Home Office

NON-TECHNICAL SUMMARY

Misfolded proteins in neurodegenerative diseases

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
- (c) Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Aging, Neurodegeneration, Therapeutic, Alpha-synuclein pathology, Gut-brain axis

Animal types	Life stages
Mice	Neonate, Juvenile, Adult, Pregnant adult, Aged animal

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Our aim is to understand the processes that lead to nerve cell death in mouse models of Parkinson's disease (PD) and test interventions at early stages that decrease the severity of the disease and help the development of treatments against PD.

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?

PD is the most common brain disease that causes impaired movement. It is caused by progressive nerve cell loss (termed “neurodegeneration”) and has no cure. According to the Parkinson’s UK website, around 166,000 people live with PD in the UK, and someone is diagnosed with PD every 20 minutes. The World Health Organisation (WHO) estimated in 2019 that around 8.5 million people were living with PD but by 2050, the number of people with PD worldwide will increase to 25 million due to the increase in the aging population. Current treatments focus on the movement symptoms of the disease, and aim to replenish the loss of dopamine, (an important chemical needed for proper brain function) whose level is diminished because of the death of nerve cells that produce and release it in the brain to control muscle activity and movement. However, these treatments are often prescribed too late to prevent the loss of nerve cells in the first place, and they also neglect the earliest non-movement aspects of PD that herald the onset of the disease. We need to be able to detect the disease before irreversible nerve cell death occurs to understand what causes its onset and thus prevent its progress.

The protein alpha-synuclein (aSyn) is the major pathological cause of PD due to its tendency to abnormally accumulate and form toxic clumps -a process known as “aggregation”. Since its discovery in 1997, aSyn clumping has been identified in other neurodegenerative diseases (Dementia with Lewy Bodies, Multiple System Atrophy, and even Alzheimer’s disease) that are now defined as Synucleinopathies, thus widening the importance of understanding its roles in disease.

Our research will focus on why and how abnormal aSyn accumulation and clumping occurs to identify new approaches and treatments which may lead to the development of new therapies for PD and other synucleinopathies.

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

Diseases like PD, which kill nerve cells and lead to great physical and mental disability, are currently not curable as the available treatments only manage to reduce the severity of some of the symptoms. Treatments are being sought all the time but further understanding of the core processes causing the disease are needed to develop effective drugs to prevent the disease. In this project, we will continue our research which aims to understand the earliest changes that occur in the brain and the rest of the body that lead to PD. Then, these changes can be targeted with treatments, leading to halting or delaying disease progression so that people can lead a healthy life for longer. By introducing the disease-causing gene for human aSyn into the mouse DNA (forming transgenic aSyn mice, abbreviated tg-aSyn), we have created unique mouse models of PD that develop core features of the disease that are also found in humans.

We have developed a number of novel mouse models to study various aspects of aSyn toxicity. In one set of models, tg-aSyn clumps form in the brain cells that produce dopamine (because the neurons that produce dopamine are the ones that are responsible for the motor symptoms and die in PD), enabling us try to prevent dopamine loss and nerve cell death. In another set of models, tg-aSyn begins forming in the gut and in the nose. The gut location model was developed to test the leading hypothesis that PD begins in the gut (causing early problems with constipation and related symptoms) and spreads into the brain through nerves that connect the two tissues. Expression of tg-aSyn in the nerve cells in the nose is consistent with the fact that most PD patients lose their sense of smell even before motor symptoms develop. The nose nerve cells connect to the olfactory bulb, which is the part of the brain responsible for sensing smell.

Many PD patients also develop problems with anxiety and depression. There are simple non-invasive behavioural tests to study these symptoms in mice. By studying these behaviours in our mouse models, we will also understand the steps that lead aSyn to spread from the gut and nose into the brain, and identify which species of aSyn clumps are responsible for disease spreading and for the different symptoms seen in PD. We expect to test new compounds that inhibit aSyn accumulation and assembly into clumps and it's spreading to delay the development of disease (the study of disease is called pathology). Understanding these processes may also benefit clinical diagnosis by identifying new participants early in the disease process including molecules in body fluids (known as biomarkers) that can be used for early detection of the disease.

By the end of this project, we will acquire important information on (1) how protein clumps of aSyn are formed, (2) how are they involved in the death of nerve cells, and (3) how can they be targeted to stop nerve cell loss.

Our findings will be made publicly available by publishing in open access journals, presenting our findings at open meetings, by collaborating with colleagues who use techniques that complement our range of methods that can add value to our findings, and with the pharmaceutical industry.

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

In the short term the research community will benefit from our outputs by developing more refined models, providing new insights into mechanisms of disease progression and identifying the molecules

that can be targeted for novel treatments. Using more refined models, we can pinpoint the specific disease mechanisms to be targeted, without additional adverse symptoms.

Through our work, we will have contributed to long-term societal benefits by improving the quality of life and well-being of PD patients and those suffering from other synucleinopathies, especially within the aging population, since age is the highest risk factor for development of dementias and loss of mental functions. Due to mutations in aSyn that are incorporated in our transgenic mice, which are responsible for early onset dementia, our research will also benefit younger patients with inherited conditions.

Lastly, by refining our mouse models and further testing them, we will gain a better understanding of the models themselves and which tests to use to gain valuable data in the least invasive manner. As such, mice and the research community using them will also benefit.

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

Outcomes of our research, including unsuccessful approaches, will be reported in conferences and webinars and in open-access, publicly available databases.

Several collaborations are in place and will be developed to obtain new tools to further the understanding of these pathological processes.

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 mice because their nervous system is sufficiently similar to that of humans and the causes of neuronal cell death are almost the same. In addition, mouse behaviour is well characterised, and our mouse models mimic the behaviour of the human disease. Furthermore, mice can be genetically altered, allowing hypotheses about the molecules and pathways of disease development to be tested.

The highest risk of developing neurodegenerative diseases including PD is the aging process. Therefore, we aim to study the mice up to 24 months, to allow the body to respond to the pathological processes that occur during aging and thus discover new pathways that may be impacted. While the time of onset of PD and other synucleinopathies cannot be predicted in humans, the time-course of accumulation of aSyn clumps can be carefully monitored in mice during the aging process and linked to the other disease-linked deficits (like constipation, loss of smell sense, anxiety) that are occurring during this time. By studying how the disease develops throughout the lifetime of the mice, we will

acquire new insights into what causes aSyn to clump and become toxic. These insights will enable the study of the effect of potential cures that delay the beginning of these diseases and promote healthy ageing.

Since most of our PD mouse models have been developed by our group and thus are not commercially available, we breed our colonies in-house and have a number of juvenile animals in our colony at any given time.

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

Most of the PD mouse models used in the studies covered by this licence will be bred in-house and aged until defined time-points (maximum of 24-months of age), which correspond to different stages of disease. During this time, we will use three main approaches to understand the disease mechanisms mimicked in our mice: 1) generation and maintenance of genetically altered animals (GAAs), 2) behavioural testing and/or biofluid collection via different routes and 3) treatment with different substances and surgical procedures to modify the disease processes. While each of these procedures can be applied on its own, a mouse may also experience multiple procedures.

Generation of the GAAs can be done through standard breeding of transgenic or non-transgenic males and females, leading to pregnancy and birth of the offspring. In some cases, the offspring may be ear-notched for collection of a small piece of skin tissue to be used for genotyping. Ear-notching happens at the minimum age of three weeks. In the cases of unwanted genotypes, pups may be humanely killed. In the cases where a pup needs to be killed at an earlier age than 10 days, decapitation will be used as the method, since scientific guidelines advise against the use of carbon dioxide. Sharp surgical scissors will be used by a trained operator to rapidly decapitate the pup.

In some cases, breeding may not be achieved through traditional methods, in which case in-vitro fertilisation will be used, with a vasectomised male employed to cause pseudo-pregnancy, followed by the surgical (SET) or non-surgical (NSET) transfer of the embryo will be performed. Breeders and vasectomised males will be kept alive, since these procedures cause no long-lasting harm.

Behavioural testing related to PD, such as motor dysfunction, sense of smell and anxiety, will be performed to determine the progression of PD-like symptoms.

Motor impairment will be tested using the balance beam, and/or treadmill (DigiGait), and/or rotarod, which are non-invasive methods that match those most used in PD research worldwide. Each test measures different features of locomotion. Beam walking tests fine balance and coordination as the mouse walks along a static round wooden beam slightly elevated from the ground, with soft bedding placed underneath in case the mouse falls off the beam. DigiGait analyses the finer aspects of walking (stride length, stance, swing, and propulsion for each paw) by imaging underneath the animals as they walk on a transparent treadmill belt for 5-10 seconds. The rotarod tests balance, grip strength, and motor coordination by trying to keep hold of a rotating beam that is slowly accelerated. Each test session is preceded by a practice session, so the mice familiarise themselves with the task. If all 3 tests are required, they will be conducted in series from the least to the most demanding, e.g. beam walking on day 1, DigiGait on day 3, then rotarod on day 5 -with a rest day after each test.

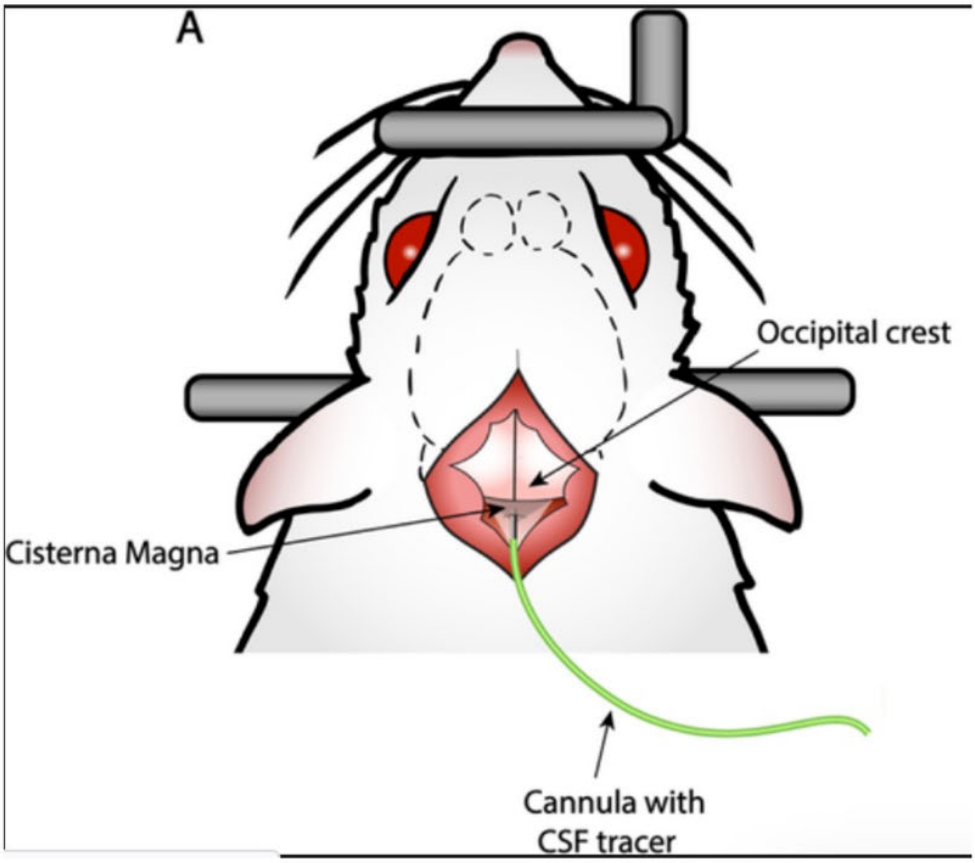
Olfactory dysfunction will be studied using tests that distinguish between general odour detection, odour discrimination between two different chemicals, and social odour (pheromone) sensing. The reason three different tests may be needed is that each one reports on a different area of the brain that may be affected by the disease. These tests are either conducted in a standard home-cage or a T-shaped box, with mice removed from their home-cage for no longer than 20 minutes. The odours used in these test are natural chemicals mice encounter in their daily lives and thus not harmful.

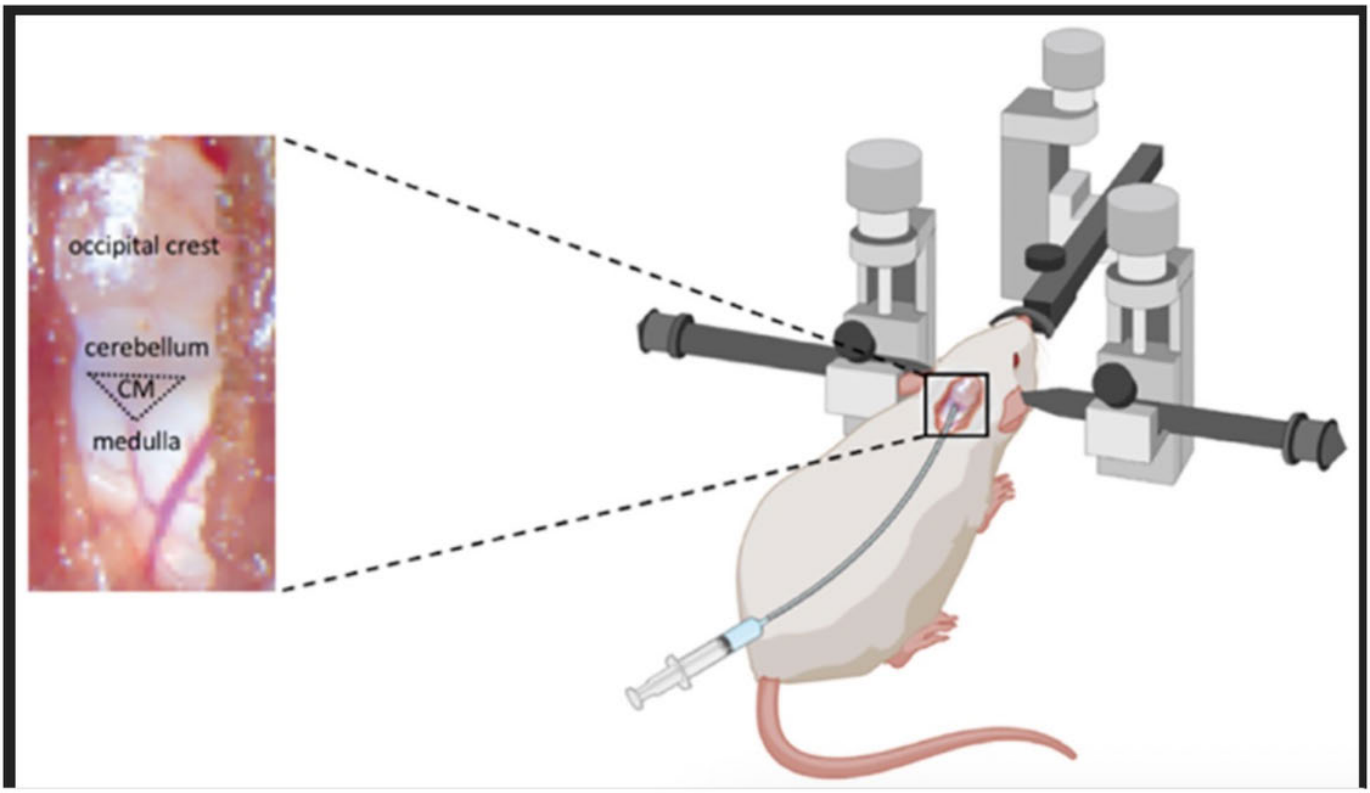
Anxiety related tests will be performed to study if the emotional changes observed in PD patients are also seen in the mouse models. We will test anxiety using three different methods, as each one is directed against a different area or function of the brain affected in PD: the elevated plus maze measures anxiety by using the natural curiosity of the mice and aversion to open and elevated spaces by measuring the time in the open (more risky) and closed (less risky) arms of the maze. While it is very rare for the mice to fall off from the elevated maze (50 cm above the floor), soft bedding is placed underneath to protect the mice in the case of a fall. The open field measures locomotion and anxiety in mice by comparing the time spent exploring different parts of a square shaped field. Anxious mice tend to stay in one place and explore less. Along with anxiety, open field is also useful for measuring the activity levels of mice. Lastly, the marble burying test relies on the natural tendency of mice to bury objects in their bedding; it is a non-invasive way to measure stereotypic (repetitive) behaviours in mice and are controlled by the mechanisms related to anxiety.

At any given day, only one of the behavioural tests will be applied to the mice. In experiments with multiple behavioural tests, they will be performed in the order of least stressful to most stressful to avoid any impact on the test from the previous one. After tests with motor burden, which may cause physical stress, mice will be given a rest day before the next test is applied. Subsequently to behavioural testing, tissue from these animals will be collected and investigated to identify the routes that molecules take (e.g. molecular pathways) that lead to protein clumping and its behavioural outcomes.

In addition to behavioural testing, biofluid collection from the mice will also inform us about disease processes. Cerebrospinal fluid (CSF), and/or interstitial fluid from the brain, and/or blood, will be collected from the mice using the least-invasive methods as follows.

CSF is a special fluid that flows around the brain and spinal cord. It collects the liquid secretions from the brain and circulates this fluid to enable extra communication. It is altered in all neurodegenerative diseases including PD in a disease-specific manner so is collected to look for signs of disease and/or inflammation, and for molecules that could be biological markers that identify a certain stage in the disease. To collect CSF, the least harmful route is through the Cisterna Magna (shown in the figure below) as it does not damage the skull. Anaesthetised mice are placed in a stereotactic instrument (a frame that positions the head of an anaesthetised mouse in a fixed orientation to allow the precise location of a brain region without having to remove the skull, shown in the figure), and a needle is inserted into the Cisterna Magna after gently parting the neck muscles.





CSF collected from the Cisterna Magna without damaging the skull, while the mouse is under anaesthesia, immobilised with a stereotaxic frame, allowing precise manipulations.

Interstitial fluid is a special fluid found in the spaces between the cells in the brain, where compounds released from cells, such as dopamine (DA), are located. In some of these experiments, mice will undergo one session of sampling of the brain neurotransmitter dopamine, because the neurons that produce dopamine are the ones that die in PD, causing the behavioural deficits. In this experiment, a guide cannula is implanted into the brain under anaesthesia using a stereotaxic instrument and the mice are allowed to recover. The next day, a small volume (10 -20 µL) of brain fluid is collected via a microdialysis probe (microdialysis is conducted using a probe with a tiny semipermeable membrane that excludes large molecules such as proteins) is positioned in the desired tissue over 3 hours without causing any pain to the mice, after which the mice will be humanely killed under anaesthesia. The fluid is analysed chemically for dopamine content and the brains will be analysed to determine disease stage. Mice are constantly watched during these procedures for any signs of discomfort such as lack of movement, excessive grooming, or vocalisations, in which case they will be immediately humanely killed. Single stereotaxically-guided injections to anaesthetised animals without an implanted cannula may also be used for local delivery of compounds or vectors that deliver genes that may be used to treat or label these regions without their spreading to the entire brain. Mice will be under constant supervision to ensure they awaken and function without any signs of discomfort.

Blood may be sampled at certain time-points to detect molecules that may serve as biological markers of the disease, thus predicting the disease in humans before it fully develops. Blood collection will be performed using saphenous bleed, which is from the saphenous vein in the hind leg. Urine will be collected by holding the mouse over a tube and lightly stroking the belly of the animal.

For studying how aSyn influences gut function, we will use two gastrointestinal performance tests: Total transit time measures how long it takes a non-toxic dye to appear in the first expelled faecal pellet after inserting the dye into the stomach through their mouth via a soft flexible plastic tube (a procedure known as oral gavage also used in humans).The colon motility tests how many faecal pellets an animal produces over a fixed time period.

Once we have mapped how these molecules and behaviours appear during the aging process of the mice, using the methods explained above, some mice may be given treatments at early stages that may slow down or accelerate the clumping of aSyn, in order to further study the disease mechanisms. There are multiple routes for delivery of these treatments, including (1) mixing with their food (such as giving the mice a diet with higher fat concentration as a treatment to induce inflammation) or water, (2) direct injection to the brain under anaesthesia followed by analgesia using the cannulation method explained above, or direct access through the Cisterna Magna, (3) injected into the body (under the skin, or into the muscle, or into the blood stream, or (4) via a small drop in one or both nostrils to ensure that the compound’s effects are limited to the brain region connected to the nose and not the rest of the body (this method is known as intranasal injection). This method is especially relevant to the mouse models where aSyn originates in the nose.

One of the hypotheses we will test in this project is that PD develops first with aSyn in the nerves that line the gut (the enteric nervous system), mainly the intestine (constipation is prevalent among PD patients) that then spreads to the brain. The main route of spread from gut to brain is via the vagus nerve, the most important nerve connecting these two organs. As such, separating the vagus nerve from the brain by cutting it on one side (a procedure known as vagotomy) under anaesthesia with painkillers may prevent aSyn transport from the gut to the brain and prove that this is the route of aSyn spreading, and thereby delaying disease progression. Vagotomy may also prevent other molecules that accompany/aid aSyn movement to the brain from entering the brain. Vagotomy is a procedure used in humans, who can live many years after vagotomy.

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

Based on our previous experience with these mouse models, we do not expect any additional adverse effects on our transgenic mice, apart from the usual aging-associated deficits listed below. Moreover, the behavioural tests we propose to use are non-invasive and ample recovery time is given between the different tests to avoid fatigue. Hence, while we expect some age-related deterioration, based on our experience, these will not be any more severe in the transgenic mice.

There are adverse effects associated with aging. These may include tumours, fur loss, sensory deficits, weight loss, and movement difficulties. In order to make sure that the mice are not suffering from any of these, after 16 months of age, mice are monitored closely on a regular basis and any mouse showing these symptoms are humanly killed.

Administration of a high fat diet may cause itching/scratching due to a greasy coat although we have not encountered this so far. In recent trials, all the mice on the high-fat diet gained weight during the treatment period while the mice on the control diet initially lost weight. We discovered that the loss of weight is due to the control diet's texture being very hard. In order to solve this problem, we have mixed the control food with warm water and placed it inside a small dish in the cage, making it easier for the mice to chew. This has solved the problem of weight loss. High fat diet will also be presented on a dish.

The administration of therapeutic compounds could lead to unforeseen adverse events: thus far we have tested 3 chemical compounds and while none has shown adverse effects impacting all the treated mice, about 5% of the mice in one of the experiments showed significant weight loss. In order to minimise the weight loss, treated mice will be weighed daily and mash (food pellets ground up and mixed with water) will be given in case of loss of more than 5% of weight. Mice losing more than 15% of the starting body weight will be humanely killed.

Mice undergoing stereotaxic injections under surgery may experience temporary discomfort displayed as reduced movement, hunching, and itching (for which topical analgesia will be administered), however these animals should fully recover in less than 3 hours with no lasting effect on their general well-being. About 1% of the surgeries cause excessive bleeding during the surgery. When this happens surgery is stopped and the mouse is killed using a humane method. Similarly, in about 1% of the cases, mice are found to be paralysed after waking from anaesthesia, in which case again they are immediately killed using a humane method. The same approach will be used as soon as such a problem occurs.

PD animals undergoing vagotomy are expected to recover from surgery without immediate adverse effects due to the procedure itself but over 6 months all are expected to experience moderate adverse effects, such as weight loss. All animals reaching 15% weight loss from baseline with be humanely killed, as will those showing further clinical signs such as piloerection (hair standing on end), hunched posture, or bloating.

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)?

Mild 75%

Moderate 25%

What will happen to animals used in this project?

- Killed
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse
- Used in other projects

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?

It is not currently possible to accurately re-create a functioning nervous system outside an animal that mimics the complex interactions between different brain cell types, and the other tissues and organs of the body. Therefore, to understand the mechanisms behind disease progression and full therapeutic effectiveness of relevant drugs, an animal model is needed.

This project will avoid unnecessary duplication as it is based on our current work. Existing data was considered to prevent duplication when calculating the animal numbers and protocols.

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

We are currently growing human cells in dishes (cell culture) to understand how aSyn clumping is directly toxic to cells. This procedure involves taking human skin samples and converting them into the different cell types that reside in the brain to form “mini-brains” that resemble some of the complexity of the human brain. The advantage of this approach is that skin cells can be taken from people who have an inherited from of PD because they have a mutation in the DNA of the aSyn gene, so they can be compared to those from healthy people. We also use techniques that promote clumping of aSyn in a test tube to test compounds that might prevent this.

We are also using systematic review tools such as SyRF, databases (EURL ECVAM, Norecopa) and other search strategies in order to find ways to reduce and refine animal use when possible.

Why were they not suitable?

Cell culture and test tube technologies are very useful; however, they complement but do not replace the study of animal models. Both non-animal approaches have limitations: the mini-brains lack a blood supply through which natural nutrients and hormones are provided that change how the brain reacts to its environment, including the rest of the body, especially in the context of disease mini-brain architecture is very primitive and does not mimic the intricate 3D structure of the brain, which develops slowly over several years and is built on the experience that people have during their life time. Regarding the test tube work, as yet the clumps in the test tube do not accurately replicate the shape of the clumps in the brain so the drugs tested may not be as powerful against human aSyn clumps. The cell responses that are a crucial part of the toxic process is missing from work in the test tube. To offset this limitation to some extent, we feed the mini-brains with clumps from human disease brains, but these experiments can only extend to a year at most because the mini-brains die, thus limiting the contribution of the aging process in the disease.

PD is a complex disease, impacting the entire body and not just the brain. As such, mouse models complement these cellular and test tube models (and vice versa) and together they allow us to understand fundamental PD mechanisms.

Reduction

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

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

We have several years of experience in using these models of synucleinopathies. We have been reducing animal number year on year as we learn more about the models and understand the disease process better, along with careful planning based on our experience. During the tenure of our last project license, we reduced the number of animals significantly by using longitudinal behavioural studies (in which the same mouse is tested at different ages), hence decreasing the number of animals needed. For example, a mouse can be tested at young, middle, and old age, allowing us to see how the disease symptoms change in that mouse specifically. One possible limitation of this type of experimental design is “cross-talk” which refers to possible changes in results at later time-points due to previously being tested. We carefully design the experiments and specifically use tests that cause minimal cross-talk, allowing us to test the same mouse more than once. Importantly, this is not re-use, since all the time-points of testing belong to a single experiment. Longitudinal experiments also increase the ability to detect statistical differences between the groups using smaller numbers of mice.

We have also optimised our tissue processing techniques, for example by employing new tissue staining methods increasing sample quality, which decreases waste in tissue usage and the need for collecting tissue from more mice.

We have optimised our breeding by identifying the disease-relevance of heterozygous and homozygous transgenic mice (heterozygous meaning that the gene comes only from one parent and the mouse has just a single copy, while “homozygous” means that both parents pass on the altered gene, so two copies exist). Mice that do not carry any altered genes are considered “wildtype” and used as controls in the experiments.

These optimisations mentioned above, along with planning for future work in terms of the number of students and post-doctoral researchers our group predicts to employ during the duration of this project, allows us to calculate accurate numbers for mice to be produced.

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

For best experimental design we referred to the PREPARE guidelines checklist (https://norecopa.no/prepare/prepare-checklist), which considers the steps needed to ensure that statistically meaningful results are obtained while using the minimum number of animals. We also use the NC3Rs Design assistant (https://eda.nc3rs.org.uk › node › 358) Taking these into consideration, we carefully weighed the balance between harm and benefit based on several years of experience in using our mouse models of synucleinopathy.

We determine group sizes to minimise variability and randomly allocate the animals between experimental conditions. Mice are tested in a random order without the experimenter knowing which

group they are in (known as blinding).

We have been continuously improving our understanding of how the animals respond during the development of the disease process, allowing us to reduce animal use year on year through careful maintenance of colony size and breeding. By applying the most appropriate power analysis statistics to the experimental design, we ensure that we will obtain meaningful results, using the minimum number of animals, avoiding both unnecessary animal use and experiment repetitions.

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

Our aim is to gain the most amount of data from a single mouse available, without reuse and/or causing any suffering to the mouse. To achieve this, we have established collaborations to perform analyses which we cannot perform in our own laboratory. These include gut bacteria analyses, transcriptomics (a technique that identifies the genes that are active in any given cell or tissue at the time of sample collection) and advanced imaging using specialised microscopes to further characterise the aSyn clumps. Through these collaborations, we make sure no part of a single mouse is wasted. Indeed, we have been collecting brain, liver, kidney, spleen, stomach, intestine, muscle, and fat tissue from our mice, along with faecal and blood samples for these analyses.

Since we have been working with these models for a number of years now, our pilot experiments characterising their basic pathology is complete, allowing us to know the important age-points to further study them. As such, we focus on these specific ages, further reducing the number of mice at other ages. However, we will still conduct pilot studies when new behavioural and/or treatment strategies are being developed. These studies will use a small (but representative) group of mice that are carefully monitored during and after testing to identify any adverse effects and find ways to minimise harm before a larger number of animals are tested. We also discuss these new procedures with the animal care team (NACWO) and the university veterinarians (NVS).

We have identified a lack of pathology in the heterozygous mice in some of the models, which we now use as controls along with the non-transgenic control (wildtype) mice, which further reduces the number of mice wasted due to their being the wrong genotype.

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.

To be of use for PD research, disease models need to express the disease from birth, just like humans who have PD. We therefore cannot turn the genes on and off but rather need the disease to develop as the animal ages. Our PD models have subtle signs of disease so do not develop any lasting harm.

The behavioural and surgical methods we propose to use have been perfected since they were developed by our group, so we know what to expect and how to deal with adverse events in the most humane manner. We have yet to have had to kill an animal undergoing behavioural studies, and no lasting harms have been caused. The olfactory tests we propose to use rely on naturally occurring chemicals such as ones found in the urine of the mice, thus minimising adverse effects of other tests such as those using noxious chemicals like lemon juice which can cause distress. For gavage we have changed our practice from metal needles to flexible needles coated with a natural lubricant such as milk shake. For CSF collection and delivery of compounds to the CNS, we are proposing a new method, which gains access to the nervous system by parting the neck muscles, so not damaging the skull like other methods used for CSF collection. The microdialysis protocol has not given rise to any signs of harm or distress and the aftercare has been optimised e.g. by delivering water bottles instead of relying on the automatic water system to stop the tubing from getting caught. We also avoid distress by habituating a mouse to its surroundings before behavioural tests are conducted.

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

There are other animal models of PD, including fruit flies and worms. While these are valuable to ask specific questions about disease processes that don’t involve the whole body, the biological and behavioural alterations seen in them due to aSyn clumping are not representative of the human disease. For example, one of the disease processes we specifically study is the starting of aSyn clumping in the gut and spreading to the brain. Due to physiological differences between worms/flies and humans/mice, these models cannot be used to study this disease mechanism. They also do not live long enough to tie the disease into the aging process. We cannot use terminally anaesthetised animals for this project because anaesthesia works by paralysing the nerve cells.

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

We will continue to enrich the environment with nesting and tunnels. Induction of inflammation by diet change instead of chemically induced inflammation is a more refined and physiologically relevant method. As we come to understand the later stages of pathology, this will guide us as to what to look for at earlier stages of the disease, thus avoiding the development of the most severe clinical disease.

We are also constantly improving the handling methods of the mice. For example, using a tunnel to pick up mice initially to reduce anxiety because picking up by the tail enables a hind limb clasping test without causing. (https://www.nc3rs.org.uk/mouse-handling-webinar). We will also refer to the RSPCA website (https://science.rspcs.org.uk/sciencegroup/researchanimals) for further information.

In behavioural tests, mice will always be habituated to the experimental apparatus first, to reduce stress. When more than one behavioural test is applied to a single mouse, they will be ordered from least to most stressful, and one rest day will be given after each test. When mice receive injections using needles, the needle will be changed after each mouse.

By continuous training of the scientists working under this license, we will use the most up-to-date protocols reducing the pain and suffering of the animals. For example, the cupping technique is now used to pick up the mice, to cause the least possible amount of stress and discomfort. We will also be using plastic flexible needles for oral gavage, instead of the metal ones, as the plastic ones are shown

to be less invasive. Moreover, we are also proposing to use a new CSF collection method, using access through the Cisterna Magna on the neck, which is not damaging to the skull and thus less invasive.

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

We will follow the NC3Rs guidance notes and webinars (https://www.nc3rs.org.uk/welfare-assessment) and those from the Jackson Laboratories (https://www.jax.org/news-and-insights/jax-blog/2016/march/experimental-design-top-four-strategies-for-reproducible-mouse-research). Advice and notes provided by the LASA guidelines will be also consulted (www.lasa.co.uk/publications.html) and we will follow the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments). The guidelines provide a checklist of recommendations to improve the reporting of research involving animals to maximise the quality and reliability of published research, and enable others to better scrutinise, evaluate and reproduce it.

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

We will continue to routinely access the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) website for information and advice and spread this knowledge among all members of our group. We also follow Norway's 3R centre (NORECOPA) guidelines (https://norecopa.no/about-norecopa). We also have access to internal resources and receive communications on 3Rs matters provided by the establishments Named Information Officers (NIOs).