NON-TECHNICAL SUMMARY

Metabolic basis of microglia function in development

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
  - (ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Neuroimmunology, Neuropsychiatric disorders, Maternal immune activation, Maternal infections, Maternal diet

<table>
<thead>
<tr>
<th>Animal types</th>
<th>Life stages</th>
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<tr>
<td>Mice</td>
<td>neonate, pregnant, adult, juvenile</td>
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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 objective of this work is to determine how the metabolism of immune cells present in the brain of developing mice regulates their activity during normal development and after the activation of the immune system in their mothers (i.e., using mouse models of “maternal immune activation” or MIA). The ultimate goal of this project is to identify cellular and molecular pathways that can be modulated in immune cells to understand and treat major neuropsychiatric disorders.

A retrospective assessment of these aims will be due by 20 January 2029

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?

Evidence suggests that neurological and mental disorders (altogether “neuropsychiatric disorders”), such as autism spectrum disorders (ASDs), schizophrenia, and depression, are linked to maternal infection. Scientists analysed the medical records of ≈ 1.8 million pregnant women and their children and found that infection diagnosed in pregnancy increased the risk of children to develop ASD or depression in the following 41 years. Specifically, researchers found that there was a 79% increased risk of ASD among children and adults exposed to any maternal infection during pregnancy. This means that while 2/1,000 children developed ASD if there were no infections during pregnancy, this number increased to 5/1,000 if there was any maternal infection. Similarly, researchers found that there was a 24% increased risk of depression, which increased the number of depressed individuals from an average of 10/1,000 to 14/1,000 in case of any maternal infection. Several studies in animal models have confirmed that acute inflammatory episodes in pregnant females are sufficient to trigger brain alterations in the adult offspring, especially in those specific areas that are found to be affected in psychosis. From a mechanistic perspective, strong evidence supports the involvement of inflammatory
molecules released by the pregnant female during infections (e.g., cytokines, such as interleukin 6 and leptin, an hormone with effects on inflammation), which can reach the foetal brain leading to long-lasting detrimental effects. These data strongly support the idea that maternal infections (resulting in the activation of the immune system in the mother) can lead to the development neuropsychiatric disorders in children and adults later in life.

Additional evidence also suggests that maternal diet during pregnancy may play a role in the development of neuropsychiatric disorders in children. Maternal diet can influence inflammation in the brain, which in turn causes adverse neurodevelopmental outcomes such as ASD. Obese/overweight pregnant women show indeed an increase in circulating inflammatory molecules. A recent meta-analysis confirmed a significant increased risk for ASD in children conferred by being overweight and/or obese during pregnancy. These data suggest that the high-fat diet (HFD), which is becoming widespread in most countries, could be one of the causes of the rising incidence of ASD in the general population.

Therefore, key areas for research are to understand the role of maternal infections and maternal diet in inducing neuropsychiatric disorders in the offspring.

Previous studies identified “dark microglia” (DM) as a specific immune cell type that is nearly absent from the brain of healthy young adult mice and is instead significantly increased in conditions of brain damage or inflammation. The number of DM sharply increases in mature mouse offspring after the activation of the immune system in their mothers (i.e., in mouse models of maternal immune activation or MIA). Further work also showed that DM are present in the human brain of patients with neuropsychiatric disorders, where they contact and interact with the blood vessel and brain cells. Most importantly, recent data show that DM have a distinct way to produce energy (metabolism) and accumulate glycogen granules - a glucose storage - which is associated with a pro-inflammatory damaging activity of the cells of the immune system.

With this license, we wish to further explore how MIA caused by infection or diet regulates the interactions between the brain and the different types of immune cells (with a specific focus on DM). We will concentrate our efforts on studying the way DM produce energy to carry out their complex activities. This will create new opportunities to test new therapies that can stop inflammation, brain damage, and the subsequent development of neuropsychiatric disorders in the offspring.

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

The main output of this project will be new information on the mechanisms driving MIA in the mother that lead to brain inflammation and damage in the offspring. Additional outputs will include the publication of data from experiments in scientific journals. Datasets containing a large amount of information will be made accessible. We will use these datasets to apply for funds for future projects. These datasets will also be useful for other scientists and doctors studying neuropsychiatric disorders. Additional products from this project will be (1) tools to study immune and brain cell function or deliver therapies, and (2) technologies to identify if therapies are effective.

Ultimately, our approach will lead to the identification of new and important interactions of the brain and immune system. We can then create new methods to target these interactions and modify current approach to pregnancy to prevent neuropsychiatric disorders in the offspring later in life.
Who or what will benefit from these outputs, and how?

In the short term, the main beneficiaries of this project's outputs will be scientists from academic institutions and from pharmaceutical companies. In the medium term, the outputs generated by this project will help the NHS and the patient community. These outputs will advance the prevention and understanding of neuropsychiatric disorders. In the long term, these outputs will help us to create new tools that can be used to predict the risk of developing neuropsychiatric disorders in children and adults later in life by studying the metabolism of immune cells. In addition, we will better understand how to prevent the damage and brain reorganisation caused by MIA. This will lead to the identification of new targets that in the long term will be possibly modulated via specific therapies (e.g., anti-inflammatory) and/or dietary changes in pregnant mothers.

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

The outputs of this work will be distributed to academic scientists throughout the duration of the licence. We will communicate regularly with organised research networks and laboratory groups in the field of neuropsychiatric disorders and beyond. These networks will allow us to share workloads and ideas, but also to avoid repeated experiments, thus accelerating the progress in these fields.

Preliminary data will be shared at national and international conferences and/or workshops to gain valuable feedback from peers. This will also provide us the opportunity to build new collaborations locally and internationally, thus improving the quality and rigor of our research for the duration of the licence. We will also present our findings ahead of publication on preprint servers.

When finalised, all our data will be published in peer-reviewed scientific journals. This data will include gene, protein, and metabolic datasets. We will also make these datasets available on appropriate databases for other research groups to access freely.

We are committed to publishing both positive and negative results. This will increase awareness and inform the community of how our findings fit into the wider field of study. It will also help in determining which experimental outputs are worth pursuing further and those we feel would not be worth further exploration.

Finally, we will ensure that the published results are made available to the general public. This will maximize their impact and increase global awareness to both the public and fellow scientists.

Species and numbers of animals expected to be used

- Mice: 1,340

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 pregnant female mice because currently they are the most used animal model of human maternal immune activation research. It is indeed extremely difficult to model the complexity of maternal-offspring interactions during pregnancy using cells in a dish. Therefore, using mice we can study many of the changes caused by maternal immune activation in the offspring. These include the presence of inflammatory immune cells, changes to the brain cells and blood vessels. Additionally, the possibility to use genetically modified mice to target DM is particularly important, as this will allow to study the role of DM in maternal immune activation and possibly identify a new therapeutic target (or biological readout) to prevent neuropsychiatric disorders in children and adults later in life.

Since ASD are more common in men than women, some of our studies on the offspring will reflect this. Here, we will use an increased proportion of male mice to female mice in our long-term experimental assessment of the offspring. Additionally, we will also use both young and old mice (up to 6 months) as controls to document the role of the alterations induced by maternal immune activation vs infection and dietary changes occurring later in life.

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

Mice will be subjected to maternal immune activation induction via either exposure to Polyinosinic:polycytidylic acid (Poly(I:C)) or exposure to a high fat diet (HFD) during pregnancy.

Poly(I:C) is a chemical compound made of RNA that is used to simulate viral infections as it is very similar to the genetic material found often in viruses (i.e., double-stranded RNA). To model the Poly(I:C)-induced maternal immune activation, one or two female mice will be first paired with a male. At 12-16 hrs after mating, researchers will inspect the females for a vaginal plug (a gelatinous secretion deposited by a male into a female genital tract, such as the vagina, after successful mating). When a plug is seen, female mice will be weighted. Nine days afterwards, pregnant female mice will be weighted again and, if found to have gained >1.7g from their weight, they will receive one single injection of Poly(I:C) (or a control non-toxic solution) within the peritoneal cavity (the area that contains the abdominal organs). Pregnant female mice will be monitored for general health and weight twice a day in the 24hrs post-injection and afterwards on daily basis for a week. After birth, the male will be either killed or kept for future breeding. The females that received the Poly(I:C) injection will be killed after weaning of the litter, while the offspring will be kept until the end of the experiment.

The western high fat diet (HFD) is a special diet that has a high content of fat (60%, compared to normal diet that contains only 10%), which is used to study diet-induced obesity. To model the HFD-induced maternal immune activation, female mice will be given the experimental HFD diet (or experimental control ketogenic, or control normal diet) starting 4 weeks before mating. In the first 2 weeks, they will receive a 50%-50% mixture of HFD and normal diet. Mice will be checked on daily basis for general health and weight during the 1st week, and 3 times during the 2nd week. Female mice will be then given only HFD and checked on daily basis for the 1st week and then 3 times during the 2nd week. After these 4 weeks, one or two female mice will be paired with a male and kept on HFD (or control diets) until the birth of the litter. After birth, the male and the females will be either killed or kept for future breeding (in case of females, mice can be used again as breeders in protocol 2 of this PPL for a maximum of 3 litters). The offspring will be kept until the end of the experiment.
The study lengths for both maternal immune activation models may be as short as 21 days or as long as 6 months (maximum limit) to study both early and delayed inflammatory responses. Age and sex matched adult mice obtained from conventional breeding (i.e., without maternal immune activation) and exposed to either one single injection of Poly(I:C) within the peritoneal cavity or experimental diet can be used as further controls. Blood can be collected from these control and experimental mice using a superficial vessel during the experiment (once a week). The duration of each experiment is determined prior to the use of any mouse study and is variable in length. At the end of the experiment the offspring will be humanely killed, and tissues and organs collected. Tissue and organs collected this way will be stored in a solution that preserves their structure until follow-up analyses.

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

Within the Poly(I:C)-induced maternal immune activation protocol, mice are expected to display some signs of pain after injection, but this resolves quickly. Since this is an experimental modelling of a viral infection, the female mice are expected to develop symptoms within 24-48 hrs post injection, with a peak sickness response within the first 24hrs post-injection followed by a progressive improvement of health status within a week. Therefore, all pregnant female mice will be monitored for general health and weight at the time of injection and twice a day in the first 24hrs. After the first 24hrs, monitoring will depend on the symptoms mice display. These symptoms are classified as:

“No symptoms”.

“Stage I”, which are mild and expected. Stage I signs/symptoms include hunched posture, raised hair on the back, reduced activity compared to normal mice, weight loss (<10% from pre-injection weight).

“Stage II”, which indicates moderate signs/symptoms. Stage II signs/symptoms include dehydration, significantly reduced activity, repeated coughing or sneezing, diarrhoea, blood or discharge from vulva, weight loss (>10% but <15% from pre-injection weight).

“Stage III”, which are severe signs/symptoms that will require killing the female immediately. Stage III include coldness to the touch, continuous open-mouthed breathing for at least 5 minutes, immobility, vaginal or uterine prolapse lasting more than 1 hr, difficult birth (dystocia) lasting more than 1 hr, more than one generalized (“grand mal”) seizures, weight loss (>15% from pre-injection weight).

The high fat diet (HFD)-induced maternal immune activation is associated with an increased risk of depressive-like behaviours and obesity. Due to the disruption of normal feeding patterns in the initial first week of a full HFD introduction, mice can lose weight. Therefore, to prevent dietary change impact shock, experimental mice will be eased into this special diet starting 4 weeks prior mating. During pregnancy HFD is usually tolerated, but it can increase the size of the foetuses. This can lead to difficult birth (dystocia) or vaginal/uterine prolapse after birth. In case of difficult birth, female mice will be given maximum 1 hr to complete the delivery, if this does not happen, mice will be culled humanely. In case of vaginal/uterine prolapse after birth, female mice will be given maximum 1 hr to heal, if this does not resolve, mice will be culled humanely.

For both the Poly(I:C)-induced maternal immune activation and the high fat diet (HFD)-induced maternal immune activation experimental and control newborn mice are supposed to develop normally and have a normal life span after birth. In these mice, collection of blood using a superficial
vessel will only lead to temporary discomfort for the mouse (equivalent of a very fine needle jab). The majority (>90%) of these mice remain healthy up to 6 months of age.

**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: Moderate 90%

Mouse: Severe 10%

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

- Killed

**A retrospective assessment of these predicted harms will be due by 20 January 2029**

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?**

Neuropsychiatric disorders have received increasing attention from researchers during the past decades. The burden of disease is substantial, with more than 164 million people in the EU alone suffering from these diseases.

The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2019 showed that the most disabling mental disorder is depression, ranked among the top 25 leading causes of burden worldwide in 2019. While depression can occur at any age, ASD are the most common neuropsychiatric disorders of the childhood. It is estimated that worldwide about one in 100 children has ASD. This estimate represents an average figure, and reported prevalence varies substantially across studies. Some well-controlled studies have, however, reported figures that are substantially higher. Little was known about the causes of these disorders, until recent evidence suggested that events occurring during pregnancy can later cause neuropsychiatric disorders in the offspring. These events include stresses and stimuli like infections and inflammation that increase the activity of the brain immune cells (microglia) in the foetal brains.
Given the complexity of the mother-foetal interactions, the use of an animal experimental model in mice is necessary in this project to examine in a longitudinal manner the cellular and molecular mechanisms that underlie the normal developmental period, onset, and progression of neuropsychiatric disorders, as well as treatments promoting beneficial microglial activity. In fact, complete in vitro approaches to model these aspects do not exist. The choice of the mouse model is justified not only by the excellence of the experimental model, but also by the need to use transgenic mice that can be used to remove certain cells (like "dark microglia") from the brain. This will be important to identify new players and targets for treatment.

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

Over the years our team has refined and improved upon our models of cells grown in lab culture dishes. This has allowed us to test whether treatments will be (1) safe for cells and (2) how effective the treatments are before testing them in a mouse. Additionally, we have developed a new model of maintaining and expanding human immune cells and human stem cells in plastic dishes that does not involve the use of mice.

This new system allows us to (1) capture the response of human cells in a dish and (2) design/perform experiments to test ideas about the response of cells without having to extract these cells from mice beforehand. We also investigated previously published gene expression datasets (RNA sequencing) maternal immune activation models, but single cell RNA sequencing approaches at different stages of maternal immune activation are lacking.

**Why were they not suitable?**

Immune cells grown in lab culture dishes are useful for studying some aspects of immune activation. However, they cannot replicate the complex changes that occur in the cells and tissues of a living organism, nor the interactions between the pregnant mother and the offspring.

These include changes to the cells of the brain and spinal cord (neurons) and to the immune cells of the brain (microglia). Immune cells and neurons grown in culture also behave differently to those found in a living organism, as they show loss of cellular heterogeneity (i.e., the unique identity of individual cells) and loss of communication with other cell types.

It is therefore necessary to use animal models. This allows us to assess the complexity of biological and behavioural responses throughout the animal lifespan.

**A retrospective assessment of replacement will be due by 20 January 2029**

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?

Mouse numbers were estimated based on data reported in the literature and data provided from our collaborators. With the new mouse colony management system in use, we now have access to our total mouse usage year-over-year.

For the Poly(I:C)-induced MIA protocol, we are expecting to use $n=120$ female mice for pregnancy/breeding. With an average of 5 mice per litter, this will lead to 300 offspring. Control adult mice will be 200. Over the course of this five-year licence we will use an estimated total of 620 mice in this experimental protocol.

For the HFD-induced MIA protocol, we are expecting to use $n=120$ female mice for pregnancy/breeding. With an average of 5 mice per litter, this will lead to 300 offspring. Control adult mice will be 300. Over the course of this five-year licence we will use an estimated total of 720 mice in this experimental protocol.

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

The National Centre for the Replacement Refinement & Reduction of Animals in Research (NC3Rs) experimental design assistant is a tool which we constantly use to help design and further refine our experiments. We also reference the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines.

According to our lab standard operating procedures (SOPs), experiments are constantly assessed at the pilot stage first. This is when a first experiment is conducted with a reduced number of mice to adjust any aspects before running the full experiment. This guarantees that we are using the correct number of mice to achieve reliable statistical results when experiments are ready to be conducted in full. Mice are then placed in the experimental groups randomly, which helps to ensure treatment and non-treatment groups are evenly distributed. Treatments are given ‘blind’. This means that the person giving the treatment (or a vehicle, as control) have been given no access to the information related to the treatment they are giving. Unblinding (i.e., informing the scientists of the treatment given) is the responsibility of the principal investigator and it is done only after the experiment is concluded and results are analysed. This is to avoid any bias in the generation of the results.

Specifically for female mice in Protocol 1, we noted that the presence of a plug (i.e., a gelatinous substance that covers the entry of the female reproductive tract after mating) is not a definitive indicator of true pregnancy, particularly in inbred mice (in which false-pregnancy rates have been reported to be 50% or higher). This could mean that female mice may receive the Poly(I:C) injection even if they are not pregnant. To reduce this occurrence, we have implemented our approach by using data from literature showing that if female mice are found to have $>1.7g$ weight gain in the first 10 days
of pregnancy, the false-positive rate (i.e., the rate of false pregnancies) is reduced to 10.5%, without excluding any pregnant mice. Thus, we have included a threshold of 1.7 g weight gain at day 9 in our mice, as a further check before giving the poly (i:c) injection.

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 always perform pilot studies before undertaking a full experiment to ensure that larger studies are as accurate as possible. These pilot studies allow us to assess the experimental design and identify potential problems, as well as implement improvements early on in the licence. We are also coordinating with other groups to share animal tissues - including tissues from genetically modified mouse lines and post-mortem tissues - to further reduce overall mouse numbers.

A retrospective assessment of reduction will be due by 20 January 2029

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.

The maternal immune activation hypothesis proposes that inflammation in utero can affect fetal neurodevelopment, and evidence from human epidemiological studies supports an association between maternal inflammation during pregnancy and offspring neurodevelopmental disorders. Rodent models of maternal immune activation are increasingly used as experimental tools to study these neuronal and behavioural dysfunctions in relation to neurodevelopmental disorders.

One of the most widely used maternal immune activation models is based on gestational administration of Poly(I:C), a synthetic analogue of the genetic material found often in viruses (double-stranded RNA). This challenge induces a viral-like acute phase response that causes neuropathological alterations in the offspring. Another most widely used maternal immune activation model is based on changing maternal nutrition, which is critical for proper fetal development. While increased nutrient intake is essential during pregnancy, an excessive consumption of certain nutrients, like fat, can lead to long-lasting detrimental consequences on the offspring. Animal work investigating the consequences of HFD revealed in the offspring a maternal immune activation phenotype associated with increased inflammatory signals.
While invertebrate organisms share some elements of metabolic control with humans, to obtain meaningful data we must use mammalian models of maternal immune activation. Rodents have the enormous advantage of being readily susceptible to genetic manipulation enabling precise alteration in the function/expression of specific genes and the creation of animal models of relevant human diseases. In addition, the complexity of the mammalian brain is key to model central metabolic-sensing circuits, and this research question cannot be addressed in lower organisms.

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

We are extremely limited in the use of invertebrates (e.g., worms), fish, or amphibia. These animals are not fully suitable for the development and testing of new therapeutic targets for humans with neuropsychiatric disorders. Some preliminary work on regenerative biology can be done in non-mammalian species. However, the complexity of the interactions between the maternal and foetal immune system and the brain in the context of infection and diet can only be studied in mammals. This is because they possess a body structure with similarities to the human central nervous system and immune system. We also cannot rely completely on animals that have been terminally anaesthetised as we need to produce offspring from the pregnant females that will undergo treatment.

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

To cause the least pain, suffering, distress, or lasting harm to the animals, we will adopt several refinement steps, as follows:

Minimising suffering. In designing experiments, we use standard operating procedures and non-invasive techniques whenever possible to keep suffering to a minimum. Early detection of side effects to prevent animals’ suffering will be performed and analgesics will be used when appropriate.

Improving environmental factors. When using single housing, we always add old bedding to maintain the olfactory environment, add old standard nesting and enrichment material, and keep the old cage for when the animals return to group housing if appropriate. We will regroup mice whenever possible after short term single housing sessions. In these cases, we will monitor for potential aggressive behaviours.

Refining the use of gene inducing agent (Protocol 2 only). We are optimising the use of gene inducing agents to avoid side effects and increase efficiency using pilot studies and trying ways to limit weight loss (supplementation with diet mash during tamoxifen administration).

Welfare assessments. Weight loss will be used to assess welfare in the majority of the cases. However, when physiological response occurs in response to scientific procedures, weight loss is not an indicator of well-being. In mice presenting weight loss, we will always determine whether the presence of other clinical signs justifies the interruption of the experiment and seek the advice of the NVS to avoid unnecessary animal waste.

Further refinements will include increased monitoring and pain management.
Before starting any study plan, we will discuss all experimental methods with the appropriate staff within the animal unit. This will guarantee that all the necessary equipment is in place to perform procedures under optimal conditions and/or supervision. This is to maintain the best health and welfare of the animals. Prior to running studies, we will determine if the necessary staff and expertise is available to successfully run the whole study. This way we can ensure that no skills are missing to guarantee the study is successful and that relevant equipment is available to process samples under optimal conditions.

Once the study has started, we will rely on our established step-by-step care packages. This will minimise the harm to the mice and ensure that the welfare of the mice is never compromised.

Refinements will be centred around the housing of mice experiencing expected adverse effects and to the daily care and monitoring of the mice, which include the following: (1) providing bedding that does not inhibit the free movement of these mice, (2) heating pads fixed to the bottom of the cages to maintain stable core body temperature, (3) placing wet mashed food on the cage floor to encourage eating and allow ease of access to disabled mice, and (4) providing cardboard houses.

If needed, we will also use pain medications to ease disease complications (after NVS approval), and perform fluid replacement through subcutaneous (i.e., under the skin) injections if dehydration is present. As part of our daily monitoring, mice will have their abdomen checked by an experienced user to identify signs of infection or dystocia.

Finally, full training will be provided to new technicians who are unfamiliar with these procedures. This helps new technicians to learn how to assess our mice correctly. This guarantees that mice recovering in our experiments receive the same high quality and consistent level of monitoring and care they need.

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

We plan our experiments in accordance with the guidance provided in the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines. This will guarantee we use the minimal number of animals to answer our objectives and ensure our results are both robust and reproducible. We will follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines when preparing our data for publication. In so doing, we will ensure our published findings are complete and clearly presented and easily accessible to other groups. This will lead to a reduction in the unnecessary duplication of animal experiments.

Excellent information is available on our establishment website, which is routinely updated with new 3Rs information. The NC3Rs website will be regularly consulted to be sure that we are applying the latest recommendations for the refinement of our experiments. We will also consider any new publications in a peer-reviewed journal relevant to our field offering refinements to our protocols.

**How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?**
Our establishment offers continuous training and recommendations via the animal facility and from animal care staff located within. We will keep informed of any changes to animal welfare guidelines by regularly consulting the website they provide. This will ensure that we maintain compliance should any new updates be posted.

The NC3Rs will be the main reference to assess whether our experiments match the highest standards of 3Rs. We will adapt our protocols if the recommendations evolve throughout the duration of this project. Regular consultations on the latest practical guidance from Laboratory Animal Science Association (LASA), Institute of Animal Technology (IAT), and the Royal Society for the Prevention of Cruelty to Animals (RSPCA) will provide additional sources of new recommendations and advances in animal techniques and clinically applicable models.

Training records for all personal licence holders will be kept up to date using a centralised database. Senior group members will provide extensive training on the relevant regulated procedures to all new lab members who will be working with animals. Further, new lab members will be informed of the mandatory training services available to them. This will guarantee that general practices are firmly adhered to and will ensure the welfare of the animals is consistently upheld. As a licence holder, it is my own responsibility to stay updated on published best practices. This will be done by consulting information for licence-holders provided by our establishment and by speaking to other project licence holders.

A retrospective assessment of refinement will be due by 20 January 2029

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?