Home Office

NON-TECHNICAL SUMMARY

Embryogenesis, stem cells and cell fate decisions

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

Stem cells, Sex determination, Cell fate decision, Embryogenesis, Endocrinology

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

Retrospective assessment

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

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

To understand how cells choose and maintain specific fates during development of the mammalian embryo and in the adult animal, how this leads to disease when the processes go wrong.

A retrospective assessment of these aims will be due by 22 January 2026

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve it's 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?

During the development of an animal, cells have to undergo decisions of cell fate, choosing which path to follow. Similar decisions are made throughout life by stem cells and progenitors in many tissues. These decisions rely on intrinsic factors, such as transcription factors, and extrinsic signals, which together establish gene regulatory networks that define specific cell states. These have to be coordinated in time and space to generate functional tissues, organs and the animal, where little in the latter is static: cells are constantly having to be replaced due to normal wear and tear and to cope with changing physiological states, trauma, and disease. The main purpose of the work to be conducted under this Project Licence is to provide fundamental knowledge on cell fate decisions in specific biological systems, notably the gonads, CNS, pituitary, gut, and sensory systems, which is relevant to how these develop, mature, and age normally. However, there are many situations where decisions of cell fate are aberrant or discordant. This can lead to infertility or embryo failure, to congenital disorders, disorders affecting maturation, health span or aging, or to cancer. There can also be aberrant responses to environmental factors. Improved understanding of underlying mechanisms can lead to improved diagnosis and/or novel forms of treatment.

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

1. Improved understanding of gene regulatory networks in the early gonad will not only inform clinical cases of Disorders of Sex Differentiation (DSDs), but be of broad significance in studies of organ formation.

2. Improved understanding of gene regulation during gonadal development will also inform clinical cases of Disorders of Sex Differentiation (DSDs), as it is become apparent that many are due to regulatory mutations. We also anticipate that this work will provide general insights into both temporal and spatial control of gene activity, especially for genes such as *Sox9* which are active and have critical roles in many tissues.

3. Improved understanding of gonadal sex maintenance and reversal will lead to new insights into cell-fate reprogramming and organogenesis, both of which could be of potential importance for regenerative medicine. More directly, this could be of clinical benefit for some cases of DSDs, giving new options for treating patients, such as in cases where ovotestes are present it may be possible to turn the whole gonad into a testis or ovary, or to convert the entire gonad, either to match chromosomal sex or, perhaps, gender identity.

4. Improved understanding of female reproductive function, fertility and premature ovarian failure, could help inform new strategies to manage or maintain fertility in women.

5. Developing methods to obtain gonadogenesis and gametogenesis in vitro, could provide information relevant to DSDs and to causes and potential treatments for infertility. It is very difficult to study the etiology of DSDs given that the phenotypes develop in the embryo in utero, and they are generally recognised at birth or, often, at puberty. The only alternative at present is to make and study an animal model, which, while useful, may not always accurately reflect the human situation. (N.B. Our work deriving Sertoli-like cells in vitro from pluripotent stem cells has already contributed to one study (yet to be published), where patient derived cells were unable to give rise to Sertoli cells, unlike controls, showing that the defect was in primary sex determination). Current efforts to derive sperm or eggs in vitro from pluripotent stem cells reveal that co-culture with somatic cell types from the gonad is essential for primordial germ cell-like cells to progress into later stages of spermatogenesis or oogenesis. Our culture systems, if validated in animal studies, will help these endeavours, which are important both to allow detailed study of human germ cell development and, in the long term, to provide ways to treat infertility or even as a route to correcting deleterious gene variants in subsequent generations.

6. The use of animal models to provide improved understanding of the mechanisms underlying sex bias in human diseases will be of clinical benefit in terms of improved diagnosis and perhaps options for treatments. Our current work on Hirschsprung's Disease provides an example, where two mechanisms that could contribute to the distinct sex bias have been identified. Work on sex differences in the biology of neural stem cells is of potential relevance to understanding and perhaps eventually treatment of a range of CNS disorders that affect one sex more than the other, such as depression.

7. In addition to providing novel fundamental insights, our studies on intrinsic versus extrinsic control of CNS development and disease could be of benefit in providing new options for avoiding or treating diseases that have previously thought to have their origins within the CNS.

8. Our work on the role of SoxB1 and SoxE genes in development, from the early embryo to neural stem cells in the adult, has already led both to new fundamental knowledge and to clinically relevant findings. These range from Sox2 being recognised as a gene essential for pluripotency and hence to the development of iPS cells, to studies on the decline of neural stem cell populations in ageing. We expect our further studies on these genes to continue to give new knowledge.

9. Improved understanding of neural stem cell niches will contribute to basic knowledge, but also potentially to the development of new clinical options for treatments of CNS defects and trauma. Neural stem cells have already been used in attempts to treat a range of diseases as well as conditions such as stroke, but with limited effectiveness. Providing other niche components, whether factors produced by cells or the cells themselves, may be beneficial.

10. Gliomas and glioblastomas have proved to be very hard to treat. Our work on this topic is designed to reveal more about the origins of these aggressive tumours, and the knowledge gained will potentially provide new ideas for treatment options.

11. Our work on the pituitary and hypothalamus, and stem and progenitor cells in both, has already led to new insights. Ultimately, we hope this work will lead to better, more physiological options to treat a range of clinical disorders that involve deficiencies in pituitary hormones and/or in hypothalamic function. These can be congenital or occur after trauma, disease (including cancer), or may be due to current treatment regimes.

12. Pituitary tumours are relatively common, but not well understood. Our work may provide new treatment options, especially as surgery on the organ, which is centrally located below the brain and highly vascularised, is often very challenging. For example, via the use of drugs to disrupt specific cell-cell signalling molecules that our current work suggests are required for tumour growth or that might reduce *Sox2* expression, which is also associated with tumour growth.

13. Our work on the development and function of endocrine organs and sensory systems is mostly to provide fundamental knowledge of the role of specific genes. However, this knowledge will be relevant to diagnosis and management of patients and may eventually provide new treatment options.

We will publish all of our findings in open access journals, with data in a reusable form. Moreover, any genetically altered mice produced as part of this PPL, which should be of benefit to other researchers, will be made freely available, as we have done so in the past.

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

There are likely to be multiple beneficiaries from the outputs above. These will first include other scientists and clinicians involved in similar studies or who are interested in the systems we are exploring. Benefits to patients may come within a few years, perhaps some within the time frame of this PPL, from improved diagnosis and from better management of disorders or trauma. In the longer term, perhaps in 5 to 15 years, we would expect our work to lead to new treatment options.

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

Alongside the publication of primary research papers in open access journals, we will present our work at meetings, ranging from small focussed workshops to large international conferences. Critically, we will communicate negative results and approaches as well as those that are positive.

We collaborate widely, both internally within the host establishment, and externally with scientists and clinicians based in the UK and in several other countries (including currently France, Spain, Israel, the

USA and Canada, and Australia). We share details of approaches and data generated with our collaborators, which allows for improved development of methods and better synthesis of findings.

Where we develop new transformative methods, we will publish the protocols as papers and post them on bioRxiv.

Species and numbers of animals expected to be used

- Mice: 95,700

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 for all the projects covered in this PPL. Mice are chosen because of the powerful techniques and knowledge available for this species in terms of genetics, cell biology, embryology, physiology, reproductive biology, and behaviour, but also as they have relevance to the human situation.

Evolutionary comparisons can be very informative. A separate PPL will cover our work with the chick. In addition, our mouse work will include a small number of chimeric animals that will be generated by admixing mouse embryos with cells (such as embryonic stem cells) from another mouse or small mammal, such as the rat. We will use this strategy to replace the elements of, for example, the developing brain and spinal cord so we can observe some aspects of how these systems develop, and how much is determined by the cells of the system and how much by the surrounding tissues. This approach can be used to study aspects of physiology or disease that affect other mammals where it would be difficult to conduct the experiments in these mammals. This could include humans, although this is not part of our current programme of work.

With respect to life stages, our work ranges from preimplantation embryos all the way to ageing adults. This range reflects, in part, the focus of the lab on certain genes, for example *Sox2*, that function in cell fate decisions throughout many or all these stages, but also the importance of understanding the changes that take place during organ development and maturation and altered physiological circumstances, including ageing.

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

Because we often use genetic approaches, much of our work involves breeding, including GA animals, and harvesting embryos or tissues from postnatal animals after they have been killed (using a schedule 1 procedure or fixation/perfusion) for detailed analysis of phenotypes. We will use hormone injections to control aspects of reproduction, e.g. superovulation when making genetically altered animals. Quite often we make use of substances, such as tamoxifen, to induce a genetic alteration, e.g. a conditional

loss- or gain-of-function of a specific gene, as well as to follow cell fates or isolate specific cell types (e.g. by activation of a fluorescent reporter gene).

For a smaller number of animals, we will use some surgical techniques, always employing appropriate anaesthesia and analgesia, and in consultation with the NVS. These include methods associated with the production of genetically altered mice, including chimeras, and studies on reproductive biology, such as embryo transfer, vasectomy, ovariectomy and orchidectomy. Some of these methods are also used for routine maintenance or preservation, as frozen embryos or sperm, of specific genetically altered mouse lines. We also use surgery in a small number of animals to explore the consequences of removing target organs (such as the adrenal gland) of the pituitary on the hypothalamic-pituitary axis. Our work has shown that this leads to activation and differentiation of stem cells within the pituitary. Occasionally more than one organ will be removed (e.g. adrenals and testes) for the same reason, and to explore if the effects are additive or affect distinct cell populations. As part of this PPL, due to having derived a new genetically altered mouse line that permits cell fate mapping without the use of tamoxifen (which disturbs reproductive function and the hypothalamic-pituitary axis) we will investigate the effects of changing physiology, such as puberty, pregnancy, lactation, on the pituitary stem cells. This will not necessarily involve surgery, but may involve additional injection of relevant substances including into pregnant mice, where the main object is to study the mothers rather than the embryos.

We will also use substances, such as EdU or BrdU, to look at cell proliferation in tissues after harvesting. These substances are introduced by injection or gavage, into pregnant females or live-born animals, and may be carried out multiple times over a few days, followed by a variable period prior to the animal being killed and the tissues analysed. This can be to follow cell fates over this period, or to examine the consequences of an induced mutation at different life stages, or to carry out specific assays, such as a ‘label-retaining’ assay, often used to identify quiescent stem cells.

Some projects, involving few animals, make use of conditional or inducible genetic systems or drugs to deliberately kill specific cell types. This can be to study the consequences of their loss on the organ system under study, such as the pituitary or stem cell niches in the brain. And a new project will explore the effects of radiation or anti-mitotic chemicals on the hypothalamic-pituitary axis, which is known to be compromised after radiotherapy or chemotherapy. Most of these experiments are short term, lasting days to a few weeks, before the consequences are analysed. Alternatively, these methods may be for studies using blastocyst complementation, when specific cells, such as neurectoderm progenitors, are deleted in the developing host embryo, but replaced by cells differentiating from pluripotent stem cells introduced at blastocyst stages. In this case, the effects are assessed in a stepwise manner, looking at embryos less than two thirds through gestation, then at embryos shortly before term, before allowing any chimeras to be born. If these are viable, the animals will be kept longer for subsequent phenotype analysis, but with careful monitoring.

For some projects, but again involving small numbers of mice, we need to introduce substances and/or cells into the brain, which will also involve surgical procedures, including implantation of canulae and osmotic minipumps, injection needles, and, electrophysiology. Substances and/or cells may also be introduced into the developing brains of embryos in utero, which also requires procedures to be performed on the pregnant female. Imaging methods, such as ultrasound, may be used to guide positioning of needles. For monitoring changes in physiology or hormone levels, we may implant a cannula into a blood vessel to sample blood.

A small number of control and genetically altered mice, some after surgery, may be subject to (mild) learning and memory or other behavioural tests.

Some projects are also concerned with cancer and/or ageing. In the majority of cases, those mice expected to develop tumours will do so as a result of a particular breeding protocol involving certain genetically altered strains, such as with null or conditional mutations in *p27*. In other situations, the tumours will develop through injection of cells that are known to lead to tumours, either from an original tumour, such as a gliobastoma, or from pluripotent stem cells that can give rise to teratomas or teratocarcinomas. In all these cases we monitor tumour development, using imaging methods where possible, as detailed in the relevant protocol. Extensive post-mortem tissue analysis will be performed to maximise the information obtained from each animal. For certain types of tumours, such as those developing in the pituitary due to mutations in *p27*, these generally only become apparent in older animals. The effects of ageing on stem and progenitor cells in the CNS and pituitary, and on gonadal sex reversal, also require some animals to be kept for more than a year.

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

For the majority of our experiments, wild type and genetically altered liveborn mice should experience no more than mild effects. For strains that carry harmful mutations, the lines are maintained as heterozygotes, which themselves tend to have mild or no apparent phenotypes. However, animals may be crossed to generate homozygotes or compound mutations, where stronger phenotypes occur, including embryonic or postnatal lethality, or reduced lifespan. It will be necessary to study embryonic stages and to keep some animals with harmful mutations until the phenotypes develop, in order to study how they arise. We will kill animals before end points for the relevant severity band are reached.

The types of phenotype range, according to the specific gene being altered and the type of alteration, from complete or partial sex reversal and/or infertility, craniofacial defects, loss of hearing or vision, CNS defects, abnormal behaviour and/or defects in learning and memory, epilepsy (although this can be managed by careful handling), hypopituitarism, tumours, to shortened healthspan or lifespan. With mutations affecting some genes, there may be phenotypes outside the tissues we study, which can lead to lethality. An example would be kidney defects in addition to sex reversal with mutations in *Wt1* or in addition to enteric nervous system defects with mutations in *Ret*. In these cases, the animals are killed prior to the kidney defects becoming deleterious shortly after birth. In other cases, we are interested in the origins of a deleterious phenotype, such as the failure of the enteric nervous system to colonise the distal gut in mouse models of Hirschsprung's disease, and not the problems this leads to. We therefore kill any newborn animals with the relevant genotype before symptoms of the disease, such as 'megacolon', become evident.

The frequency, type and severity of any adverse event depends on the procedures being used, together sometimes with genetic status, including the genetic background of the strain. We endeavour to minimise the chances of these occurring, but the cause is sometimes unknown, such as the occasional death after administering tamoxifen. Depending on its severity, the duration of an impact or adverse effect may range from a small number of days to a small number of weeks, or even for much longer if fertility or behaviour are affected, with the animal being killed prior to it reaching the relevant end points as defined in the protocols.

For animals undergoing surgical procedures, most should only experience transient discomfort with pain being managed by anaesthesia and analgesia. They would be expected to recover fully within a few days. For some, the outcome may be more severe, notably after adrenalectomies there can be significant weight loss associated with ‘salt wasting’. This can be managed by adding salt to their drinking water, but this fails to rescue the mice after 4-8 weeks. We therefore kill all such mice at a maximum of 3 weeks post adrenalectomy. If combined with tamoxifen treatment, animals that have been subject to adrenalectomy have a significantly increased risk of death about three days afterwards, which can be up to 50% (with no clear reason). For this reason, animals treated under this combined protocol (No. 11), which is used for a small number of animals, are maintained for no more than 7 days, and the protocol is classified as severe.

With aged animals, the chance of an adverse event, including unexplained death, is also increased. Such animals are therefore also maintained under a severe protocol (No. 9), although the majority do not exhibit severe 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)?

The expected severities for the mouse experiments are:

Mild; about 75% of the animals.

Moderate; about 23% of the animals.

Severe; less than 2% of the animals

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

- Used in other projects

A retrospective assessment of these predicted harms will be due by 22 January 2026

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?

Our work is very much embedded in mouse genetics, and this requires breeding animals. Furthermore, most, if not all cell fate decisions in the embryo and adult animal take place within a complex environment, where events intrinsic to the cells are influenced by a variety of extrinsic signals, whether these are from neighbouring cells, involve molecules, such as growth factors, cytokines and hormones (which can act over considerable distances), are commensal with the animal, such as gut microbiota, or are part of the environment, i.e. are external to the organism. Moreover, most tissues develop in a complex way in three dimensions over time in a carefully orchestrated manner. Therefore, although some aspects of certain cell fate decisions can be studied *in vitro*, and we both use and develop such approaches, it is generally essential to study them *in vivo* (as a minimum to judge the suitability of *in vitro* systems to give meaningful information). This is best illustrated with a few examples relating to our work:

(i). Several distinct cell lineages give rise to the developing gonads and their continued interactions are required for appropriate gene activity leading to the development of either testes or ovaries. For example, in the mouse, the supporting cells arise from the coelomic epithelium overlying the genital ridge, the steroidogenic cells arise from mesonephric mesenchyme at early stages, and the germ cells are specified amongst extraembryonic mesoderm in the base of the allantois during gastrulation and then migrate back into the embryo and eventually to the gonad. Some connective tissue cells arise from unspecified mesenchyme in the early gonad, whereas endothelial cells that are critical to establish an arterial blood flow in the testis migrate from the mesonephros after Sry activity has triggered Sertoli cell differentiation. Early Sertoli cells also influence germ cells to enter mitotic arrest, and actively prevent them from early entry into meiosis, which is typical of the ovary. It is currently not possible to mimic all of these cell-cell interactions using cells maintained *in vitro*. We can culture the intact early gonad for periods of two to three days, which does allow us to follow some events in real time (and reduce animal numbers), but this still requires breeding to produce the animals.

(ii). To study postnatal gonadal sex reversal, such as when *Foxl2* is conditionally deleted, cannot meaningfully be studied in any in current *in vitro* system. While it is possible to culture isolated granulosa cells for a limited time, they tend to lose expression of critical genes and their normal phenotype. The same is true of Sertoli cells. Without a robust and reliable culture system that could maintain both of these cellular phenotypes, it would be impossible to address the consequences of deleting *Foxl2* in meaningful way. Moreover, it would not be possible to investigate how other testicular cell types differentiate, nor the morphological changes that accompany the changes from ovary to testicular-like structures.

(iii). The pituitary develops through a complex series of reciprocal inductive events between the oral ectoderm and the overlying ventral diencephalon. Some progress appeared to have been made several year ago to mimic aspects of this *in vitro*, beginning with ES or iPS cells; however, the structures reported failed to reflect the cellular organisation of the pituitary, or appropriate production of hormones. Moreover, these experiments have proved difficult to replicate. We can also grow stem cells from the pituitary for a limited time *in vitro* and, by changing conditions they can differentiate into each of the hormone producing cell types typical of the anterior lobe and we use this *in vitro* model to ask some questions about factors influencing stem cell self-renewal and differentiation. However, it has not been possible to reintroduce these to the pituitary *in vivo* to test if they retain relevant functional properties. Nor are these ‘pituispheres’ likely to have sufficient complexity to model the real organ or to be useful to address issues of the interactions of the pituitary with the hypothalamus and its target organs, which requires whole animal studies.

Over the last few years we have determined that the pituitary stem cells respond to changing physiological conditions. For example, the stem cell population is mobilised by estrogen treatment of males or by gonadectomy or adrenalectomy, but it is not known how a systemic signal affects the stem cells, whether this is via other cell types in the pituitary, or the hypothalamus, etc. We have also recently discovered that the stem cell population is itself complex. To answer these questions requires *in vivo* experiments. Moreover, as we move to explore how the stem cells respond to normal life events such as puberty, pregnancy, lactation, etc, these again have to be carried out *in vivo*.

(iv). To explore the role of specific *Sox* genes in CNS development and their association with the origin of specific tumour types or with ageing, similarly cannot be adequately replicated *in vitro*. For example, there is no *in vitro* model of hippocampus development. Addressing the consequences of abnormal CNS development on behaviour or learning and memory also requires *in vivo* experiments.

(v). There is now increasing evidence that many aspects of anatomy, physiology, behaviour, pathologies, and responses to treatment, differ between the sexes; and even when these appear similar, the underlying mechanisms may be different. These differences are likely to be due to direct effects of X and Y linked genes, to sex hormones made by ovaries or testes, or both. Moreover, these effects can be organisational (i.e. they are established during development, perhaps prior to any obvious difference), or activational (require constant input). Experiments to understand the mechanisms involved, the importance of which have been widely recognised in recent years, cannot be conducted *in vitro*.

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

We complement our *in vivo* analyses with tissue culture models and organoids. These include the pituispheres mentioned above, as well as neurospheres and NS (neural stem) cell cultures for the CNS. We also make use of cell types obtained from mouse or human pluripotent stem cells (ES or iPS cells) via processes of directed differentiation *in vitro*. These can give rise to cell types typical of the CNS and, from our work, to the early gonad. We have also, with collaborators, attempted to use tissue engineering to construct 3-D models of neural stem cell niches, and plan to use similar methods to assemble gonad-like structures.

Why were they not suitable?

We can gain a certain amount of information from these *in vitro* systems, but there are serious limitations. It is not currently possible to replicate the complexity of mammalian tissue structures in culture models. In addition, while some molecular assays (e.g. RNAseq), suggest that the various cell types we have derived from pluripotent stem cells are similar to the endogenous cell types we wish to study, they are not identical. Moreover, the proof that we can derive and propagate relevant cell types, will depend on their ability to function when reintroduced into the relevant organ *in vivo*, which can be challenging and still requires animals. Finally, such cell or organoid cultures cannot permit research on aspects of biology such as reproduction, brain function, or physiology.

A retrospective assessment of replacement will be due by 22 January 2026

The PPL holder will be required to disclose:

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

Reduction

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

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

This estimate is based on several factors. It is based on our past experience, particularly over the past 5 years. We have also taken into account the number of researchers within the group who perform mouse experiments (currently 10, and it is likely to remain around this number over the next five years, with MSc students usually boosting this (currently two) for 3 to 6 months during each spring/early summer). We also continually re-evaluate the numbers of mice required for each experiment using power calculations. For this we access help from an in-house statistician when necessary. This will allow us to determine the number of animals required per experiment to give statistically valid results. Numbers of mice used for breeding are based on best practice, experience with each strain or combination of strains, and factoring in the likely proportion of the desired genotypes, including controls.

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

When designing specific experiments within the overall project we estimate the minimum number of animals required to give robust answers. Most often this can be based on our prior experience or on published data. Often it is not necessary to use statistics (e.g. three transgenic lines giving the same pattern of expression shows that this is correct), however, we perform statistical analysis whenever necessary. Where experiments involve physiological manipulation, or result in phenotypic and/or physiological consequences, for which we have little or no prior information, usually around 5 or 6 animals per treatment group (which will include sex as a variable when relevant and possible) are sufficient to obtain robust results. The design of quantitative experiments generally follows the ARRIVE guidelines and sample sizes may be set using power analysis. Any exceptions are where there is a degree of variability beyond our control (for example, where minor fluctuations in conditions together with threshold effects require more animals to be examined in order to have statistically significant results). We generally use a significance level of 5% and a power of 80%, estimating standard deviation from pilot experiments. We include advice taken from local statisticians as well as make use of online tools, such as the NC3Rs' Experimental Design Assistant.

For some important questions that we wish to address there can be a choice between using a mild procedure but many animals because the measurable effect is weak, or a moderately severe procedure with few animals because the effect is robust. Our choice will depend on the specific question and available resources, but it will most often be to use fewer animals.

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

We try to keep as few mice as possible by careful monitoring our mouse colony and good practice. Whenever possible and when there are no harmful phenotypes (or infertility) we maintain genetically altered mouse lines as homozygotes to reduce the numbers of animals required and to reduce the need to genotype. These may be crossed to another genetically altered strain or to wild type mice prior to beginning an experiment followed by intercrossing the heterozygote offspring when wild type and/or heterozygous animals are required as controls for the homozygotes. We also make use of fluorescence reporters that can also (in some circumstances) avoid the need for biopsies, especially for genotyping. To minimise breeding, lines under sporadic use are maintained at lower levels. We also use cryopreservation, such as of embryos and sperm, whenever a strain of mice is not in current use, to preserve unique alleles or allele combinations, and also to permit efficient export or rederivation of animals.

Whenever possible, we prescreen substances (including molecules to induce gene expression, cell death, mutagens, etc) and agents such as viruses *in vitro* to determine approximate doses required *in vivo*. When possible, we also test genome editing components *in vitro* (e.g. with ES cells), prior to the generation of genetically altered animals.

To maximise information gained from single animals, we use *in vivo* imaging when feasible, obtain data on as many tests of behaviour and learning and memory as possible on single animals, and obtain relevant tissue samples from multiple sites after killing. Where more than one project involves the study of an animal with a particular genotype, for example *Sox9* is relevant to studies on the CNS, pituitary and gonads, we often collect multiple tissues from single animals. Similarly, when designing new genetic tools, and maintaining animals derived with these, we will, wherever possible, do so in a way to allow them to be shared amongst as many people as possible, including making use of the host establishment sharing platform. This efficient use of animals minimizes the number used.

A retrospective assessment of reduction will be due by 22 January 2026

The PPL holder will be required to disclose:

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

Refinement

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

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

We choose well-established protocols, known to have minimal harmful effects, whenever possible. Although it is not always possible to predict the nature or severity of any defect that arises from a newly-generated genetic alteration, we take steps to minimise unwanted phenotypes and/or the number of animals exhibiting these. For example, we make use of tissue-specific regulatory elements and whenever practical, we prefer to make genetic alterations that are inducible, so that the animals do not show a phenotype until expression of the candidate gene or a deletion is induced. Animals exhibiting unexpected or detrimental phenotypes will be killed by a Schedule 1 method, or in the case of new lines or individual animals with phenotypes that may be of particular scientific interest advice will be sought from the Home Office Inspector.

When the experiment is predicted to lead to harmful effects outside the body system under study, we will provide treatments designed to alleviate these – for example, high salt will be given after adrenalectomy, and calcium lactate will be after removal of thyroid and parathyroid organs, or if tumour formation is not a desired outcome, then it may be possible to give anticancer agents (or growth inhibitors). By introducing substances, including viruses and cells, into specific tissues or cavities (such as the lateral ventricles of the brain) we minimise suffering because other body systems are not affected.

To minimise stress during breeding and maintenance we follow best practice guidelines, institute refinements and, for some strains, our own specific procedures of husbandry. These include cage enrichment, sufficient nesting material, and, for particularly sensitive strains and animals subject to specific procedures, minimum disturbance. In the case of any new strain of animal or application of any new procedure or refinement we pay special attention by increased observation and monitoring until we have become familiar with the phenotype and/or the consequences. If welfare implications are identified they will be acted upon and refinements considered in consultation with the NVS, NACWO and animal technicians.

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

A significant fraction of our research involves studies on mouse embryos prior to two-thirds through gestation. We also make of chick embryos (covered under a separate PPL), for some projects. This is partly for evolutionary comparisons, and in some respects the chick and human may be closer than the mouse is to human (in morphology of the fetal ovary or as a model for the craniofacial abnormalities associated with mutations in *Foxl2*), and partly because certain embryological techniques are feasible *in ovo*, but not in utero. However, in most cases the mouse is a better model for the human situation, and the wide range of methods and tools that have been developed for the mouse makes this a more tractable model to study. In addition, many of the systems we study including sex determination, reproductive biology, the pituitary, and the CNS have aspects that are specific to mammals.

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

For all manipulations, we will adhere to the relevant guidelines that aim to minimize suffering. We examine the animals for signs of pain and discomfort (such as grimacing), providing additional analgesia if appropriate, and monitor body condition, killing the animals if the distress is likely to be more than temporary. Many of the genetic and physiological manipulations, as well as the administration of substances, including gene inducers and repressors, viruses, cells and grafting of

tissues, are standard and previous refinements from the literature will be used and added to if possible. For novel types of manipulation, or where insufficient information is available, small-scale pilot experiments are conducted in order to determine the best conditions to obtain a sufficiently robust and meaningful response from the minimum dose, exposure time or treatment. These pilot experiments help to minimize any potential suffering.

In all surgery, analgesia will be provided according to best current practice and with advice from the NVS/NACWO. Appropriate aseptic surgical techniques, heat, and fluid therapy, will be applied as necessary. For studies involving tumours, we will check the animals every day and kill any that exhibit signs of significant illness. Where possible, we will also use imaging methods to monitor the growth of tumours.

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

These will include publications from the NC3Rs and the Institute for Animal Technology, but also relevant articles in scientific journals. In the case of cancer models, we will follow the guidelines in Workman et al, British Journal of Cancer (2010), 102, 1555-1577 (PMID: 20502460); or any subsequent updates as appropriate.

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

We stay up to date via regularly communication with animal facility staff at the host establishment, other scientists in our fields, via e-mail and other updates and publications from, and occasional attendance at meetings held by, the NC3Rs, the Institute for Animal Technology, and the International Society for Transgenic Technology, and through regular visits to their websites:

https://www.nc3rs.org.uk/3rs-resources

https://www.transtechsociety.org/

https://www.iat.org.uk/

A retrospective assessment of refinement will be due by 22 January 2026

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?