Home Office

NON-TECHNICAL SUMMARY

Defining the molecular mechanisms underlying neonatal brain injury

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

neonatal brain injury, biomarker, therapy, mitochondria

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

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

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

The aim of our work is to discover mechanisms underlying brain injury which occurs following birth asphyxia, a condition where the baby's brain is deprived of blood flow and oxygen during birth. We will use this knowledge as the basis for identifying biomarkers to predict the extent of the injury and for developing novel neuroprotective therapies to prevent lifelong consequences.

A retrospective assessment of these aims will be due by 13 August 2030

The PPL holder will be required to disclose:

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

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

Why is it important to undertake this work?

Asphyxia (restricted blood flow/oxygen to the brain) during birth occurs in 2-3 term babies per 1000 in the UK, leading to the development of a condition known as hypoxic ischaemic encephalopathy (HIE) and permanent, life-long brain and motor disorders such as cerebral palsy. Following asphyxia, there is a delay of a few hours before the majority of brain cell death occurs, providing clinicians with a valuable treatment window. There is still only one available treatment for this injury, therapeutic hypothermia. This is not always successful and new treatments are urgently required. However, therapeutic hypothermia does prove that intervening with treatment after the injury has occurred and within the treatment window can be effective.

This project is designed with the overall aim of understanding the basis of neonatal hypoxic-ischaemic (HI) injury, using these data to identify biomarkers to non-invasively measure injury severity as well as explore novel therapeutic avenues. The basic science outlined in the project will be of substantial interest to all researchers in the field of brain development and mitochondrial biology. However, primarily and most importantly, the success of this project will provide far-reaching, long-lasting improvements in the lives of significant numbers of babies and their families.

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

Outputs from this project are likely to be in the form of new data, publications and potentially new collaborations. We will be investigating the basic biology underpinning the injury, focussing on mitochondria, which are structures contained within all brain cells which provide the energy required for cell survival and which are susceptible to damage following birth asphyxia. Using these data to subsequently focus on translational science, we will use a combination of techniques to identify processes that are targets of the injury and which can report on the extent of the injury. In pursuing both avenues, we will provide basic and translational information to colleagues working in the fields of mitochondrial biology, cell biology and neonatal brain injury. We will make our data available via recognised routes e.g. publications, conferences and uploading of datasets to recognised repositories. If applicable, we will also consult with technology transfer colleagues to pursue any therapies in collaboration with pharmaceutical companies.

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

Hypoxic-Ischaemic Encephalopathy (HIE; a consequence of asphyxia during birth) moderately or severely affects 750,000 babies worldwide per year. In the most wide-ranging study available, it is estimated that neonatal HIE was associated with 2.4% of the Global Burden of Disease. Therapeutic hypothermia is the only treatment available, which improves outcome for only 1 of each 7 infants treated; there is a critical unmet need as 28% of babies with HIE will die, and 24% will develop lifelong neurocognitive impairment.

This project has three objectives i) to characterise mitochondrial dysfunction and impaired mitochondrial dynamics following HI, ii) to investigate a novel biomarker that would help to rapidly assess infants for enrolment into the therapeutic hypothermia protocol (within the 6h cutoff window after birth), and iii) to investigate the molecular mechanisms of neonatal brain injury to identify potential avenues for treatment.

Short term (approx. 3 years), the basic cellular science outlined in the project will be of substantial interest to all researchers in the field of brain development and mitochondrial biology.

Medium term (approx. 5 years), we will provide evidence of a novel biomarker to be used for defining injury severity and prognosis. In addition, we will also identify potential therapies through repurposing existing drugs or developing novel mitochondria-based interventions.

Longer term (> 5 years), and most importantly, the success of our project will facilitate development of a point-of-care device for rapid evaluation of brain injury severity as well as drug candidates for clinical trial. We hope our research will make long-lasting improvements in the lives of significant numbers of babies and their families who suffer the devastating consequences of birth asphyxia.

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

We will maximise the outputs of this work in a number of ways, building on our previous experience. We have been successful in sharing our work at conferences (e.g. the Hershey Conference on Developmental Brain Injury, June 2024, Sweden). Presentations in this environment have generated new collaborations and we expect this to continue. We will place any large datasets in appropriate repositories for use by the wider scientific community and will publish robust data (including any *in vivo* negative data) in well-respected, open access, peer reviewed journals in the field.

Species and numbers of animals expected to be used

- Mice: 2360

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 mice for this project. Mice are easy to handle and a wealth of information is already known about their genetics and physiology which assists in the interpretation of data and the planning of future experiments. Brains of mice continue to develop after they are born so we will be predominantly using mice at the life stage of post-natal day 9 as this is the point at which their brain development is closest to that of a human newborn at full term. The mouse model of hypoxic-ischaemic brain injury outlined in this protocol is highly relevant to the human condition as it was used in the development of therapeutic hypothermia, currently the only therapy available to babies who have suffered from asphyxia during birth.

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

Mice will be bred in social housing conditions and may be ear notched for identification purposes.

Typically a post-natal day 9 mouse pup will be rapidly anaesthetised and undergo surgery. The average time for this is approximately 10-15 min and the pup recovers in a warmed recovery box until the rest of its littermates have been through surgery. The entire litter is then returned to the mother for an hour. For the second phase of the protocol, the pup is placed in a warm low oxygen chamber for between 30-60 min (for C57Bl6 mice this is usually 40 min). During this time, the pup may experience mild seizures similar to those experienced by the human newborn following birth asphyxia. However, these usually do not last once the pup is removed from the chamber. The pup may optionally be administered a neuroprotective drug and will be returned to the mother. Pups remain with the mother until the end of the experiment (usually 1 day to 1 week) or until weaning when they are subsequently maintained for non-invasive behavioural experiments or until the experimental time point of interest. Each mouse will experience this protocol only once.

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

No expected impacts or adverse effects are expected for genetically altered mice undergoing the breeding protocol.

During the hypoxia-ischaemia protocol, recovery from surgery may include pain around the site of incision, although analgesia will be in place to mitigate for this. From observations, symptoms of pain are not normally apparent and would not be expected to last beyond 3h after the surgery. Exposure to hypoxia (30 - 60 min) may cause the pups to experience seizure-like behaviour. However, this very

rarely lasts beyond the duration of the hypoxia and if so, will be resolved within 1h of completing the hypoxia step.

Following surgery, these mice may generally have a low level of weight loss but the weight gain trajectory is entirely normalised within a week. After this time, it is difficult to distinguish the experimental animals from the control, untreated animals. All pups undergoing surgery will be regularly monitored.

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)?

All genetically altered mice that are being bred and maintained for the project (protocol 1) will experience mild severity only.

Mice in hypoxia-ischaemia protocol 2 will experience severe severity as they undergo general anaesthesia for recovery surgery and it is not possible for the seizures to be prevented. Unfortunately, sometimes the surgery or the seizures result in mortality. Once the protocol is completed, recovery is usually complete by 24h post surgery, and the actual severity is mild; the behaviour of mice is largely indistinguishable from their control litter mates. There are subtle behavioural differences, for example, forepaw preference can usually be observed in mice following the surgical procedure.

What will happen to animals used in this project?

- Killed
- Used in other projects

A retrospective assessment of these predicted harms will be due by 13 August 2030

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 project aims to understand the mechanisms involved in perinatal hypoxic-ischemic brain injury, to identify biomarkers and to test neuroprotective strategies. As such, we need models that allow us to mimic human perinatal brain injury. Animals have to be used, as to validate a mode of action, experiments are required that cannot be conducted in humans for ethical and scientific reasons. In

addition, interaction of the biological systems in whole organisms, with intact physiological barriers and excretion mechanisms, is key to inferring the potential of candidate therapies.

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

We have considered the feasibility of achieving our purpose by not involving animals at all, for example by using cell lines or *in vitro* recombinant methods, but no such alternatives are able to reproduce the brain injury we aim to investigate in this proposal.

However, where possible (for example, in altering gene expression in vitro or for testing the specificity of pharmacological activators/inhibitors), we will replace whole animals studies with primary cell preparations or experiments in appropriate cell lines (e.g. neuronal SH-SY5Y or C17.2, microglial BV2, oligodendrocyte CG4 lines).

Why were they not suitable?

Our project ultimately aims to identify neonatal neuroprotective strategies formulated from evidence using *in vitro* cell systems. However, *in vitro* systems alone cannot mimic the unique and complex environment that exists within the neonatal brain. The brain is comprised of many cell types and *in vitro* systems cannot model the physiological interactions and communication between diverse cell populations. In addition, we are aiming to discover therapies beneficial to the neonatal brain, the environment and developmental trajectories of which are still being determined. Therefore to generate clinically relevant data, *in vivo* neonatal models must be used.

A retrospective assessment of replacement will be due by 13 August 2030

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?

Protocol 1 (breeding): We have estimated 960 animals based on 8 breeding pairs per year, with two GA lines over 5 years, assuming an average litter size of 6.

Protocol 2 (hypoxic-ischaemia): We have estimated 1400 animals based on our Home Office returns over the course of the previous licence, with adjustments for COVID and the refinements we have

made to the protocol.

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 Experimental Design Assistant to generate experimental procedure numbers and have taken the advice of the RVC Chartered Statistician. We will also use mice of both sexes in pilot studies, and where there are no discernible differences in the outcomes, data will be pooled to reduce the number of mice required. If the conditions of the pilot studies are unchanged, these data will be incorporated into the main study.

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

Through a collaboration, we intend to use computational modelling to identify pharmacological compounds already in clinical use and with a validated safety profile for our repurposing study. We will also test these compounds *in vitro* to determine any cell-specific toxicity prior to commencing *in vivo* neuroprotection studies.

For pups receiving the surgery (protocol 2), we will take post-mortem blood samples (min. 50µl) as well as brain tissue so that we can use the same cohort of mice for Objectives 1 and 2. Other tissue samples will be used for *ex vivo* analyses and mitochondrial function testing (e.g. liver).

A retrospective assessment of reduction will be due by 13 August 2030

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 will predominantly be using mice at an early life stage. The brains of mice develop late in relation to birth and at postnatal day (P)9-P12, mouse brain development approximately corresponds to the term human brain. Importantly, they share several important features with the human brain with regard to brain complexity and injury response in white and grey matter and thus can be considered a valid model in which to deliver the objectives of the project.

We will use the Rice-Vannucci model of neonatal hypoxic-ischaemic injury to mimic birth asphyxia and its consequence, which represents the closest rodent model to the human condition. In our previous licence, we have refined the anaesthesia reducing the overall surgery time and imposed a minimum weight limit below which, surgery will not take place. Where it is possible to do so, we also reduce the hypoxia time (e.g. for C57Bl6 mice this is now 40 min). We now perform surgery in batches if the litter size is greater than 7 so as to minimise time away from the dam. Using these conditions, the mortality rate remains low.

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

We are not able to use animals that are at a more immature stage of life as we are already using neonatal pups to reflect the brain injury we wish to model. We also cannot use animals that are less sentient or mice that have been terminally anesthetised because we need a model with a brain structure that is as close to term human brain as possible and in which an infarct will develop over time, mimicking the human condition. We also need to model both hypoxia and ischaemia and for this reason, need to use an animal with a circulatory system amenable to ligation. The original use of mice (and rats) in the Vannucci model (described in protocol 2) provided the first compelling evidence that therapeutic hypothermia would offer neuroprotection in human neonatal patients. Therefore the mouse pup represents a relevant environment for exploring new therapeutic options for neonatal HI.

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

There is increasing evidence that vocalisation may provide an early indication of distress in young mice in situations where accepted behavioural characteristics highlighting suffering or pain may not be so obvious. In collaboration with the BSU, we will continue to explore how we may be able to use recent advances audio technology to make monitoring more effective e.g. by using iPhone-mounted bat monitors.

Equally, for young animals we will minimise the potential of rejection by the mother by rubbing the hands of the experimenter in bedding prior to handling the pups, to reduce transfer of unfamiliar smells. To this same end, routine monitoring will largely be through the side of the cage without disturbing the animals. Opening cages and handling animals will be limited as far as possible as part of the standard observation and behavioural testing procedures within the project, unless a symptom of pain or distress is observed under which circumstance it may be appropriate to increase the frequency of monitoring. For handling of mice, non-aversive handling guidance from the NC3Rs will be followed to avoid animal stress, both as a welfare issue and for a reduction in data noise (reducing animal numbers).

Over the course of the previous licence, we have worked to refine protocol 2 to minimise welfare costs. These refinements include minimising the time under anaesthesia, reducing hypoxia exposure time (whilst achieving the required outcome) and streamlining the aseptic set-up. We have also reduced gas flow and altered the input into the hypoxia chamber to prevent any temperature fluctuations. Finally we implemented a strategy so that any larger litters (>7) are processed in batches to minimise time away from the dam. These improvements have reduced the overall surgery time significantly.

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

We have always followed the published guidance of a collaborator and leader in the field, Prof Henrik Hagberg (University of Gothenburg), who refined the hypoxia-ischaemia procedure for mice including adaptations for use in P4/5 mice (Hagberg et al, 2002, Albertsson et al, 2014). We also have collaborative links with Prof Susan Vannucci (Cornell University) who originally developed the hypoxic-ischaemic protocol and continuously publishes on its refinement (e.g. Vannucci and Back, 2022). We rely on Prof Donna Ferreira's study when adapting the surgery for strain-specific differences (Sheldon et al, 2019). We and others have also noted sex-specific differences which have further influenced our subsequent experiments (Mirza et al, 2015; Kichev et al, 2018).

References

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

We will stay informed through the NC3Rs website as well as taking advantage of the advice provided by the NC3Rs representatives, AWERB emails and current advice from the BSU staff.

A retrospective assessment of refinement will be due by 13 August 2030

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?