so information can be lost. Similarly, changes that occur in a specific cell type can have a dramatic effect on immune responses and these cannot be observed in cell-basedmodels. Moreover, some of the conditions that we are interested in measuring (such as food intake), cannot be studied in isolated cells.

Flies are better than cell-based approaches because they can simulate complex effects, but the biology of flies is very different to mammals, notably reproduction. Therefore, data from flies is notimmediately comparable to humans and we need to prove it in mice.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot

studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated this number from our experience of previous similar studies that have beenconducted under our previous Home Office Project Licence.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The choice of genes that we will study is based on previous cell-based approaches and fly models. Only genes involved in controlling organ plasticity and energy metabolism will be examined. Althoughthese cell-based models cannot predict the effect of manipulating the gene in living mammals, they provide valuable clues enabling us to prioritise experiments and only use a minimum number ofanimals

We plan to obtain the maximum amount of data from a single animal. All studies and analysis of dataare designed so that the information we gain is robust while using the minimum number of animals.

We have used the NC3Rs experimental design assistant allowing us to calculate the best sample sizebased for each experiment. This will ensure that we are using the fewest number of animals and that each animal is being utilised to its full potential.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We will take the below measures to optimise the number of animals in our project.

When breeding genetically modified mice we will use strategies to maximise the use of offspringwherever possible.

For some of our investigations, whenever possible procedures will be combined in sequence, andlong-term studies on the same mice will reduce the overall numbers of mice required to reach the scientific end-points.

We will always strive to use the most efficient techniques available to reduce animal numbersthrough improved accuracy of measurements.

To reduce animal usage, where it is anticipated that a genetically modified line will no longer be ofimmediate interest embryos may be collected and stored (frozen) to reduce the need to continue 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why

these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice both wild type (normal) and genetically modified. Both types used are healthy, withnormal appearance and behaviours. The methods for getting these mice are either by breeding (natural mating) or by purchasing from a commercial supplier. Natural mating is the most refined breeding method as it causes no harm, suffering or pain to mice.

We will use various methods to give substances to mice, such as adding substances to food or drinkand injections. Food and drink routes won't cause any harm, pain, suffering, distress. Most injections will be quickly done while animals are awake but restrained. Mice may feel brief sharp pain but otherwise no suffering and lasting harm, which is similar to human's reaction to injections. However,administration through food or drink will be always used whenever possible.

Some procedures such as surgeries may cause more pain and stress than others, therefore we will dothese procedures while mice are asleep (anaesthetised). Mice will breathe in a vaporised sleeping medicine and wake up quickly once this stops. Mice recover quickly without lasting harm from this anaesthesia method. Pain control such as administration of painkillers to animals during and after surgeries is another refined method to minimise pain, suffering, distress to animals. All tools and materials used in recovery procedures are sterile to reduce the chances of infection.
The imaging methods which will be used in this project cause minimum harm and suffering to mice. Forsome imaging we use an instrument called echoMRI, where the mice are held in a tube and imaged forless than 5 minutes. This is a quick imaging method which causes little stress and suffering to the mice. For longer imaging methods mice will be anaesthatised to reduce stress. Mice won't suffer any pain or stress during imaging under anaesthesia nor any lasting harm afterwards.

We will use refined methods (e.g. BioDaq for assessment of food intake and feeding behaviour, metabolic cages); these are the tests that produce the best scientific results and the procedures arerefined in administration and monitoring.

Where a procedure(s) involves several steps, we will set a minimum recovery time between steps in order to reduce distress and harm to the animal. For example, a minimum one week will be maintainedbetween 'tolerance' tests and 48 hours between imaging experiments.

Why can't you use animals that are less sentient?

We study organ plasticity, reproduction and metabolic regulation in mice with a view to understandingthe potential causes of metabolic diseases such as obesity, diabetes in humans. In order to carry this out we need to study free-living animals with a similar level of complexity and metabolic regulation(control) seen in mammals and humans. Thus, we cannot use species that are less sentientthan mammals.

In some cases, we need to undertake studies on the same animals over time to see how theirmetabolism changes, and so these studies cannot be done in terminally anaesthetised mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We will take the below measures in order to refine the procedures to minimise the harms for theanimals. Different measures will be applied depending on the animal experience.

We will increase monitoring level (e.g. twice daily instead of daily for mice receiving DSS treatment)so we can prevent animals develop clinical signs before we finish experiments.

Anaesthesia, where used, will be of depth sufficient to prevent the animal being aware of pain arising from the procedure. Mice will be kept warm and monitored regularly until they have recovered from the anaesthesia.

Pain management will be applied when clipping ears, blood samplings through tail veins and forsurgical procedures. Different management will be adapted depending on the characteristics of theprocedure. For example, applying local anaesthetic cream on ears for genotyping while supplying anaesthetic gas to animals for blood samplings or surgeries.

We will train mice to acclimatise to the environment and equipment when they are singly housed forexperiments. As a refinement method this will reduce the stress of mice in the new environment whichultimately benefits us by providing reliable data which is not affected by the animal being stressed.

We will use pregnant mice in our project and will handle dams (mothers) prior to pregnancy so theyare acclimatised before they undergo procedures whilst pregnant.
What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow government guidelines such as the Guidance of the Operation of the Animals (Scientific Procedures) Act, the ARRIVE guidelines, LASA guidelines, the relevant published literature on studyingmetabolism in mice as well as the PREPARE guidelines, NC3Rs website and locally published guidelines from the 3Rs advisory group of our establishment.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We consult the NC3R database, our establishment’s 3Rs advisory group, our establishment’s namedanimal care and welfare officers (NACWOs) and veterinarians about the best practice and potential further refinement of our procedures.

We use our weekly group meetings to discuss and update 3Rs related to this project. This will ensurethat researchers who carry out animal work in this project are conscientious in implementing 3Rs throughout the project.

Where possible we will implement advances in the published literature and we will follow the publishedbest practice.

To facilitate implementation of the best practice, our institute has recently employed an animal facilitygovernance specialist who will oversee the delivery of this project and the competency of researchersin implementing 3Rs in their animal work throughout the project.

SAFETY OF BIOPHARMACEUTICAL MEDICINAL PRODUCTS

Project duration

5 years 0 months

Project purpose

- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aimsmentioned in paragraph (b)

Key words

Biosafety testing, Regulatory authority, Contract Testing, Adventitious agents, Biopharmaceuticalproducts

Animal types	Life stages
Mice	neonate, adult
Domestic fowl (Gallus gallus domesticus)	embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this licence is to conduct regulatory safety tests on human and veterinary biopharmaceutical products, as a contract service, in order that regulatory approval may be obtainedfor their use in clinical trials or as a licensed product.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 safety testing is required as part of a package to ensure that biopharmaceutical medicines for human or animal use are characterised and free from contamination. The work performed in the facilityis used by our clients to make regulatory submissions in support of biopharmaceuticals which are in various stages of clinical testing and also to aid batch release of licenced products.

What outputs do you think you will see at the end of this project?

This license will provide safety testing for clients wanting to submit their product for regulatory approvaland validate a number of products which are now licensed by the European Medicines Agency (EMA), US Federal Drug Administration (US FDA) and Japanese Ministry of Health and Welfare. Safety testingwill help to ensure that biopharmaceutical medicines for human or animal use are free from contamination and safe for use.

Who or what will benefit from these outputs, and how?

The benefits of the project are evident in that the safety testing will help to ensure that biopharmaceutical medicines for human or animal use are free from contamination. The work performed in the facility is used to make regulatory submissions in support of biopharmaceuticals which are in various stages of clinical testing and also to aid batch release of licenced products. Thework performed is for a number of different sponsors and therefore supports a wide range of biopharmaceutical products and ensures they are free from contamination and safe for use.

How will you look to maximise the outputs of this work?

As a Contract Testing Organisation with expertise in manufacturing and safety testing of biopharmaceuticals, the company has been successful in forming lasting partnerships with many of themajor international biopharmaceutical companies and biotechnology companies.

We have a 4Rs workstream in which all company In Vivo animal facilities worldwide share their bestpractices to implement animal welfare improvements and welfare practices. We will look for opportunities to share our refinements outside the business at local and international animal welfareand laboratory animal science conferences through presentations and posters.

Species and numbers of animals expected to be used

- Mice: 102,500
- Domestic fowl (Gallus gallus domesticus): 61,620

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The types of animals and life stages are specified with in the European and worldwide guidelines andregulations that we perform our work in line with for our clients.

Typically, what will be done to an animal used in your project?

Typically, an animal will receive 1 set of injections which can be given into the brain or abdomen. Theymay also be administered substances by the oral and nasal routes as required by test specifications. After the dose, they are checked for any signs of ill health

until the end of the study.

What are the expected impacts and/or adverse effects for the animals during your project?

In most studies procedures will produce transient and mild discomfort such as bruising, and short livedsigns of discomfort as a result of the substance administration. If animals show more substantial signsof ill health such as loss of appetite, difficulty breathing etc, they will be humanely euthanized.

In one protocol (4) the studies look to see if a therapy may have the ability to form tumours, although this is expected to occur very rarely. As a comparison in this study, a small number of animals will begiven an injection that will grow 1 superficial tumour under the skin, as a comparison to the test group(positive control)

For eggs throughout the licence, adverse effects other than mild and transient inoculation trauma arenot 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 animaltype)?

All of the mice used in this licence will be under a moderate severity, and all of the eggs will be under amild 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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

For our clients, animal testing is an important part of ensuring that their products are safe for humans and/or animals. The regulators (e.g the FDA, EMA) of human and animal drugs require that the compounds have been thoroughly tested for their safety before allowing them to be released onto the market. A large amount of the required testing that is done in the laboratory without the use of animals.There is still a small amount of testing done for methods where there is no non-animal alternative available yet.

Which non-animal alternatives did you consider for use in this project?

Current legislation for the safety of biopharmaceuticals requires animal testing. The legislation and guidelines are evaluated constantly for changes to the requirements for animal testing and where possible, alternatives to animal testing will be performed. Changes to the European testing guidelines(the European Pharmacopoeia) now allow non-animal alternatives in place of some traditional animaltesting. For this reason, these

animal tests have not been included in this license.

Why were they not suitable?

The animal tests requested in this license have no non-animal testing alternatives and are required bythe relevant EU Pharmacopeia and FDA guidelines.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 is regulatory testing we use a known number of animals per study. The number of animals to beused on this licence is based on the usage on our last licence, combined with predicted business over the next 5 years. We expect to use only 120 hatched chicks, and the rest of the domestic fowl numberswill be embryonated eggs.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The number of animals used per study are specified in the relevant regulatory requirements. As the facility runs only regulatory assays, it is also in a unique position to reduce animal usage through theuse of shared negative controls. This would not be possible with in house testing performed by our sponsors as there would not be sufficient volume of the same assays to do this.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

For our regulatory testing, we will use the minimum number of animals specified in the regulations. Where possible, we will use both sexes of animals, and we will also share negative controls betweentest 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Typically, an animal will receive 1 set of injections which can be given into the muscle,

brain, or abdomen as specified by the relevant regulatory requirements. They may also be administered substances by the oral and Nasal routes as required by test specifications. In most studies procedureswill produce transient and mild discomfort such as bruising, and short lived signs of discomfort as a result of the injection. If animals show more substantial sign of ill health such as loss of appetite, difficulty breathing etc, they will be humanely euthanized.

In one protocol (4) the studies look to see if a therapy may have the ability to form tumours, althoughthis is expected to occur very rarely. As a comparison in this study, a small number of animals will begiven an injection that will grow 1 superficial tumour under the skin as a comparison to the test group(positive control).

Embryonated eggs are inoculated via the yolk sac, allantoic or amniotic route as specified by the relevant regulatory requirements. The use of early stage embryonated eggs is a refinement over laterstages of development or mammals.

Why can’t you use animals that are less sentient?

The species and life stages to be used in this licence are specified by the regulations and guidelineswe work to. A large portion of the studies will be using embryonated eggs.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We have technicians on a rota to perform out of hours additional checks if required. Refinements havebeen made to the procedures. This includes the use of single use needles (adult studies), finest gaugeneedles as the procedure allows, bespoke anaesthetic face mask, and an oxygenated recovery chamber. Animals are brought in days before the procedure to allow acclimatization to the facility.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We perform in vivo biosafety testing studies for clients using methods which are performed in compliance with relevant EU and US regulations and guidelines. Compliance with these standardsallows our clients to apply for licensing of their products with a wide range of national authorities.

The authorities include The European Medicines Agency (EMEA), The Medicines and Healthcare Products Regulatory Agency (MHRA) the US Food and Drug Administration (FDA). Testing is performed in compliance with CBER (FDA), World Health Organisation, International Conference ofHarmonisation (ICH) and European Pharmacopeia.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Our veterinarian and training officer (NVS & NTCO) regularly attends animal welfare and laboratoryanimal science conferences such as those hosted by RSPCA (Royal Society for the Prevention of Cruelty to Animals), LAVA (Laboratory Animals Veterinary Association), LASA (Laboratory Animal Science Association). Our NACWOs (Named Animal Care and Welfare Officers) are enrolled in IAT(Institute of Animal Technology) training and will attend animal welfare conferences. Information provided at these conferences will be shared amongst the local animal unit staff and wider organization.

ENTERIC NERVOUS SYSTEM: DEVELOPMENT AND ROLES 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, ortheir effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Gastrointestinal tract, Nervous system, Immune system, Development, Inflammation

Animal types	Life stages	
Mice	embryo, neonate, juvenile, adult, pregnant, aged Zebra fish (Danio rerio)	adult, embryo, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description 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 innervation of the gut forms during development and how it contributes tointestinal health and disease pathogenesis.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within 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 control of gastrointestinal function is essential for health and survival. Understanding how the nerves that control the function of the gastrointestinal tract develop

before and after birth, how they aremaintained throughout life and how they communicate with other tissues in the gut, will provide important new knowledge. Such knowledge will ultimately shed light on the cellular and molecular mechanisms that underlie clinically relevant conditions resulting from failure of these nerves to developproperly (e.g. in Hirschsprung's Disease or congenital megacolon), or function adequately (e.g. constipation associated with old age). In the longer term, information about these mechanisms in animal models will generate insights that can be harnessed for the development of novel therapies for gastrointestinal disorders. For example, understanding which and how cells in the gut control the formation of intestinal nerves during embryo development, will allow us to engineer new nerve formation in order to restore those missing or malfunctioning.

What outputs do you think you will see at the end of this project?

The expected outputs of this project are as follows:

Advances in fundamental knowledge via the identification of molecular and cellular mechanisms that control the formation of the nerves of the gut and regulate their interaction with other tissues during normal or pathological conditions. This will facilitate the identification of biological pathways thatare central to tissue organisation and function of the intestine during health or disease. We aim to disseminate our findings to the scientific community (both basic and clinical) and more widely to the public domain, by presentations in conferences and publication in preprint servers and peer-reviewed scientific journals.

Generation of experimental models of physiological processes and disease states of the gastrointestinal tract, which will allow the in depth understanding of molecular and cellular mechanismsunderlying normal states or pathological conditions (such as Hirschsprung's disease, inflammatory bowel disease, or old age-associated constipation). These valuable resources will be made available tothe wider scientific community, including clinicians.

Identification of molecular pathways that may be used in the longer term for the development of drugs that could benefit patients with gastrointestinal disorders (such as inflammatory bowel disease orsevere constipation).

Who or what will benefit from these outputs, and how?

Dissemination of our conceptual advances, experimental findings and datasets via conferences, preprint servers and peer-reviewed publications will benefit the wider scientific community. Our work, particularly any new models of gastrointestinal disorders (such as models of Hirschsprung's disease, post-infection neurodegeneration, repair of gut innervation, inflammatory bowel disease), will also benefit clinicians and the pharmaceutical industry in their efforts to develop novel therapies. We do notanticipate that the potential therapeutic benefits of our work will be fully realised prior to the completionof the project and will almost certainly require further work that will extend beyond its duration.

How will you look to maximise the outputs of this work?

We will seek to maximise the outputs of our work by making presentations in international scientific conferences and more focused workshops. We will also publish our work in international peer-reviewedscientific journals. Where appropriate, we will upload unreviewed versions of our manuscripts on preprint servers (such as bioRxiv, Wellcome

Open Research) in order to achieve rapid dissemination ofour work for the benefit of the global scientific community. Our aim is to publish not only our successful experiments but our unsuccessful approaches will also be highlighted in order to minimise future failures by other groups.

Species and numbers of animals expected to be used:

- Mice: 35000
- Zebra fish (Danio rerio): 11000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 identify cellular and molecular mechanisms which promote the development and maintenance of gut innervation. We also aim to understand the molecular and signalling mechanisms that underpin the roles of gut nerves in immunity and host defence. Although we will continue to use cell and organoid culture models, gut formation and function involve complex and multi-dimensional interactions between different tissues that cannot be reproduced in vitro. It is therefore essential that, inparallel with our cell culture/organoid experiments, our work uses animal models and tissues derived from animals. In particular, testing the function of selected genes that our *in vitro* and *in silico* studies identify as key candidate regulators of the biologically processes of interest, requires the generation, breeding and analysis of genetically modified animals. We will work with both mice (Mus musculus) and zebrafish (Danio rerio) as each species offers unique advantages for our studies. In general, mice model well the anatomical organisation as well as the physiological and pathological processes of humans in comparison to other simpler and commonly used experimental animal models, such as worms (Caenorhabditis elegans) and fruit flies (Drosophila melanogaster). In particular, the development, organisation and physiology of mouse gut nerves resemble closely those of humans andmany of the clinically relevant molecular pathways have been identified by genetic analysis in mice. It is therefore imperative that we continue to take advantage of the sophisticated genetic tools of mice in order to unravel highly complex cellular and molecular mechanisms. The parallel use of zebrafish will facilitate evolutionary comparisons and allow us to develop general principles regarding the development and organisation of gut innervation. Zebrafish also offer unique advantages for our developmental and genetic studies as it reproduces faithfully specific aspects of human gut pathology and motility disorders (such as Hirschsprung's disease-congenital megacolon). Specifically, our previous work has shown that zebrafish deficient in the Ret gene develop a condition that is remarkably similar to what has been observed in familial cases of Hirschsprung's disease, the majorityof which are associated with RET mutations. Our own studies and work from other groups, have demonstrated that normal assembly and function of gut nerves in adulthood depends on biological processes taking place at earlier stages of life. Therefore, use of tissues from embryonic, fetal, postnatal and adult life stages of mice and from embryonic, larval, juvenile and adult stages of zebrafish will be necessary in order to dissect molecular pathways and ultimately understand the causes and consequences of disease processes.

Typically, what will be done to an animal used in your project?

The majority of regulated procedures described in this project are of mild severity and involve the breeding of genetically altered animals, often associated with minor interventions, such as ear clipping,to allow genotyping.

Typically, several hundred matings of wild-type and genetically altered mice and zebrafish will be performed each year. These will be used in order to maintain specific genetic modifications using breeding strategies that prevent expression of anatomical or physiological phenotypes that are in any way harmful to the animals (for example mutant alleles of developmental genes will be maintained in heterozygous state). Our breeding will also produce mice and zebrafish of a specific genotype for basicresearch purposes using procedures of mild or moderate severity. The animals generated for basic research purposes will be used mostly for tissue characterisation at different stages. Mice will also be used for the generation of maternal immune activation and gut injury models.

Typically, for gut pathology models, juvenile or adult mice will be exposed to gut inflammation-inducingchemicals or pathogens (such as helminths or bacteria) or induced to ablate specific cell types (such as enteric neurons or glial cells) of the intestine. Following treatment, animals will be humanely killed atdifferent post-treatment stages for tissue harvesting and analysis.

Maternal immune activation (MIA) models will be used to explore the effects of immune challenges during embryonic and foetal life on the development and homeostasis of the enteric nervous system(ENS) and the contribution of enteric neurons and glia in gut immunity and host defence. For these studies, immunostimulants, such as substances that are components of the bacterial cell wall and mimic the effect of natural infections will be administered to pregnant mice (at embryonic or foetal stages) and their offspring will be humanely killed at foetal, postnatal or adult stages for harvesting tissues that will be examined by a range of histological and analytical methods. Prior to killing, adultMIA offspring may be treated with gut pathology-inducing chemicals (DSS) or pathogens (Heligmosomoides polygyrus or Citrobacter rodentium) or cell ablation in order to uncover how the organisation and function of the gastrointestinal tract changes as a result of maternal immune activation during embryonic or foetal life.
Wild-type and GA zebrafish will be mainly used for breeding and phenotyping, including harvesting ofembryonic, larval, juvenile and adult stage tissues for detailed molecular and histological analysis. A small number of larvae (up to day 15 post-fertilisation) will be used for live imaging under terminal anaesthesia. Also, zebrafish at any developmental stage, including adulthood may be exposed or administered gene expression altering or labelling substances. Zebrafish will be maintained for 18 months.

All animals will be closely monitored following regulated procedures, and anaesthetics, analgesics and/or other ameliorative procedures will be used as appropriate. In all cases, animals will be humanely killed if there are signs of pain, distress, suffering or weight loss above the agreed limits ornot feeding. The project will use the minimum numbers of control and experimental animals that are compatible with statistically valid conclusions.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected adverse effects of the wildtype and genetically altered mice in this project are mild to moderate loss of body weight, sickness behaviour, and symptoms associated with gut pathology, such as inflammation. Typically, for maternal immune activation (MIA) models, adverse effects relate to transient and mild weight loss and sickness behaviour

(such as piloerection, hunched posture, reducedlocomotion) of pregnant females. If weight loss continues and reaches the agreed limit of 15% and animals fail to return to normal behaviour after 2 days, they will be humanely killed. No adverse effects are expected for the MIA offspring unless they are used for induction of gut pathology. Adverse effects of weight loss and gut discomfort are expected to develop with the models of gut pathology to be used in this project, such as DSS administration, infection with pathogens (helminths or Citrobacter rodentium) or gut cell ablation. If weight loss reaches the agreed limit of 20% for these models or animals exhibit signs of pain, distress or suffering, they will be humanely killed. Adverse effects on zebrafish are generally expected to be mild as these animals will be used mainly for tissue harvesting and analysis. Novel genetic modifications that we have no previous experience with, may result in adverse effects associated with developmental or adult stage phenotypes. In these cases, GA animals will be monitored closely and if signs of pain, distress, or suffering are observed they will be humanely killed. The maximum expected level of severity for any procedure conducted within this project is moderate and follows strict guidelines in accordance with the Home Office. At the end of procedures, all animals will be humanely killed by an approved method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

We anticipate that the majority of animal procedures will be at the sub-threshold level of severity butsome animals will undergo procedures at the mild or moderate level of severity. Breeding and maintenance of genetically altered mice at subthreshold level will account for ~60% ofthe animals used in this project because it can take several generations to build the complex genotypes required for our research. Rarely, breeding of genetically altered mice will fall into the category of mild severity (<5% of these animals). The remaining ~40% of animals will undergo basicresearch procedures, such as gut pathology induction and infection with pathogens, which are categorized as moderate severity.

Breeding and maintenance of genetically altered zebrafish at subthreshold level will account for ~80% of the animals used in this project because the genotypes of animals used for our research are complex and can take several generations to build. In rare cases, breeding of genetically altered zebrafish will fall into the category of mild (<5%). The remaining ~20% of animals will undergo basic research procedures for phenotypic purposes, such as exposure to labelling substances or substancesinducing gene expression changes, which may reach 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 haveconsidered 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 development of the enteric nervous system and its multipleroles in intestinal physiology and pathophysiology because these processes are very complex and aresubject to regulation by multiple signals from several other tissues. Consequently, the available cell culture models cannot reproduce this level of complexity and are unable to mimic accurately the interactions between different tissues at different developmental stages. Therefore, studies in the context of the whole animal are required in order to advance our understanding of the molecular mechanisms that integrate the ENS into the physiology of the intact gastrointestinal tract.

Which non-animal alternatives did you consider for use in this project?

Literature searches, discussion with colleagues at conferences and our own previous studies have been used as sources of information for the development and use of non-animal research models. Among those are mammalian cells maintained in culture and organoids, which have already been introduced in the laboratory. Another alternative is human tissue from surgical samples and we are inthe process of introducing this tissue source in our studies.

Why were they not suitable?

The use of mammalian cell cultures in 2D or 3D models (some of which have been pioneered by our group) can provide a certain amount of valuable information and can be used to generate hypotheses, but the interactions of the ENS with other tissues within the gut wall are highly complex and cannot be reproduced by the available cell culture models. Nevertheless, more sophisticated stem cell-based cellculture protocols continue to be developed and some of those are being introduced into our laboratory work. Such models can be used to formulate plausible and focused hypotheses, but eventually they will have to be tested in animals. Our lab has also developed enteric glia-derived primary cell culture models of enteric neurogenesis (called ganglioids), in which enteric glial cells are induced to proliferateand differentiate to neurons. Such ganglioid cultures will be used to screen the effect of genes in enteric neuron and glia differentiation and only those genes with a discernible effect in culture will be tested in GA animals. Although ganglioid cultures cannot be propagated indefinitely in vitro, this strategy will help us replace the use of animals in research. Ultimately, cell culture models are not suitable for addressing all our questions as they cannot reproduce the spatial interactions between of the diverse tissue of the gut wall and the microbiota of the lumen. We have also considered human tissue for our studies and we are currently in the process of introducing this non-animal material in the group. Although we anticipate that human samples and human genetics literature will be a valuable tool and resource for our research, they cannot be used to test directly the function of specific genes. Therefore, only a subset of pathways and mechanisms can be identified using cell culture models and human tissue, making it essential that we use experimental animal models to reach our defined goals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals required to achieve our aims is based on previous usage and the number of breeding lines that we are currently maintaining. Our aim in every experiment is to use the smallest number of animals necessary to give meaningful and clear scientific results. We estimate that typicallyfor our studies, 8-10 animals for the experimental and control group or genotype are required to achieve statistically significant results. Our previous experience indicates that this number is likely to be increased if the observed phenotype is less robust than expected due to redundancies or compensation by genes or factors independent from the one tested, or from mixed genetic backgrounds. In some instances, for example when we address the function of a novel gene or signalling pathway, we may carry out pilot studies using a relatively small number of animals (3-5) for the experimental and control group or genotype. We will consult with statisticians throughout the lifetime of this project to ensure that animal numbers are regularly assessed and adjusted to the minimum number required to achieve our scientific aims.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The sample sizes used in the animal work on this project was reached by combining our previous experience of effective sizes and consultations with statisticians on site, who we will continue to consultthroughout the duration of the project. Our design will be based on PREPARE guidelines and sample sizes may be set using power calculations, using a significance level of 5%, a power of 80% and a least practicable difference between groups of 20%. We aim to use the smallest number of animals to gain a meaningful conclusion based on our own past experience and available literature. We will also take advantage of online tools, including the NC3Rs Experimental Design Assistant, randomisation tools for the allocation of animals to groups. We will also use the ARRIVE 2.0 reporting guidelines for the design of optimal experiments with the smallest number of animals necessary to achieve statistically meaningful results. In general, 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. Our extensive expertise and excellent understanding of the physiological system under investigation (the ENS) will help us to discern true effects of the tested parameters from random variables inherent to the system, while the Experimental Design Assistant will be valuable in the planning and can suggest analyses in order to account for variables and covariates within the experimental design.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Wherever possible we will use cell culture and organoid models in order to generate hypotheses that can be critical for the success of the project. Our laboratory has developed an enteric glia-based primary cell culture model of enteric neurogenesis (called ganglioids), in which enteric glial cells are induced to proliferate and differentiate to neurons. Ganglioids will be used to screen the effect of genesin enteric neuron and glia differentiation. Where appropriate we will also use stem cell based organoid models of ENS development. These cell culture models will allow us to replace some of the mouse experiments that would have been necessary for the initial exploratory phase of hypothesis building.
An important aspect of minimising the number of animals is the efficient breeding and maintenance of genetically modified animals, which represents a major part of the regulated procedures in this project.With relation to this, we routinely review our transgenic and mutant lines and the best efforts are madeto keep the minimum number of animals

required for experiments. In addition, we regularly use cryopreservation to archive mouse and zebrafish lines in order to maintain only those needed for ongoing experiments. Finally, we reduce the number of breeding lines by sharing, wherever possible, with other labs.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use mouse and zebrafish models to study the effects of gene activity on enteric nervous system development and function using gene silencing or overexpression models. In the caseof these models, wherever possible we are using tissue and/or cell type specific genetic manipulationsas they are less harmful in comparison to germ-line gene deletions or ubiquitous overexpression.

In the case of maternal immune activation (MIA) models, we are treating pregnant females with immunostimulant compounds as they provide an excellent model of the consequences of early life infection on the offspring. This strategy allows us to titrate the dose of immunostimulants to levels thatcause minimal sickness behaviour and weight loss to pregnant females and yet elicit quantifiable responses in the offspring.

In the case of mouse models of gut pathology and inflammation, we have chosen to employ well established and widely used methodologies that, depending on the dosage, can result in the least possible pathology (for example administration of DSS and the pathogens, such as Heligmosomoides polygyrus and Citrobacter rodensii. These procedures have been extensively characterised and refinedfor many years in laboratories around the world in order to minimise pain, suffering and distress. Gut pathology in mice will also be induced by ablation of gut cell types, using inducible genetic systems that can be titrated to reduce to a minimum the number of ablated cells. Animals with gut inflammation and pathology will be monitored frequently and will be humanely killed at timepoints that will be earlier or equal to the time when they exhibit adverse symptomatology.

Wild-type and GA zebrafish will be used primarily for breeding and phenotyping, including cellular/molecular analysis of tissues.

Why can’t you use animals that are less sentient?

We will use cell culture models to replace some of the animal procedures that would otherwise be required to deliver our objectives. In commonly used invertebrate models (such the fruit fly Drosophila melanogaster or Caenorhabditis elegans) the development and function of gut innervation is fundamentally different from that of vertebrate organisms and therefore are not suitable to achieve our aims. In addition, zebrafish and mice share very similar genes, developmental mechanisms and physiological processes with humans

making them suitable models for providing insight into human disease and ultimately the development of therapies. Both mice and zebrafish have well established laboratory procedures and sophisticated genetics which help to advance our understanding of biological mechanisms within a relatively small time frame. In all cases, animal suffering will be minimised by following strict guidelines in accordance with the Home Office and by regularly monitoringanimals in consultation with a named animal care and welfare officer and a named veterinary surgeon.
For molecular profiling, tissues will be collected from humanely killed animals at different developmental stages (including adult). Terminally anaesthetized mice will be used for severalprocedures such as exsanguination and perfusion fixation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We will minimise the adverse effects associated with genetic alterations by using, wherever possible,inducible or conditional alleles to delete gene activity from specific tissues rather than from the entire animal. To minimise stress during breeding and maintenance, we will follow best practice guidelines and implement local refinements of husbandry such as environmental enrichment and sufficient amounts of nesting material available to mice. On generation of a new line, we will minimize sufferingby ensuring increased observation and monitoring (in particular for signs related to the physiological system that is likely to be affected by the specific genetic alteration), until a detailed phenotypic analysis is accomplished. If any welfare implications are identified, they will be acted upon and refinements considered in consultation with the NVS and NACWO.

I will ensure that all personal licence holders working on this project are made aware of and receive training in the published best practice guidance for animal monitoring, aseptic surgery, post-operativecare and the minimization of pain (see section below). Most of the manipulations during animal procedures as well as the administration of gene activity inducers/repressors or other agents are standard and therefore previous refinements from the literature will be used. If, however, there is insufficient information available, new procedures or new drugs/substances will be screened in small-scale pilot studies to obtain indications of the minimum dose and exposure time that is likely to be effective, thereby minimising suffering.

In all surgery, analgesia will be provided according to published best practice and also advice from theNVS and NACWO. Good aseptic surgical techniques, heat & fluid therapy will be provided. During theproject, I will consult with our NVS to optimise and improve surgical methods and communicate this effectively to the personal licence holders working under this project licence.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Experiments will be planned in accordance with the PREPARE guidelines (1). For the refinement of procedures for the administration of substances we will also consult https://doi.org/10.1258/0023677011911345. For surgical procedures, we will follow the guidelines setout in LASA Guiding Principles on Preparing for and Undertaking Aseptic Surgery (https://www.lasa.co.uk). In addition, I will also keep myself and the personal licence holders workingunder my project licence updated with the latest refinement advancements in the use of mouse and zebrafish models by attending conferences, reading journal articles, collaborating with experts and monitoring the NC3Rs website and Resource Hub (https://nc3rs.org.uk/3rs-resources).

1. Smith, A. J. et al. PREPARE: guidelines for planning animal research and testing. Lab Animals 52,134–141 (2018).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will keep up to date with the latest 3Rs developments via the NC3Rs website (https://www.nc3rs.org.uk), complemented by information we obtain from regular newsletters preparedby our animal facility and mandatory annual meetings for all project and personal licence holders. In addition, there will be regular meetings between the project licence holder and all personal licence holders working on this project to ensure efficient communication and discussion on the 3Rs and any amendments or legal changes to the project licence and its implementation. The 3Rs will also be discussed with colleagues and collaborators at lab meetings, departmental meetings and conferences and the group will be attending seminars and other events covering 3Rs topics. Finally, the group willcontinue to do literature searches for 3Rs advances in our (and related) research fields.

DEVELOPMENT AND VALIDATION OF IN VIVO PRECLINICAL MODELS TO SUBSTANTIALLY REFINE AND REDUCE THE NUMBER OF ANIMALS SUBJECTED TO SEVERE PROCEDURES IN SNAKEBITE ENVENOMING RESEARCH

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir 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

Antivenom, Venom, snakebite envenoming, neglected tropical disease, refinement

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required,and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop a refined mouse model of envenoming which accurately reflectsreal-world scenarios and will allow:

- flexibility in testing different therapeutic formats (antivenoms and other drugs) at

differenttimepoints after envenoming,
- substantial reduction of the total number of animals required for robust efficacy testing,
- a maximum ‘moderate’ severity rating to be applied, once established, and validated.

A retrospective assessment of these aims will be due by 29 September 2028

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Snakebite is estimated to kill approximately 140,000 and maim a further 400,000 people every year.Globally, all snakebite envenoming therapies, both existing and in development, are assessed for efficacy using a 40-year-old, World Health Organization (WHO)-endorsed model of “neutralisation ofvenom lethality”. Whilst simple, this assay is not reflective of human envenoming and requires large numbers of mice (n=25/experiment/venom) to be subjected to highly distressing severe procedures. The objective of this proposal is to develop and validate a new *in vivo* model of envenoming which more accurately reflects a human snakebite scenario, which will have a maximum severity limit of ‘moderate’, and will require considerably fewer mice/experiment. Ultimately the new assay will providemore pathologically relevant data on the efficacy of current and future envenoming therapies.

What outputs do you think you will see at the end of this project?

The development of a suitable, fully validated and established model of envenoming has been identified as an urgent need in the development and assessment of treatments for envenoming (Knudsen, et al,*Toxins* 2020, 12(9), 528). The development of envenoming models has been progressing ad-hoc in many recent publications, but differences in experimental methods and lack ofpublished data on the development of these models hampers confidence and their wide-spread adoption, especially by that of regulatory authorities. Furthermore, many of the newer models in use still rely on the requirement for establishing median lethal doses and nearly all rely on the use of lethality as an endpoint. These assays are becoming challenging to justify to authorities regulating animal experimentation in increasingly more countries.

Here, by focusing on optimising and fully validating a new model of envenoming, which is more reflective of real-world envenoming, and by providing full transparency in its development and demonstration of its reproducibility, we hope to develop an assay that can be used by academics, industry, and key stakeholders globally. Importantly, it will aid in the progression of the next generationof envenoming therapies, which will be required to progress through more rigorous pre-clinical testing,as compared to existing envenoming therapies, before progressing to human clinical trials.

Who or what will benefit from these outputs, and how?

Potential 3Rs impact: Our laboratory routinely uses 300-800 mice/year in severe rated conventionalassays, which equates to approximately 3-5% of the UK total of mice used in severe experiments.

Establishment of a new, refined assay would reduce severe mouse use in our laboratory to 0, substantially reducing total UK academic severe mouse use values, and result in using an estimated40% fewer mice/experiment at a lower maximum severity limit.

Academically, the conventional assays and their modifications are used routinely. To provide context, arecent review of pre-clinical testing of antivenoms for just sub-Saharan Africa, examining only the neutralisation of lethality (thus excluding the mouse determination of lethal doses required for the assay) identified 18 papers in which 3,930 mice were used (Ainsworth, et al 2020, *PLOS Neglected Tropical Diseases* 14(8): e0008579). If these experiments were to be repeated using the proposed model, we estimate this would require 15 mice/experiment maximum, 40% fewer mice than used previously (a saving of 1572 mice).

Industrially, the WHO recommends full *in vivo* testing of each batch of new or existing antivenoms. There are approximately 50 antivenom manufacturers worldwide, producing in the region of 120 antivenoms. It is difficult to accurately assess the global numbers of mice used in the current conventional assay, as it will vary substantially on the basis of how many venoms the antivenom is indicated for. However, a personal communication with a single antivenom manufacturer detailed theuse of 2,000 mice per month to fulfill batch release criteria. Thus, numbers of mice globally subjected to this severe assay annually for fulfillment of antivenom batch release regulatory requirements by antivenom manufacturers is likely to number into several hundreds of thousands, annually. If all industrial manufacturing were to adopt the proposed model, after establishment, and if performed in the same manner as proposed, we would expect an estimated 40% reduction in mouse numbers usedworldwide.

Potential snakebite victim impact: As results from murine pre -clinical efficacy assays remain the primary means to assess/predict antivenom performance prior to their licensed use in humans, despitesuch animal assays not accurately reflecting a snakebite or snakebite pathology, products lacking efficacy still find their way to market. Improved, more robust models of assessing antivenom efficacy will have the potential to reduce the number of poorly efficacious antivenom available, leading to greater confidence in therapeutic efficacy and will aid in reduction of death and morbidity due to snakebite.

How will you look to maximise the outputs of this work?

In addition to publication of protocols, we will perform three key activities to encourage wider uptake ofthe improved model.

Presentation at key international conferences. At least one of three key international conferences onvenom biology will be targeted; The bi-annual Gordon Research Conference on Venom Evolution, Function and Biomedical Application, The bi-annual International Society on Toxinology conference, orthe Annual Oxford Venoms and Toxins conference. These three conferences are well attended by key stakeholders, including academia, industry, governments and the WHO. In addition to orally presentingthe model, we will approach organisers to hold breakout/target sessions on venom *in vivo* experiments.

We will commission a professional scientific communicator to generate simple, clear trainingmaterials for application of the new technique in other laboratories, in addition to encouraging adequate reporting and improved consideration of the 3Rs.

We will organise a virtual workshop to communicate a) the new model, b) to discuss the wider issues with *in vivo* venom pre-clinical testing and c) to discuss the need for more systemic adoption of3Rs principles in animal envenoming research. We will invite potential end users, antivenom manufacturers, clinical specialists, regional/global patient stakeholders (e.g. WHO/MSF/Ministries of Health) and key regulatory bodies (e.g. WHO, UK Medicines and Healthcare products Regulatory Agency, etc) to actively contribute to the discussion.

Species and numbers of animals expected to be used:

- Mice: 2400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult (18-22g) male CD1 mice have been the model of choice for envenoming studies globally. To reduce variables and to build on extensive prior literature and expertise gained using these models, wewill develop our new model of envenoming using CD1 mice. The main alteration from the existing normwill be to use animals from both sexes in these experiments. By using a strain that is commonly used globally in such experiments, it will be much easier to encourage uptake and implement the refined protocol in these laboratories once established.

Typically, what will be done to an animal used in your project?

Mice will receive an injection of venom, which if left untreated, would lead to death within a specified time frame. Mimicking real-life envenoming scenarios, mice will be treated with a therapy (antivenomor other) after a specified delay, in order to reflect the time it takes for snakebite patients to reach hospital.

The experiments will last no longer than 1 day, and will terminate early if animals progress to humaneendpoints to ensure no animal succumbs to the effect of envenoming.

With the exception of a small number of mice at the outset of the project or working with a previously untested venom, all other mice will be given long-lasting pain relief prior to venom injections. These mice are to assess the effect of pain relief on envenoming progression, as previous experiments havedemonstrated some pain relievening drugs can worsen or accelerate envenoming (ibuprofen, asprin, buprenorphine).

We also plan to explore a further refined assay, performing similar experiments with mice under terminal anaesthesia, to allow detailed monitoring of vital signs during envenoming and treatment, withthe animal being unaware of any venom effects. It is currently not possible to complete the majority of this work in terminally anaesthtised mice as our ability to assess response to treatment currently relieson the behavouir of conscious animals. It is

hoped the pilot work performed in this project using terminally anaesthetised mice will enable progression to a greater proportion of future assessment of envenoming therapies being performed in this manner.

What are the expected impacts and/or adverse effects for the animals during your project?

Despite the use of long-lasting analgesia, we expect the mice to feel some pain and discomfort duringthe procedure. During the study, it is likely that some envenomed mice may experience the effects of the specific venom they have received. This can include difficulty with breathing or mobility, and reluctance to display normal behavioral traits, such as eating and nesting. We expect the effects of enevenoming to either i) subside due to successful intervention, ii) persist at a moderate severity untilthe end of the short experiment, or iii) progress in severity to a humane endpoint, where the mice will be humanely euthanized.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Initally, during pilot study experiments, where we attempt to optimise envenoming route, dose and perform detailed observation on progression of envenoming (allowing for identification and validation ofearly humane endpoints), we expect the severity limit to be classed as severe. We expect about 20% of the mice to be returned under a severe category. However, once the optimal route, venom dose andearly humane end points have been established for a particular venom, enabling the experiment category using that venom to be rated as moderate, we will seek to amend the severity category of future experimentation with that venom to moderate.

For mice under general anaesthesia, the severity limit will be non-recovery, i.e. the animals willenvenomed and treated and subsequently put to sleep whilst under anaesthesia.

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 29 September 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animalswere affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

Snake venoms contain a multitude of toxins that work simultaneously on many physiological systems to synergistically cause pathology/death. Consequently, it has proven impossible (despite extensive efforts by our laboratory – see the section "Replacement") to devise i*n vitro* tests that accurately reflectvenom lethality or neutralising agent efficacy.

Which non-animal alternatives did you consider for use in this project?

We and others have considered many other models for pre-clinical testing of envenoming, and continue to do so. These include - 'antivenomic' methods, chick embryo models, wax-moth larvaemodels, *ex vivo* human and mouse tissues and chick-bi venter preparations.

Why were they not suitable?

Despite extensive attempts, the vast majority of *in vitro* approaches developed have not been able to provide a satisfactory correlation to *in vivo* efficacy models. This is likely due to the way snake venomsimpart their effect, with different toxins found in the same venom acting on different targets in different organ systems simultaneously. There is therefore the requirement for any therapy to be able to neutralise pathologies in multiple compartments simultaneously, and thus animal models, despite their drawbacks, remain essential for efficacy outputs, as they are considerably more informative than singlereadout *in vitro* or limited *ex vivo* models.

It is clear that murine models of envenoming will remain essential for regulation, translation and clinicaldevelopment of snakebite therapeutics into the future. Due to the inevitability that mice will continue to be used in these experiments, it is ethically justified that attempts to refine and reduce their use are robustly explored.

A retrospective assessment of replacement will be due by 29 September 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

All the numbers below are estimates based on assumptions and will be refined as pilot and variabilitydata becomes available. Fot the total project, we estimate we will use 2100 mice.

<u>Protocol 1</u>
We have estimated approximately 1480 mice for the development and validation of the envenomingmodel proposed in this project (protocol 1).

100 mice for the establishment and validation of reference physiological ranges in CD1 mice,including validation of analgesia on reference ranges and validation of point of care tests.

300 mice for Development of a novel *in vivo* model of murine envenoming. We estimate to investigate five injection routes - assuming 15 mice/route = 75 mice x 4 venoms = 300 mice

1080 mice for examining therapeutic efficacy in the model. We estimate 15 mice/therapy/venom.We wish to establish efficacy of 6 antivenom and 3 small molecule inhibitors (9 therapy total) vs.6 venoms = 15 x 9 x 6 = 810.

Protocol 2

We have estimated approximately 620 mice for the development and validation of the non-recoveryenvenoming model proposed in this project (protocol 2)

100 mice for the establishment and validation of references physiological ranges in CD1 micewhilst under anaethesia

300 mice for development of a novel *in vivo* non recovery model of murine envenoming. We estimate to investigate five injection routes - assuming 15 mice/route = 75 mice x 4 venoms =300 mice

270 mice for establishing the efficacy of therapeutics in the non recovery model of envenoming with 2 venoms and 9 thearpeutics (as above). Therefore 15 mice x 9 therapeutics x 2 venoms =270 mice.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The main step we have taken is to reduce the number of mice required for assessment of a therapy. The current median effective dose assay requires 25 mice per venom per antivenom. By taking an alternative minimum anticipated biological effect level (MABEL) and gold standard approach, we are confident we can reduce the number of animals required to demonstrate therapeutic efficacy overall by40%

Experimental numbers will be refined as pilot and variability data becomes available, in consultationwith specialist statisticians and through use of tools, such as the NC3Rs Experimental Design Assistant.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We are taking the following measures to optimse the number of animals used in our project.

Pilot studies to validate reference values, dose routes and variation in the new model will beperformed. Results from these pilot experiments will be used to inform improved statistical analysis in order to optimize the number of animals used.

A mouse envenoming tissue bank will be established and made available for academics to use, with the objective of reducing unnecessary mouse experimentation in the future. This

will includeplasma for coagulation and immunoprofiling in response to different venoms, tissues for RNA profiling and spleens for naive B-cell mouse library controls.

Finally, fresh mouse skin will be made available to colleagues to aid development of *ex vivo*
mouse models of local envenoming.

A retrospective assessment of reduction will be due by 29 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be developing a new animal model of envenoming during this project. The current widely usedmodel is rated as 'severe', does not use analgesia and requires large numbers of mice. The model weintend to develop will use analgesia by default, and be based around the discovery and validation of early humane endpoints. We hope this will enable a study whereby the highest severity level experienced by mice will be 'moderate', thus ameliorating the substantial pain, suffering and distress experienced by many animals in the current and globally used assay.

Why can’t you use animals that are less sentient?

Models of envenoming in non-sentient or less-sentinet animals have been developed (such as *ex ovo*,*in ovo*, wax moth larvae or zebrafish larvae). However, they frequently lack one or more requirements for demonstrating therapeutic efficacy against venoms which simultaneously targets multiple systems.

For example, *in ovo* models are unable to demonstrate the neutralisation of one of the most medically relevant venom components (three finger toxins), while many insect models lack the vertebrate- specific targets of many venoms and typically require substantially more venom to induce insect death.

The reality is that despite extensive efforts over the past two decades, no murine alternative model ofenvenoming has been developed that satisfies the requirements of regulators, and is unlikely to change in the near future. As these murine models remain the primary mode of assessment of envenoming therapies prior to their use in humans, it is essential that models used can provide an accurate representation of envenoming, which is currently not-possible with less-sentient or more immature life stages. However, in this

project, we are exploring the development of an assay using terminally anesthetised mice, with a view to this refinement being implemented more widely in future.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

This project is centered on refining the currently globally used standard envenoming protocols. The major refinement will be for improved and validated monitoring which will enable the implementation ofendpoints at a lower severity level than is widely practiced. Other refinements will be for the absolute requirement for analgesia use in any developed protocol. Experiments using terminal anethesia have not been performed previously, and will provide valuable information about potential further refinementusing this "less sentient" model.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow current LASA publications on best practice and NC3R's continuously updated guidancedocuments which can be accessed via their respective web pages.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

This work is funded by the NC3Rs, and as such we are obliged to not only remained informed aboutadvances in the 3Rs and implement them, but to champion them to wider audiences as well.

We will stay informed through subscribing to both the NC3Rs and LASA websites, routinely attendingNC3Rs seminars, and attending (and presenting) at suitable conferences (e.g. International Conference on Laboratory Animal Science and Welfare).

A retrospective assessment of refinement will be due by 29 September 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved forfuture work of this kind? During the project, how did you minimise harm to the animals?

Home Office

PREPARATION OF XENOPUS LAEVIS EGG EXTRACT FOR DNA REPLICATION AND DAMAGE RESEARCH

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

DNA replication, DNA damage, protein biochemistry, cancer development, cancer therapy

Animal types	Life stages
Xenopus laevis	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

We use cell-free *Xenopus* egg extract model system to study process of DNA replication: egg extracthas the ability to support a whole round of DNA duplication in a test tube, which is regulated in a surprisingly similar manner to human DNA replication process. The aim of this project is therefore to generate the material needed to produce egg extract system: *Xenopus* eggs and *Xenopus* sperm.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Every cell has to duplicate its DNA before cell division. During DNA replication all of the DNA has to beduplicated, just once and without any mistakes. When mistakes made during DNA replication are not immediately repaired this leads to mutations, which in turn can lead to development of cancer, aging and other disorders. It is essential therefore that we understand in depth the mechanisms that drive and regulate DNA replication and DNA repair. The process of DNA replication that happens in *Xenopus*egg extract duplicating

Xenopus sperm DNA is remarkably similar to the mechanism of replication happening in our cells, we can use it therefore to discover novel regulators and novel pathways that can be potentially useful in the future to target in cancer and other disorder therapy.

What outputs do you think you will see at the end of this project?

The output of our work will be in the form of new information on novel mechanisms of DNA replicationand DNA repair, which we will publicise through publication in peer reviewed manuscripts.

Who or what will benefit from these outputs, and how?

In short and medium term our outputs will have scientific impact - we will generate new knowledge thatwill impact on researchers in DNA replication and DNA damage fields.

In long term, which are beyond the scope of this application, the mechanism and proteins we studyand characterise have potential to serve as bio-markers or targets for cancer therapy or therapy of other disorders.

How will you look to maximise the outputs of this work?

We will collaborate with a network of researchers we have already assembled and will continue towiden.

We will publish our findings in open access peer-reviewed journals. We will publish in open access journals or public depositories (BioRxiv) the discovery of unsuccessful approaches. We will deposit allof our large datasets in public depositories, so that they can be accessed and re-analysed by others.

We disseminate findings and knowledge generated through attendance at conferences, social mediaand interaction with public.

Species and numbers of animals expected to be used

- Xenopus laevis: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 our research is to understand better the process of DNA replication and repair. Our cells duplicate DNA only once they decide to divide and on average the process of DNA replication takes a relatively short time of cell life. In a mixture of growing cells only some of them will undergo the processof DNA duplication at any time. It is therefore difficult to study these few cells in the mixture of others.
In result, it is very useful to have a system where DNA replication process can happen quickly and efficiently in a tube, in the extract, without the need to grow cells. *Xenopus* egg extracts are the onlyhigher eukaryotes system that is capable of efficiently supporting DNA replication and DNA damageprocesses in the tube, and is hence indispensable for a

biochemical study of these processes. For production of egg extract we need grown up females able to lay eggs.

We use demembranated *Xenopus* sperm as a source of DNA in our assays as it is a physiological substrate for egg extract. For preparation of demembranated sperm we use grown up *Xenopus* males.

Typically, what will be done to an animal used in your project?

Female frogs will undergo injections of pregnancy hormone in order to induce egg maturation and second, larger, dose of hormone that induces egg laying. Food will be withdrawn for the duration of theprocedure (up to 5 days), as contamination of buffer with regurgitated food or faeces leads to deterioration of egg quality.

This procedure will be repeated a maximum of 10 times with a minimum of 3 months between, andonly if the frogs are confirmed as healthy by the Named Veterinary Surgeon.

Male frogs will undergo injections of pregnancy hormone in order to induce sperm maturation and thenthey will be killed using a humane method and the testis collected post-mortem.

What are the expected impacts and/or adverse effects for the animals during your project?

Injection of hormones will lead to transitory pain and discomfort. No adverse effects are expected fromthe hormones.

Food withdrawal does not affect frogs behaviour as they do not require to food intake daily and adult frogs are most often fed twice a week. The withdrawal of food for the procedure therefore means thatthey miss one feed.

Frogs can retain eggs longer as they get older, which usually does not lead to discomfort. However, allfrogs will be monitored and removed to isolated tank if any signs of discomfort are visible. The isolatedfrogs will be carefully monitored and humanely killed if discomfort persists.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

mild (100%)

What will happen to animals at the end of this project?

- Kept alive
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you

haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Xenopus egg extract is a cell-free system, which is able to synchronously and efficiently replicate DNAtemplate in a tube. Importantly, the undertaken DNA replication is executed and regulated in manners remarkably similar to replication in human cells. *Xenopus* egg extract presents therefore an excellent biochemical system to study process of DNA replication that cannot be achieved by other systems.

Xenopus eggs have to have accumulated all the material needed to run the first 12 cell divisions. In result frog eggs are full of concentrated replication factors. When we take these eggs and separate outthe liquid part from them (the egg extract), it can support the process of DNA replication in the tube.
When we add DNA to the egg extract, the nuclear envelope is formed around the DNA and inside this newly formed nucleus a whole round of DNA replication happens. The nuclei form very rapidly and synchronously and in result the process of DNA replication is quick, efficient and synchronous. As thereare no other processes happening in the extract apart from nuclei formation and DNA replication, it is very easy to manipulate these processes and enforce direct effects. Importantly, years of studies have shown that DNA replication in egg extract is govern by the same rules as DNA replication in cells. It is a unique system for biochemical studies of DNA replication in higher eukaryotes.

To prepare *Xeno*pus *laevis* egg extract we need *Xenopus laevis* eggs, and these can be produced onlyby live adult female frogs of this species. *Xenopus laevis* sperm DNA is a natural substrate for replication in egg extract and it can be produced only by adult male frogs.

Which non-animal alternatives did you consider for use in this project?

Immortalised human cell lines.

The only other cell free system to study DNA replication is based on purified recombinant proteins from budding yeast. Mixing about 50 purified proteins in the correct order can provide DNA synthesis ina tube.

Why were they not suitable?

Cells duplicate their DNA in preparation for cell division. The process of DNA replication takes a relatively short time. In a population of growing cells only small proportion (about 20% of growing culture of cells derived from cancerous samples and about 5-10% of immortalised non-cancerous cell population) undergoes the process of DNA duplication at any time. It is therefore difficult to study specifically this small proportion of cells amongst the others that do not replicate their DNA at that time.Such asynchronously growing population of cells can be forced to undergo DNA replication more synchronously, but the methods used to synchronise cells induce stresses to cells (e.g. mitotic spindle stress, replication stress) and lead to the stress responses within cells. They are therefore not appropriate for studies of unchallenged, natural DNA replication process. Moreover, cells present a very complex system with a multitude of simultaneous processes undertaken at any time e.g. generation of energy, endocytosis, transcription, DNA replication, synthesis of proteins etc. It is challenging to study any of these processes in isolation. When cells are treated with drugs or inhibitors,these substances can affect any of these processes and observed phenotypes are necessarily due to direct effect on the process studied.

Yeast reconstitution system does not allow to discover new factors and new regulatory mechanismsas it is pre-defined by what we already know, what factors are included in the mix. The *Xenopus* egg extract has the full set of egg extract protein present and allows for discovery science and screening for new factors. Moreover, *Xenopus* egg extract is the only higher eukaryotic system capable of in vitroreplication. Budding yeast reconstitution system is based on yeast proteins and it does not allow for research into regulation of DNA replication and damage repair specific for higher eukaryotes, includinghuman. It is therefore not adequate for our research objectives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 used 15 years of experience of preparing extract form *Xenopus* eggs for estimation of how manyanimals we will need for the duration of this project license.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Separation of the liquid part of the frogs egg in the way that it remains active to support DNA replication is a multistep lengthy process. At each step we lose part of the material due to retention on tubes sides and specific procedures that avoid contamination of egg extract with enzymes inducing DNA cleavage rather than replication. These enzymes are most abundant in immature and apoptotic eggs. In result, the quality and quantity of used eggs is a key factor for good extract preparation. We carefully separate "bad" eggs from the good ones. We usually process eggs from 8-10 females at onceso that at the end of the procedure we get enough extract to be able to carry out a number of experiments.

Moreover we store the extract in frozen form maximising the number of experiments performed withthe material produced.

The egg induction and collection has also been optimised - stimulation of egg maturation before the egg laying procedure leads to at least double quantity of laid eggs and therefore reduces the numberof animals used. We also optimised the temperature for egg laying (22-23^{o}C) to achieve the best quality and quantity of laid eggs and buffer composition to collect eggs in, that best preserves eggs during the procedure.

We have established experimentally that priming males with low dose of pregnancy hormone to maturemore sperm leads to higher sperm yield and therefore reduces the number of animals used to generate the quantity of sperm required for the experiments.

This project does not involve observing a phenotype within the animals and we are restricted with thegender of the animals we can use, as only females can lay eggs and only males can produce sperm.Most of the online tools for animal experimental design are not relevent here.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Reduction in animal use is achieved by maximising egg yield in the procedure by optimising husbandryand handling. Stressed frogs do not lay many eggs nor produce much sperm, therefore maintenance of good health and providing optimal condition of housing is essential for good quality and quantity of obtained material. We ensure also that all the injections are completed with minimal handling, quickly and efficiently, to reduce the stress from handling.

Both males and females are primed with a low dose of hormone which induce sperm/egg production and maturation, but not egg laying, to increase the yield from each procedure. We have tested in the past that this step increased the quantity of laid eggs by about 100% and quantity of extracted sperm.

The injection of hormone and subsequent egg-laying is a relatively minor procedure with minimalcumulative impact over the time scales involved. For this reason, the females are reused as this reduces the total numbers of naive animals required by around 10-fold. Re-use also allows the femalesto settle into an egg laying cycle, maximising the likelihood of successful egg laying.

We also keep track of quality and quantity of eggs laid by each batch of females, so that we can remove females from the experiment that are consistently not producing good quality and quantity ofeggs, rather than continuing to put them through fruitless procedures.

We do also use other model systems to investigate the elements of the DNA replication process we areinterested. For example, we use human immortalised cell lines, when appropriate, to follow up our discoveries from *Xenopus* egg extract. Finally, also study purified recombinant proteins, when appropriate for the research question.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We chose *Xenopus* laevis due to their long term established procedures for egg laying and egg extractproduction and higher yields of eggs than *Xenopus* tropicalis. In the wild *Xenopus* can breed up to 4 times a year and females are stimulated to lay eggs by the mating ritual. As we wish to collect unfertilised eggs, the females need to be stimulated to lay eggs in an artificial way by providing them with pregnancy hormone.

The animals (both females and males) are injected with a small volume of hormone solution. The procedure of hormone injection is very quick and any local administration of

anaesthetic is likely to stress frogs more and has the possibility to negatively affect the egg and sperm yield. Immediately after hormone injection that induces egg laying in females, the animals are placed in buffer containingsalt that relaxes them and stops bacterial infections.

Why can't you use animals that are less sentient?

Fully grown female *Xenopus laevis* frogs are the only form of the species that is capable to lay eggs forwhich they need to be conscious. Testis are collected from the adult males post-mortem.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The hormone injection procedures have been refined over the years to ensure only minimal and transitory discomfort to the animals. This includes covering the head so the animal remains calmer as itperceives darkness, and administering the injection on the dorsal surface in a position that avoids the sensory line so as to avoid unnecessary pain. The finest gauge needle possible is used and needlesare changed between animals to ensure sterility.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

The work is work is performed according to the relevant sections of the ARRIVE2.0 and PREPAREguidelines.

We are in continous contact with other groups using *Xenopus laevis* egg extract as a model system todiscuss improvements to husbandry and procedures.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Continued review of the scientific literature will be undertaken on regular basis in order to identify anynew emerging technologies and models that could be potentially adopted in order to replace in vivo animal use.

We are also in continuous contact with other groups in UK and worldwide using Xenopus laevis eggextract and exchange advice and ideas for improved husbandry, procedures and extract usage.

Finally, we attend a number of DNA replication and DNA damage conferences each year - if a novelsystem replacing egg extract will be developed we will find out from the replication community.

NOVEL TREATMENTS FOR ISCHAEMIC STROKE

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Ischaemic Stroke, Focused Ultrasound, Targeted Delivery, Neuroprotection, Neuroplasticity

Animal types	Life stages
Mice	adult, juvenile, neonate, embryo, pregnant
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

We aim to develop novel treatments for ischaemic stroke which take advantage of the latest developments in targeted drug delivery as well as recent advances in pharmaceutical therapies forneuroregeneration.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Stroke is one of the most common causes of disability in our society, yet we have very few pharmaceutical agents which can reduce stroke severity or improve recovery. Developing

newtherapies for stroke is an essential task for modern medicine.

What outputs do you think you will see at the end of this project?

This investigation will result in a set of *in vivo* preclinical data assessing the efficacy of novel drug candidates for the treatment of focal ischaemic stroke, which we will administer both systemically and using a cutting-edge targeted delivery approach using focused ultrasound. We will assess the safety and efficacy of this approach for stroke treatment. This will pave the way for future investigations usingsimilar technologies and compounds with the eventual goal of clinical translation. Our investigation willalso result in an extensive dataset of recordings of the electrical activity of the stroke-injured rodent brain which will help understand the mechanisms of stroke damage and aid the development of novel treatments for stroke in humans.

All of these findings will be made publicly available through publication in peer-reviewed journals.

Who or what will benefit from these outputs, and how?

In the short term, the scientific community will benefit from the new data and knowledge showing the efficacy of new drugs for treating stroke, as well as the safety and efficacy of using focused ultrasound-induced blood brain barrier opening for targeted drug delivery following stroke. We will seek to further the impact of our work by making the data available to both current and future collaborators.

In the long term, these results will form the basis for further preclinical and clinical trials to develop aclinical therapeutic treatment strategy for ischaemic stroke.

How will you look to maximise the outputs of this work?

We will collaborate with several labs working both within our department and at other institutions, withwhom we will share our findings in the short term. We will also publish our results in peer-reviewed journals and present our findings at conferences and scientific meetings.

Species and numbers of animals expected to be used

- Mice: 2000
- Rats: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.
Stroke is a very complex pathology which imparts damage to the brain via a range of complex and interacting mechanisms. Testing novel treatments in animal models is therefore necessary to maximisethe translational relevance of novel treatments, as it is impossible to replicate all of these mechanisms in vitro. We will investigate these complex mechanisms in adult mice and rats as both their behaviour and the organisation of their brains is similar to ours, whilst being a low order animal. We will use mice as there are a

many genetic tools available in mice, as well as several other techniques being optimised for use in mice such as the ultrasound-induced drug delivery we will be using. In certain cases, we will use rats due to their larger brain size and their suitability for behavioural tests which are relevant to human stroke.

Typically, what will be done to an animal used in your project?

In a surgical procedure lasting 1-2hrs, we will create a small, controlled and highly repeatable injury in the animal's brain which models some of the characteristics of a stroke in humans. This will typically beseen in the behaviour of the animal as a slight loss of control of one of it's forepaws, but otherwise the animal should remain healthy. The animal will experience some level of pain or discomfort as it recovers from the surgery, so it will be given appropriate analgesics.

We will then treat the animal with drugs which we hope will help the animal's brain to recover. Typically,animals will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal). Some of the drugs will be paired with an ultrasound treatment, in which the animal is exposed to a non-harmful dose of ultrasound which will help the drugs enter the brain tissue. This treatment will be repeated no more than daily in the weeks following injury, up to a maximum of 60 doses in total.

Over this time, we will assess how well the treatments have worked by measuring how quickly and to what extent the animal regains use of its forepaw, which it lost as a result of the stroke. Some of theseexperiments will require animals to perform many repetitions of a laboratory task. To get animals to practice a task many times, we will have to limit their access to fluids while they are in their cage: instead, animals will get a fluid reward after each successful trial in the laboratory. We will ensure thateach animal receives the appropriate amount of fluid every day by giving them an extra volume to achieve a healthy minimum, when necessary. It is to be expected that the animal loses some weightbecause it is drinking less, but this is monitored closely and is not allowed to exceed 20% of the original weight.

The tasks may also require the animal to have a head fixation device implanted, which will be performed in a separate surgical procedure lasting 1-2hrs. We will typically also create small windows in the skull to gain access to the brain, to see how the cells in the brain are reacting to the injury and totreatment. We will insert small electrodes into the brain whilst the animal is performing tasks, causing no damage to the brain (up to 120 recording sessions). Alternatively, rather than inserting electrodes, we may use a microscope to view the activity of cells in the brain.

The behavioural training phase and the post-lesion recovery phase will typically last no longer than 8-10 weeks in total.

At the end of experiments, terminal procedures may be carried out such as performing neural recordings under terminal anaesthesia, tissues may be removed for ex vivo analysis or the animal maybe killed by perfusion fixation.

What are the expected impacts and/or adverse effects for the animals during your project?

In order to motivate animals to perform the repetitive actions required for training in behavioural tests,access to water in their cage is restricted and used as a reward in

training. In this case, the animal's body weight will be closely monitored to ensure that the animal remains at a healthy weight and the impact on the animal is minimised. This phase of training and familiarisation with the behavioural testing and recording environment will typically last less than 2 weeks. Some of the behavioural tests also require a device to be implanted to keep the animal's head steady whilst it moves its body. In certain cases, for the animal's safety and recovery from this surgery, the decision may be taken to house the animal by itself, although this time will be minimised to reduce any stress this may cause.

The induction of a model stroke will cause animals to suffer some of the effects of a human stroke, such as a partial loss of function of one of their forepaws. The severity of this injury will be very carefully controlled, to minimise the impact on the animal. The surgery associated with these procedures will cause the animals some level of discomfort and the animals may lose up to 15% of their body weight, so they will be given painkillers and monitored closely, just like people in hospital.The animals' recovery will then be monitored over the following weeks (typically a maximum of 6-8 weeks).

We may also administer drugs which may affect the animals' behaviour and may cause some anxiety,although the duration of these adverse effects will always be short and the animals will be monitored closely the entire time.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

mice 10% mild, 90% moderate; rat 10% mild, 90% 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 haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Stroke is a very complex injury involving many interacting pathways of damage, and this makes it veryhard to model with a computer simulation or in cells grown in a lab. Only in animals which have similarbrains and physiologies to ours can we test potential treatments for this complex injury. The drug treatments we are hoping to administer also work in many different ways, helping the brain to regrow and repair and helping the immune system to function healthily. This would be impossible to model accurately outside of a living animal.

Which non-animal alternatives did you consider for use in this project?

We have considered the following alternative approaches:

computer modelling,

cell cultures and brain slices

non-protected species.

Why were they not suitable?

Computer modelling: Where possible, we will use computational models to test hypotheses before doing experiments on animals. However, scientists do not yet know enough to simulatethe entire brain and how it reacts to an injury such as stroke, so we need animal models to answer these questions.

Cell cultures and brain slices: Understanding how the brain recovers from stroke requires it to beconnected to a body to assess how recovery in the brain translates into functional recovery of deficits in the body. In some cases however, when we need to assess how modified versions of drugs interact with tissue, this will not be done in live animals.

Non-protected species: These species have brains which are very different from ours, so they arenot suitable for helping us understand stroke in humans and developing new treatments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 mice and rats based on the anticipated numbers of experiments, thenumbers of experimental groups, and the numbers of animals in each group. These estimates are based on the standard number of animals used to show statistically significant effects in our field.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We carefully plan all our experiments to minimise the number of animals we need to use to get the most information possible. In many cases, we will obtain answers to multiple research hypotheses within each animal, for example assessing the efficacy of a drug through behavioural testing whilst simultaneously recording electrophysiological 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. All experiments will be designed insuch 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 ofanimals 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 overdifferent experimental sessions. This will often include the same animal taking part in two phases of a study that are aimed at answering interrelated scientific questions. We will use consistent experimentaltechniques 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 efficacy of administering a drug at a particular time point post-injury) beforeincluding more animals to reach statistical significance.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice and rats are the most suitable models for this type of research because they share close resemblance to human biology, and their behaviour is relatively similar to ours. Mice will be used whenever possible, with rats only used whenever unavoidable (for instance due to their larger brainsize and suitability for certain behavioural tasks).

The stroke models we will use are among the most minimally invasive and highly controllable models available to cause a highly repeatable stroke injury. This is in contrast to more traditional models of stroke, which carry a much higher risk of mortality. Both models achieve small and very localised lesions in the brain which minimises the distress caused to the animals and allows a highly repeatablefunctional deficit to be induced between individual animals.

The proposed fluid control strategy should avoid clinical signs of dehydration or significant weight loss,because mice will receive a sufficient volume of water each day. By working with fully grown adult mice(age 3 months or older), we will avoid interfering with their normal development.

Overall, animals will be constantly supervised by trained individuals and advice will be sought from theveterinary team if there is any cause for concern. We will use anaesthesia, analgesia, and humane endpoints to limit suffering.

Why can’t you use animals that are less sentient?

Less sentient animals are further apart from human biology. In contrast, mice and rats are also mammals and have brains, nerves, sensory receptors and immune systems that are similar to ours. The way they experience the world and behave are also quite close to the way humans do. We needto work with animals that are fully grown both from a practical perspective and to be able to translateour results to adult humans.

How will you refine the procedures you're using to minimise the welfare costs

(harms) for theanimals?

Refinement measures carried out in this project will include use of anaesthesia and pain-relief medication as well as enrichment of the animal's environment. We will frequently monitor animals, especially after they have received the model stroke injury, and will increase monitoring if any adverse effect manifests; depending on the severity of harm, the animal will be treated following a veterinarian'sinstructions or humanely killed.

Animals taking part in behavioural experiments will first be accustomed to people, to reduce their stress levels. During any such experiment, we will closely watch all animals for any sign of discomfortand distress and either stop the experiment or humanely kill any animals suffering unexpectedly.
Throughout the project, we will continue to look for new ways to refine our experimental protocols andmake animals more comfortable.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow published best practice guidance for how to design, perform and report experiments tothe highest standard including the NC3Rs PREPARE and ARRIVE guidelines, as well as the LASA guidelines for aseptic surgery and administration of substances.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will keep informed about any advances by reading scientific publications and speaking with colleagues within our own establishment and elsewhere, including not only other scientists but those directly involved in the care and welfare of animals. We will constantly review our protocols and experimental design to reduce, refine and replace the use of animals. We will also attend the frequentmeetings held at our institution on the advances in the 3Rs and their implementation.

DEFECTIVE REGULATION OF PANCREATIC HORMONE SECRETION IN DIABETES

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Diabetes, Pancreatic islets, insulin, Hyperglycaemia, Hypoglycaemia

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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify and characterise the regulation of pancreatic hormone release and how it becomesdefective in diabetes with a view to developing improved therapies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Diabetes is a very common disease that results in substantial morbidity and mortality. In the UK ~2.9million people currently have diabetes and a further 850,000 are undiagnosed.

Diabetes affects every cell of the body, which explains the wide spectrum of diabetic complications (increased cardiovascular mortality, kidney disease, blindness, lower limb amputation and neuropathy).
Two hormones play a central role in diabetes: insulin and glucagon. They are secreted by the beta- and alpha-cells of the pancreatic islets. There are two forms of diabetes. In type-

1 diabetes, the insulin-producing cells are destroyed. In type-2 diabetes, the beta cells remain but their capacity to release insulin is impaired. In addition, both forms of diabetes associate with defects of glucagon secretion thatexacerbate the effects of the lack of insulin and complicate diabetes therapy. These considerations provide a justification for the planned work, which aims to explain how the release of these two hormones is normally regulated and why/how it becomes impaired in diabetes.

What outputs do you think you will see at the end of this project?

At the end of this project, the likely outputs will be:

Publication of research elucidating the causal mechanisms of dysregulated islet hormone secretion in diabetes.

Novel animal models for diabetes research that will be shared with the research community.

New research techniques that will be shared with the research community.

Information that guides the design of clinical trials for novel treatment strategies of diabetes.

Who or what will benefit from these outputs, and how?

We perform mechanistic studies to elucidate the physiological regulation of pancreatic hormonerelease with a view to unveiling how they become disrupted in diabetes.

We publish the results of our studies in the scientific literature and this will benefit the wider scientificcommunity and may lead to the development of new pharmacological treatment of diabetes.

We will establish new mouse models of type-1 diabetes that will facilitate the elucidation of the cellulardefects underlying the secretion defects. Because type-1 diabetes involves many different genes, thiswill require advanced mouse genetics. These models will be shared with our colleagues.

We strive to develop new experimental techniques to explore pancreatic islet function. We will continueto share these techniques and reagents with our colleagues worldwide.

Finally, work in experimental animals will guide clinical trials/experimental medicine research studies.Many of the compounds we use for our experiments are already in clinical use (but sometimes for other indications than what they are used for experimentally) and this will accelerate the translation ofour experimental studies in animals to work in humans.

In summary, our work will benefit the scientific community (short-term), the pharmaceutical industryand clinical colleagues (medium term) and – ultimately – diabetic patients (long-term).

How will you look to maximise the outputs of this work?

This project is a collaborative effect of several advanced groups in islet physiology and mouse genetics. The combined expertise will enable efficient execution of the project,

maximising the outputs.The project will also generate novel animal models that will be very useful for research in broader scientific communities. This will lead to new collaborations and have a continuous impact in the field.

Finally, the ultimate goal of our work is to provide improved therapy for people with diabetes. We are inactive discussion with the industry to translate novel findings into real-world applications.

Species and numbers of animals expected to be used

- Mice: 7500

Predicted Harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The choice of using mouse models is generally based on the considerations of feasibility andconsistency.

Feasibility. The architecture and function of the pancreatic islet are highly conserved across species, especially in mammals. Mouse models have been widely used in the study of islet physiologyand there is a wealth of pre-existing data. Experimental mice are easy to house and fast to breed. There are well-established mouse models for diabetes. These models would allow detailed mechanistic studies that are practically impossible to carry out only using diabetic human islets (given the limited access to these islets).

Mice are also relatively easy to genetically manipulate for specific gene expression in the islets. Thiswill allow detailed studies to collect data from cellular to whole-body phenotype. In principle, such studies could be conducted using human islets. However, limited and irregular access to human pancreatic islets make it impossible to build a research programme on human islets (although they represent a very valuable resource). Although there are reasonable insulin-secreting cell lines, thereare no good glucagon- or somatostatin-release cell lines.

Consistency. Mouse cohorts offer the possibility of perfect matching of sex, age and geneticbackground. These are essential conditions for acquiring consistent data.
All mice will be used when they reach adulthood (i.e. when they are 12-14 weeks of age) for acquiring fully mature/developed islets. Mouse models of diabetes will be used as soon as is logistically possibleafter they develop diabetes (blood glucose: 30 mM), to minimise the discomfort associated with the disease.

Typically, what will be done to an animal used in your project?

This license is for both breeding genetically modified mice and some functional tests on a limited number of mice to perform detailed metabolic profiling in vivo. The level of severity of these tests willnot exceed ‘moderate’.

Most of our functional tests will be carried out in test tubes with isolated tissues/cells after the mice arehumanely killed. Whole-body phenotyping will be performed in a limited

number of animals to test the whole-body metabolism and the efficacy of compounds in reversing diabetic phenotypes. This will include injections of glucose, hormones and/or pharmacological agents; and tests of glucose and hormones in small-volume blood samples collected throughout the experiments.

For mouse models of diabetes, mice will develop spontaneous diabetes as they age. The experimentswill be completed one week after diabetes has presented after which the animals will be humanely killed (to allow ex vivo analysis).

Non-diabetic mice will not be kept past 15 months of age (but in most cases, the mice will be usedbefore they are 4-5 months of age).

What are the expected impacts and/or adverse effects for the animals during your project?

Most transgenic animals will be phenotypically normal (essentially the same as wild type animals) andthey are unlikely to require any treatment.

If we have reason (based on pre-existing information) to suspect a new mouse model to have a diabetic phenotype, the animals will initially be monitored on a daily basis. The duration of the experiments will be no longer than 1 week once diabetic phenotypes are visible (as determined bymeasurements of glucosuria or abnormal wetting of the cage bedding).

Certain experiments may require food restriction (but with free access to water) for a short period during mice's sleep period. Mice are not expected to experience hypoglycaemia under this condition.

Some experiments will involve administering substances that can transiently (<2 hours) increase or decrease blood glucose (either with intraperitoneal injection or oral gavage). Normally healthy blood glucose levels will be restored within the period of the experiments. If hyperglycaemia or hypoglycaemia persists beyond the duration of the experiments, experiments will be discontinued andremedial action will be taken to restore normal blood glucose.

Some experiments will involve taking small-volume blood samples from the mice (<10% of total bloodvolume) within a duration of 2 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 animaltype)?

- Mice: 36% Sub threshold, 24% Mild, 40% 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

haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Pancreatic islets are highly conserved across species and the mouse is the lowest vertebrate with enough aspects of its genetics, anatomy, physiology and embryonic development shared with humansto generate biologically relevant data that ultimately can be extended to improved understanding of diabetes in humans.

Therefore, mouse models have been widely used in the study of islet physiology and there is a wealth of data from previous studies that form excellent references.

Experimental mice are easy to house and fast to breed, and there are established mouse models for diabetes. It is also relatively easy to genetically manipulate mouse models for specific gene expression in the islets.

Finally, mouse cohorts offer the possibility of perfect matching of sex, age and genetic background.These are essential conditions for acquiring consistent data which is critical for our studies.

Which non-animal alternatives did you consider for use in this project?

Many of the experiments will be performed on human pancreatic islets obtained from a clinical islet transplantation programme. However, the supply of human islets is limited and some experiments aretechnically not feasible using human islets.

Certain experiments will be performed using clonal cell lines, but these cells do differ in many waysfrom the primary islet cells. Whilst it is a common practice to use them for proof-of-principle studies, they cannot replace rodent islets for detailed physiological analyses.

Finally, we are developing human cell lines derived from human induced pluripotent stem cells (hiPSCs). These cells will provide excellent models for human islet cellular physiology and overcome the limited supply of human islets. However, the crosstalk among different types of islet cells, which isimportant for islet function, is currently difficult to achieve in hiPSC-derived islet cells.

Why were they not suitable?

For human islets, availability is not sufficient.

Clonal cell lines are tumoural and their properties are different from primary cells. For example, theirglucose metabolism is very different, which is a major concern when studying an organ involved in metabolic regulation.

Human stem cell-derived islet cells is an exciting development and we are actively involved in theseefforts but this technology is still in its infancy.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals is estimated based on our previous studies during the last 40 years.

Hormone secretion experiments normally require a large number of islets. Islets are pooled from several mice to increase the robustness of data and reduce the impact of individual variability. Eachexperiment series (involving up to 10 different treatments) will require 8-10 mice.

Electrophysiology and imaging experiments require much fewer islets and they will be taken from theislets isolated for secretion experiments. This will limit animal usage and enable a direct correlation ofthe responses obtained with the different techniques.

A large number of islets will also be needed for fluorescence-activated cell sorting (FACS) sorting andsubsequent genetic studies (e.g., RNAseq). It usually requires 5-10 mice to collect enough cells for transcriptomic analysis.

For in vivo studies, each experimental condition will require 10 mice (again based on prior experience).

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?
Our experiments are performed according to best practices and state-of-the-art methodology.Sample sizes are estimated using power calculation based on data from previous studies.

When using diabetes animal models, the development of diabetes will be monitored by frequent measurements of glucose using urine or blood samples. Mice will be used as soon as logisticallypossible after they develop diabetes, to minimise the discomfort associated with the disease.

Experimental efforts will be coordinated to ensure that tissue samples can be used for multiple types ofmeasurements.

All experiments will be conducted following the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

The group has established the approach of using mouse models in which specific cell types have been‘tagged’ with fluorescent proteins. This allows cell identity to be established before the experiments commence. This will increase the success rate and thus result in a reduction in the number of animals used.

GA colony management is carried out via the Mouse Colony Management System (MCMS) and activecommunication with animal technicians at the animal facility. Breeding is limited to sustaining experimental cohorts sufficient for experiments planned. Small scale pilot studies (including using clonal cell lines) will be carried out to establish the

proof-of-principle and optimise experimental protocols before large scale experiments are conducted.

Although we are primarily interested in the pancreatic islets, we make an effort also to harvest otherorgans known to be affected by diabetes (including the heart and kidneys).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically altered (GA) mouse models that have fluorescence-tagged islet cells but no harmful phenotype. We have recently extended this approach to a type 1 diabetes mouse model (thenon-obese diabetic [NOD] mouse) and this will dramatically improve the analyses of the cellular defects that contribute to the hormone secretion defects associated with insulin-dependent (type 1) diabetes. NOD mice develop autoimmune diabetes (detected as high blood sugar and increased urination) whenthey are ≥12 weeks of age. Although mice tolerate high blood sugar well, they will be used for experiments as soon as logistically feasible after diabetes has been confirmed.

Most functional/histological experiments will use pancreatic islets isolated from the animals after they are humanely killed. A small number of experiments will use phenotyping methods in live animals to assess how well animals can regulate their blood sugar under different conditions. This will involve injections of glucose and/or substances and small volume blood sampling but will cause no more thantransient discomfort and no lasting harm. In most cases, mice will only be subjected to these experiments one time during their lifetime.

Why can’t you use animals that are less sentient?

The mouse is the lowest vertebrate with enough aspects of its genetics, anatomy, physiology and embryonic development shared with humans to generate biologically relevant data that ultimately canbe extended to improved understanding of diabetes in humans. Adult mice are required because fullydeveloped islet function/structure is essential for the study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Animals will be closely monitored and if any become unwell they will be humanely killed and an examination performed to identify the cause of illness and to inform subsequent experiments. If animals exhibit diabetes, measures will be undertaken to minimise the consequences of this, such as changing wet bedding (or using ultra-absorbent bedding), frequent refilling of the water bottles and reducing the numbers of animals housed per cage if appropriate. When introducing new models, the progression of diabetes will be

carefully monitored primarily non-invasively by measurements of sugarin the urine using urine testing strips. Mice that undergo in vivo experiments will be monitored after experiments are completed.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow guidance published by the National Centre for the Replacement Refinement and Reduction of Animals in Research (www.nc3rs.org.uk), Norecopa (https://norecopa.no) and LaboratoryAnimal Science Association (https://www.lasa.co.uk).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

All personal licensees of the team will be regularly updated on the advances in the 3Rs through 1) monthly internal 3Rs meetings, 2) termly 3Rs Newsletters, and 3) annual 3Rs symposiums organised by Named Information Officer. We also have access to a NC3R's Regional Manager. New approachesto Replace, Reduce or Refine animal experiments will be tested and applied during the project.

Home Office

DEVELOPING MINIMALLY INVASIVE INTERVENTIONAL CARDIOLOGY TECHNIQUES FOR STUDYING AND TREATING ATRIAL FIBRILLATION AND OTHER ARRHYTHMIAS IN THE HORSE

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, ortheir effects, in man, animals or plants

Key words

Horse, Heart, Electrophysiology, Therapy

Animal types	Life stages
Horses	adult, aged
Ponies	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required,and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Uses Cats, Dogs or Equidae

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

Heart rhythm abnormalities in the horse have a rare but very important impact on horse welfare andhuman safety.

The aim of this project is to develop safe and minimally invasive imaging techniques to investigate theelectrical and contractile activity of the heart, so allowing better evaluation

and treatments for heart rhythm abnormalities in horses. Such techniques are routine in humans, and sometimes used in dogs,but the technology is only recently sufficiently advanced to allow their use in horses.

A retrospective assessment of these aims will be due by 30 September 2028

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Treatments for rhythm abnormalities in horses are very basic and lag well behind the options for humans or even dogs. For example, while many horses with persistent atrial fibrillation can be treatedand successfully return safely to their previous use, recurrence is a problem in a large proportion, withfigures of up to 64% reported. Intermittent rhythm abnormalities can be particularly frustrating to evaluate and treat. Treatments to prevent recurrence of persistent AF and better evaluate and treat these intermittent problems are therefore required so that horses can be restored to normal heart function. While some horses can be left in atrial fibrillation or tolerate abnormal rhythms, restoringnormal heart function is optimal to improve welfare and is likely to reduce the likelihood of sudden cardiac collapse and/or death.

What outputs do you think you will see at the end of this project?

By the end of this project we should have:

- developed minimally invasive techniques that allow us to safely investigate and better treatabnormal rhythms (including atrial fibrillation) in live horses
- developed a better understanding of the physiology of the normal horse heart
- developed laboratory based techniques that allow us to investigate heart rhythm abnormalities inthe horse

Who or what will benefit from these outputs, and how?

The primary benefits will be horse welfare and human (owner) safety. Atrial fibrillation (AF) is the mostcommon heart rhythm abnormality in the horse (prevalence up to 5%) and usually causes dullness, unexpected poor performance or unwillingness to exercise. It can also predispose to exercise-inducedbleeding from the nose and it may be one of the causes of sudden death in horses. While collapse and sudden death is rare it has serious impact for owners and rider safety

A better understanding of diseases like AF in the horse will also benefit our understanding of AF in thehuman and dog. The horse is unusual in that it has a high prevalence of this condition, without significant evidence of underlying heart disease. A better knowledge of the physiology of the equine heart and the development of pathologies such as atrial

fibrillation will bring tangible benefits to our general understanding of AF in particular.

Once the techniques are developed and proven to be safe and effective, they will be offered as a cinical service to horse owning clients. This substantially increases the options for treatment of arrhythmias in the horse, providing as yet unavailable treatment for intermittent rhythm abnormalitiesand perhaps preventing the recurrence of problems like atrial fibrillation (up to 64% recurrence following treatment according to some studies).

How will you look to maximise the outputs of this work?

We will collaborate with others in the UK and the world doing similar work to ensure that the development of these techniques is optimal. Experiences will be published, whether successful orunsuccessful.

Species and numbers of animals expected to be used:

- Horses: 8-12
- Ponies: 8-12

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Only horses are suitable as subjects for this study due to the need to develop this as a clinical technique for horses. The physical size and species-specific differences in heart anatomy and physiology from other large animals (e.g. bovine) make this necessary. Lone (also known as idiopathic) atrial fibrillation appears to be unique to horses and some human athletes. As a consequence, the information gained during development of these techniques, from horses without AF, will be crucial to our understanding of this important disorder. Bovine species for example do notdevelop AF without significant underlying disease.

Typically, what will be done to an animal used in your project?

This study will use normal horses that are destined for euthanasia due to chronic intractable problems that are affecting their health and/or welfare. Veterinary clinicians with no interest in this study, throughdiscussion with the owners, will make the decision for euthanasia. Any horses with acute clinical conditions where immediate euthanasia is required, or those horses with conditons that preclude travelfor euthanasia, will be excluded from the study. There will be informed client consent. There will be noeconomic incentive to the owners, other than the fact that the costs of euthanasia will be borne by the project. Horses will have intravenous catheters placed as per normal for euthanasia. Instead of euthanasia, a general anaesthetic will be given to render the horses unconscious, without stopping theheart. Up to six further intravenous or intra-arterial catheters will then be placed while the horse is anaesthetised to allow internal access to the heart. Electrodes will be used to detect electrical activity and contraction patterns within the right heart. The electrodes will then be placed into the left heart, again to detect electrical activity and contraction. Finally special electrodes will be used to cauterise areas in the heart that are known to cause arrhythmias in humans. The total time of the

procedure willbe maximum 6 hours, after which the horse will be euthanised.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects will be minimal. A single intravenous catheter in the left jugular vein will be placed byan experienced veterinary clinician and personal licence holder. All subsequent vascular access will be under general anaesthesia, so the horse will be unaware of the procedures. If there are any adverse effects from the procedure, e.g. it induces dangerous heart rhythm problems or serious bleeding, the horse will immediately be euthanised as planned.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

All horses will be non-recovery.

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 30 September 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animalswere affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The proposed techniques must be developed in live horses to enable translation into a safe andeffective technique for use in clinical practice.

Which non-animal alternatives did you consider for use in this project?

Post mortem specimens

Why were they not suitable?

No realistic and complex enough models exist for the horse heart; and post mortem specimens would not accurately represent the live technique proposed for treatment. We aim to also develop a laboratory model that uses hearts dissected after death. This should provide us with a convenient toolfor investigation the basic electrophysiology of the equine

heart, minimising use of future live horses. Comparison of this laboratory technique/tissue with the live, but anesthetised, animal work is thereforecrucial.

A retrospective assessment of replacement will be due by 30 September 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Because these techniques are new and the purpose of this project is development, it is very difficult toestimate the number of animals required. Experience from developing other clinical techniques wouldsuggest that 8-12 animals should allow acquisition of the necessary skills and data. After this numberwe will also have knowledge of the inherent variation in horse heart tissue and effects of the procedure.

Should the necessary expertise and knowledge be acquired before this upper number is reached, oralternatively in the unlikely event that the technique be deemed impossible, the project work will be stopped.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

A minimal number of experimental animals will be used, allowing development of the technique while also generating scientifically useful data. One of the team is an experienced human electrophysiologist who has already translated skills to experimental large animals (pigs). The applicantis an experienced equine cardiologist. Sequential experiments will be performed to a set degree of confidence in the technique, while also gaining knowledge of inherent tissue electrophysiological variation and procedure effects, estimated to be maximum 12 animals.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Some aspects of the techniques will be optimised prior to experimental work in either clinical cases (non-invasively) or in post mortem specimens. For example optimising cardiac imaging to enhance placement of necessary electrodes, practising the transfer of electrodes from the right side of the heartthrough the septum to the left. Although not equal to the live situation, this preparatory work will maximise the chances of effective development in the live horses.

A retrospective assessment of reduction will be due by 30 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Only horses are suitable as subjects for this study due to the need to develop this as a clinical tool for horses. The physical size and species-specific differences in cardiac anatomy and physiology from other large animals (e.g. bovine) make this necessary. The choice of horses already destined for euthanasia and the non-recovery protocol will minimise suffering while also allowing us to measure theeffects and the outcomes of our techniques. From the horse perspective, this is no different from the intended euthanasia, with one intravenous catheter inserted before the horse is rendered unconsciousfor the procedure.

Why can't you use animals that are less sentient?

Non-recovery anaesthesia protocol opted for in this study to minimise harm. Immature animals wouldnot be appropriate, nor would other species for reasons already covered.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

As per our usual protocol for anaesthesia and/or euthanasia of clinical cases, a single intravenous catheter in the left jugular vein will be placed by an experienced veterinary clinician. For horses that are averse to intravenous injections, we have in-house behaviour expertise to help minimise stress andtrain horses, e.g using overshadowing techniques. All subsequent vascular access will be under deep general anaesthesia. The horses will be anaesthetised by highly trained anaesthetists, with significant experience in equine anaesthesia, ensuring they maintain unconsciousness until euthanasia.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

The following sites provide excellent resources from which we will seek guidance for best practice inrefining the experimental protocol: https://nc3rs.org.uk/ ; and https://norecopa.no/prepare

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Maintaining awareness of these concepts and advances by using sites such as: https://nc3rs.org.uk/

A retrospective assessment of refinement will be due by 30 September 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved forfuture work of this kind?
- During the project, how did you minimise harm to the animals?

CELLULAR MECHANISMS OF ADAPTATION IN THE BRAIN

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Neuroscience, Memory formation and loss, Synaptic connections, Epilepsy

Animal types	Life stages
Mice	neonate, juvenile, adult
Rats	neonate, juvenile, adult,

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The overall aim of the present project is to understand how the contact points between nerve cells, called 'synapses', are modified during brain activity and how this may contribute to learning, health anddisease, in particular epilepsy. To achieve this, we will use cutting-edge methods in optics and electronics, genetic modification and refined animal models.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

How cells and molecules contribute to learning and memories at the level of dividual cell-cell connections is poorly understood. The present project aims to bridge this knowledge gap with novel and cutting-edge experimental tools and techniques. The expected results should provide basic neuroscientists with novel insights into the workings of brain machinery while

equipping experimentaland clinical neurologists with potentially important information and tools.

What outputs do you think you will see at the end of this project?

The key outputs of the present project will be:

peer-reviewed scientific publications in high-profile academic journals,

development and dissemination of novel experimental and theoretical methods for cutting-edgeneuroscience research,

dissemination of obtained results at research meetings, through the internet-aided tools, and viascientific collaboration,

scientific training and enabling of experimental researchers in the field of neuroscience andneurology.

Who or what will benefit from these outputs, and how?

The present project will generate new knowledge about fundamental mechanisms of brain function. Representing the building blocks of the brain machinery, this knowledge should directly benefit a largecommunity of basic and translational neuroscientists, experimental and practicing neurologists.

The potential benefit of the expected results to clinical practitioners lies in identification of new drug target candidates. Many of these mechanisms are likely to be important in common neurodegenerativediseases such as Parkinson's, Alzheimer's, and age-related dementia, as well as epilepsy.

How will you look to maximise the outputs of this work?

Our group has consistently been engaged in productive research collaboration with leading neuroscience researchers and laboratories worldwide, since at least 1999. More than 70% of all research articles published by us have been a result of collaborative research. Part of the present project is purposely funded by a five-year collaboration initiative among several univeristies. The present project will continue this trend, to boost our research output. We will disseminate new knowledge through high-profile publications, international seminars and meetings, internet and mediatools. We have in the past published a number of papers dealing with advantages and flaws of key experimental methods that we have developed (including high-impact articles in Nature Methods, Nature Protocols, etc.) and plan to continue this important dissemination route.

Species and numbers of animals expected to be used:

- Mice: 1100
- Rats: 400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The species of rats and mice are the most suitable for the proposed project.

Scientific studies such as these have high relevance to human health. The majority of basic physiological concepts dealing with communication among brain cells have been established using rodents, and the key techniques relevant here have been tested vigorously in rats and mice. Importantbiology tools including genetic modifications have also been developed and established in these species.

For the procedures described here, we perform experiments in one of two life stages. We will study adaptation in adult animals, where learning mechanisms have developed to maturity and we can studythe intact nervous system in vivo. We will also use ex vivo brain slice preparations from juvenile animals (typically 21-28 days). Brain slice preparations are more robust at this stage and this will allowus to perform more sophisticated electrophysiology.

Typically, what will be done to an animal used in your project?

These animals will undergo one or two surgical procedures involving anaesthesia, depending on the experiment. The primary purpose of these procedures is to (a) add a light-emitting protein to specific cells in the brain and (b) use sophisticated microscopes to record the light emitted from such proteins.These proteins are engineered by biological engineers in companies and universities around to world and relay information about the activity of brain cells. We will collect these microscopic images alongside other relevant data such as electrical signals in the brain, to build an elaborate and sophisticated dataset on brain activity during learning and memory formation. The duration of the imaging experiments can vary; in some cases, an animal will be imaged once; in others, we may repeatedly image over the course of weeks or months, allowing the animal to interact and behavenormally in between.

What are the expected impacts and/or adverse effects for the animals during your project?

There are some impacts associated with the surgeries that are expected and well-managed, such aspain, distress, weight loss and chance of infection. These are common to many surgeries and will bemanaged with close monitoring, painkillers and nutritional support. These effects are not long-lastingand resolve within 2 or 3 days of the surgery.

The imaging experiments themselves are low impact and do not lead to lasting harm or distress in theanimals. In a small subset of animals (100), we will examine how the learning mechanisms are impacted by or can contribute to the onset of seizures. These animals may experience fatigue and distress that can last for up to 24 hours but are not expected to experience pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

No severe protocols will be used in the proposed project, with 'moderate' severity expected in approximately 77% of animals. The remainder of animals will be subjected to non-

recovery proceduresunder terminal anaesthesia only.

	Mild	Moderate	Severe	Non-recovery	Total
Mice	0	890 (81%)	0	210 (19%)	1100
Rats	0	260 (65%)	0	140 (35%)	400

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Understanding the basic biology that underpins brain machinery is not possible without at least someanimal experimentation. These experiments will allow us to observe and manipulate the activity of brain cells in their native context. Other reduced preparations, such as cell and tissue culture, or computational models, are useful and will be used in this laboratory. Unfortunately, these lack the fullcomplexity of structures seen in the brain that give rise to its many functions and features. Our group will be able to build upon our extensive expertise and experience in using these animals in an optimaland least harmful way.

The species of rats and mice are the most suitable for the proposed project, for several reasons. Firstly, scientific studies at the cellular level have repeatedly demonstrated their relevance to human health. Secondly, the majority of basic physiological concepts dealing with communication among braincells have been established using rodents, and the key techniques relevant here have been tested vigorously in rats and mice. Thirdly, critical molecular biology tools including targeted genetic modifications have also been developed and established in these species.

Which non-animal alternatives did you consider for use in this project?

Over the past decades, we have developed and implemented a wide range of computational tools andplatforms that provide realistic brain models relevant to our research objectives. We have employed extensive simulations of molecular events in and around brain cell connections to better predict and understand brain function.

Computer simulations are and will be continuously used in our work to optimise experimental design and reduce the number of animals used in our experiments. We have been using these modelling approaches to test our hypotheses, particularly at levels of resolution that go beyond physical experiments. This approach has allowed us to rule out unfeasible lines of experimental study, replacingand/or significantly reducing animal experimentation. The present project will build on and use full advantage of this research strategy.

We are also collaborating with experienced clinicians to establish methods for recording

from human tissues. These methods will directly replace certain rodent recordings while also more directly relevantto questions of human health research.

Why were they not suitable?

The methods and research strategies as described above have been and will be fully suitable to replace animals for a significant part of, but not all research objectives of the present project. The models also require real world data inputs to improve their accuracy and relevance, and where newlines of evidence emerge in our models, they are extremely useful in informing future animal experiments. These two approaches go hand-in-hand in our laboratory.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissueand reuse.

How have you estimated the numbers of animals you will use?

Our group contains several highly experienced scientists with backgrounds in neuroscience, molecularbiology, physics and computational biology. There is a strong emphasis on statistical and computational methods in our group that has helped us determine the optimal number of animals for each experiment. We have consulted with the biostatistical and animal welfare support teams in our university to confirm optimal numbers.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We used our extensive previous experience and related records to estimate the numbers of animals forthe present project which will involve several separate lines of study. Experimental designs and reporting are continuously refined in accordance with the NC3Rs' Experimental Design Assistant and ARRIVE guidelines: the use of multifactorial statistical designs and unbiased estimators including Monte Carlo simulation-based has been inherent to our workas refelcted in multiple research publications.

As per these guidelines, we have implemented a range of design elements intended to optimise the number of animals to be used. Randomisation, blinding and error prediction will be routine to all our experiments. We have gathered a lot of datasets in our previous project licences that enable us to incorporate relevant and accurate measures of noise, variability, effect size and so on. These will helpdetermine optimal sample sizes for each given experiment.

In some of our studies, we have implemented a longitudinal study design that will further reduce thenumber of animals needed.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We will make use of a limited number of pilot studies with specific objectives, particularly when applying new experimental tools with unknown effects. We have also developed a wide range of computational tools that provide realistic physical models relevant to our research objectives and will inform and bolster our animal experiments. Our research strategy to use or develop non-animal alternatives for our work is reflected in our recently awarded a research grant focused on incorporationof additional research approaches, including human samples and in silico analyses.

We will use both males and females, and have implemented measures to reduce the attrition rate in our experiments. As we hold several research grants involving animal experimentation, we have beenactively sharing tissue and animals within and between labs, and maximise data sampling from individual animals, often running numerous, separate lines of inquiry from the same animal without theneed to increase the number of animals, or procedures per animal. For example, we can optically monitor cerebrovascular responses during adaptation, without the need for additional recordings, or post-mortem tissue can be used for ultrastructure 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use rats and mice, the animal species most suitable in the present context, for various reasons.As mammals, findings of fundamental importance made in these species have been relevant to humanbiology and health. Many essential physiological concepts in brain communication have been established through experiments with these species. Additionally, the molecular biology tools (includingtargeted genetic modifications) and experimental techniques that are relevant to this proposal have been developed and validated in mice and rats, removing the need for additional experiments in settingup our methodology.

Why can’t you use animals that are less sentient?

Key physiological concepts pertinent to the brain machinery have been established through experiments in rats and mice. In the context of our scientific aims, less sentient species would carry therisk of being irrelevant to the cellular mechanisms of memory formation and loss in humans.

In a large number of the experimental procedures, animals will be terminally anaesthetised, wherethe procedure is performed under anaesthesia but the animal is not recovered.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We have sought to refine the procedures outlined here for many years before this application, and willcontinue to do so. We have been doing this in collaboration with technical staff, veterinarians and welfare officers at the establishment. The refinements include updates to animal husbandry, nutrition, postoperative care and redesign of experimental apparatus, implants and recording devices. We haveimplemented habituation procedures, non-aversive training and non-aversive handling methods. We have added new forms of enrichment in the animal's cage, and ensured that the bedding and nesting material is appropriate for any postoperative animals.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow the NC3Rs internet resources which provide up-to-date advice and practical guidance onthe experiment refinement, such as 'Refining rodent stereotaxic surgeries' (University of Edinburgh), links to Norecopa PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) Guidelines, 'Humane Endpoints in laboratory Animal Experimentation' (3Rs-Centre Utrecht), and other practical information. We will carry out systematic reviews of our procedures and always ensure that refinement methods are discussed with the NamedAnimal Care and Welfare Officer (NACWO) and Named Veterinary Surgeon (NVS) at the university.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We have been acutely aware of the advances in the 3Rs through the efforts of NC3Rs organisation,the local animal welfare board, through our peers and through continued professional development offered in our establishment.

The NC3Rs website and its 3Rs resource library provide a wide scope of up-to-date information andpractical guidance pertaining to the evolving 3Rs issues. I and team members are subscribed for theNC3Rs e-newsletter, to stay informed about advances in the 3Rs.

ONCOLOGY PHARMACOLOGY STUDIES

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Cancer, Therapy, Tumour models, Pharmacodynamics, 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 notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

This project will support oncology drug discovery and development. It will be used to determine whether potential new anti-cancer drugs can stop tumours from growing and to understand how the drug is working. It will also be used to support the use of oncology drugs in the clinic e.g by providingdata to influence dose, schedule and disease setting.

This project will also be used to develop new cancer models for use in future research into new cancertreatments.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is a complex disease and despite an increase in the number of new drugs available to treat patients, not every patient will respond to existing treatments and be

cured. Resistance mechanisms toexisting therapeutics are also emerging.

It is therefore important to continue to support the discovery and development of new anti-cancerdrugs.

What outputs do you think you will see at the end of this project?

Outputs from this project will be:

- New information on oncology drug targetsNew oncology drugs entering clinical trials New registered oncology drugs
- Publications in peer-reviewed journals in the field of oncology researchPresentations at national and international oncology conferences

Who or what will benefit from these outputs, and how?

The work carried out under this licence will provide benefits:-In the short term by supporting:

Basic research in the field of cancer (1-5yr timescale)Cancer drug discovery (1-5yr timescale)
In the medium term by sharing cancer research data which will increase the understanding ofcancer biology (1-5yr timescale)

In the longer term by supporting the clinical development of new drugs for the treatment of cancer, which may not be realised during the lifetime of this project (>5yr timescale)

It is expected that output from this project will benefit researchers in the field of oncology.

As a number of drugs research programmes supported by this service licence will be successful indeveloping new cancer drugs, patients will also benefit from this project.

How will you look to maximise the outputs of this work?

As an organisation working under confidentiality agreements, we provide a service to clients, and as such, we will not own the data generated nor have the right to publish the data. Clients will be providedwith the data, which they will use to inform their drug discovery and development process. At an appropriate time in the drug discovery process, it is in the best interests of the client to publish their data, whether this is in relation to their novel therapy, or a fundamental break-through in their basic research. At this point, we will support clients and their publication strategy.

In addition, we will take every opportunity to publish where there is no breach of client confidentiality oruse of proprietary data e.g in relation to 3Rs or where new models have been developed.

Data will be placed in the public domain by:

Publication in international peer-reviewed journals in the fields of oncologyPresentations at national and international conferences.
Publication of data will allow others in the industry and academia to benefit from the research. It will also allow clinician and patients to learn about potential drugs of the future,

enhancements to oncologytherapies and re-purposing of existing drugs that have been found to have utility in disease models aside from the ones they were registered for.

The information from this research may be directly submitted to ethics committees to justify efficacy claims and to regulatory authorities (e.g. FDA, EMEA). These studies are not required to meet specificregulatory requirements and are not covered by specific regulatory guidelines, however details of the research will be published as a consequence of any regulatory submission.

Species and numbers of animals expected to be used

- Mice: 20,500
- Rats: 1900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The overall plan of the work is to support the discovery and development of novel cancer drugs tobenefit human health in the treatment of cancer.

Using non-mammalian species is not possible because they lack relevant tissue physiology andtherefore cannot replicate human physiology

Only rats and mice, including strains which lack an immune system, are used on this project.

Mice will be used in the majority of studies unless there is a scientifically relevant reason that micecannot be used.

The most appropriate species and strain of mice and/or rats will be chosen based on previous dataand choice of tumour model.

To study human tumour growth in rats or mice, strains lacking an immune system are required. Theleast immune-deficient strain required to promote good, reproducible tumour growth will be used.

Typically, what will be done to an animal used in your project?

Animals will be kept in a purpose built facility.Animals will be housed in groups.
Tumour growth in animals will be induced by chemical carcinogens or more typically by injecting tumour cells or pieces either under the skin or in breast tissue. Injections require the use of needlesand in some cases minor surgery.

The majority of animals will be given the experimental drug every day for the duration of the experiment, using experimental drug delivery methods or methods as used in patients. In the majorityof cases this will involve the use of needles only. Drug delivery can be for example once weekly, twiceweekly, once daily or twice daily.

A small number of animals will have minor surgery to implant a device under the skin which can slowlyrelease the test substance, this may happen twice during the experiment.

Blood samples will be collected during the experiment. Up to six blood samples may be taken over a24hr period, or up to 12 over a 28 day period.

An animal's clinical condition will be monitored on a daily basis for the duration of the experiment.

The weight of an animal will be assessed daily, twice weekly or three times a week depending on theexperimental design.

At the end of the study it is necessary for the animals to be humanely killed and tissues taken foranalysis after death.

What are the expected impacts and/or adverse effects for the animals during your project?

The initiation of tumour growth may require the use of needles and in some cases minor surgery. Theuse of needles will cause brief discomfort or pain. Where minor surgery is required, animals are expected to recover quickly and will be given painkillers during and after the surgery and post- operative care just like people recovering in hospital.

Delivery of experimental drugs will in the majority of cases involve the use of needles only, which will cause brief pain or discomfort. In a small number of cases, brief local irritation of the skin where the drug has been injected may occur. A small number of animals will have minor surgery to implant a device under the skin which can slowly release the experimental drug. Animals are expected to recoverquickly and will receive painkillers during and after the surgery and post-operative care, just like peoplerecovering in hospital.

Animals will be regularly monitored for weight loss and general condition.

Animals may become unwell as a result of the test drug. Signs that the animals are starting to becomeunwell can include weight loss, deteriorating coat condition, reduced movement, reduced social interaction.

Animals will be humanely killed if signs of being unwell persist.

Blood samples will be collected during the experiment, which will cause brief discomfort or pain.

At the end of the study it is necessary for the animals to be humanely killed and tissues taken foranalysis after death.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The expected severities on this project are mild or moderate.

>90% of animals will undergo experimental procedures which are classified as moderate

severity suchas implantation of tumour cells, minor surgery, use of repeated anaesthesia.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered 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 a complex process. It involves lots of different cell types including the immunesystem, which cannot be recreated in non-animal alternatives or non-protected species.

Therefore, protected animals are needed for the studies proposed in this project.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives including cell line assays, biochemical, binding and enzyme assay have beenconsidered

Why were they not suitable?

Non-animal alternatives are used in the identification and selection of test substance/s and generally include measurements of the likely effect of the agent on the target cells, by assessing the potency of test substance in relevant cell line assays. In addition, target engagement can also be demonstrated inassays such as biochemical, binding and enzyme assays. Activity in particular in vitro assays and cell types however, cannot predict the likely in vivo activity given the complexity of issues such as bioavailability, metabolism and elaborate physiological interactions associated with tumourgenesis andtherefore the whole animal is needed for the studies proposed in this project.

Non-animal alternatives maybe used in the identification and selection of test substance/s for pharmacokinetic assays. These may be computational/in silico assays e.g homology modelling, structure activity relationship models and predictive metabolism models or in vitro assays, which measure parameters such as hepatocyte or microsomal stability and solubility. Wherever possible, computational compartmental kinetic modelling will be used to predict the consequences of alternativedoses and multiple dosing protocols. However, due to the complexity of the processes involved, there is currently no viable in silico or in vitro alternative to modelling the total in vivo PK profile of a given test substance.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals estimated to be used on this project is based on the ongoing animal usage onan existing project licence with a projected increase to account for the increasing demand from clientsfor the service we provide.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

To maximise the scientific integrity of data generated and to use the minimum number of animals, statistical expertise will be applied to all experimental design and analyses. Statistical advice will be sought from experts in the field, e.g internal or external statistical experts, as well as using tools suchas the NC3Rs Experimental Design Assistant (https://www.nc3rs.org.uk/our-portfolio/experimental- design-assistant-eda) and reference to the ARRIVE guidelines (https://arriveguidelines.org/arrive- guidelines).

Where plausible the following statistical guidelines will be used to minimise the number of animalsrequired for each procedure:

Meaningful biological change and measurable endpoints will be defined.

Estimates of biological variability will be used in sample size and power calculations.

Where possible, animals will be randomised into groups based on estimates of biological variability established from accrued historical databases, pilot studies or published data.

Regular monitoring and updating of biological databases with regular review of group sizes. Typically, groups sizes vary from 6-10 animals per group, depending on model variability andexpected magnitude of test substance effect.

One-sided (rather than two-sided) statistical tests will be used wherever appropriate (e.g. whenidentifying inhibition rather than change).

Statement of intended statistical analyses and justification for use, if any, of transformed data(e.g. tumour growth data may be analysed on the logarithmic scale if the variance of tumour measurements increases with the mean).

Statistical power will be set to a minimum of 80% (e.g. at least an 80% chance of declaring thedefined ‘meaningful biological change’ as being statistically significant).

Multiple treated groups will be compared against one control to reduce the number of studiesperformed. Group sizes may be weighted to reflect this.

The design and analysis of studies to compare combinations will depend on the number of testsubstance/s being used and by defining appropriate control groups.

Principles of Factorial Experimental Design (FED) may be applied.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We will only purchase animals from establishments with the authority to breed, maintain

and supply experimental animals. We will purchase the number of animals required to carry out the work required,thus keeping the number used under the project to the minimum required

Where appropriate:

We will use pilot studies to understand the robustness and variability of models and methodsused.

We will work with in house statisticians to analyse data outputs from pilot studies

We will maximise the output from every study by collecting relevant tissue and by sharing tissue withother departments within the company e.g blood samples may be taken from tolerability studies to enable assessment of the PK/biodistribution of test substances rather than running a separate PK/biodistribution 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 welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

All work will be conducted with reference to best practice guidelines such as:

Diehl et al (2001). A good practice guide to the administration of substances and removal ofblood including routs and volumes. J Appl Toxicol. 21:15-23

Folts and Ullman-Cullere (1999). Body condition scoring: A rapid and accurate method for assessing health status in mice. Lab Animal. 28 (4):28-32

Workman et al. (2010). Guidelines for the welfare and use of animals in cancer research. BritishJournal of Cancer 102(11), 1555-1577

PREPARE guidelines (https://norecopa.no/PREPARE)

Only rats and mice including immune-deficient strains are used on this project.

The most appropriate species and strain of mice and/or rats will be chosen based on previous data that has been used to generate single agent efficacy data. The choice of strain will be driven by the choice of tumour model. For human tumour lines immune-deficient animals are required to support the growth of the tumour, the least immune-deficient strain required to promote good, reproducible tumourgrowth will be used.

Typically, animals will be implanted with 1 tumour per animal. Animals will be humanely killed before the tumour burden of the 1 tumour exceeds 10% of body weight for mice or 5% of body weight for rats

On occasions, a maximum of 2 tumour implants per animal may be required and animals will be terminated before the combined tumour burden of both tumours exceeds 10% of body weight for miceor 5% of body weight for rats. Two tumours per animal will only be used when justified to meet the scientific endpoint of the study

Tumour measurements may be assessed using non-invasive methods such as calipers for tumoursimplanted subcutaneously or in the mammary fat pad and the tumour volume calculated using the following formula for tumour volume based on measurements of length(l) and width(w): volume= (pi/6)*l*w2, in accordance with published methods (Lab Animals: 46, 207-201 2017)

Using this formula, a tumour which measures 15mm by 10mm will have an estimated volume of 1cm3.

Based on initial pilot growth curve studies, with tumours implanted subcutaneously or into the mammary fat pad, we can then correlate estimated tumour volume with actual tumour weight which allows an accurate assessment of tumour burden for each individual animal. In our experience we have found that in the majority of cases 1cm3 tumour volumes weighs approximately 0.5g, however asan extra precaution we implement a stricter criteria and assume 1cm3 weighs 1g to ensure that study endpoints are implemented below 10% tumour burden limit.

The use of bioluminescent/fluorescent imaging to assess tumour volume/burden will be limited to circumstances where it is required to accurately assess tumour volume/burden, or where the imagingdata produced will add value to the analysis and interpretation of study data.

Where bioluminescent/fluorescent imaging is required to accurately assess tumour volume/burden, acorrelation between bioluminescent/fluorescent signal and tumour burden will be established for eachmodel in pilot growth curves.

Animals will only be dosed with an activating agent/imaging agent/imaging probe or contrast agentwhen this is required to visualise the imaging signal

Tumour burden and tumour condition is monitored closely daily. We will use the least invasive tumoursite/line that will achieve the scientific aims and will apply the earliest endpoints to meet the scientific requirement of the study.

Partial and transient skin erosion may occur. In certain circumstances, skin erosion may be associatedwith treatment with the test substance. In these cases, if the erosion is improving, animals will continueon study. However if tumours are ulcerating and weeping, or if there is evidence of infection the animals will be culled.

In some tumour types e.g melanoma, a small dry scab may develop which does not cause any adverse effects to the animal, in these cases the animals will be closely monitored and maintained forthe duration of the study, if there are no additional adverse effects seen that are attributed to tumour condition. If we were not able to include these in the analyses, then we would have to significantly increase the number of animals in both control and treatment groups on studies to allow for the attritionof tumours due to this skin erosion. Any other signs of adverse effects will result in the culling of the animal.

None of the tumours used under this licence will be cachectic.

Studies will be continually monitored and once the scientific endpoint has been reached studies will beended. Humane end-points will be used to control severity to the least severity consistent with the scientific aim.

To minimise the use of satellite animals and to produce a more refined data set, where each animalacts as its own control, blood samples will be taken via direct venepuncture by micro-sampling methods unless prohibited by the required sample volume.

Multiple tissue samples for ex vivo PD analysis will be taken from the same animals where possible

Normal tissue pharmacodynamics biomarkers will be explored in vivo based in vitro or in vivo data or literature and may be established in either immune-competent or immune deficient strains. The need toestablish a relationship between normal tissue pharmacodynamic biomarkers and tumour efficacy and reference to literature data will be used to influence the strain of choice.

Mice will be used in the majority of studies unless there is a scientifically relevant reason that micecannot be used, for example, metabolism issue with the test compound.

A number of factors will be used to determine the test substance vehicle, such as route of administration, test substance solubility and animal species. Where a choice of vehicle is possible, themost innocuous vehicle will be chosen.

Where novel vehicles are required to achieve a suitable formulation for administration in vivo, it may benecessary to assess the tolerability of the vehicle alone ahead of dosing with test substance.

Where novel test substances are encapsulated in novel vehicles e.g nanoparticles, it may be necessary to assess the biodistribution of the novel vehicle alone ahead of dosing with test substance.

Test substances will be administered by clinically relevant routes, or by routes required by novel drug delivery systems. A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. J. Appl. Toxicol. 21, 15–23 (2001) will be referred to when determiningdose volumes and frequency

Radiotherapy will be given as a single dose or fractionated dose over a period of days, at doses thatare required to achieve the scientific endpoint.

For anaesthetic protocols the optimal anaesthetic regime relevant for the species/strain will bedeveloped and used in conjunction with the NVS.

Analgesia will be given peri operatively and maintained after surgery for as long as is necessary to alleviate pain. Animals will be closely monitored for signs of pain and discomfort e.g hunched posture, including use of Mouse or Rat Grimace Scale. Appropriate levels of analgesia will be given, as advisedby the NVS. All recovery and long-term non-recovery surgery will be done aseptically as outlined in the LASA guidelines Guiding Principles for Preparing for and Undertaking Aseptic Surgery 2nd Edition April 2017

(www.lasa.co.uk/pdf/lasa_guiding_principles_aseptic_surgery_2010.2.pdf) and by the

Home Office Minimum Standards for Aseptic Surgery
(www.procedureswithcare.org.uk/ASMS2012.pdf)

Animal suffering will be minimised and will be achieved byAcclimatisation of animals prior to entry onto study.

Use of group housing unless single housing is required for study or welfare reasons.All animals will have free access to food and water

Enrichment such as houses, nesting materials, chew sticks will be provided in every cage. Application of best practice and use of most refined methods in all experimental protocols.

Why can't you use animals that are less sentient?

Using non-mammalian species of lower neurophysiological sensitivity is not possible as they lackappropriate tissue physiology.

Although exact replication of all pharmacokinetic parameters between species is not possible, manyfeatures of human PK can be predicted from those observed in small mammalian species unlike effects seen in lower organisms.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We will continually monitor and review the models and methods we use to ensure we continue tominimise the welfare costs.

We will increase welfare monitoring in situations where we believe earlier intervention would minimisethe welfare costs.

Through active engagement with the NACWO, NVS and other named individuals we will implementimprovements to procedures for example to post operative care and pain management.

Through sharing of best practice, we will apply potential refinements to this project

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

All work will be conducted with reference to:

Diehl et al (2001). A good practice guide to the administration of substances and removal ofblood including routs and volumes. J Appl Toxicol. 21:15-23 Folts and Ullman-Cullere (1999). Body condition scoring: A rapid and accurate method forassessing health status in mice. Lab Animal. 28 (4):28-32

Workman et al. (2010). Guidelines for the welfare and use of animals in cancer research. BritishJournal of Cancer 102(11), 1555-1577

Prepare guidelines (www.norecopa.no/prepare)

NC3Rs guidelines for example www.nc3rs.org.uk/3rs-resources/introduction-systematic-reviewsand www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda

LASA guidelines for example
www.lasa.co.uk/PDF/AWERB_Guiding_Principles_2015_final.pdfand
www.lasa.co.uk/pdf/lasa_guiding_principles_aseptic_surgery_2010.2.pdf

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Ongoing systematic reviews by use of tools such as the NC3Rs systematic review tool (https://www.nc3rs.org.uk/3rs-resources/introduction-systematic-reviews), review of literature/websitesand attendance at conferences/training events will be undertaken to:

Keep up to date with advances in the 3Rs Keep up to date with all relevant regulations.

Understand any advances in in vitro/in silico/ex-vivo techniques which may provide a suitableand reliable replacement for work carried out under this licence.

How to best apply any advances to the models and methods on this project.

In addition, through membership of the Institute of Animal Technology and the establishment AWERB,best practice and advances in the 3Rs will be shared and implemented where possible to this project.