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NON-TECHNICAL SUMMARY

Regenerative Neuroimmunology Applied to Progressive Multiple Sclerosis Research

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, Progressive MS, Brain-Immune Interactions, Regenerative Medicine, Therapy

Animal types	Life stages
Mice	Adult, Aged animal

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 this project is to identify and manipulate important biological processes that control the coordinated actions of the immune system and the brain in the context of progressive multiple sclerosis (MS). The ultimate goal of this project is to increase the brain’s own ability to heal the damage caused by MS, which will prevent the accumulation of disabilities and slow down the progression of the disease.

A retrospective assessment of these aims will be due by 8 December 2031

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?

This work is important because it establishes a reliable foundation for research into neurological diseases. Studying cellular responses to inflammation in vitro (experiments done outside an organism) helps identify key processes and potential treatment targets early in the research. Additionally, this work will improve disease models and enhance understanding of normal development and how a disease works in the body (disease mechanisms) in both young and adult mice. Confirming experimental targets will guide future studies and increase the relevance of findings to human health. Ultimately, the goal is to better understand how chronic inflammation affects the body, especially in conditions like multiple sclerosis (MS), and to contribute to the development of more effective treatments. This research has significant potential to advance medical science and improve patient outcomes.

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 function and therapeutic potential of mechanisms that lead to continued brain inflammation. 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 progressive MS. Additional products from this project will be (1) patents to protect the main discoveries, (2) tools to study immune and brain cell function or deliver therapies, and (3) technologies to identify if the 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 use new experimental methods to target these interactions. These experimental methods could then be tested in patients with progressive MS.

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. Specifically, these outputs will advance treatments for progressive MS that are aimed at slowing down disease progression and promoting brain repair.

At an experimental level, the behavioural-cognitive tests will help us understand the role of immune cells in cognitive decline in progressive MS and thus, develop therapies to precisely target them. Furthermore, the use of imaging methods that can be used in the clinic will help us to achieve a better understanding of how neuronal circuits are organized and which brain regions are mostly affected by cognitive impairment. In the long term, this combined approach will allow us to find new ways to slow the damage to brain caused by brain inflammation and help the ever-growing senior population maintain healthy brain function.

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 organized research networks and laboratory groups in the field of progressive MS and beyond. These networks will allow us to share workloads and ideas. This depends on the expertise of each group, avoiding repeat experiments, and accelerating the progress in these fields.

Preliminary data will be shared at national and international conferences and/or workshops in order to gain valuable feedback of the work from peers. This will also provide us the opportunity to build new collaborations locally and internationally. This will improve 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 (such as arXiv, bioRxiv, or PeerJ).

When finalized, all our data will be published in peer-reviewed scientific journals. This data will include information about genes, proteins, and how the body uses energy. 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.

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

Species and numbers of animals expected to be used

- Mice: 2500

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 new-born mice that have been produced on a different licence to collect cells to grow and keep in a dish to do experiments.

We are using mice because currently they are the most commonly used animal in human disease research. It is extremely difficult to accurately and fully model the complexity of MS using cells in a dish. Therefore, using mice we can study many of the changes caused by MS disease in the patient's cells and tissue. These include the formation of scar tissue, the presence of inflammatory immune cells, and damaged nerve fibres. Additionally, the possibility to use genetically modified mice to study target proteins and pathways involved in the MS disease process is particularly useful.

Since MS is 2 to 3 times more common in women than men, our studies will reflect this. Here, we will use an increased proportion of female mice to male mice in our experimental protocols. We will also use both young and old mice. This will allow us to investigate the biological impact of human adulthood and human ageing more accurately on the progression of brain and spinal cord inflammatory damage. This is highly relevant as progressive MS affects older adults more and is a chronic, life-long disease. Additionally, we now know that ageing plays a significant role in the ability of the brain to heal itself. This further emphasises the need to study the disease using both young and aged adult mice.

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

Mice will be subjected to a disease called experimental autoimmune encephalomyelitis (EAE) or will be subjected to Lysophosphatidylcholine (LPC) induced loss or damage of the myelin sheath, the protective wrapping around nerve fibres, which impairs the transmission of nerve signals. Mice may also undergo behavioural testing without EAE or LPC induction in order to compare the cognitive abilities of mice with a different genetic profile or condition and establish if the observed effects are unique to EAE and LPC or shared among other conditions The EAE model is used to replicate the immune components of MS and the LPC model is used to replicate damage to the nerve wrapping (myelin sheath) and see if it can repair itself. Mice will be subjected either to EAE induction or LPC induction but not both.

EAE is widely used to investigate aspects of the human disease multiple sclerosis. In mice, EAE disease results in immune cells normally found outside the brain and spinal cord, collectively called the central nervous system (CNS), invading the CNS. Here, immune cells interact with cells of the CNS to create an environment that is destructive to the tissue. This results in the mice displaying clinical signs of disability similar to those observed in MS patients. These include impairment in walking, paralysis of limbs, and issues with bladder control and output. These mice also display tissue level signs of the human disease such as the accumulation of inflammatory immune cells normally found outside the CNS, damage to nerve fibres, and destruction of the protective wrapping (i.e., myelin sheath) of nerve fibres.

EAE disease induction in mice is as follows. On day 0, mice will be maintained under anaesthesia and loosely restrained and positioned for injections. A protein of the myelin sheath will be mixed with an oil-based solution that has been supplemented with a heat-inactivated version of a disease-causing bacteria. This will create a dense mixture that will be injected under the skin of the mouse. This mixture will produce a local autoimmune response that results in the generation of inflammatory immune cells. These immune cells eventually enter the brain and spinal cord and start a neurological disease like in MS. The mixture will be injected under the skin at three separate locations on the mouse: 1) in front of the base of the tail, 2) in two sites near the left and right shoulder blades. Mice will also receive two consecutive doses into a vein of a solution containing a bacterial toxin. These doses will be given on the day of the mixture injection (day 0) and two days later (day 2). Following each of these injections, mice will be closely observed while they recover from the light anaesthetic until fully awake and responsive. The weight and overall condition of the body of the injected mice will then be monitored daily throughout the length of the study.

The Lysophosphatidylcholine (LPC)-induced MS-like lesion model is as follows. Mice will be anaesthetised and remain unconscious throughout the surgery. Mice will be given both pre- and post-surgery pain relief. Anaesthetised mice will then be securely fixed in a device to maintain a stable body position. Then, a small portion of the thin membrane covering the upper and middle part of the spinal cord will be exposed to reveal the underlying tissue. This way we can cause the localised destruction of the myelin sheaths. This is done by slowly injecting a small volume of a chemical that is known to be toxic to myelin sheath forming cells only (i.e., lysophosphatidylcholine-LPC) into the spinal cord tissue through a syringe and ultra-fine needle. Following the injection, the needle will be slowly removed. Mice will receive post-surgery care that includes pain medication, soft bedding, and access to (wet) mashed food.

Depending on the experiments, EAE and LPC-lesioned mice may receive additional injections. These injections can be intravenous (e.g., into a vein), intraperitoneal (e.g., into the abdomen), subcutaneous (e.g., under the skin), intrathecal (e.g., into the fluid filled spaces of the spinal cord), intracerebroventricular (e.g., into the fluid filled spaces of the brain), intraparenchymal (e.g., into the central nervous system tissue), and local into the spinal cord or brain. Injections will include either substances (such as drugs, beneficial small molecules, and agents to induce gene modification), viruses (as a vehicle to artificially carry foreign genetic material into cells of the mice), and cells. These substances are all meant to encourage the interactions between the immune system and the brain in a way that results in regeneration or less damage of the brain and the spinal cord. Fibre optics, thin light-carrying cables, may be placed into specific areas of the brain so that light or other signals can be delivered directly to those areas to control brain cell activity

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Mice will undergo behavioural-cognitive tests to evaluate memory, learning, anxiety and depression. These tests will involve placing mice in an open field box, an open circular platform, or an apparatus that has three arms for **no longer than 10 minutes**. We will assess if mice can remember a place they visited before or recognize objects they have seen, how they interact with another mouse that is not housed in the same cage, or if they prefer to drink sweetened water. The behavioural-cognitive tests will not cause any pain or distress to mice and anaesthesia will not be required. Mice subjected to behavioural cognitive tests may be single housed for 2 days before the last testing session of the whole study period. In case of tests assessing depression (sucrose preference test), mice may need to be single housed up to 6 hours (while the test and the relevant measurements are being performed). Mice from protocol 3 will undergo the same experience.

Mice may also undergo imaging of the central nervous system (brain and spinal cord), such as Positron Emission Tomography (PET) or Magnetic Resonance Imaging (MRI), to obtain data that can be readily applied to human studies. During imaging studies, mice will be under anaesthesia for typically 90 minutes, and the needle of a syringe may be inserted into a vein (e.g., tail vein or femoral vein, with a cannula), intraperitoneally, subcutaneously, or intramuscularly. This will be done to inject contrast agents (i.e. a substance that helps us distinguish different internal structures of an organ or the whole body, such as gadolinium-based contrast agents) or metabolic tracers (i.e. substances that can be used to see how the tissues consume and produce energy). In some mice the needle of the cannula or syringe may also be inserted into an artery to collect blood and measure the levels of the injected substances. In case specific imaging agents need to be used, animals may be subjected to up to 12 hours of food withdrawal (with continuous access to water), which is necessary to make sure that the obtained data are valid. Food intake can increase the levels of sugar in the blood which can in turn reduce the amount of imaging agents taken up by the tissues leading to incorrect conclusions.

Finally, to validate and identify biomarkers of brain and spinal cord damage and their role in the regenerative process, we may also collect bodily fluids from live mice. These include blood and cerebrospinal fluid (CSF). CSF is a clear fluid that surrounds and cushions the brain and spinal cord from injury. These fluid samples are important for verification or discovery research using technologies that enable the rapid testing of large numbers of substances and molecules for activity. Blood is obtained from a surface vein observable to the naked eye using a fine needle. CSF is obtained via a quick, minimally invasive surgical procedure under general anaesthetic so that the mouse is unconscious.

The study lengths for experiments using EAE or the LPC-induced MS-like lesion model may be as short as 7 days or as long as 6 months (maximum limit) to study both early and delayed regenerative responses. 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 all mice will either be humanely killed or tissues and organs collected under deep, terminal anaesthetic unconsciousness. 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 EAE protocol, mice will present with limb weakness that progresses to total paralysis. Total paralysis of a limb or limbs often leads to weakness in other functional limbs. The effects of EAE begin with weakness of the tail muscles that spreads towards the back legs. This weakness can also spread to the front legs. The severity of the weakness or paralysis can last from 10-35 days after the first sign

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of disease are observed. The first signs of disease are usually observed at 10-15 days following injection with the disease-causing mixture. Within 21 days from the injection of the disease-causing mixture, over 90% of mice show a long-lasting and irreversible disease course. All mice with the disease gradually lose body weight following injection with the disease-causing mixture. The amount of weight loss that occurs depends on the severity of the disease in each individual mouse. This weight loss is temporary. All mice gradually gain back the weight that was lost. This recovery of weight occurs during the 2-3 weeks after the most severe effects occur. In some cases, mice may develop skin lesions or ulcers associated with disease progression, which they may scratch and occasionally reopen.

Within the LPC-induced MS-like lesion protocol, mice are expected to display signs of pain after surgery. This can last for up to 10 days after surgery. Steps are taken to minimise the pain by providing pain medications to mice before and after surgery. Mice will also have temporary weight loss after surgery. The weight loss is expected to recover within 48 hours of the end of the surgery.

In mice that receive additional injections of substances or implantation of optical fibres, there could be temporary pain and discomfort. This will last for a maximum of 48 hours (depending on the route of injection). Collection of blood through the vein in the tail will only lead to temporary discomfort for the mouse (equivalent of a very fine needle jab). Collection of cerebrospinal fluid may cause changes in (1) food/liquid intake, (2) normal expected weight gain, and/or (3) temporary (max 48 hours) pain at the site of collection. Analgesics will be provided to manage any resulting discomfort or pain.

Within this licence, some mice used within the EAE and LPC-induced MS-like lesion protocol will be aged. The majority (>90%) of adult mice remain healthy up to 15 months of age. Mice aged past this point (up to 24 months) may develop health problems due to their old age. This can include: (1) the growth of small, non-cancerous tumours under the skin (10%), (2) hair loss (10%), (3) loss of fur colour (i.e., greying of fur) (10%), and obesity (i.e., non-healthy weight gain) (10%). This should not cause any adverse effects or suffering in the mice.

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 25%

Mouse: Severe 75%

What will happen to animals used in this project?

- Killed

A retrospective assessment of these predicted harms will be due by 8 December 2031

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?

Progressive MS in humans leads to permanent and irreversible damage to the brain and spinal cord. This leads to a life-long decrease in the quality of life of patients and their carers. Therefore, we need to develop treatments that are able to reduce the impact of non-stop inflammation on the human brain and spinal cord to promote its healing. This is a main unmet need for patients with progressive MS. However, no experimental treatment can be tested in humans without first testing its safety and effectiveness in relevant animal diseases that reproduce aspects of the human disease. Any treatment developed to work on cells grown in a lab dish has to be tested and refined in animals. This way we can see how the treatment works in the complex environment of the adult brain and spinal cord. We can also determine if the treatment causes a recovery of functions.

To confirm findings using our cells in a dish, we need to use validated and widely accepted mouse models of diseases that reproduce aspects of MS seen in humans. This allows us to test whether the responses of immune cells in a dish and the effect of treatments are similar to the effects that occur in a mouse with the disease. The response of immune cells in disease is very different in a mouse compared to cells in a plastic dish. In a mouse with MS-like disease, there are many responses by the cells, tissues, and organs of the body which directly impact the ability of the brain and spinal cord to recover. These include inflammation, the formation of scars, and loss of nerve fibres necessary for normal movement of the body's limbs. It can also affect how well a treatment works. Further to this, injury to the brain and spinal cord and ageing also affects many other cell types that are present. This is a further challenge that limits our ability to study cells in a dish.

We are using mice because currently they are the most commonly used animal in human disease research. It is extremely difficult to accurately and fully model the complexity of MS using cells in a dish. Therefore, using mice we can study many of the changes caused by MS disease in the patient’s cells and tissue. These include the formation of scar tissue, the presence of inflammatory immune cells, and damaged nerve fibres. Additionally, the possibility to use genetically modified mice to study target proteins and pathways involved in the MS disease process is particularly useful.

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 the treatments will be (1) safe for the 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. After the identification of targets, we will then proceed to test our results in our mouse models of disease.

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Why were they not suitable?

Immune and stem cells grown in lab culture dishes are useful for studying some aspects of progressive MS and ageing. However, they cannot replicate the complex changes that occur in the cells and tissues that support the function of the brain and spinal cord. These includes changes to the supporting cells of the brain and spinal cord (called glia). This can lead to the formation of scar tissue, the recruitment of immune cells into the injury site, and damage to nerve fibres that is typical of progressive MS. Immune and stem cells grown in culture also behave differently to those found in a living organism. Here, they show loss of cellular heterogeneity (i.e., the unique identity of individual cells) and loss of communication with other cell types.

A retrospective assessment of replacement will be due by 8 December 2031

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 a combination of the retrospective review, annual return of procedures, and estimated mouse usage for the duration of the project. The retrospective review refers to looking back at records from previous years to understand how many mice were used in similar studies and how often procedures were performed. With the new mouse colony management system in use, we now have access to our total mouse usage year-over-year.

For the experimental autoimmune encephalomyelitis (EAE) protocol, we are expecting to use 300 mice/year based on our usage over the last 5 years. Each study typically involves 50 mice and lasts approximately two months. Therefore, we can run six studies per year that includes 300 mice. Over the course of this five-year licence we will use a maximum total of 1500 mice in this experimental protocol.

For the lysophosphatidylcholine (LPC)-induced MS-like lesion protocol, we are expecting to use 100 mice/year based on our usage over the last 5 years. This will provide us a maximum total of 500 mice used across in this experimental protocol.

For the behaviour testing protocol, we are expecting to use 100 mice/year based on our usage of other protocols over the last 5 years. This will provide us a maximum total of 500 mice used across 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. The NC3Rs Experimental Design Assistant (EDA) (https://eda.nc3rs.org.uk/) guides researchers through a structured workflow, prompting consideration of key design elements such as ran. We also reference the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines.

Power calculations were conducted using G*Power to determine the minimum sample size needed to achieve statistically significant results. These calculations were informed by data from previous studies and, when available, pilot data. Assumptions regarding expected variability, significance level (e.g. $\alpha = 0.05$), and desired statistical power (e.g. typically 80–90%) were incorporated into the calculations. The appropriate statistical test for each experimental design (e.g. independent t-test, one-way ANOVA, two-way ANOVA with repeated measures) was selected prior to running the power calculation, as the required sample size is test-dependent. This approach aligns with the Animals (Scientific Procedures) Act 1986 (ASPA) requirement to use the minimum number of animals necessary to achieve the scientific objectives, while avoiding underpowering — which risks missing true effects — and overpowering — which would expose unnecessary animals to procedures. Where pilot data were not yet available, conservative estimates of effect size and variability were used to avoid underestimating the required sample size, thereby preventing the need for unplanned repeat experiments at a later stage, which would itself result in greater animal use.

According to our lab standard operating procedures (SOPs), experiments are first assessed at the pilot stage. This is when the 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. The pilot stage also serves to validate that the variability and effect sizes assumed during power calculations are realistic, allowing sample size to be confirmed or adjusted in a transparent and justified manner before the full cohort is committed. Mice are then randomly assigned to the experimental groups, which helps ensure that treatment and non-treatment groups are evenly distributed. Treatments are given ‘blind’. This means that either the person administering the treatment (or a vehicle as a control) or the surgeon performing the injections has been denied access to the information related to the treatment they are administering. Blinding is also applied to post-mortem tissue and molecular studies. Unblinding is the responsibility of the principal investigator, and it is done only after the experiment is concluded and the results are analysed. This is to avoid any bias in the generation of the results.

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 - in order to further reduce overall mouse numbers.

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A retrospective assessment of reduction will be due by 8 December 2031

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.

Mice will be subjected to a disease called EAE. This is a widely- and consistently used method to investigate aspects of human MS disease. It has undergone continual refinements over decades of research. The induction of EAE disease is the most refined for the purpose to cause the least pain, suffering, and lasting harm to the mice. Despite these refinements, mice that develop physical signs of EAE inevitably suffer and are in pain. We minimise and manage pain by providing mice with pain medications as needed throughout the course of the study. The substances used to induce EAE disease are certified as high-quality from trusted suppliers. The substances do not contain impurities and other potentially toxic components which could impact on the welfare of the mice. To reduce the chance of ulceration (i.e., open wounds forming) from injections, the area is wiped down with a sterile cleaning solution. The substances to induce disease are administered under the skin and into the vein and result in minimal and temporary discomfort.

The lysophosphatidylcholine (LPC)-induced MS-like lesion requires surgery to inject a toxin that damages the protective covering of nerve fibres. This injection is in the bottom part of the spinal cord. This model allows for the study of tissue damage (loss of protective covering of nerve fibres) and regeneration (regrowth of the protective covering of nerve fibres) in the spinal cord. The toxin-induced lesions very closely resemble the lesions observed in MS patients. This method induces moderate pain and suffering with no lasting harm. We minimise and manage pain by providing mice with pre- and post-surgery pain medications. Pain will be managed during the period of study by administering medication as needed. This MS-like lesion rarely results in mice developing clinical signs or complications from surgeries. This produces extremely limited suffering to the mouse.

Behavioural testing will be done to study how the immune system and brain cells interact and how this affects memory, behaviour, and mood in mouse models relevant to different types of dementia. Mice will undergo well-established behavioural tests that are designed to cause minimal stress and rely on natural behaviour; these tests are performed on dry land, require little movement or training, and do not involve swimming, electric shocks, or food or water deprivation. Where necessary, mice may be housed individually for a short period to allow accurate measurement of brain activity. Imaging agents and other substances will be administered using refined, established methods to minimise discomfort,

with anaesthesia used when appropriate. During scans, mice will be kept warm and closely monitored, and after each procedure they will be allowed to recover fully with access to food and water.

Within our mouse models of MS and MS-like lesions we will also be using aged mice (i.e., older than 15 months). This is the closest way to study how increasing age impacts brain recovery. Mice will be naturally aged (i.e., no manipulations to the mouse to force accelerated ageing will be performed). This is the most refined for the purpose to produce the least suffering.

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 treatments for humans with progressive MS. Some preliminary work on regenerative biology can done in non-mammalian species. However, the complexity of the interactions between the immune system and the brain and spinal cord in the context of tissue damage and regeneration can only be studied in mammals. This is because they possess a body structure and immune system with similarities to the human brain, spinal cord, and immune system.

We also cannot rely completely on animals that have been terminally anaesthetised. This is because we need to assess the long term behavioural and pathological outcomes of our manipulations and interventions. We need to know if they promote regeneration of the damaged and diseased brain and spinal cord. Therefore, we need the animals to remain alive for several weeks-to-months after the onset of disease or after making a lesion in the spinal cord using a toxin.

Finally, we need animals with cells that have reached a mature stage of development. This is because we need the cells to be representative of the cellular make-up present in adult human disease. Therefore, we will use adult and/or aged animals for all pre-clinical animal models in this project. The use of animals during the immature stages of life will be restricted to the collection of cells to be grown in lab dishes where indicated and appropriate.

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

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 determine which skills are missing to guarantee the study is successful. As well, which relevant equipment is available to process samples under optimal conditions. Refinements to the study design will be guided by prior literature (e.g. the study by Wolfensohn S et al. Reducing suffering in experimental autoimmune encephalomyelitis (EAE). J Pharmacol Toxicol Methods. 2013 May-Jun;67(3):169-76. doi: 10.1016/j.vascn.2013.01.009). This paper provides practical recommendations for refining EAE protocols to minimise animal suffering, several of which have informed the refinements described in this section.

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 that the welfare of the mice is never compromised. Over the years

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we have made significant refinements to our EAE mouse model. These refinements cause the least amount of pain, suffering, and distress to mice. As a result, we have put together a dedicated and comprehensive standard operating procedure (SOP) that provides a detailed step-by-step care package.

Refinements to this model are centred around the housing of mice experiencing expected adverse effects and to the daily care and monitoring of the mice. Refinements made to the housing of mice experiencing expected adverse effects 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. While housing all EAE mice in dedicated heating chambers would represent an ideal refinement, this is not always feasible in practice due to limited availability of heating chambers, particularly when studies involve large cohorts (>50 mice) or when multiple cohorts are run in parallel, with total numbers approaching 100 animals. Based on our experience, heat pads fixed to the bottom of the cage provide sufficient and effective thermal support for mice scoring 3–3.5, including during the acute stage of disease. However, as an additional refinement, mice approaching the humane endpoint will be transferred to a heating chamber for a minimum of 24 hours to ensure optimal thermal support during this critical period; (3) placing wet mashed food on the cage floor and dry food pellets directly on the cage floor or hand/syringe feeding during a 24-hour monitoring period if experiencing higher clinical scores to encourage eating and allow ease of access for disabled mice; (4) using cardboard eco-dishes with the sides cut down to improve accessibility to feed, and (5) providing cardboard houses to mice with ulcers to distract the mice from continuing to itch and re-open the wound. Mice have a natural instinct to gnaw, and cardboard offers an appropriate outlet for this behaviour. Engaging in chewing and manipulating the box can serve as a behavioural distraction from ulcer-associated pain or irritation, thereby reducing attention directed toward the affected area.

For the daily care and monitoring of the mice we have made a number of improvements. We have increased the number of daily checks to ensure the health and welfare of the mice is maintained. We now use pain medications to ease disease complications, and perform fluid replacement through subcutaneous (i.e., under the skin) injections if dehydration is present. Mice can experience bladder dysfunctions such as increased frequency of urination with decreased urine output. Mice can also have issues with urinating that results in the build-up of urine in the bladder. Here, as part of our daily monitoring, mice will have their abdomen checked by an experienced user to identify signs of an obstructed bladder. If the bladder cannot be emptied by manual stimulation through the skin, a veterinarian will be contacted to provide immediate care.

Having a dedicated SOP ensures that the highest quality of care is provided to mice throughout the course of this severe study. The SOP also significantly minimises the suffering and improves the welfare of mice experiencing expected adverse effects. This SOP has been generated and further refined following recommendations from animal technicians, named animal care and welfare officers, and the named veterinary surgeon. The SOP includes a comprehensive score sheet to support these animals. The SOP and protocol for EAE induction are stored on a dedicated external storage device as a living document. The SOP and EAE induction protocol are constantly updated so that any refinement procedure that is found to be beneficial is kept and stored in our system for training (and access) by future users. For example, our new protocol for EAE induction has led to the reduced incidence of ulcer formation. It is now standard in our lab and is used by all new trainees and existing users.

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In case of the LPC-induced MS-like lesion model, or any surgery, we have put in place post-operative assessment sheets specific to our model of injury or disease. These assessment sheets help the technicians and users to better monitor the recovery of the mice after surgery. This post-operative assessment sheet is constantly refined during our work, depending on our observations and in collaboration with the animal unit staff. Incidence of pain during the post-surgery recovery period will be controlled by the administration of pain killers, as directed by the named veterinary surgeon. Surgeries will inevitably cause temporary pain that is gone within 48 hours. This temporary pain will be minimised by the use of pain medications. Regular discussion with a named veterinary surgeon will allow us to improve the management of pain if any new and more suitable recommendation appears during the work. In the case mice suffer from complications after surgery, appropriate guidelines are in place for humane endpoints.

To further minimise any stress during the assessment of memory/learning, as well as anxiety/depression, mice will be accustomed to being handled by the experimenter before each behavioural-cognitive test. Upon completion of these tests (or between trials of the same test) gel products can be provided in the cage to guarantee additional hydration and nutrients. As further refinement, the possibility to use lower amounts of immunogen (a substance able to trigger an immune response) for EAE induction (aiming to reduce motor severity while conserving EAE incidence) may be also investigated for cognition studies.

Additionally, it can be tested if the duration of single housing can be reduced to one day (instead of two days according to the literature) without compromising the quality of the data acquired for a marker of neuronal activation called c-Fos.

In imaging studies, fine non reusable needles will be used to minimise any damage to the vein or tail. Cannulation will normally occur while the animal is under anaesthesia, thus minimizing animal discomfort. In case of unsuccessful cannulation attempts, local analgesics will be applied to reduce any pain. Analgesia will be provided, when necessary, to reduce any pain. During the scans, mice will be placed on scanner beds, which are pre-warmed. Upon completion of the scanning, the cannula will be removed, and bleeding will be stopped before the animal is returned to its cage. Mice will be then kept in an individual warm environment with access to food and water to recover from the anaesthesia.

Adult mice undergoing surgical procedures will rarely have complications 48 hours post-surgery. However old mice (15 months and older) may have an increased risk of complications. This includes a lack of recovery to normal baseline of activity levels and food intake. Proper precautions are in place to handle old mice after surgery. This includes daily welfare checks to ensure mice are not suffering, in pain, or distressed until fully recovered. If necessary, pain medications will be given either via (1) an injection under the skin by an experienced user or (2) as a voluntary treatment to be eaten by mixing pain medication via flavoured jelly, paste, or milk shake. Appropriate humane endpoints are defined if old mice are unable to recover.

All substances intended for use in mice will be reviewed using current published and unpublished data to assess safety and efficacy. For substances that are novel or for which insufficient safety data exist, their chemistry and formulation will be reviewed and verified by subcontracted Contract Research Organisations (CROs) prior to use in animals under this Project Licence. This step ensures that any unknown tolerability risks are identified and addressed before animals are exposed to such substances.

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Full training will be provided to new technicians who are unfamiliar with these procedures, as we have filmed previous study procedures to show how we expect our mice to recover. 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 National Centre for the 3Rs (NC3Rs) website will be regularly consulted to be sure that we are applying the latest recommendations for the refinement of our experiments. The Laboratory Animal Science Association (LASA) website provides updated information, especially regarding best research practices to perform aseptic (i.e., germ free environment) surgeries. We will also consider any new publications in a peer-reviewed journal relevant to our field offers 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. The establishment’s Named Information Officers will share 3Rs updates and guidance with our team. 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 National Centre for the 3Rs (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 surgical or non-surgical 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.

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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 8 December 2031

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?