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NON-TECHNICAL SUMMARY

Mouse developmental genetics to study neurodevelopment and disease

Project duration

5 years 0 months

Project purpose

- (a) Basic research

Key words

neurodevelopment, stem cell biology, regenerative medicine, cerebellum, brain injury

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This project aims to identify and study the cellular and molecular mechanisms that regulate neural stem cell behaviours during development, adulthood and upon injury to the brain. Neural stem cells are specialized cells of the brain that are able to give rise to neurons and glia, cells of the brain that are crucial for its function. Neurons transmit information from the body to the brain and back to the body so we can run, smell or think, whereas glia are the supporting cells of the brain which help the neurons to function efficiently. Neural stem cell function is crucial during development to ensure our brain is formed properly and also later in adulthood to ensure our brain continues to function efficiently. However, generally in the brain, our cells do not get replaced efficiently and cell loss after injury or disease is detrimental. This project utilizes the mouse brain, and specifically the cerebellum, a critical brain region that is important for motor, cognitive and social behaviour, basically how we move, think and interact with others, as a point of entry to study neural stem cells in mammalian systems. This understanding is the crucial first step to designing stem cell-targeted therapies that facilitate the repair and regeneration of brain 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?

Brain injuries have devastating outcomes due to the loss of cells followed by inefficient repair. Currently, there are 1.4 million people that are living with acquired brain injuries and 40,000 deaths only from stroke annually in the UK, and an estimated 69 million people suffer from traumatic brain injuries and 14 million new incidents of stroke globally, each year. This highlights the desperate need to develop novel regenerative therapies. However, our knowledge of the stem cells in the brain that can facilitate repair, as well as the molecular and cellular mechanisms that regulate neural stem cell populations' behaviours, such as how they divide or make neurons are limited. Lack of this understanding impairs the development of stem cell-targeted therapies that are desperately needed to replace and repair the injured cells in the brain upon injury. Successful accomplishment of this project will provide the crucial knowledge to design therapies that can activate stem cells of the brain to make new neurons after injury. Additionally, the cerebellum a region of the brain that is critical for motor and cognitive functions develops later than the rest of the brain and therefore is susceptible to injury around birth. Indeed, cerebellar defects are the second leading risk factor for autism spectrum disorders. Therefore, in addition to representing a powerful model to study neural stem cell biology, understanding the regenerative potential of the cerebellum has an impact on newborn health and development.

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

In the short term, the successful completion of this project will provide much-needed new information on the molecular and cellular events that govern stem cells during development, adulthood and upon injury to the mouse brain. In light of this information, we will be able to design and develop approaches to understand how these mechanisms are conserved in humans and how we can facilitate

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regeneration in the human brain. We will disseminate the results of this project throughout the project period via presentations at international conferences and publications in peer-reviewed journals. We anticipate attending at least one conference each year and publishing 3-5 peer-reviewed articles at the end of the project period.

Importantly, in the long term, this project will provide a springboard to utilize endogenous neural stem cells as therapeutic entry points for regenerative medicine in the brain.

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

In the short term scientific community, particularly groups of scientists who are interested in the fields of neurodevelopment, stem cell biology and regenerative medicine will benefit from the data and the tools generated as a result of this project. In the long term, this data will provide fundamental knowledge on the development of the brain, and particularly the cerebellum, identify and describe neural stem cells and provide tools/methodology to manipulate these cells. Furthermore, experimental models that utilise human cells to study the cerebellum are limited. The knowledge that will be generated as a result of the project will also inspire studies that will assess how conserved these mechanisms are in humans and enable the establishment of more efficient systems to study human biology. Finally, in the long term, this understanding may also lead to the development of therapies to facilitate regeneration, in other words, new neuron production after injury to the brain, benefiting many who suffer from brain injury.

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

We will openly share all data, a detailed explanation of the animal work and procedures in all our publications publicly via open-access journals. We will present our unpublished findings at conferences with the greater scientific community. We will also openly share the resources generated prior to publications with collaborators upon mutual agreements in order to minimize duplication of efforts and facilitate the efficient distribution of resources within the scientific community. We will also disclose unsuccessful approaches and results on our websites or via publications when possible. The large/high throughput data sets such as genomics data generated as a result of this project will also be uploaded into public domains.

Species and numbers of animals expected to be used

- Mice: 4500

Predicted harms

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

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

The major goals of our studies are to elucidate how the mammalian brain and its underlying cellular and functional complexity are established during development, and how signalling factors regulate stem/progenitor cell behaviours in developing and adult organs during regeneration. Developmental processes and stem cell responses cannot be fully replicated with any of the existing tissue culture technologies since a complex set of cellular interactions must occur in a precise three-dimensional organization and over an extended time period. Furthermore, these processes and cellular behaviours cannot be modelled on the computer. Transgenic technologies provide ingenious ways of studying the control of gene expression and of following the consequences of altering or blocking specific gene products leading to the discovery of gene function. By analysing the outcome of such manipulations using various transgenic mice, we can address questions of how development occurs or diseases form. By being able to label and visualize normal and mutant cells amongst their neighbours, and following their development or response to injury, we can determine the cellular movements and interactions that underlie normal development and disease. This line of research is required to understand the molecular mechanisms that govern neural stem cells and establish the necessary knowledge required to develop future therapies against human disorders.

Our previous studies have highlighted that the neonatal mouse cerebellum (postnatal (days after birth) day 0 – 5) is highly regenerative and can recover from the loss of its neurons at birth, whereas the adolescent or adult mouse cerebellum loses this ability dramatically. In order to understand the age-dependent changes in the repair capacity of the brain, our research involves studying various stages, particularly early neonates (Postnatal 0 - 5 days) and early adulthood (4-8 weeks).

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

Experiments will involve the following procedures:

1) Breeding and maintaining of genetically engineered mouse models (~7-8 lines, that consist of cell type specific Cre/CreER and Flpo/FlpoER animals and R26 Cre/Flp dependent reporters, floxed alleles for key genes of interest. These genetic mouse lines allow us to label and follow cells of interest and their daughter cells). We will collect tissue for genotyping in-house. Optimize our breeding to maximize the use of litters and minimize the cages needed for maintaining our animals outbred.

2) Genetic inducible fate mapping: The majority of our work involves studying which cells do neural stem cells generate under different conditions. Experiments will involve Tamoxifen (a drug) injection to induce specific labelling of neural stem cells at multiple different ages based on the experimental paradigm. The majority of our time points are postnatal and we have not had adverse side effects of Tamoxifen at these doses/time points. Some of our experimental paradigms also involve transectional approaches that require the administration of Doxycycline (a drug) in the drinking water for defined periods that will allow us to achieve specific labelling of cells that would otherwise not be possible.

3) Injury models: Our work involves studying the stem cell responses to injury at various postnatal ages. We will use established injury models such as localized mild stroke models that are carefully designed to ensure animal welfare and minimise pain.

a. Irradiation to the neonatal mouse: A single dose (4Gy) cerebellum targeted (via specific collimators or body shielding) irradiation at postnatal day 1 to anaesthetized pups. The neonatal cerebellum can recover from this injury very efficiently and no visible behavioural effect has been

observed. We also do not observe any immediate morbidity in the pups due to the procedure and no associated adverse effects.

b. Targeted stroke model (photothrombotic injury): Anesthetized mice are injected with Rose Bengal (a drug) and a cold LED light source is shined over the skull (upon incision) for 10-15 minutes. This causes local blood clothes and a mini stroke-like injury. This is a widely used and published model. We have optimized the parameters to ensure the strokes are mild, animals recover well and do not show obvious physical and behavioural damage upon injury. In the case of adverse effects, animals will be humanely killed.

c. Targeted cell killing: Using transgenic mouse lines, we selectively express diphtheria toxin receptor in the cell types of interest to allow their killing. Diphtheria toxin will then be injected (30 ng/g) and the response to injury will be assessed. The paradigms we will use involves the selective killing of small subpopulations within the brain and we do not observe mortality/ lack of fitness in these animals upon injury. Importantly, the dose of diphtheria toxin used for these experiments is low and is not harmful to humans, therefore experiments can be performed within specific biosafety requirements.

4) Intracranial (in to the brain) injection of cells or other biologicals (such as lentivirus): Cranial windows will be opened (Skin incision and drilling of the skull (adult animals)) and small volumes (1-5ul) of cells or inactive lentivirus will be injected into target regions using a stereotactic frame that immobilizes the head of the animal. Proper analgesic regimens will be given. This is a well-established procedure and used in the neuroscience field for decades.

5) Intraperitoneal/subcutaneous injection of various chemicals: small molecules, BrdU, EdU.

6) Tissue collection for data analysis: Brain tissue from various stages will be collected from all the above paradigms (except for 1) for downstream analyses such as histology or other molecular biology approaches. Estimated tissue collection times after the experimental procedures explained in (2-5) vary between 1-2 hours to up to 2 months after the procedure)

7) Fresh tissue collection for primary culture establishment/cell isolation from various ages.

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

Our research involves multiple cell ablation and injury models. Our previous research shows that neonatal mice recover from injury well and these procedures do not cause obvious defects in the cerebellar cytoarchitecture or mouse behaviour. On the other hand, the injury paradigms used in adult mice and other surgical procedures used at all ages may cause mild to moderate pain in animals until the experimental endpoint or briefly after the procedure, respectively. Adult mice that have undergone a surgical procedure will be killed within 4-8 weeks unless they do not show any severe complications, weight loss and signs of distress. Neonatal animals will also be closely monitored for their feeding behaviour and growth, as well as signs of distress. All animals will be immediately killed if they exhibit signs of distress.

Some of the mutant animals (generated as a result of genetic loss of function studies) may cause developmental abnormalities. We will utilize genetically engineered mice that bears Cre-lox (or Flpo-FRT) systems that are temporally controlled, when possible, to restrict the effect of the mutation on the

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cells of interest and time in order to minimise the systemic effects. When needed we will perform pilot experiments to assess the severity of the phenotype to determine humane experimental endpoints.

Importantly, we are looking forward to sharing the mouse lines, experimental models and procedures that we will establish during this project and the specimens and data generated from these experiments open with the scientific community. We believe that this will prevent duplication of efforts and increase the rigour and reproducibility of our and others' research while allowing refinement and reduction in our experiments.

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)?

Species	Severity	Percentage
Mouse	Mild	80%
Mouse	Moderate	20%
Mouse	Severe	0%

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

- Killed
- Kept alive

Replacement

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

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

A major goal of our studies is to elucidate how signalling factors regulate stem cell behaviours in developing and adult organs, and how do these behaviours change upon injury. Developmental processes, stem cell responses and injury response/regenerative processes cannot be fully replicated with any of the existing tissue culture technologies since a complex set of cellular interactions must occur in a precise three-dimensional organization over an extended period of time. Furthermore, these processes and cellular behaviours cannot be modelled on the computer. By analysing phenotypes in transgenic mice, we can address questions such as what a gene’s function during tissue development and homeostasis or in disease is. By marking normal and mutant cells amongst their neighbours, and following their development, we can determine the cellular movements and interactions that underlie

normal development and disease. These tools are required to assess the behaviours of stem cells and to be able to devise ways to manipulate them for therapeutic purposes to facilitate regeneration.

The availability of mouse models that are genetically well characterized and the wide range of genetic manipulations that can be performed make mice ideal for studying the genetic basis of brain development and postnatal stem cell biology. Since the DNA sequence of the mouse genome is available, any region of the genome can be experimentally manipulated in any desired manner. Moreover, the relative ease of housing and maintaining a large number of mice compared to other mammalian species makes the mouse the animal of choice for the described studies.

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

In vitro (cell and tissue culture) models of mammalian stem cells that allow cells to be cultured in self-renewal or differentiating conditions have been a gold standard of stem cell research. Furthermore, current brain organoid models are good alternatives to animal models since they recapitulate cell-to-cell interactions better.

Why were they not suitable?

Although cell culture models are very powerful, it is not possible to study the full spectrum of developmental and regenerative signalling, and injury response in such systems because they lack critical microenvironmental components and the tissue dynamics that drive development and repair. Importantly, faithful multicellular tissue culture systems that recapitulate neonatal cerebellum development are not available. Our research, in addition to accomplishing our aims described here, will also provide a springboard to establish faithful cerebellar in vitro models for future use of the scientific community and may help replacement efforts in the long run.

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?

Experimental animals utilised in the procedures (Sum of all protocols except for protocols 6-8):

3800 animals

The number of animals is estimated using power calculations or based on previous studies that utilized similar techniques to identify the necessary number of animals to achieve the aims of this licence. Per the experimental paradigm, we anticipate using ~5-10 animals/condition. Details of power calculations are provided under each protocol.

Number of animals required for the generation of lines and continues line breeding (protocols 6-8):

700 animals

Considerations for general breeding for maintaining colony and generating experimental breeding pairs:

We plan to have about ~10 lines at a time. For each line, the breeding pairs (1-3) will be continuously bred to produce ~12 litters/year. We also will keep one stock cage of males and females each. Some of these animals will be used to establish primary cell cultures to reduce the number of animals needed to achieve the goals of this licence.

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

We have utilized online tools (NC3R's experimental design assistant, PREPARE guidelines) and performed power analysis to assess the minimum number of animals required to fulfil the objectives set in the licence.

Furthermore, when possible, we will generate primary cell lines from mouse brains which will allow us to test some of our hypotheses in vitro prior to performing elaborate experiments that may require the generation of new transgenic animals, therefore reducing the number of animals needed to fulfil the objectives we set up in this license.

Finally, we also carefully designed our experiments to obtain the maximum amount of data to be generated from each animal killed and hence reduce the total number of animals needed to achieve our goals.

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

1) We will perform pilot studies and in vitro tests to allow validation prior to moving to large scale studies.

2) We will share tissue with neighbouring labs from our studies that we do not anticipate using.

3) We will optimize our breeding scheme to allow maximum usage of the animals and allow us to provide correct littermate controls.

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.

We will use the newborn mouse as a model to study how the brain develops, and the repair mechanisms after injury to the brain. Our research involves identifying and isolating brain stem cells, following their behaviours through development and in adulthood or after injury to the brain with the goal to understand how we can facilitate repair in the human brain. Our research involves genetically engineered mice to allow the labelling and manipulation of stem cells in the animals, and several different injury models that are carefully designed to mimic devastating human disorders that cause neural loss.

The mouse brain is a great model for studying complex stem cell behaviours at different stages of life and in disease. Such responses cannot be studied in other organisms or cell, or tissue culture models as previously mentioned. Over the years we carefully optimised our injury methods and experimental paradigms to cause the least amount of pain and distress to the animals, compared to some other previously published work that leads to robust injuries to the mammalian brain.

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

The efficient repair processes (regeneration) are observed in newborn mouse brains and the impaired repair phenomenon is a disease of the mature brain, therefore we cannot perform studies at a more immature life stage.

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

Handling: Animals will be handled minimally following the best practice guidelines to exhibit the minimum stress possible.

Food and drink: Some of our experiments may require Tamoxifen or Doxycycline given in the drinking water or chow. We closely monitor weight and food intake to ensure animals are feeding properly.

Solitude and companions: Unless otherwise needed (such as fighting) animals will be housed in groups to ensure social interaction.

Play: Our cages will be enriched to ensure play time for the animals.

Post-surgical care: After surgery, animals will be closely monitored. Proper anaesthesia and analgesic cocktails will be used as described in the protocols and these will be regularly revised in collaboration with the veterinary staff.

We have previously optimized the stated procedures to induce minimal pain and discomfort to the animals, but in the unlikely event that we observe an increased frequency of unanticipated signs of pain and distress, we will stop our studies and evaluate the procedure in question.

Importantly, some of our protocols involve the use of neonatal (newborn) animals. Assessing distress and welfare in pups could be more difficult. We will closely monitor feeding behaviour (such as the

appearance of white milk in the belly and weight gain patterns) as well as whether the dams are taking care of the pups or whether any rejection after the procedure. In case of rejection, foster dams will be utilized.

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

We have and will continue to follow the PREPARE and ARRIVE guidelines. Additionally, we will closely follow resources such as the Breeding and colony management for genetically altered mouse guidelines by NC3R and humane endpoints in laboratory animal experimentation by 3R-Centre Utrecht Life Sciences.

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

1) We will perform monthly literature searches to assess whether there is any new knowledge in the field that will allow us to modify our plans and improve our research with respect to the 3Rs.

2) We will analyse our data and assess our outcomes regularly and frequently (at least monthly) to make sure that our proposed objectives are still accomplishable or if we need to revise our experimental plan/abort studies.

3) We will follow the NC3R e-newsletter for the latest developments and implement the relevant updates immediately.

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