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NON-TECHNICAL SUMMARY

Investigating developmental neural stem cell development to better understand congenital disorders.

Project duration

5 years 0 months

Project purpose

- (a) Basic research

Key words

Nervous system, Organ development, Cell biology, Stem cells

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We aim to understand how cells organise to form tissues of the central nervous system, particularly the eye and spinal cord, during fetal development. We will focus on how neural stem cells balance decisions between proliferation to allow growth of the organ, and specialisation to generate the mature cell-types of the adult organ, e.g nerve cells.

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

Why is it important to undertake this work?

Neural stem cells are cells in the developing nervous system (brain, eyes and spinal cord) while the baby is in the womb. They have two important properties; firstly, they multiply and increase in number allowing growth of the developing nervous system. Secondly, they can transform and specialise into all the different kinds of nerve cells found in the adult brain, eyes and spinal cord. These properties must be balanced to generate a fully developed and functional nervous system. This is controlled by signals from the surroundings of neural stem cells and also the genes inside them. Sometimes, issues arise during pregnancy, disrupting the proper multiplication and specialization of neural stem cells, which can lead to disorders of the central nervous system. For instance, some inherited eye diseases, like microphthalmia (patients have small eyes) and coloboma (patients have under-developed eyes) may arise from a lack of neural stem cell growth. These conditions affect about 1 in every 10,000 births and are a major cause of childhood blindness. Although we know about 100 genes that can cause these issues, we don't fully understand how they work during eye development. So, it's crucial to study how neural stem cells balance growth and specialisation during normal central nervous system growth and how things can go wrong in these developmental disorders. Our research aims to uncover: 1. Important behaviours of neural stem cells and signals that guide central nervous system development. 2. The genes and processes that don't work properly during development, leading to congenital eye disorders.

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

The outputs of our study include new information and publications on:

1. How neural stem cells balance growth and specialisation during organ development.

2. Understanding the reasons behind the congenital eye disorders microphthalmia (patients have small eyes) and coloboma (patients have under-developed eyes). These are the most common cause of childhood blindness.

We will generate new animal models of congenital eye disorders. These will contribute knowledge of genes that are candidates for human inherited eye diseases and a better understanding of the

processes that these genes control.

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

We will provide important information on stem cell decisions and tissue development. In the short-term this will benefit other researchers in the field. Although it is not directly translational, it may be of clinical use in the future, beyond the length of the project, in stem cell treatment/replacement therapies in degenerative diseases or damage in the central nervous system.

Our research in to congenital eye disorders will be relevant to clinical research scientists and clinical geneticists. We aim to test genes linked to eye disorders but also novel genes that have been predicted to be involved from whole-genome sequencing of patients. As such, we will expand the number of genes associated with congenital eye disorders providing new genes for clinicians to screen their patients for mutations.

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

We will present our research at national and international conferences and as invited speakers at Universities and research institutes. This allows direct exchange of information, including best practices and meeting new researchers to expand our collaborative network. We currently collaborate nationally and internationally with other scientists working on neural development and have direct collaborations with clinicians working on eye diseases. This will help bring maximum impact of our work.

Species and numbers of animals expected to be used

- Mice: 3550
- Zebra fish (Danio rerio): 6650

Predicted harms

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

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

We are using two vertebrate species, zebrafish and mouse, to model the development of the central nervous system. We use immature and embryonic stages, both in zebrafish and mouse, which reduces suffering as embryos do not have a mature nervous system.

Refinement is central to our project as we use zebrafish as the least sentient model animal that shows a similar eye structure, organisation and cell composition to human, thereby reducing mammal use. Zebrafish can also have coloboma and microphthalmia, the disorders of interest in the study. We use zebrafish to screen for new genes associated with human eye disorders. This is because zebrafish lay

multiple eggs externally and we can simply collect them from their home tank, without harming the female.

We use mouse to model all regions of central nervous system development, particularly the eye and spinal cord. We follow up on the genes identified in zebrafish and test their function in higher vertebrates, but also explore more well established pathways and genes that are known to be important for central nervous system development. Mice are an important experimental system for neural development because they;

1. Share the same developmental brain regions as humans

2. Share similar gene patterns during neural stem cell maturation and specification of major nerve cell types.

Years of study of mouse neural development has generated a plethora of genetically altered mice, to a degree not available in other species. For instance, we will make use of mice with key neural development genes tagged with fluorescent proteins, allowing us to monitor and probe developing nerve cells under the microscope, a key step in our objectives.

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

We study development of the central nervous system so the vast majority of our work will be on embryonic or larval stages in zebrafish and mouse. We will use genetically altered animals for natural mating to generate eggs (zebrafish), larva (zebrafish) or embryonic tissue (mouse) to be used in vitro research or to maintain the stock of animals.

Zebrafish

Typically, we will mate genetically altered zebrafish to obtain eggs and larva. The genetic alterations include reporter animals to aid detection of specific cell types, mainly neural stem cells, under the microscope. No phenotype is expected in these animals. Additionally, we will use animals with knockout and gene changes (mutations) in genes found in human eye disorder patients. We expect the severity of these animals to be mild as we expect some visual impairment, but not severe enough to affect feeding. Genetic alterations will be induced using microinjection of wild-type zebrafish eggs.

Mouse

We propose to use two types of genetically altered animals.

1. Most of these genetic alterations (90%) are to aid detection of neural stem cells and our proteins of interest under the microscope. No phenotype is expected in these animals. Genotyping will generally be undertaken using surplus material from ear notching for identification. Typically, we will mate genetically altered mice and obtain embryos or tissues after humane killing of the pregnant female mice. The embryos or tissue will then be used for in-vitro experiments.

2. For some strains (<10% of breedings), we may induce gene expression changes in the embryos by administering an inducing agent such as Tamoxifen or Tetracycline to the pregnant female, wait typically 1-4 days and then isolate embryos after Schedule 1 killing of the female. The downstream

gene chances will occur in the embryos and we will administer between E8.5-E12.5 at unprotected stages. Most (85%) embryos will be typically collected at unprotected stages, but a subset of embryos, around 15% will be taken to protected stages up to E17.5 and then subject to schedule 1 cull.

The gene changes induced by Tamoxifen or Tetracycline will cause either a) activation of a fluorescent protein in neural stem cells in the embryos to help identification of these cells under the microscope or b) knockout of a gene of interest in neural stem cells in the embryos. The genes of interest will affect how neural stem cells communicate with each other.

Tamoxifen is a drug frequently used to treat breast cancer in humans and Tetracycline is an antibiotic commonly used in humans for acne. They will be administered by the most appropriate method. Where possible through the diet, but when we need a greater control on the timing of administration by injection into the body cavity (intra-peritoneal injection) of the pregnant female or gavage (oral administration).

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

Mouse

We will use two types of genetically altered mice;

1. Reporter mice - mice containing genetic reporters to aid detection of specific cell types (mainly neural stem cells) and proteins under the microscope. No phenotype is expected in these animals because the addition of the reporter does not affect the function of the protein or cell.

2. Mice containing Tamoxifen or Tetracycline responsive genes. The transgenes and gene changes induced by Tamoxifen or Tetracycline will cause either a) activation of a fluorescent protein in neural stem cells in the embryos to help identification of these cells under the microscope or b) knockout of a gene of interest in neural stem cells in the embryos. The genes of interest to be knocked out will affect how neural stem cells communicate with each other. Most (85%) embryos will be typically collected at unprotected stages, but a subset of embryos, around 15% will be taken to protected stages up to E17.5 and then subject to schedule 1 cull. We do not expect an impact from the embryonic gene changes on the pregnant mothers. For the embryos taken to protected stages, there may be some alteration in the number or position of nerve cells generated, but we do not expect any adverse effects more than mild.

The routes of administration of inducing agents such as Tamoxifen and Tetracycline to mice are associated with a minor discomfort and animals may experience stress due to restraint and handling (up to 100%). Tamoxifen can be harmful to the pregnant mouse if administered too early during development (less than 7.5 days after conception). Therefore, we will administer Tamoxifen after this point to reduce harm to the pregnant female.

Zebrafish

We will use two types of genetically altered zebrafish; 1. Zebrafish containing reporter transgenes to aid detection of specific cell types, mainly neural stem cells, under the microscope. No phenotype is expected in these animals as the addition of the reporter does not affect the function of the cell. 2. Zebrafish with knockout and gene changes (mutations) in genes found in human eye disorder patients.

We expect these animals to have smaller and underdeveloped eyes in either 1 or both eyes, which may lead to slight visual impairment.

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

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

Mouse - Mild - (100%)

- Reporter mice - No phenotype is expected in the Reporter mice and embryos as the addition of the reporter does not affect the function of the cell or protein.

- Pregnant female mice that will be induced to alter gene expression are expected to experience mild severity due transitory pain associated with administration of Tamoxifen or Tetracycline into the body cavity (intra-peritoneal injection) or mouth (oral gavage). For the embryos with altered gene expression due to Tamoxifen or Tetracycline and taken to protected stages, there may be some alteration in the number or position of nerve cells generated, but we do not expect any adverse effects more than mild.

Zebrafish - Mild 100%

- Reporter zebrafish - no phenotype is expected in these animals as the addition of the reporter does not affect the function of the cells.

- Zebrafish with knockout and gene changes (mutations) to genes found in human eye disorder patients. We expect these animals to have smaller and underdeveloped eyes in either 1 or both eyes and we expect the severity of these animals to be mild as we expect some visual impairment, but not severe enough to affect feeding.

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

- Used in other projects
- Killed

Replacement

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

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

Our studies investigate the complex interactions between cells and their patterning and organisation into tissues and organs during fetal development. There are replacement approaches such as in vitro organoid models of neural development and mathematical modelling. In vitro organoids are clumps of cells that resemble an organ and are grown from stem cells in a petri dish. Indeed, we use human

retinal organoids and mathematical modelling in the lab. However, these approaches do not completely model the complexity of human or other vertebrate eye development.

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

We considered using human retinal organoids and mouse embryonic stem cell protocols to study neural development and found relevant protocols through literature searching.

Why were they not suitable?

Human retinal organoids and mouse embryonic stem cell protocols do not re-capitulate the complexity of cell and tissue types found in the developing vertebrate nervous system or the 3D morphology and tissue shape changes that occur during central nervous system development.

For example, current protocols for human retinal organoids generate the neural, light-sensitive tissue but not together with other eye tissues e.g lens, retinal pigmented epithelium and surrounding mesenchyme which are known to influence eye development. Human eye disorders can arise from problems in multiple tissue types, not just the retina. So to fully understand the processes that lead to eye disorders, vertebrate animals are required. Furthermore, patterning of the tissue, such as patterns of gene activity, and patterning of cell types is crucial to generating a functional and structured organ. However, it is currently missing in in vitro organoids. For example, human eyes have a central macular, an area of increased light sensitivity, which is missing in retinal organoids. Therefore, in vitro alternatives cannot be used to study patterning.

Reduction

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

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

We have estimated the numbers of animals that will be used based on 1.Requirements for strain maintenance and 2. The number of breeding pairs required to generate the embryonic tissue for invitro studies:

Zebrafish

- the number of tanks needed for breeding to maintain a strain (based on 40 zebrafish per tank) per year, per strain. We plan to use 5 zebrafish strains.

-the number of tanks needed for generating a genetically modified zebrafish line. We plan to generate 3 out of the 5 strains.

-the number of breeding pairs required to generate enough embryos and larva for our in-vitro studies. We have based this on our previous experience experiments to determine how many zebrafish crosses are required per experiment.

Mice

-the number of breeding pairs needed to maintain a strain, per year, per strain. We plan to use 5-6 strains, with 2 breeding pairs set up every 4-5 months.

-the number of breeding pairs required to generate enough embryonic tissue for our in-vitro studies. We have based this on our similar published experiments to determine how many embryonic mouse litters are required per experiment. We have additionally taken into account the frequency of false pregnancies (mouse) we have observed over the last 5 years.

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

We have used the NC3R's Experimental Design Assistant website and ARRIVE 2.0 guidelines for advice on randomisation and blinding, sample size calculations and appropriate statistical analysis methods.

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

We will use several strategies to reduce numbers of animals. While producing genetically altered animals, we will use breeding schemes that maximize the number of animals with the correct hereditary traits that are produced, this will ensure that as few as possible genetically altered animals are born unnecessarily. Colony numbers will be managed efficiently, in mice we will generally use 2 breeding pairs every 4-5 months to keep numbers low. To minimise the use of mice and mouse embryos for preliminary studies we use embryos of zebrafish, a lower vertebrate.

We will maximise use of tissue by freezing and banking it within our lab and also share it wider in the Establishment's tissue sharing resource.

Refinement

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

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

We are using two species, zebrafish and mouse, to model the development of the central nervous system. We will collect embryonic tissue after natural mating in both models. Refinement is central to our project as we use zebrafish as the least sentient model animal that shows a similar eye structure, organisation and cell composition to human, thereby reducing mammal use. Furthermore, we use embryonic stages, both in mouse and zebrafish, when embryos do not have a mature nervous system and so suffering and distress is reduced.

In generating embryonic mouse tissue we will occasionally (~10% of matings) need to induce gene activity in the embryos by administration of an agent. We will use the most appropriate and refined method, including by diet, intra-peritoneal injection or oral gavage. When we want to study processes that happen within a short time window during development, then oral gavage or injection are necessary because they induce a rapid change in gene expression.

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

We already use embryonic stages that are less sentient. Zebrafish embryos will be used replace and reduce mouse wherever possible; they will be used to screen genes associated with human congenital eye disorders because they provide a high-throughput approach with a reduced welfare impact compared to the mouse. We can't use less sentient experimental models to investigate human congenital eye disorders as C. elegans have no eye structures and Drosophila have compound eyes which have very different structure to humans. Therefore they do not represent human eye disorders.

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

Historically administration of inducing agents to pregnant females has been done by injection into the body cavity of the female (intra-peritoneal), we will refine this by testing and implementing alternative less invasive routes, e.g by diet and oral administration.

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

We will follow the best practice guidance from the Home Office on Animal Testing and Research from the Home Office, LASA, RSPCA, local AWERB guidelines, ARRIVE 2.0 guidelines of the NC3Rs and the PREPARE guidelines to ensure our experiments are high quality and the most refined.

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

We are committed to seek and review advances that can improve our experiments throughout the project duration. We will keep abreast of reduction and refinement advances by attending workshops and seminars run by the Establishment as well as signing up to NC3Rs newsletter and IAT bulletin. We collaborate with groups that aim to improve organoid and stem cell models of neural development. By meeting with these researchers and attending conferences we will keep up-to-date on replacement approaches that avoid the use of animals.