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NON-TECHNICAL SUMMARY

Nutrient sensing in the brain

Project duration

5 years 0 months

Project purpose

- (a) Basic research

Key words

obesity, appetite, diabetes, brain, nutrient sensing

Animal types

Life stages

Mice

Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

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

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to understand how the brain controls our appetite, weight and health. In particular, we want to understand how the brain detects the different types of food that we eat and the different types of energy that we have available in the body, and uses this information to regulate appetite, weight and health.

A retrospective assessment of these aims will be due by 11 July 2027

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 when brain pathways regulating appetite and energy use in the body are not working, this can cause diseases like obesity, diabetes and cardiovascular diseases like heart disease or atherosclerosis. Understanding these pathways will help develop new more efficient and safer therapies to lift the huge burden that these diseases represent (63% adults in the UK are overweight or obese, causing over 30,000 deaths each year and £6 billion of related health care cost).

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

I hope to discover pathways that the brain uses to control appetite and weight that we could target to treat obesity and metabolic diseases.

I hope to produce new databases of genes involved in how the brain detects the nutrients we eat or have available in our body. These databases will be made available to others. I hope to produce a detailed characterisation of the mechanisms through which 3 of these pathways work, providing a description of how neuronal circuits contribute to energy information processing in the brain. In the end, I may demonstrate that targeting these pathways is an efficient strategy to treat obesity and related health complications, leading to new research to develop new anti-obesity drugs. Such findings may be of interest for the pharmaceutical industry and I will submit patent applications when relevant. Data arising from this research may support future funding applications/clinical trials.

This work is important because our understanding of how the brain controls appetite and metabolism is very limited and as a consequence, there are only a few drug options to treat obesity. Obesity is a major threat to public health and costs billions of pounds each year in healthcare treatments. Most of

the available pharmacological options have limited efficiency. Our new results indicate that we have identified one of the mechanism of action of the new anti-obesity drug Zepbound, which we want to further pursue. In addition, we are testing dietary interventions which are relevant for the human population (high protein diets, yoyo diets).

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

The annual UK-wide NHS costs attributable to overweight and obesity are proposed to reach £9.7 billion by 2050. Most of this cost relates to dealing with the metabolic diseases associated with obesity, including type 2 diabetes, fatty liver disease and cardiovascular disease. This license will support research that will help generate new insights into the causes of type 2 diabetes and other common metabolic diseases associated with obesity, and to potentially reveal novel therapeutic drug targets.

In the short term, the scientific community will gain new information on the pathways and genes that we will investigate. The proposed work will increase our knowledge of how the brain works which will be useful for other researchers in neuroscience but also researchers who study nutrition and interactions between the body and the brain.

Clinical researchers may benefit from this work, in particular if we show that some of the pathways we identify can help reduce obesity.

Eventually this may benefit patients and society as a whole if we manage to decrease the burden of obesity and metabolic diseases.

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

We will maximise research output from each individual animal, for example using repeated assessments in single animals.

We will disseminate all our findings, including negative results and unsuccessful approaches, in local, national and international scientific conferences and seminars, and via publications in specialised open access peer-reviewed journals.

We coordinate with other researchers in the university and outside our institution internationally to share tissue samples and mouse models. This helps maximise the output of each animals or model we are using to prevent wastage.

We will engage with clinical researchers to initiate the translation of our work.

Species and numbers of animals expected to be used

- Mice: 20,800

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.

Our goal is to produce knowledge that can be applied to improve human health. Therefore we need to use an adult mammalian organism. Mammals have unique sophisticated pathways to regulate feeding and metabolism. We cannot use a lower life-form, e.g. fish or insects, as they regulate energy balance, and in particular the expenditure side of the equation, very differently from mammals.

Rodents allow the study of whole-body control of energy balance in a manner relevant to humans, as pathways involved in the control of appetite and body weight are largely similar between rodents and humans. Rodents allow access to several tissues critical to the control of metabolism (brain, pancreas) that are inaccessible in humans. Rodents are amenable to genetic manipulations, offering endless possibilities to characterise mechanisms underlying diseases in a specific and relevant manner.

We need to understand how an adult mammalian organism functions as a whole to obtain and use energy. This happens at the level of the “whole organism” and not simply at a cellular level. Therefore, in the majority of the cases we cannot perform experiments in a dish.

We will focus on juveniles and adult mice because before weaning, the way pups obtain and use energy is very different. Also, fat mass storage occurs typically in the adult and cannot be studied in younger animals. Last, younger animals have different nutritional needs as they are still growing. We will also study pregnant females to study the metabolic adaptations that occur during pregnancy, a physiological state during which energy balance is challenged and which can help us understand underpinning pathways.

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

Firstly, we will use established standardised breeding protocols to generate genetically altered mice.

Before performing large scale studies, we will use small groups of animals (pilot study) to confirm the details of our experiments (dose, timing), identify potential problems, confirm the experimental design, as well as implement improvements early on in the licence using a reduced number of animals.

Mice will typically be group-housed in their home cages while being supplied with a regular diet or a modified diet enriched or depleted in specific nutrients for the entire lifetime. Mice may be exposed to dietary modifications, typically once in their lifetime but up to 7 times. They may be housed at different ambient temperatures ranging for 4C to 30C, typically for 4 weeks, and may be single housed typically for up to 8 weeks but sometimes for their entire lifetime. Mice may also be housed on modified flooring with a continuous pattern of holes. Mice may be food restricted overnight, typically on 4 occasions, or in some cases may be calorie-restricted typically for 4 weeks.

Mice on moderate severity protocols will typically undergo 1 surgery to inject substances into the brain, the blood or the intestine, and/or position permanent cannula for repeated injections in awake animals.

- In brain surgery (up to 2 times in the lifetime of the animal), mice will undergo general anaesthesia for up to 30min. A small hole in the skull will be performed and a cannula will be inserted temporarily for the purpose of an acute injection, or secured permanently. This allows local manipulation of brain activity, local injection of molecules or local recordings of activity in awake animals. The same animal can be used for repeated measures, reducing variability.
- For in-vivo two photon calcium imaging, the mice will undergo general anaesthesia typically for 60 minutes. A small incision will be made on the scalp, and a small hole will be drilled in the skull. Afterwards, a virus will be injected in the brain, which makes neurons glow for a moment whenever they're active. Next, a special cylindrical lens, called gradient index (GRIN) lens, is inserted in the brain. GRIN lenses allow us to see whenever neurons glow deep in the brain without distorting the image. Finally, a head holder is attached to the top of the skull, to allow the mouse's head to stay still during recording sessions. Using a specialized microscope, we will record the activity of many neurons over several recording sessions, allowing us to precisely observe when each neuron is active or not.
- Vascular surgery (once per animal) will typically last 60 min and involves general anaesthesia, the opening of the upper body to isolate the jugular vein and carotid artery and the insertion of a permanent catheter into these blood vessels.
- Intestine surgery (once per animal, 30min per procedure) involves general anaesthesia, the exposure of the intestine after opening of the body cavity, and the insertion of a permanent cannula.
- Surgery for the insertion of a minipump or a telemetric device involves general anaesthesia and typically takes up to 5 min per procedure. Minipumps are used to deliver a substance in a continuous manner typically for 2 weeks in awake animals. Telemetric devices are used to measure body temperature and activity in a continuous manner in awake animals. Both these surgeries involve the opening of the body cavity or a small incision of the back, the insertion of the device, and closure of the cavity with sutures.

Alternatively, mice doses with tamoxifen via oral gavage might be on a moderate severity protocol because of the transient adverse effects produced by the required dose of tamoxifen through this route.

The majority of the mice will receive substances administered either into a vein, into the brain, into the intestine, into the body cavity, under the skin and/or via a tube inserted via the mouth onto the stomach dietary. The total number of substance administration through all these different routes will typically be 20 and in rare cases up to 60 over a minimum of 50 days.

In the majority of the mice, we will collect blood from either a superficial vein or via a pre-inserted catheter.

In some cases, we will collect cerebrospinal fluid (CSF) from the magna cisterna via a pre-inserted catheter in terminally anaesthetised animals.

Some mice may be tested in bespoke experimental chambers that allow advanced measurements or experimental paradigms. These include:

- behavioural chambers: typically, animals will be acclimatised to these chambers and trained to perform a task of drinking or eating a predefined amount of diet or solution. This may be in a conditioning paradigm where the animals need to perform a task before accessing the diet or solution. Animals will be tested typically once a day (up to 2 times a day) and each session will last typically for 1h, typically for 8 weeks. Mice might be food restricted (80% of normal intake) in these experiments.
- calorimetry chambers: mice will be housed in a home-cage based system measuring gas consumption to determine how the body uses energy. Mice will be housed in these chambers typically for 3 days.

Typically, mice may need to have 2 scans in their lifetime to measure nutrient absorption in specific tissues or determine the amount of fat or muscle in their bodies.

Female mice will receive a gene-inducing substance prior mating and undergo measurements of energy intake, energy expenditure, body composition and nutrient handling during pregnancy or lactation. For the purpose of energy intake and expenditure measurements, mice might be singly housed.

At the end of the experiment, animals will be humanely killed, tissues will be collected under deep, terminal anaesthetic unconsciousness by first removing the blood by pumping a salt-containing liquid through the blood vessels, called perfusion, followed by tissue preservation in a fixative solution for follow-up analyses. We will coordinate 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.

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

Our studies will involve the following:

1. Blood sampling with its associated handling stress and transient discomfort.
2. Injections with their associated handling stress and transient discomfort.
3. Surgeries that will produce moderate transient discomfort. All surgical procedure will be completed under general anaesthesia so that the animal will remain in a state of sleep/unconsciousness throughout.
4. Some mice may develop diabetes and other metabolic complication which will result in increased drinking and urine production. If this is noted, we will monitor blood sugar levels and if they go too high the animal will be killed.
5. They will experience stress related to experimental procedures in bespoke environmental chambers or equipment and social isolation, and during procedures performed in awake mice and repeated over time in the same animal, producing mild transient or long-term harm. However animals will not need to be restrained during imaging sessions.

6. Mice will also experience the cumulative effect of successive procedures producing mild and transient discomfort. All studies involving multiple steps will be performed in a longitudinal manner. Mice will be allowed most of the time 1 week (minimum 3 days) recovery between distinct steps and their welfare will be constantly monitored.

7. In some cases, specific mouse strains need to be dosed with a high dose of tamoxifen through oral gavage, which might produce transient moderate discomfort, and weight loss up to 20% from baseline, and therefore justifying a moderate severity limit.

8. For mice used during in vivo calcium imaging, will experience moderate transient discomfort related to head-fixation acclimatization and recording. Furthermore, these mice will experience social isolation due to the need to keep them single-housed. Single housing of animals at this stage is preferred because of the possibility of animals fighting or interacting in any way in which the head holder may be damaged or dislodged, or the GRIN lens is exposed and damaged. While it is difficult to provide an exact duration of how long the animals need to be single housed, a reasonable estimate would be around 12-15 weeks, considering time for experimentation and familiarization to the recording setup.

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)?

Mice :

- Mild 62%
- Moderate 37%
- Severe 1%

What will happen to animals used in this project?

- Killed
- Used in other projects

A retrospective assessment of these predicted harms will be due by 11 July 2027

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?

The control of energy balance and metabolism occurs at the level of the “whole organism” and not simply at a cellular level. Therefore, the scientific understanding of these processes requires the study of whole organisms and cannot be studied in a dish.

We want to understand how the human body uses and obtains energy and the role of the brain in these processes. Therefore, we need to use a specie where the interactions between the brain and the body are similar. Also the interactions between the brain and the body are integrated multi-organ processes that require the study of live animals and whole organisms.

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

As much as possible we will study our research questions in a dish. In particular, we have recently developed the use neuronal cell cultures that we can study in a dish. These are useful to test sensing mechanisms with a number of different assays. They enable us to test the role of candidate pathways in a dish before investigating their role in whole organisms. In addition, we have developed the use of brain tissues in a dish. In this model, we can test nutrients or hormones and measure a number of metrics relevant to brain function.

We use genetic and health data obtained from large group of human participants to guide our research in rodents.

We obtain tissues from collaborators as often as possible and share tissues as well.

Why were they not suitable?

We cannot use a lower life-form, e.g. fish or insects, because in these species, the way the body uses energy is different from mammals. Our goal is to produce data that are relevant to humans, therefore we need to use species where energy use is similar to that in humans.

A retrospective assessment of replacement will be due by 11 July 2027

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?

I used data from the past 4 years of my research to estimate numbers given the expected level of funding I will have in the next 5 years. Numbers are being adjusted in Dec 2022 following the obtention of new research funding. In these studies, we will use transgenic mice with 4 transgenic alleles, 2 of which have to be maintained in heterozygous for breeding efficiency (ob allele- ob/ob being infertile- and myrf-flox allele). This created complex breeding schemes with a high number of animals with unwanted genotype. Numbers are adjusted again in Apr 2024 following obtention of new research funding, with experiments requiring mice bred in the heterozygous state to create homozygous and wild-type littermates.

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

In this project, we have the option to use diverse "phenotyping steps" in phenotyping protocols. Phenotyping steps are procedures where we measure how the body obtains and uses energy. They allow us to characterise the role of a pathway and the mechanisms through which this pathway eventually regulates body weight and health. The specific phenotyping steps we will use in a given study will depend on our specific experimental goals, varying from study to study. For example, we may find that one pathway modifies body weight by increasing the addiction to sugar and decreasing how the body burns fat. We may find that a pathway increases diabetes by increasing the preference to sugar and decreasing how the body uses sugar. Therefore, it is difficult to predict which steps may be used together. For that reason, we chose to propose modular phenotyping protocols, allowing us to optimise the quantity and type of information we will obtain from each study. In our experience, such modular approach will promote reduction.

In addition, we included a number of generic phenotyping steps aimed at measuring covariables that can explain variability in our main outcome measures. These steps will increase the quality of the datasets we collect and promote reduction.

To further reduce the number of animals necessary to reach statistical significance and demonstrate an effect, we:

- 1) consult a senior biostatistician to advise us on statistics and experimental designs,
- 2) reduce experimental variability using procedures, designs and technologies that limit variability in the measures,
- 3) acclimatise animals to stressful procedures such as injections (using mocking injections where the animals are handled as during actual injections but do not receive any substance). This allows to familiarise the animal to the procedure, decreasing stress during the actual experiment where data are collected, and therefore decreasing variability,
- 4) maximise the information that can be recovered from a single animal by testing several conditions and measuring multiple variables in the same mouse. See also above paragraph on modular protocols
- 5) use the guidelines and online tools from NC3Rs and PREPARE to design experiments

- 6) We use in vivo imaging technologies and measure in awake animals to obtain allowing for longitudinal assessments. Particularly, the use of calcium imaging, allows the study of a large number of cells, whose activity can be recorded longitudinally over several days. This approach reduces the number of animals needed and enables statistical within-animal comparison, leading to a higher statistical power.

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

We use randomisation, blinding protocols, and crossover designs when possible. To further reduce variability, we use inbred strains, littermates, body-weight-matched, gender-matched and age-matched groups. We acclimatise animals to experimental procedures to reduce stress-induced variability. For example, for animals who will receive substances through a pre-implanted brain cannula, we habituate the animals to the injection paradigm with "mocking brain injections" during which we connect the mice to the injection system without delivering any substance. We do this on 4 consecutive days before the actual substance administration. In behavioural experiments, will acclimatise animals to modified testing conditions and train them to perform expected tasks in order to increase the quality of the data collected.

We measure production and breeding performance and ensure the minimum numbers of animals are used. Whenever possible, we maintain homozygous lines. We also reuse WT animals for embryo recipients and in the generation of vasectomised males. However, it is best to keep cre alleles hemizygote, which we use routinely. Because we use bi- or tri-allelic strains, a number of animals with unwanted genotypes are being created.

Data collection from behaving undisturbed animals (telemetry, behavioural or calorimetric chambers) significantly reduces stress-induced variability. These technologies often enable continuous data collection, a way to detect small dynamic changes. Using these technologies, each animal can be used as its own control, which negates the need to have a second cohort of control mice running alongside.

Surgical procedures also promote reduction by allowing the administration of substances in freely moving animals (undisturbed), hence reducing stress and variability in collected experimental data, therefore reducing the number of animals required to reach good statistical power. To reduce the number of animal needed in surgical procedures, we implement strategies to maintain the potency of implanted catheters or cannulas, including regular flushing, and we use of surgery bedding.

Systematic use of body weight loss as humane endpoint sometimes leads to the loss of precious experimental animals otherwise clinically fine. Therefore, we use adequate techniques to assess welfare depending on each procedure (body composition scoring, assessment of clinical signs of suffering). Experience has enabled minor interventions to enable animals to continue to be used without additional suffering. In addition, strain-specific baselines for clinical signs improve our assessments of animal welfare. For disease models (i.e., ob/ob mice), different endpoints are necessary to allow us to study the disease state. These mice will be used under a specific age and allow to display frank diabetes for a maximum period of 8 weeks.

BC 1



Mouse is emaciated.

- *Skeletal structure extremely prominent; little or no flesh cover.*
- *Vertebrae distinctly segmented.*

BC 2



Mouse is underconditioned.

- *Segmentation of vertebral column evident.*
- *Dorsal pelvic bones are readily palpable.*

BC 3



Mouse is well-conditioned.

- *Vertebrae and dorsal pelvis not prominent; palpable with slight pressure.*

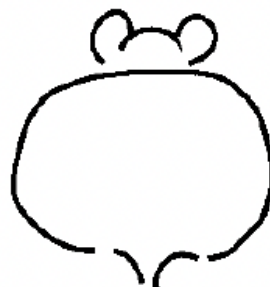
BC 4



Mouse is overconditioned.

- *Spine is a continuous column.*
- *Vertebrae palpable only with firm pressure.*

BC 5



Mouse is obese.

- *Mouse is smooth and bulky.*
- *Bone structure disappears under flesh and subcutaneous fat.*

A "+" or a "-" can be added to the body condition score if additional increments are necessary (i.e. ...2+, 2, 2-...)

A retrospective assessment of reduction will be due by 11 July 2027

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.

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. We will use genetically altered mice or viral vectors to manipulate the activity of pathways of interest.

We use and continuously develop non-invasive technologies that enable the collection of data without killing the animals (e.g., use of a scan to determine the amount of fat in the body). For example, we have recently developed the use of telemetry to monitor body temperature in behaving animals in their normal housing environment, and we are planning to use telemetric devices to monitor blood glucose continuously.

In mice dosed with Tamoxifen through oral gavage, we acclimatise the mice to the scruffing and supplements 1 week prior dosing, we monitor weight loss closely and administer mash, hydrogel, peanut butter and heating pads to mitigate weight loss.

Cannulas inserted into a blood vessels, into the brain or into the intestine, although invasive, permit the development of refined animal models with discrete manipulation of candidate pathways, which helps for the generation of data more easily interpretable. During brain infusion of large volumes ucb (>2ul), infusion flow rate will remain below 1ul/min to avoid sudden increase in CSF pressure. Cannulas inserted into a blood vessel also enable decreased suffering in protocols with repeated blood sampling and delivery of substance into the blood. We are developing the use of new technology that use transgenes to insert proteins into some cells which then allow to rapidly turn on and off these cell populations. These tools permit reversible and discrete pathway manipulations, therefore minimally affect the phenotype of the animals. We constantly improve our surgical techniques, for example with the use of magnetic access buttons, allowing reduced catheter flushing frequency. These magnetic vascular access buttons allow a magnet-enabled clean and easy connection of an implanted catheter to a syringe for direct access, but otherwise maintain the catheter in a closed system, allowing long-term patency, low-stress sampling and infusion, and group housing of implanted animals. We are also currently developing the use of programmable minipumps, which are small devices that we insert

under the skin or in the body cavity and deliver drugs continuously. The programmable versions refine chronic drug delivery by enabling pulsatile delivery, better representing how the body works. We use in vivo imaging technologies to obtain more physiologically-relevant data in behaving mice, and as much as possible perform measurements in awake mice to allow for longitudinal assessments. To limit the adverse events related to the use of tools to determine how neurons in the brain are connected together, we are continuously integrating new tools that are less toxic to the brain. When implementing two-photon calcium imaging in rodents, we will collect more physiologically relevant data within a same animal over sessions with different experimental treatments, refining the type of data we collect. There are already plenty well-characterized tools, refined experimental protocols and well-established analysis pipelines that will help us maximize the amount of data and quality of each mouse and minimizing the total number of animals needed.

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

We need to use animal models relevant to 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.

Very rarely we can use terminal anaesthesia to measure how the body uses energy, but often anaesthetics inhibit the brain control of metabolism and are therefore not appropriate. In addition, when we study behaviour, terminal anaesthesia are not appropriate either.

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

To improve the quality of life of the animals we:

- reduce stress by group housing where possible to keep singly housed mice to a minimum, using buddies when needed.
- use environmental enrichment (EE), within what is available to us at our animal facility. In general, EE is an animal housing technique composed of increased space, physical activity, and social interactions, which in turn increases sensory, mental, motor, and social stimulation. Igloos, running wheels, saucer wheels, fun tunnels, and other objects in the housing environment provide stimulation by promoting exploration and interaction. EE can be maintained when animals are handled (e.g. handling tunnels), thus minimising stress when for example an injection is needed. In mice housed on grid flooring (typically 35% of the surface punctures with 5mm diameter holes), a plain nesting area will be provided to mitigate harm.
- use a series of non-invasive methods for characterising mice so that we can generate useful data without killing the animals, thus minimising the number of mice needed (telemetry etc..)
- use pain killers to lessen pain.
- provide 'behavioural' training to mice undergoing specific procedures (e.g. acclimatisation to single housing in cages used to measure food intake and energy burned).

- use scoring sheets to monitor the health of animals undergoing procedures.
- monitor harm and potential adverse events with daily observations and provide energy-dense enrichment when appropriate.
- provide additional intervention in post-surgical conditions (post-operative bedding, nesting, heating pads and mash)
- provide medicated palatable substances for voluntary treatment such as flavoured jelly, paste or milk shake liquid, when necessary.
- before and during tamoxifen delivery by oral gavage, provide energy-dense supplements, mash and heating pads as needed to minimise weight loss.
- During brain infusion of large volumes through the intracerebroventricular route (higher than 2 microliters), infusion flow rate will remain below 1ul/min to avoid sudden increase in CSF pressure.

In experiments with LPS administration we will always test new batches in pilot studies, adjust doses based on strain, age and gender and typically kill the animals at 48 to 72h after a single injection.

- Two-photon calcium imaging techniques reduces the number of animals needed, allowing us to maximize the amount and quality of data recorded from each mouse, leading to an overall reduction in the total number of mice used. Given the nature of the data that will be generated, results will be reported in accordance with the ARRIVE guidelines, maximizing the information from the research performed, while minimizing unnecessary experimentation. .
- animals implanted with a head holder will be acclimatized over a period of several days until they are comfortable with the experimental setup.
- For imaging experiments, we will first perform experiments in a small subset (between 2 - 3 mice), and we will refine the experimental procedures whenever its possible or necessary (e.g., refinement of acclimatization protocol) before resuming experiments with other mice in the same experimental protocol. We will seek further opportunities to refine procedures with consultations with animal technicians as well as the Named Animal Care and Welfare Officer (NACWO) and the Named Veterinary Surgeon (NVS), as well as other colleagues with previous experience.
- During acclimatization for the head fixation setup, the recording setup will include a running wheel, and adjustable tilt and height position, allowing the animal to be as comfortable as possible, since it allows the animal to express its natural behaviour of locomotion. Once we provide the means for the animal to have voluntary movement, it can help to minimize the stress of being in an unfamiliar experimental setup.
- For acclimatization, we will use a score system to assess how comfortable the animals are in the setup. In this system, we gradually introduce the animals into the head-fixation setup. Comfortable animals tend to remain still or to run forwards, while animals that are stressed or uncomfortable will tend to try to run backwards and bend their bodies to escape. By keeping a track of the proportion of time when the animals are running forwards or still vs. the amount of time they try to move backwards, we can assess the progress during training and take corrective measures (i.e., to reduce the time of

exposure, to allow the animal to freely explore and become more familiar with the setup, a small reward) when necessary.

- To minimize the risk of losing the head holders the animals will be housed individually. This is done to avoid interactions with other mice (such as fighting) that might damage or dislodge the head holder. However, this also means that the animal will be subject to the stress of social isolation. To mitigate this situation, we will ensure that within the cage the animal has sufficient enrichment and include items such as additional nesting material, chew blocks and whenever possible, a running wheel to improve welfare and minimize the effects of isolation.

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

Laboratory Animal Science Association (LASA) guiding principles documents of aseptic technique (https://www.lasa.co.uk/current_publications/)

ARRIVE (Animal Research: Reporting of In Vivo Experiment) guidelines for preparing papers for publication (<https://www.nc3rs.org.uk/arrive-guidelines>)

PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for planning our experiments (15 topics including formulation of the study, dialogue between scientists and the animal facility, and methods) (<https://www.ncbi.nlm.nih.gov/pubmed/28771074>).

Refining the use of head fixation and fluid control in rodents: <https://nc3rs.org.uk/3rs-resource-library/refining-use-head-fixation-and-fluid-control-rodents>

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

To be informed about latest advances we will primarily use the National Centre for the Replacement and Reduction of Animals in Research (NC3R) website (<https://www.nc3rs.org.uk>) and 3Rs tools in-house tool and external resources such as Norecopa <https://norecopa.no/databases-guidelines>; <https://resources.jax.org/>; Systematic Review Facility (SyRF). It provides an extensive library of 3Rs guidelines, resources, practical information and themed hubs. It also provides links to publications, other online resources, and video and training materials.

Implementation of the advances will be defined on a case-by-case basis and will be informed by the latest NC3R recommendations.

We also share expertise across our institute with regular internal seminars and have an external seminar series so hear from other experts on a regular basis.

We have a team of researchers at our institute who specialise in using mice in obesity and diabetes research and they regularly provide us with updates on new advances.

A retrospective assessment of refinement will be due by 11 July 2027

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?