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NON-TECHNICAL SUMMARY

Regulation of the mammalian reproductive axis.

Project duration

5 years 0 months

Project purpose

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

Key words

Infertility, Reproduction

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of the project is to study how mammalian reproduction and fertility are regulated by the interplay between different genes.

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

Why is it important to undertake this work?

Globally, infertility affects around 5% of all couples so the number of individuals with fertility problems is enormous. In many cases, the underlying cause of the infertility is not known reflecting out incomplete understanding of the factors that can cause these problems. A number of infertility problems will be caused by genetic mutations and it is important to study these to try to identify ways in which the infertility can be treated.

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

The main output from this research will be a contribution to scientific knowledge including data sets and publications in peer reviewed journals and presentations at scientific meetings. Specifically, we will generate novel data and knowledge about the role of specific genes in mammalian fertility. For example, we will define the genes that control the activity of *Kiss1* neurons and how these might change during puberty. We will also define the way in which neurons involved in fertility are regulated by hormones (eg sex steroids) or peptides in the brain (neuropeptides). We will also define the function of the *Hiat1* gene in controling male fertility.

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

The immediate short term benefit of this research is that it will provide training to stakeholders that are involved with the project and contribute to our knowledge of the mammalian reproductive axis and how it is controlled. The project will contribute to the research training of undergraduates, PhD students and post-docs. In the longer term, the project will also provide a greater understanding of clinically relevant disorders such as precocious or delayed puberty and may allow the development of treatment regimes. The knowledge from this project might also be of benefit for the development of approaches to treat disorders in women (eg cyst formation in the ovary or post-menopausal hot flushes) or might allow the development of novel male contraceptives by phamaceutical companies.

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

The outputs will be disseminated through peer reviewed publications, reporting findings at scientific meetings, on the departmental web site and via social media. Where possible, we will also try to report

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negative findings in peer reviewed publications and on my personal web page which is accessible to all.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

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

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

Genetically modified mice are a powerful resource to understand the function of a gene in a whole animal system as the technology exists to allow the distruption of a specific gene and examine the consequences of this. This project is examining puberty and fertility so it is necessary to use mice at these stages (prepubescent and adult).

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

The majority of the mice (5000, 90% of total number) are part of the breeding colony to generate a smaller number of mice (750, 15%) for experiments. The genetically modified mice in the breeding colony will not usually have any procedures performed on them, unless a second earclip is required for genotyping, and will not have any detrimental effects. From the breeding colony it is estimated that 350 mice (7% of total number) will only have injections of substances, and a small number (400, 12.5% of total number) will have surgery with recovery. A vaginal lavage will be taken from some mice to determine the stage of the estrous cycle or to determine if cycling has stopped after ovariectomy. This method is non-invasive as it involves depositing a small volume of fluid onto the entrance of the vagina and then collecting the liquid. The liquid is placed on a glass slide and the cell types present examinied to determine the stage of the reproductive cycle of the mouse. Samples may be collected daily for several weeks to monitor the regularity of cycles.

Some mice will have small microchips implanted subcutaneously to allow future identification or for monitoring temperature. These chips are around the size of a grain of rice and widely used in veterinary practice and animal husbandry. They will be injected under general anaesthesia with subsequent recovery.

Most protocols will only take a few weeks to complete although mice undergoing pharmacological treatments will be kept for longer to allow recovery after any surgical procedure. Within the pharmacological treatment protocol, 350 mice will have minor procedures (injection and/or blood sampling). 400 (12.5% of total number) mice will have one surgical procedure (ovary transplantation or gonadectomy or substance delivery into the brain - icv). Up to 50 mice (1% of total number) will have two surgical procedures (gonadectomy and icv delivery). The mice that have undergone surgery may be kept for 12 months to allow long term monitoring of reproductive parameters.

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

Breeding of the GA mice is not expected to be associated with any detrimental effects or losses apart from those normally associated with maintaining a breeding colony.

The method of vaginal lavage is similar to that described by A C. McLean et al (2012) in J Vis Exp. Advice from the Home Office Inspector (23rd March 2023) is that this method is considered to be less invasive than vaginal swabbing and unlikely to exceed the threshold requiring regulation. Consideration will be made however, if repeated lavage over several days might cause stress that would cross the threshold for a regulated procedure in which case it will be regarded as such. In general, however it is considered that the severity of vaginal lavage will not be a regulated procedure in most circumstances.

Subcutaneous implantation of microchips will be carried out under sterile conditions and are not expected to have any detrimental effects on the mice. Side effects might include tissue tenderness and bruising and these will be monitored after the injection and appropriate analgesia provided if necessary.

Surgical procedures will be carried out using aseptic techniques. In the uncommon event of postoperative complications, animals will be killed unless, in the opinion of the NVS, such complications can be remedied promptly and successfully using no more than minor interventions. In case of wound dehiscence, an uninfected wound may be re-closed on one occasion within 48 hours of the initial surgery and the NVS informed.

Peri- and post-operative analgesia will be provided in accordance with a regime agreed in advance with the NVS

Animals are expected to make a rapid and unremarkable recovery from anaesthesia. In the uncommon event that animals fail to do so, or exhibit signs of pain or distress or of significant ill health, they will be killed by a Schedule 1 method.

Unless otherwise specified, the administration of substances and withdrawal of bodily fluids will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm. We have not observed any adverse effects from administration of hormones, which are normally given in the normal physiological range. Any animals showing any deviation from normal health or well-being will be killed.

A minority of the animals may experience weight loss within 15% (measured against their weight at the beginning of the protocol or compared to age matched controls, as appropriate), mild piloerection, intermittent and moderate hunching, and reduction in their activity within the cage. From the time point of the surgery when these symptoms may be expected to occur, animals will be closely observed every 4 hours during the day to ensure that the symptoms would not last for more than 12hours. If these signs persist for over 12 hours or begin to deteriorate the animals will be killed by Schedule 1 methods. This is not expected to occur in more than 1% of the animals.

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

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

Mild 5100 mice (92% of total number)

Moderate 450 mice (8% of total number)

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

- Killed

Replacement

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

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

There are no suitable alternative systems that can recapitulate the complex interplay of the different tissues (brain/pituitary/gonads) of the reproductive axis or the hormonal feedback mechanisms involved in its regulation.

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

The only non-animal alternatives that could be considered are using tissue explants or established cell lines. Established cell lines that could be used to model the physiology of *Kiss1* neurons include the mHypoA-50 and mHypoA-55 lines and the KTaV-3 and KTaR-1 lines. While these lines show some responses found in *Kiss1* neurons in the mouse such as a response to estrogen, which makes them suitable for some experiments, they cannot model the neuronal circuitry found in the intact brain.

Why were they not suitable?

Tissue explants are not suitable for this project because they cannot be maintained in culture for long periods, they will change as soon as they are removed from the animal, they do not recapitulate the reproductive axis and are not subject to the normal regulatory signals (eg fluctuating hormone levels) that occur in a whole animal.

Reduction

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

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

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

The number of transgenic mice that will be used over the 5 year duration of this project is based on the number of mice that have been used each year on my previous PPL. Over the course of this licence, we will generate around 5,000 mice (using approximately 170 mice each year for experimentation). Of these, the majority (around 4,150) are used for breeding and these will have no detrimental effects caused by the genetic alteration. As we are studying mutations that affect fertility, the mutant mice are sterile so we have to breed from heterozygous pairs which means that many mice will be wild-type. A further 100 wild-type mice will be used each year for the generation of genetically modified mice and these will not have any surgical procedures performed on them as I reoutinely perform non-surgical embryo transfer. Surgical procedures (mainly removal of testes or ovaries) will be performed on around 400 mice with delivery of substances into the brain performed on around 150 of these.

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

When appropriate, the NC3R's Experimental Design Assistant (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda) and the NC3R's Breeding and Colony Management Resource (https://nc3rs.org.uk/our-portfolio/breeding-and-colony-management) will be used to plan an experiment and to define the minimum number of mice in each treatment group to achieve statistical power. We also have standard operating procedures (SOPs) in the laboratory to help ensure standardisation between different staff.

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

To minimise the number of surplus mice that are bred and not used in experiments, we routinely keep breeding pairs together for only a couple of months and then stop breeding until we require new offspring. We actively monitor and manage the number of breeding pairs so that we only have the number that is required to generate a cohort of offspring for a specific experimental plan. Pilot studies might be undertaken to get preliminary data on the variability of experimental measurements to allow us to perform Power calculation to determine the minimum number of mice required for statistical integrity. We use surplus wild-type mice from our breeding stocks for control groups rather than buying in additional mice. We also provide surplus mice for tissues to 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 welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

Transgenic mice with disruptions of specific genes involved in fertility (*Kiss1, Kiss1R, Hiat1*) will be used in this project. Mice are an excellent model to study mammalian reproduction. The main physiological processes are conserved between mice and humans and the hormonal regulation of fertility in particular is almost identical between these species. In addition, the ability to generate transgenic mice, in which a single gene has been altered or removed, provides a very powerful tool for studying the role of a single gene in reproduction. They represent much better and more specific models to study brain function than models involving crude destruction of brain areas. The genetic approaches to generate these models are very well established in the mouse and are efficient enought to allow transgenic mouse generation while keeping the mouse numbers low. In addition, the short gestation period of the mouse allows us to study aspects of reproductive function relatively quickly.

The transgenic modifications carried by the mice do not cause any detrimental effects or pain - the vast majority of the mice will only be killed and used for tissues. Vaginal lavage will be used to determine the estrous cycle stage of the mouse. This is a considerably more accurate method than by visual inspection of the vagina and provides a quantitative and permanent record of the estrous stage. Visual inspection is usually only 50% accurate, even with experienced users and is particularly difficult in agouti mice.

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

It is not possible to use a less sentient model organism (eg nematode worms, *Caenorhabditis elegans* or fruit flies, *Drosophila melanogaster*) to study the mammalian reproductive axis. They do not have the appropriate tissues that are found in the mouse or the same hormone systems.

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

We have undertaken a number of refinements in our animal welfare procedures and will strive to continue to do this. For example:

We have developed an ultra-sensitive method for measuring a hormone in the blood, which works with very small volumes of blood (5 ul) so that we can reduce the amount of blood taken from the tail vein.

We will retain existing ear clip tissue taken for identification purposes and use this for identifying the genetic make up of the mice [and thus eliminate the need to re-earclip for genotyping alone.

Transfer of embryos into recipient female mice to generate genetically modified mice will normally be performed using an NSET (non-surgical embryo transfer) device, which is a less invasive method than surgical transfer of embryos.

Where possible, we will use genetically sterile mutant male mice instead of vasectomised males.

When performing vaginal lavage, the mice will be handled regularly to habituate them to the procedure to minimise stress. The procedure normally involves scruffing the mice but this is only for a few

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seconds. We will also determine the feasibility of taking a vaginal lavage while restraining the mouse in a tube which should be less stressful than scruffing.

We also want to test whether subcutaneous implants can be used to accurately monitor body temperature in the mice. This might provide a number of refinements. It might be possible to use changes in body temperature to define the stage of the mouse estrous cycle so that the mice do not need to be handled or have vaginal lavage performed. An additional benefit is that the implant might allow us to monitor the temperature of mice that have had surgical procedures to ensure that there are no welfare issues following the surgery.

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

We will use the NORECOPA online resource (https://norecopa.no/prepare) for guidance on quality assurance and animal management and as *an aide* memoire for the topics that should be considered when planning experiments.

We will refer to the various publications on the LASA web site (https://www.lasa.co.uk/current_publications) for information and specific guidance about many aspects of animal research.

We follow the ARRIVE guidelines for publishing our work.

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

My establishment has a knowledge distribution system (mainly via e-mail and a Biomedical Support web site) to keep all PPL holders informed about advances in the 3Rs. In addition, I frequently check the NC3R's website for advances and subscribe to their Newsletter. I will also have regular discussions with the Named Persons and animal technicians to review current approaches and whether there are any new 3R opportunities. I have also subscribed to the Norwegian NORECOPA Facebook page as this organization provides useful information and resources about the 3Rs. I will also check the LASA web site (https://www.lasa.co.uk/) for information and I have set up an e-toc alert for the ATLA (Alternatives to Laboratory Animals) publication.

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