Refinements to mouse high-yield behavioural experiments involving head fixation and fluid/food control

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Abstract

High-yield behavioural experiments with mice, in which the animals perform hundreds of trials in one session, are increasingly common, particularly in the field of sensory neuroscience. Despite this, there is little guidance on how best to perform these types of studies to optimise both scientific outcomes and animal welfare. This article summarises current practices and provides recommendations to improve animal wellbeing and data quality, based on a survey of the community, literature reviews, and the expert opinion and practical experience of an international working group convened by the UK's National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs). Topics covered include head-fixation surgery and post-operative care, habituation to restraint, and the use of fluid/food control to motivate performance. We also discuss some recent developments that may offer alternative ways to collect data from large numbers of behavioural trials without the need for restraint. The aim is to provide support for researchers at all levels, animal care staff, and ethics committees to refine procedures and practices in line with the refinement principle of the 3Rs.

Keywords: 3Rs, animal welfare, guidelines, mice, neuroscience, restraint, water restriction

Contents

1. Introduction

1.1. Background

Mice are increasingly used in research to investigate the neural circuitry of perception and cognition, owing to the availability of genetic tools and perturbation technologies (Navabpour, Kwapis, & Jarome, 2020), brain atlases (Wang et al., 2020), advances in neural measurement (Dana et al., 2019; Jun et al., 2017; Peron, Chen, & Svoboda, 2015; Steinmetz et al., 2021), and growing awareness of their sensory (Seabrook, Burbridge, Crair, & Huberman, 2017) and learning capabilities (Nakajima & Schmitt, 2020). High-yield methods for probing mouse behaviour involve tasks that often result in, or require, a large number of trials in each session ("high-yield" methods, Burgess et al., 2017). The number of trials that the mouse needs to complete varies depending on the experimental power needed but is often many hundreds. This approach is common in sensory neuroscience where, for example, brain activity is recorded in response to stimuli, and a large number of presentations may be required to collect reliable readouts of sensory function in a sufficient number of trial types. For example, many different stimuli may be used in different locations and/or combinations, requiring a large number of trials to be completed to have sufficient replicates of each possible permutation.

The increasing use of mice, and to a lesser extent rats (Schwarz et al., 2010), in high-yield behavioural experiments has highlighted possible animal welfare concerns associated with restraint, surgically implanted head fixation devices, and fluid/food control. Establishing best practice for these experiments represents a refinement opportunity in this growing field.

Head fixation is used not only to control the sensory and motor environment, but also to allow techniques that would be difficult in a mobile animal, such as two-photon calcium imaging (Dombeck, Khabbaz, Collman, Adelman, & Tank, 2007) and patch-clamp recording (Margrie, Brecht, & Sakmann, 2002). Head fixation can therefore be necessary for consistent and reproducible measurements to be taken, but it is generally aversive to rodents. Without proper habituation, restraint is a source of stress, inducing rapid increases in heart rate, stress hormones and overt signs of distress (Keim & Sigg, 1976; Pare & Glavin, 1986). This is not only a welfare concern but is also likely to impact on the ability of the animal to perform tasks and provide reliable data. In the absence of a behavioural task, acute restraint generates a negative affective state in rats (Stuart, Butler, Munafo, Nutt, & Robinson, 2013) and repeated restraint, for as little as 10 minutes per day, is known to induce behavioural despair, while repeated exposure to restraint is a commonly used rodent model of depression (Chiba et al., 2012). Whilst there are potential species differences and mice are more routinely used in head-fixation studies than rats, mice that are not engaged in a task also show markers of stress following chronic restraint, including elevated levels of corticosterone and impacts on hippocampal volume (Woo, Hong, Jung, Choe, & Yu, 2018; Yun et al., 2010).

For a rodent to be restrained by its head, an initial surgery is required to implant a head-fixation device. This may be combined with other surgical interventions, for example injection of virus for gene change induction or the implantation of electrodes to record brain activity during the subsequent head-restrained task (e.g. Li et al., 2018; Radvansky & Dombeck, 2018; Williams, Speed, & Haider, 2018). Again, this surgery is necessary for the scientific outcome, but needs to be performed in a way that does not unduly compromise the welfare of the animal. There are existing guidelines on how to perform rodent stereotactic surgery in a way that is aseptic (Lilley & Berdoy, 2017) but there is less specific guidance when it comes to the surgical and post-operative care of rodents with head implants.

Increasingly, head fixation is used in tasks that require a behavioural response from the animal and/or the animal to navigate through a virtual space (Thurley & Ayaz, 2017). This is enabled by using equipment such as treadmills or 3D tracker balls, often in conjunction with screens and projectors that display the virtual space the animal is navigating through, all whilst the rodent is restrained by its head (e.g. Havenith et al., 2018; Pinto et al., 2018; Radvansky & Dombeck, 2018; Sato et al., 2017; Sofroniew, Cohen, Lee, & Svoboda, 2014). Whilst this widens the possibilities for the application of these approaches, it may also

lead to more sources of concern for the care of the animal. Conversely, providing a means by which the animal can move whilst being head restrained may represent a refinement over whole body restraint.

Some head-restrained tasks are motivated by reward, including fluid or food rewards. Typically, fluid rewards are preferred for experiments as the size of reward can be finely titrated; it can be difficult for a rodent to chew a food pellet whilst head restrained, and the time taken to consume this reward would run counter to the need for as many trials as possible to be completed within a session. Performance is therefore often motivated by controlled access to water in the home cage with small volumes of water used as the task reward (e.g. Galinanes, Bonardi, & Huber, 2018; Han, Zhang, Zhu, Chen, & Li, 2018; International Brain Laboratory et al., 2021; Li et al., 2018; Mayrhofer et al., 2013; Murphy et al., 2016; O'Connor et al., 2010; Radvansky & Dombeck, 2018; Sanders & Kepecs, 2012; Sariev et al., 2017; Sato et al., 2017; Sofroniew et al., 2014; Williams et al., 2018). However, food rewards can be delivered not only as solid pellets (e.g. Sauerbrei et al., 2020) but also, and more commonly, as caloric liquids (e.g. Nashaat et al., 2017; Phillips, Heath, Ossowska, Bussey, & Saksida, 2017; Pinto et al., 2018; Poort et al., 2015). That food control is more commonly used to motivate other kinds of behavioural tasks in rodents means greater expertise is often available for this approach. However, both fluid and food control present several potential welfare concerns if poorly managed or even well-handled, but for prolonged periods, as is typical in rodent behavioural experiments.

1.2. The Working Group

The NC3Rs is an independent, scientific organisation established by the United Kingdom (UK) Government in 2004 to lead the discovery and application of new technologies and approaches to replace, reduce and refine the use of animals for scientific purposes. In 2018 the NC3Rs convened an expert Working Group with the following terms of reference:

1. To review the use of head fixation and fluid control in rodent high-yield experiments.
2. To identify the animal welfare issues.
3. To recommend opportunities for refinement.
4. To publish the deliberations of the Working Group and promote its recommendations within the international research community.

The overall aim was to identify and collate best practice for rodent high-yield behavioural experiments and to support the international community to improve animal welfare whilst sustaining or increasing the value of the science. The Working Group consisted of experts from academia around Europe, many with experience of working in the USA, members of the UK pharmaceutical industry and staff of the NC3Rs. In addition to researchers with years of practical experience designing and running high-yield behavioural experiments, the group also included representatives with professional expertise in animal welfare and care, alternatives to head fixation, and general rodent behaviour.

1.3. Scope of this study

In this paper, we discuss each of these aspects of rodent high-yield behavioural experiments, focussing on mice, and give recommendations for the most refined approach currently available, based on the expert advice and experience of the Working Group, the results of the survey conducted, and the available information in the literature. Where there is published experimental evidence to support a specific recommendation the citation is given. We also identify questions and areas where further research is required to identify and validate refinements. These will be of principal interest to those engaged in rodent studies requiring head fixation and/or fluid or food control, or planning to adopt these procedures. Separately, these two focal areas may also be of interest to a wider audience, particularly the use of fluid control, which is being adopted increasingly to motivate behaviour in other types of tasks.

2. Methods

The Working Group engaged in several activities which informed the recommendations in this paper. Multiple meetings of the members allowed for deliberation and discussion of their collected expert opinions. This included sharing common and best practice from their own laboratories as well as those of their collaborators internationally. Data gathering exercises were also performed, including two systematic literature searches investigating the use of head fixation and fluid control, and an online survey.

2.1. Literature searches

The systematic literature searches were conducted in March 2019 using the databases PubMed, Web of Science, Scopus, and Ovid. In the case of Ovid, both Medline and Embase indexes were used. Details of the keywords and search strategies used are given in an appendix (Appendix 1: Search strategies for the systematic literature reviews). Duplicates were removed, then titles and abstracts of the papers retrieved and reviewed for relevance before further exclusion criteria were applied. For the head fixation search, results were excluded if the experiments were conducted under anaesthesia (terminal or otherwise) or if, despite the search criteria, the experiments used species other than mice or rats. Finally, we focused on papers that specifically addressed methodological details or presented alternatives to traditional head fixation. Full text copies of 85 articles published between 1998 and 2020 were then obtained and screened for methodological details on how the head fixation was achieved, any information on the animal welfare impact, and reports of alternative ways to achieve high-yield behavioural data without the use of restraint.

For the fluid control search, results were excluded if they concerned pups or cross-generational studies or strains of mice or rats not typically used in behavioural studies (e.g. the Brattleboro rat); if fluid control was combined with other manipulations to induce dehydration (e.g. a high salt diet) or invasive surgical procedures (e.g. adrenalectomy); if the study was principally concerned with establishing the toxicity of a novel compound; or if, despite the search criteria, the experiments used species other than mice or rats. Finally, papers reporting physiological impacts from fluid control and measures of the HPA axis during fluid control were focused on, in addition to those performing head-fixed experiments. Full text copies of 128 articles published between 1947 and 2020 were then obtained and screened.

2.2. Survey

An online survey was conducted between April and July 2020 to establish current practice in the field and identify refinements. The survey questionnaire was developed by the Working Group and piloted by selected members and their close collaborators. A copy of the final questionnaire is given in an appendix (Appendix 2: Survey questionnaire). The questions concern protocol details that are frequently not reported in published papers, but are nonetheless crucial in conducting successful studies, as well as animal welfare implications that are often a focus for institutional ethical review committees. Ethical approval for the survey was granted by the University of Oxford's Medical Sciences Interdivisional Research Ethics Committee (IDREC) Central University Research Ethics Committee (CUREC), reference R68817/RE001.

The survey was administered via SurveyMonkey. Participation was voluntary, and responses were submitted anonymously after completing a consent statement. As responses were anonymous, multiple responses per research group were possible, but respondents were requested to be "the lead person responsible for carrying out the research or the person chiefly involved in the care of the animals involved." This means that duplicate responses could be possible but would likely reflect differing practices between individuals within a larger group. The data acquired were managed according to a data management plan for NC3Rs office-led data sharing projects available on request from the corresponding author or enquiries@nc3rs.org.uk.

Participants were recruited principally by direct email from members of the Working Group. The survey link within the email was not restricted to the recipient to allow for a "snowball" of further recruitment. The survey was also advertised on the NC3Rs website, Twitter accounts of the NC3Rs and Oxford3Rs, the NeuroMethods Slack channel and the LinkedIn groups for the Society for Neuroscience, the British Neuroscience Association, the Federation of European Neuroscience Societies, Animal Models in the Neurosciences and Laboratory Animal Veterinarians. These adverts identified head fixation and fluid control as focal areas, but the survey was open to those performing rodent behavioural studies that employed only one or even neither of these approaches.

A total of 137 survey responses were returned for analysis. The survey responses represented a wide geographical distribution with responses from 20 countries in Europe, North America, South America, Africa, Asia, and Australia. Most respondents were researchers, including 38 laboratory heads, 40 post-doctoral researchers, six laboratory technicians and 14 graduate students, but some responses were also received from animal care staff (nine) and veterinarians (seven) who routinely cared for animals undergoing the procedures of interest.

The raw data were downloaded to Excel and summarised for analysis. Only anonymised data are reported here; any free-text responses that could identify individual facilities have been redacted. Results are reported below as absolute numbers as well as percentages since some respondents did not answer all the survey questions. Many questions asked for the frequencies of certain events to be reported as "never", "rarely", "sometimes", "usually" or "always". When necessary, these responses are reported as the median of the weighted average ± the interquartile range (IQR). These weighted averages were calculated by assigning the numerical values of 1, 2, 3, 4 and 5 to the responses never, rarely, sometimes, usually, and always, respectively.

For much of the data presented in this paper, we focused on responses from researchers employing head fixation, i.e., selecting "Head fixation device" as one of their responses to question 8, "What permanent devices are typically implanted? Select all that apply" (41 of 78 responses) and/or selecting the "Head fixation" option for question 57, "Which of the following are routinely paired with the behavioural testing of your animals?" (27 of 68 responses). This resulted in a pool of 43 respondents that form the focus of the data presented, but we also identify areas where their responses differ greatly from those of the remaining respondents who did not use head fixation.

Of the 43 respondents involved with head-fixed work, the majority were researchers, including 12 laboratory heads, 13 post-doctoral researchers, two laboratory technicians and five graduate students, but some responses were also received from animal care staff (seven) and veterinarians (four) who routinely cared for animals undergoing the procedures of interest. They predominantly worked in the UK (21, 49%) or USA (15, 35%) and overwhelmingly used mice in their research (37, 86%). 24 of 35 (69%) respondents also made use of fluid control, while 15 of 34 (44%) used food control. This level of food restriction was comparable to the population of respondents that do not use head-fixation ($\chi^2(1) < 0.001$, $p = 0.985$), but the use of fluid control was over-represented in those also using head-fixation ($\chi^2(1) = 24.32$, $p < 0.0001$).

3. Head-fixation surgery

This section describes recommendations for how to refine the initial surgery and post-operative care to allow for experiments under head-fixed conditions. While these recommendations focus on surgeries in mice, the principles also apply to rats and other small mammalian species. These recommendations are intended to supplement standard guidance on, for example, aseptic technique (Lilley & Berdoy, 2017), as well as the support offered locally. An example surgical standard operating procedure (SOP) is available in an appendix for a more detailed account of a typical approach to these surgeries (Appendix 3: Detailed surgical SOP).

3.1. Preparing the animal in the days ahead of surgery

Ensuring a good recovery from surgery requires some steps to be taken in the days before the surgery itself. An important aspect of the surgery is the pre-operative health status of the animal. The mouse to be used therefore needs to be carefully inspected before the surgery to confirm a stable condition. For instance, daily scoring of the weight and home-cage behaviours can help to detect changes in health status. Injured or sick animals should be excluded from surgical procedures. Furthermore, animals under fluid or food control need to be taken off the restriction regime for a sufficient time prior to the surgery.

Since animals undergoing surgeries are often shipped from external facilities, immediate exposure to the holding area, surgery room and interaction with an experimenter can increase stress. Careful acclimatisation to the new facilities (~5 days) and habituation to the surgery room and surgeon can help to reduce stress. Health scoring prior to surgery presents an ideal opportunity to handle the animals and begin their habituation to the surgeon.

Mice should also be habituated to the home cage that they will occupy post-surgery. If this is a new cage, it could be useful to place them in it a few days ahead of the surgery, depending on local cleaning practices. Providing mice with additional nesting material at this point and allowing time for nest building will also help maintain body temperature in the post-operative period when thermoregulation may still be compromised. Consideration should be given to what nesting material is used to minimise it tangling in the implant (Windsor & Bate, 2019), and nesting can be scored (Deacon, 2006a). Allowing time for good nesting at this point will avoid the impaired nesting that is part of mouse sickness behaviour (Gaskill, Karas, Garner, & Pritchett-Corning, 2013), further impairing post-operative thermoregulation.

Post-operative analgesia can be delivered by jelly (see Section 3.3.2) but mice need to be habituated to the non-drug form of this ahead of time to avoid neophobia. Place this and any other recovery diet in the cage in the days before surgery to avoid this.

3.2. Head fixation and stereotactic surgeries

3.2.1. Instruments and operating table

Post-operative infection, even one not apparent to the naked eye, can impact both the physiology and behaviour of rodents (Bradfield, Schachtman, McLaughlin, & Steffen, 1992). Aseptic conditions are also a key factor for the long-term stability of a chronic head implant. Asepsis will help to avoid infections, accelerate healing, and reduce animal suffering and discomfort. All of these aspects will likely have a positive impact on subsequent behavioural performance and reproducibility. Surgeries can be carried out within a local (“wound level”) asepsis scheme whereby aseptic conditions are limited to the surroundings of the head. This can be achieved by covering the rest of the body by sterile barriers, such as drapes. Nevertheless, general asepsis can be required by specific needs or local regulations. The Laboratory Animal Science Association has published detailed guidelines on aseptic procedures (https://www.lasa.co.uk/current_publications/, Lilley & Berdoy, 2017) and NC3Rs-funded video tutorials are available on the Research Animal Training website (https://researchanimaltraining.com/article-categories/aseptic-technique/).

The organisation of the operating table plays an important role in maintaining aseptic conditions. Before the surgery, the table should be free of clutter and thoroughly disinfected, ideally with a chlorhexidine solution, otherwise with 70% ethanol. Areas can also be covered in part with sterile drapes to prevent contamination of the surgical instruments. An ergonomic disposition of the surgical instruments will minimise the surgeon's need to move away during the procedures, thus reducing the risk of breaking asepsis.

All surgical instruments, glassware, and other elements, such as head-posts, implants, electrodes, glass windows etc., should be sterilised and laid out in an orderly manner. For items that cannot be autoclaved, it may be necessary to employ cold sterilant or a glass-bead steriliser for wound level asepsis. Instruments

that have been sterilised using a glass-bead steriliser need to be allowed to cool on a sterile surface before use.

The surgeons are recommended to wear a clean surgical gown, face mask and gloves following scrubbing up. Following this initial thorough clean, the hands can be sterilised by scrubbing with a chlorhexidine-containing skin disinfectant.

Although less commonly used (Table 1), a trained assistant can help minimise the chances of the surgeon breaking asepsis. The surfaces of instruments that cannot easily be sterilised, for example anaesthetic vaporisers and surgical microscopes, can be wrapped in sterilised foil or similar. If batch surgeries are carried out, separate sterilised instruments should be used for every animal to ensure uniform levels of asepsis, reducing infection rates and variability in the resulting data.

Table 1: Responses to the survey question "What steps are taken to ensure aseptic conditions? Select all that apply." by respondents employing head fixation, n = 36.

Responses are ranked by the number of positive responses, illustrating what steps are commonly used over those currently rarely implemented. Presented as percentage of responses (number of positive responses).

Response	Percentage of responses (raw number of responses)
Sterile consumables	94% (34)
Sterile instruments	92% (33)
Sterile equipment	81% (29)
Sterile surface for instruments	81% (29)
Mask	72 % (26)
Separate sterile instruments for each animal	56% (20)
Scrubbing up	44% (16)
Sterile foil	36% (13)
Separate prep area	36% (13)
A trained assistant	19% (7)
A trained anaesthetist	8% (3)

The head-post implant is one of the key elements of this procedure. Implants are typically designed according to the experimental requirements as well as spatial constraints. For illustration, Figure 1 depicts a series of head-posts utilised in mice made of different materials, shapes and sizes. When choosing the head-post material, the strength, rigidity, weight and biocompatibility must be factored in. Typically, head-posts are made of precision-machined or laser-cut metal (e.g. titanium, aluminium or stainless steel). Titanium is preferable due to its biocompatibility, reduced probability of corrosion (Goldey et al., 2014) and light weight. On the other hand, plastic materials can also be used, for example if magnetic resonance imaging will form part of the work to be conducted. Another alternative to machined metal plates are stainless steel screws that can be anchored to the skull and further cemented to increase grip, although the

use of skull screws is currently uncommon for head fixation devices, with only eight out of 42 respondents (19%) using skull screws in comparison to the wider use of bone cement (27 of 42 respondents, 64%) and dental adhesive (35 of 42, 83%).

Head-posts and any other implants need to be prepared in advance and meticulously cleaned and sterilised before use. It is highly recommended to have a sufficient number (three to four) of sterile backups ready for use before the surgery, since these small parts can easily get lost or accidentally contaminated during the procedure. Detailed instructions on how to implant a head-post can be found in the documentation of the International Brain Laboratory (International Brain Laboratory, 2020a; International Brain Laboratory et al., 2021).

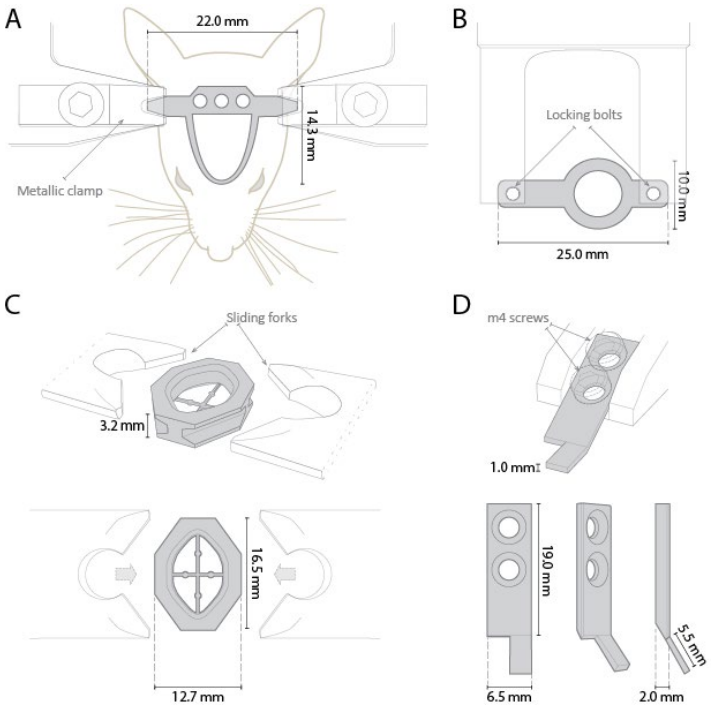


Figure 1: Examples of different head bar designs (in grey) with their corresponding locking system (in white).

A "Bar and arc" shaped head bar designed to provide access to the dorsal part of the cerebral cortex (Galinanes et al., 2018). The 430 mg head bar is machined from 1.2 mm thick titanium sheets and sits on top of the interparietal bone. The "arc" sits partly on the lateral ridges and the nasal bone providing several anchoring points for good stability. Holes in the "bar" reduce weight without compromising rigidity while providing additional entry points for the dental cement increasing bonding strength. The wings of the head bar are locked by two metallic clamps on each side of the mouse's head. **B** Similar design as A but with a round opening that allows more flexible skull positioning, for example being positioned over the parietal and interparietal bones to gain access to the visual cortex (Erchova, Vasalauskaite, Longo, & Sengpiel, 2017). The stainless-steel head bar, with a thickness of 1 mm and weight of 920 mg, is locked with a pair of stainless-steel screws. **C** RIVETS head bars (Osborne & Dudman, 2014; https://dudmanlab.org/html/rivets_fabrication.html) were conceived as a flexible system based on 3D printed head bars that can be adapted to multiple experimental needs, such as in vivo electrophysiology or imaging. **D** An angled metallic head bar design that can be positioned on virtually any part of the skull. Machined in aluminium, it weighs 16 g. The angled wing is inserted into the canal of a metallic rod and locked with a pair of m4 screws (Abraham, Guerin, Bhaukaurally, & Carleton, 2012).

3.2.2.Anaesthesia and analgesia

Implantation of a head-post is an invasive procedure that must be done under general anaesthesia. Table 2 shows an example of a balanced anaesthesia schedule based on a combination of analgesic and anti-inflammatory drugs in addition to a general anaesthetic. Other examples and further discussion can be found elsewhere (e.g. Percie du Sert et al., 2017), although the survey revealed that a general schema is followed by those employing head fixation (Table 3). This included the use of inhalation anaesthetics such as isoflurane by 80% of respondents (29 of 36 respondents), along with both NSAIDs and opioids being widely used both pre-emptively and in the immediate post-surgical period.

Table 2: Typical agents used as part of rodent stereotactic surgeries such as implanting a head fixation device.

As with any prolonged stereotactic surgery, careful consideration should be given to the anesthetic and analgesic agents to be used at every stage of the process. Best practice is to use pre-operative analgesia, local anesthetic at the site of incision, gaseous general anethesia, and post-operative analgesia that may continue to be administered for several days post-surgery.

Agent	Administration	Effect
Buprenorphine (opioid)	Pre-anaesthetic (injectable, 20 minutes before general anaesthesia)	+ Analgesic effect during the surgery + Reduces the necessary dose of isoflurane anaesthesia
Lidocaine/bupivacaine	Local anaesthetic (injectable in the field of surgery, 10-30 minutes before skin incision)	+ Local anaesthesia; lidocaine fast onset / short duration (10 minutes / 1 hour); bupivacaine slow onset / long duration (30 minutes / > 4 hours)
Carprofen (non-steroid anti-inflammatory)	Anti-Inflammatory and analgesic (injectable at the end of the surgery)	+ Prolonged analgesic effect
Isoflurane	Anaesthetic (gaseous, induction and maintenance)	+ Loss of sensation + Loss of consciousness + Muscle relaxation

Table 3: Responses to the survey question "Which of the following form part of your standard surgical drug regimen?" from respondents employing head fixation, n = 36.

The following aspects of the recommended best practice are already widely observed, with 80% of respondents using gaseous anaesthesia, over 40% using some form of pre-emptive analgesia and over 40% using post-operative non-steroidal anti-inflammatory (NSAID) treatments. Fluids are also widely delivered, typically during the surgery itself. Presented as percentage total responses (raw number of responses).

Compound	Pre-emptive	During surgery	Post-operative
Opioids	42% (15)	28% (10)	33% (12)
Sustained-release opioids	6% (2)	0% (0)	3% (1)
NSAIDs	47% (17)	17% (6)	42% (15)
Steroids	14% (5)	0% (0)	6% (2)
Other anti-inflammatories	8% (3)	6% (2)	11% (4)
Local anaesthetic	42% (15)	31% (11)	6% (2)
Inhalation anaesthesia	53% (19)	81% (29)	3% (1)
Injectable anaesthesia	8% (3)	8% (3)	3% (1)
Fluids	28% (10)	53% (19)	36% (13)
Routine antibiotics	3% (1)	0% (0)	19% (7)

3.2.3.Health care under anaesthesia

Anaesthetised animals must be continuously monitored and cared for in a number of ways. In mice and many other species, the eyelids stay open under anaesthesia. The eyes therefore need to be protected either by applying sterile ophthalmic ointment, petroleum jelly, or eye drops. Monitoring vital signs, such as respiration and heart rate, is key to ensuring a healthy animal and appropriate depth of anaesthesia. Sufficient depth of anaesthesia should be confirmed, particularly before any painful procedure, for example by checking the toe-pinch withdrawal reflex. Respiratory distress can occur if the anaesthesia level is too deep or air pathways are blocked (e.g. by tongue retraction), so experience and competence in interpreting and responding to these signs is crucial. Advice on anaesthetic monitoring and intraoperative care, along with other aspects of anaesthesia, is given in the e-learning modules available from the NC3Rs and Research Animal Training: https://nc3rs.org.uk/e-learning-resources

Since thermoregulation is reduced in anaesthetised animals, body temperature must be artificially maintained at 37°C with closed-loop controlled heating pads or using fixed temperature systems (e.g., recirculating warm water blankets). Animals can be covered with sterile drapes to further reduce heat dissipation whilst simultaneously providing a mechanical barrier, reducing the likelihood of breaking asepsis. Transparent drapes achieve this without impeding visual access for inspecting vital signs, such as breathing rate. Use of cling film, some brands of which are available sterile on purchase, may better retain heat compared to other drape materials (Celeste, Emmer, Bidot, Perret-Gentil, & Malbrue, 2021).

If the procedures are performed under aseptic conditions, bacterial infections rarely occur with immunocompetent mice. Prophylactic administration of antibiotics is therefore generally not required. Nevertheless, subsequent infections of the skin or areas below the implants can occur over long periods of

time and specific antibiotic treatment may become necessary. Case-by-case analysis with the local veterinarian will help to determine the best treatment and most refined delivery method.

For surgeries lasting longer than 30 minutes, active fluid-replacement with sterile isotonic saline should be considered. The administration of subcutaneous (s.c.) or intraperitoneal (i.p.) injections at the beginning of the surgery is recommended. For mice, a single 1ml injection should be sufficient. For surgeries lasting longer than an hour, a second 1ml injection at the end of surgery should also be considered. Notably, many survey respondents deliver fluids during the surgery itself (19 of 36, 53%; Table 3), which for shorter surgeries could be replaced by these pre- and post-surgical injections to reduce the time the animal is under anaesthesia, as well as simplifying the surgery itself. The mean length of surgery reported by 37 survey respondents employing head-fixation was 110 minutes, ranging from 45 – 300 minutes, suggesting that pre- and post-operative fluids would be widely applicable.

3.2.4.The surgical procedure

Once the anesthetised animal is mounted on the stereotactic frame, the surgery begins by making an incision in the scalp to provide access to the skull for implantation of the head-post. In addition to aseptic practice, a strong bonding between implant and skull is another crucial factor for long-term stability, avoiding loosening or rejection of the device and the animal welfare implications of this. This is achieved by removing the periosteum and using a bone-compatible cement (typically dental cement or dental acrylic on top of a cyanoacrylate layer). Adherence to the cement is increased by etching the skull with a drill or scalpel. Etching can be enhanced with agents such as ethanol or dilute peroxide, but these would have to be used with extreme care to avoid contact with the animal's skin, particularly with peroxide.

Notably, results have been seen to vary with different brands of cement. If problems are encountered, trying a different formulation may improve the outcome. Members of the working group have had most success with Superbond C&B dental cement from Parkell, marketed as C&B Metabond in some countries.

Once the skull is exposed and prepared, it is typically covered with a layer of a priming agent such as cyanoacrylate glue (i.e. veterinary tissue glue). At the same time, the head-post can be positioned to its final location and held in place until the glue has cured. The head-post and skull are then covered with a layer of cement. Head-post surgeries are often performed simultaneously with other surgical procedures (e.g. Holtmaat et al., 2009; Holtmaat et al., 2012), so areas for subsequent craniotomies should be spared. From respondents to the survey, the most common combined procedure was viral delivery of genetic material (28 of 42 respondents, 67%) and/or lesioning of a discrete brain area (11 of 42, 26%), although 24% of respondents (10 of 42) did not combine the implantation with any other surgical intervention.

Additional anchoring screws can be used to obtain stronger bonding, but this is uncommon and typically reserved for rats or especially large implants. If screws are to be used, special care must be taken to adjust the screw length to avoid damaging the underlying dura or brain.

Finally, covering the edges of the skin and hair with primer and cement can create an ideal seal to protect the wound and avoid infections.

At the end of the surgery, animals are removed from the stereotactic frame. Excess petroleum jelly or eye ointment should be removed with wipes or cotton buds. It is also important to ensure that eyes, whiskers and fur are not obstructed with any cement or glue applied during the surgery. The animal should be placed in a heated recovery cage with access to sufficient water and food (Table 4). It should be kept in isolation and observed regularly until full recovery from anaesthesia.

3.3. Post-operative care

3.3.1. Steps to improve post-operative outcome

As detailed in Section 3.1, additional nesting material, food, and food types should be present in the recovery cage to encourage good thermoregulation and maintenance of body weight. Normal chow can also be placed on the cage floor so that this is easily accessible, a particularly important consideration with larger head implants. However, wet chow will be easier for the animal to eat, as well as providing a further source of fluids.

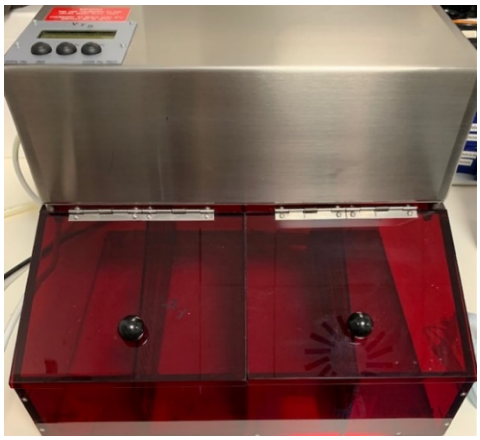
Analgesia should be considered before, during, and after surgery (Table 2) and used in all instances where it will not interfere with the scientific outputs. Administration of one or more analgesic agents is therefore likely to be a part of the immediate post-operative care, and ideally continues for several days post-surgery. This may result not only in faster recovery but also less variable research results (Peterson, Nunamaker, & Turner, 2017). Post-surgical pain can be evaluated cage-side through score sheets and grimace scales, available for mice (Cho et al., 2019; Langford et al., 2010) and rats (Sotocinal et al., 2011), as well as other species, not only at this point but in the days following surgery. Analgesia should be delivered by the least stressful route of administration. An effective method is the voluntary consumption of individual doses of palatable analgesics (e.g. in flavoured jelly, Flecknell, Roughan, & Stewart, 1999). This method of delivery can be easily continued in the days following surgery, but mice should be habituated to the non-drugged form of this jelly before surgery to avoid neophobia as discussed in Section 3.1. Notably, even with habituation some vehicles such as MediGel are not well accepted by mice, limiting the amount of analgesia that will be consumed (Hovard, Teilmann, Hau, & Abelson, 2015), whereas highly palatable substances, such as chocolate-hazelnut spread, are readily consumed without habituation (Kalliokoski, Jacobsen, Hau, & Abelson, 2011). Dosing of the palatable base should therefore be adjusted based on expected intake.

To prevent hypothermia immediately after surgery, temperature in the post-operative room should be monitored. In addition to keeping the room warm, local sources of heat such as heated cabinets or heating pads should also be used (Table 4). If possible, the animal should be able to leave the heated area once recovery starts, for example through placing a heated mat below half of their cage, giving them the choice to be close to this heat source or moving away from it once their natural thermostasis is restored. If possible, allow recovery in the home cage to reduce stress, both through familiarity as well as reducing the amount of handling of the animal.

Also consider lighting conditions during this recovery period. Of the 30 respondents that indicated when testing took place, 11 used a reversed light cycle (37%) and a further three used reversed light for some studies (10%). Dimmer conditions will be preferable for nocturnal mice, but this will be especially important if the rodents are on a reverse light cycle as the movement into a brightly lit room will interrupt their circadian rhythm.

Table 4: Different options for the housing of post-operative mice in the early recovery period.

Each option presents different pros and cons; in particular the space required to house each option varies greatly, which may further dictate where animals are housed during this period in which close monitoring is required. Images used by kind permission of Vet Tech Solutions and Techniplast.

Option	Example image	Pros	Cons
Recovery chamber. These can be used with veterinary bedding, which will ensure the animal is comfortable during recovery. Temperature can usually be changed but they should nonetheless be used only for a limited time.		▪ Whole animal is warm. ▪ Temperature can be changed depending on the animal's needs.	▪ Animal does not have the choice to leave heated area, limiting the time it can be used for.
Heated recovery cabinets These cabinets have a speed-controllable fan for heating and a HEPA filter. Typically, they can be temperature controlled and can be mobile.		▪ Whole animal is warm. ▪ Temperature can be changed depending on the animal's needs. ▪ Animal can recover in their home cage.	▪ Animal does not have the choice to leave heated area, limiting the time it can be used for. ▪ Costly. ▪ Large, requiring dedicated space.
Heated shelving units: Racks where part of each shelf is heated and temperature controlled. These can house home cages and are often mobile.		▪ Whole animal is warm. ▪ Animal can recover in their home cage.	▪ Costly. ▪ Require dedicated rack space.

3.3.2.Long-term husbandry

Following the initial days of recovery, close monitoring of the animal should continue. Other steps such as easily accessible food can also be continued to encourage maintenance of body weight. Appropriate analgesia may be required post-operatively, but its use should not normally be necessary beyond two to three days. Routine antibiotics should not be required with good aseptic technique and their use is not widespread (Table 3), but if needed, the most refined route of administration should be used, which may include delivery via water bottle.

There has been a general reluctance to group house animals with head implants, but this is now being successfully practiced by some (Table 5). Group housing animals avoids the detrimental effects of single housing over prolonged periods. The welfare impact in male mice is most well studied (Kappel, Hawkins, & Mendl, 2017), but effects of single housing on behavioural performance in cognitive tasks and neurobiological measures of plasticity have been shown in both male and female mice (Liu et al., 2020). Complications, such as implant loss, are a major source of concern and yet they are not observed any more frequently by either group- or pair-housing compared to single housing (Table 6). To minimise aggression, only animals that were cagemates before surgery should be housed together and their time apart (if singly housed in the immediate post-operative period) should be minimised. Further advice on minimising aggression in groups of male mice is given by Lidster and colleagues (2019).

Table 5: Responses to the survey question "How are animals typically housed during an experiment?" from respondents employing head fixation, n = 35.

Whilst group housing is widespread with stock animals, it is less common with those with head-fixation devices fitted. Nonetheless, single-housing post-surgery is only practiced by approximarely 55% of respondents. Presented as percentage total responses (raw number of responses).

Housing	Singly-housed	Pair-housed	Group-housed
Before surgery	0% (0)	17% (6)	83% (29)
Immediately after surgery	77% (27)	6% (2)	17% (6)
Following recovery from surgery	54% (19)	14% (5)	31% (11)

Table 6: Weighted averages for responses concerning the loss of implants or other damage that could relate to cagemate activity, split by post-operative housing method.

The assumed increased risk of adverse outcomes with the group housing of animals with head-fixation devices is often a barrier to avoiding the single-housing of these post-operative mice. However, data from the survey does not support an increase in these adverse outcomes being observed. The weighted average was derived from responses of Never/Rarely/Sometime/Usually/Always being given the numerical values of 1/2/3/4/5. Expressed as median of the weighted average ± IQR (n).

Post-operative housing	Loss/repair needed of head cap	Wound rupturing/loss of stitches	Removed [from study] due to ill health/implant complications
Singly-housed	3 ± 1 (18)	2.5 ± 1 (18)	3 ± 0.25 (16)
Pair housed	2 ± 1 (5)	2 ± 0 (5)	2.5 ± 1.5 (4)
Group housed	2 ± 0.5 (11)	2 ± 1 (11)	2 ± 1 (9)
Kruskal-Wallis across three groups	*H(2) = 1.341, p = 0.521 (not significant)*	*H(2) = 0.345, p = 0.841 (not significant)*	*H(2) = 0.803, p = 0.669 (not significant)*
Mann-Witney U across two groups (singly-housed versus [pair or group housed])	*Standardised U(1) = 1.158, p = 0.247 (not significant)*	*Standardised U(1) = 0.548, p = 0.584 (not significant)*	*Standardised U(1) = 0.885, p = 0.376 (not significant)*

3.3.3.Monitoring of post-operative animals

Practices such as providing additional palatable food are already commonplace (Figure 2) and no single adverse outcome was commonly reported as part of the survey. Amongst those that were more routinely seen were scabbing/wounding around the headcap (median of weighted average 3 ± 1, n = 33), wound rupturing, implant damage, a reluctance to move, a hunched posture, a lack of grooming (all 2 ± 1, n = 33), and piloerection (2 ± 1, n = 32). This suggests that monitoring of the site of surgery as well as the general condition of the animal are both required to assess the health of the mouse following surgery.

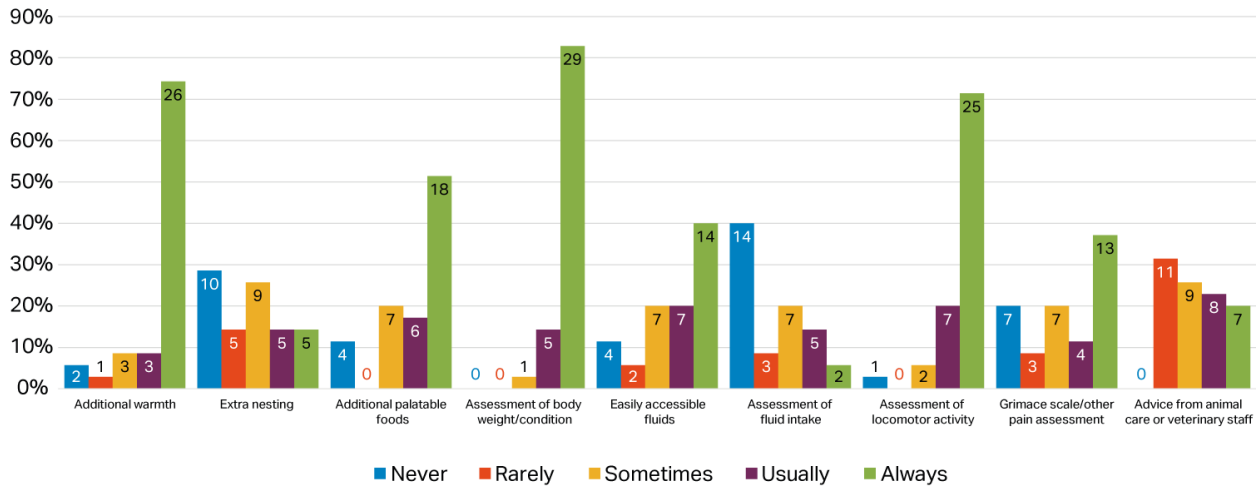


Figure 2: Responses to the survey question, "In addition to the drugs detailed above, which of the following additional steps form a part of post-operative care immediately after surgery (i.e. for the first day or several days post-surgery)? Select all that apply" from respondents employing head fixation, n = 35.

Steps such as providing additional sources of warmth, palatable food, and assessments of body weight/condition and locomotor activity are commonly practiced with post-operative animals, whereas provision of extra nexting material, steps to ease and measure fluid intake, and assess pain are less widespread. Plotted as percentages of responses with raw response numbers displayed.

Typically, post-operative welfare assessments are conducted daily (20 of 35 respondents, 57%) and consist of a check of the site of surgery for infection, the body weight and condition of the mouse, and an assessment of locomotor activity. Body condition scoring (Ullman-Cullere & Foltz, 1999) relies on a visual inspection of the mouse, scoring it from 1, emaciated, to 5, obese, thereby providing a rapid assessment that correlates well with body weight. Laboratory mice ideally have a body condition score of 3, although frequently mice can get towards a score of 4 or above as they age. Locomotor activity assessment is sometimes further divided into spontaneous and provoked activity. “General appearance” or more specific references to grooming or coat condition were also common. Some also required checks for clinical signs such as respiratory distress or seizures, although such overt adverse outcomes were rarely seen in practice. Such assessments can be completed in minutes and are widely used, the weight of the animal often serving as the key metric for decision making regarding the health of an animal.

Survey data suggests that these checks typically continue for the first two – four post-operative days (18 of 38 respondents, 47%) but may continue for a week or more (a further 11 of the 38 respondents, 29%, monitor for 5 or more days). Assessment of body weight and condition and locomotor activity may continue as a part of routine monitoring in the long-term when using mice with head fixation devices (21 and 16 of 35 respondents, 60 and 46%, “always” continue to monitor body weight/condition and locomotor activity as part of long-term care, respectively).

Whilst some groups record the results of this monitoring on scoresheets, either generic ones for all post-operative animals (13 of 35 respondents, 37%) or specific scoresheets for head-fixed work (11 of 35 respondents, 31%), the use of lab books (15 of 35, 43%) or cage cards (17 of 35, 49%) remains widespread. Using cage cards will ensure that the health records of these animals are easily accessible to others also involved in the care of these animals, for example animal house staff. Scoresheets, however, allow for more extensive, and more detailed, observations to be recorded in a consistent way that can be assessed at a glance. Scoresheets also provide a convenient *aide memoire* for the items that need to be assessed on each occasion and checking that these observations have been made is simple. We therefore encourage their use to not only assess the health and welfare of each animal but also to keep a clear record of these

health checks in an easily understood way. Guidance on developing and implementing welfare assessment protocols is given by Hawkins and colleagues (2011)(2011)(2011).

Across the working group, different styles of scoresheet were used, some involving numeric scoring systems, others simply requiring the ticking of boxes if certain clinical signs were present. Formal numeric scoring was often found to be unnecessary for decision making and to have the potential to be confusing, particularly if subtly different systems are used by different groups working in the same institution. However, the severity of signs observed can vary, from mild and only requiring monitoring to continue, to more severe and requiring input from veterinary staff or for humane killing to be considered. Taking this into account, we have developed example scoresheets that can be found in Appendix 4: Example scoresheet and heath monitoring templates. These scoresheets cover the major indicators of health and provide space for recording whether signs of concern are present in either a mild or more severe form. They are intended to be easy-to-use and easily adaptable following discussion by interested groups and their institutional ethics committees and any necessary changes can be made to ensure that they adhere to the expectations of local and national policies and legislation. Further details are given in Appendix 4: Example scoresheet and heath monitoring templates.

3.4. Recommendations to refine head-fixation surgery

- **Pre-surgical steps are key to a successful outcome, so ensure animals are healthy before surgery, habituated to the experimenter and facility, and steps have been taken to optimise the home-cage for the post-operative period. This may include habituation to unmedicated jelly to prepare for self-administering anaesthesia post-operatively.**
- **If mice are already under caloric control, return them to *ad libitum* access and allow them to regain their full weight before surgery.**
- **Good aseptic technique should always be observed.**
- **A combined anaesthetic and analgesic regimen should be followed, including pre- and post-operative analgesia. Particular care should be taken in monitoring an animal's health whilst under anaesthesia for prolonged surgeries.**
- **Deliver fluids before surgery, as well as after for prolonged surgeries, to prevent dehydration without lengthening the time spent under anaesthesia.**
- **The site of surgery and general health of the animal should be monitored closely in the days following the procedure.**
- **Group housing following implants is strongly recommended to avoid the negative welfare impacts of single housing. Group housing has not been observed to lead to greater post-surgical complications or implant loss.**
- **Welfare scoresheets are recommended for post-operative monitoring to act as a clear guide and record of the checks performed.**

4. Motivation and reward

Food or fluid control are the two primary methods used to motivate animals to perform large numbers of trials in single sessions during high-yield tasks. This immediately raises animal welfare concerns as food or fluid control are aversive and can be stressful (Rowland, 2007; Toth & Gardiner, 2000). Here we provide broad guidance on refinement and information to aid in performing a cost/benefit assessment for any scientific study.

In some cases, it is possible to conduct high-yield studies with no food or fluid control, for example when measuring innate behaviours such as odour trail tracking (Khan, Sarangi, & Bhalla, 2012), locomotion (Darmohray, Jacobs, Marques, & Carey, 2019) or predator escape (Evans et al., 2018). However, for many other behaviours, animals will not perform the required task with the required level of performance unless motivated to obtain water or food rewards. A high motivational state is also required in these animals to overcome the aversive nature of the restraint in movement such as head-fixation. Since the methods used to motivate animals, as well as levels of restriction, can vary, these options present different scientific and welfare implications (Table 7). The most refined approach, the minimum required in order to obtain the necessary motivation level, should therefore be chosen and the welfare of the animals monitored throughout the study.

Table 7: Differing approaches to food or fluid control to motivate behaviour in rodents.

The level of restriction used should be chosen based on what is necessary to motivate the majority of the animals to perform the chosen task. Greater restriction may increase motivation, but comes with a greater welfare cost, so these must be balanced to ensure the best welfare and scientific outcome. Levels of restriction needed may also be reduced by reducing stress using habituation procedures (see Section 5.1).

Restriction level	Detail	Limitations	Welfare costs
1. No restriction	Animals perform tasks for appetitive rewards with no limitations to their access to water or food.	May limit engagement with task if behaviour required is not innate. Animals may not perform at all or in sufficient numbers of trials. Higher individual and inter-session variability.	Low.
2. Time limited daily access to water or food	Animals are given fixed periods during which time they can acquire their normally daily intake of water or food with minimal impact on body weight; access is limited at other times. Could be applied following initial habituation and acquisition of the task if not successful in early stages of task.	May limit the total number of trials an animal completes as this achieves a lower motivational state than restriction regimens associated with significant weight loss. Motivational state will change over the course of the test session as the animals become satiated.	Low to moderate depending on level of restriction applied.
3. Limited quantity of water or food	Animal's normal daily intake of water or food is intentionally restricted leading to weight loss.	Achieves a high motivational state but with animals in a possible state of abnormal physiology (i.e. dehydrated or hungry). Motivational state will change over the course of the test session as the animals become satiated.	Moderate to high depending on level of restriction applied.

4.1. Diet control

Food restriction has been used extensively in behavioural neuroscience to motivate responding in tasks, whether or not any form of restraint is also used. In many protocols, animals are fed a restricted and weighed amount of their standard food each day. The amount of daily food is chosen to keep the animals at a target percentage of their free feeding weight, typically 80-85% (Table 8). During the task, a variety of caloric rewards may be delivered (Table 11). In the case of head-fixed work, this includes a 10% solution of soy milk (Poort et al., 2015), strawberry milkshake (Phillips et al., 2017) or condensed milk (Nashaat et al., 2017; Pinto et al., 2018). Solid food (e.g. small pellets) may also be used as food rewards (Sauerbrei et al., 2020), but this is less common in head-fixed high-yield studies. Daily monitoring of the weight of the animal provides a key measure of the welfare impact of the food restriction. Notably, weight loss due to food restriction has been found to impact the functioning of the visual cortex in a head-fixed study (Padamsey, Katsanevaki, Dupuy, & Rochefort, 2021), although this is likely due to the weight loss rather than the method used to achieve this.

Table 8: Responses to survey question "What is the limit for intervention, for example increased monitoring or free access to water/food?" from respondents employing head fixation.

Steps to address weight loss were typically taken when animals reached 80-85% of their reference weight, dependent on whether fluid or food control was being used. Presented as percentage (raw number of responses).

Response	Fluid control (n = 22)	Food control (n = 14)
<90% reference weight	5% (1)	0% (0)
<85% reference weight	50% (11)	21% (3)
<80% reference weight	18% (4)	36% (5)
<75% reference weight	9% (2)	14% (2)
<70% reference weight	9% (2)	14% (2)
Other proportion of reference weight	9% (2)	14% (2)

Fluid control, restricting the quantity of water or the time it is available to test subjects, is a common approach in rodent high-yield behavioural studies. The use of fluid control requires close monitoring of the animal's welfare as it may result in recurring periods of dehydration, especially in small rodents such as mice. In male CD1 mice, 24 hours water deprivation has been found to decrease plasma volume and alter blood composition, and increase plasma corticosterone and renin activity (Bekkevold, Robertson, Reinhard, Battles, & Rowland, 2013). These latter changes were also observed after eight days of restricted water access, either to 50 or 75% of *ad libitum* intake, but without altered blood composition. Of note, the 50% *ad libitum* group in this study lost approximately 11% body weight in the first seven days of restriction (Bekkevold et al., 2013), a level of weight loss that would be consistent with many high-yield behavioural studies (Table 8). In male C57BL/6J mice, plasma markers of metabolism were also altered after 24 hours water deprivation (Cui, Liu, Zou, & Li, 2015). C57BL/6 mice also showed increased urine osmolality following 12 hours water deprivation, this increase differing between male and female mice (28% versus 59%, respectively; Nair, Yanhong, Paunescu, Bouley, & Brown, 2019).

A study investigating the effect a lack of oxytocin has on stress responses also found that 18 hours water deprivation was sufficient to increase plasma corticosterone levels in male C57Bl/6 mice, although this was

driven by the exaggerated response in the transgenic mice (Mantella, Vollmer, & Amico, 2005). Taking a non-conservative statistical approach, planned t-tests of the data presented in the paper suggest that the increase in plasma corticosterone in the wildtype mice would not be statistically significant in response to either food or fluid deprivation (control 55 ng/ml ± 14 versus 93 ng/ml ± 18 in fasted mice and 130 ng/ml ± 28 water deprived mice. Water deprived mice versus control $p = 0.0521$, $t = 2.156$, $df = 12$. Fasted mice versus control $p = 0.1328$, $t = 1.623$, $df = 11$). However, this does highlight that the physiological responses to fluid control may differ between wildtype and mutant mice. This needs to be considered where fluid control is being used with mutant mouse lines.

For a majority of high-yield behavioural studies, mice are restricted to a proportion of their normal *ad libitum* daily water intake, or alternatively access to *ad libitum* water is limited to a fixed duration each day, typically 1 hour or less. When a fixed volume is used, the value used varies substantially, both in the literature as well as in the laboratories of those that took part in the survey. These are typically to ensure that mice receive a minimum amount of water regardless of performance in the behavioural task to ensure some degree of hydration. When asked what volume must be given, four of the 19 respondents had no specific amount of water that had to be delivered to mice each day, two were not sure of the amount given, and the remaining seven stated 1 ml/day must be delivered, the most common response (7 of the remaining 13 respondents, 54%).

The value of 1 ml/day was also often given as the minimum amount required for mice that are under fluid control in the documentation followed by members of the working group. This sometimes assumed a model mouse with a body weight of 25g, giving the value of 40 ml/kg of body weight as the minimum to be delivered per day. A recent study in rats indicates that renal adaptations make rodents readily tolerate a daily intake of 50 ml/kg/day, with quantities below this being required for motivation in behavioural tasks (Vasilev et al., 2021). The value of 40 ml/kg/day is often equated to approximately 25% of a mouse's normal daily intake. However, *ad libitum* intakes vary between individual mice and mouse strains (Bachmanov, Reed, Beauchamp, & Tordoff, 2002). Taking the data from Bachmanov and colleagues (2002), 40 ml/kg of body weight may on average be closer to 16% of typical intake (the average intake of all strains tested being 7.7 ml/30g body mass). This is therefore well below the quantities given in studies on dehydration in mice (Bekkevold et al., 2013), as discussed above. Although based on one study, this finding highlights the importance of carefully considering the level of restriction necessary to motivate performance, and to ensure that an individual mouse's needs are met. This may be derived from an appropriate proportion of *ad libitum* intake, but should be adjusted to account for any individual variability seen.

Ensuring this daily minimum is reached often involves a "top-up" in addition to the water earnt during behavioural tasks. Although timing of its delivery differed, 83% of respondents (19 of 23) gave a quantity of water not dependent on behavioural performance. The remaining four responses gave text responses typically indicating that the use of this top-up was study dependent. Of those indicating they deliver a top-up, the most popular timing was "some time after testing" (9 of 19, 47%). Avoiding delivering this water too close to the task itself avoids associations being made between the end of the task and a large delivery of the reward substance. If the top-up were delivered at a consistent time, there is a risk of timing behaviour developing, so varying exactly how long after testing it is given is also advisable.

Notably, systems are available that automate fluid control, even allowing for individual adjustments of the quantities delivered to group-housed animals if mice have RFID (radio-frequency identification) tags. This includes the WaterR system, an open-source and inexpensive option (https://github.com/DodsonLab/WaterR). This allows for the quantity of water delivered to each animal to be tailored to its needs based on welfare monitoring, preventing dehydration.

Measures of body weight are often relied on as an indicator of animal welfare and measure of fluid control. Restricting access to water leads to reduced food intake in mice (likely due to the dehydrated nature of laboratory animal food) and hence subsequent weight loss. Fluid intake is therefore adjusted to maintain body weight at a proportion of the mouse's weight were it to be receiving *ad libitum* water. Typically, this is

85% of the reference weight, but can range from 90 to 65% (Table 8), with exclusion from study typically occurring if animals remain below 80% of the reference value for several days (Table 9).

Table 9: Responses to the survey question, "What is the limit for removing the animal from the study? (e.g. euthanasia)" for respondents using head fixation and either fluid or food control.

When weight was used as a humane endpoint, a criterion of animals remaining below 80% of the reference weight was typically used as the endpoint, although with food control an acute drop below 85% of the reference weight was just as common. However, around a quarter of respondents did not use body weight as a factor in determining humane endpoints for their studies. Presented as percentage total responses (raw number of responses).

Response	Fluid control (n = 22, including one text-only response)	Food control (n = 14 including two text-only responses)
<85% reference weight acutely	9% (2)	14% (2)
Remain <85% reference weight	14% (3)	7% (1)
<80% reference weight acutely	14% (3)	7% (1)
Remain <80% reference weight	27% (6)	14% (2)
<75% reference weight acutely	5% (1)	7% (1)
Remain <75% reference weight	0% (0)	7% (1)
<other% reference weight	5% (1)	0% (0)
This measure not used for removal from study	23% (5)	29% (4)

This reference weight is often based on the free-feeding weight of the mouse before starting fluid control, but alternative approaches exist (Table 10), such as using age-matched weights from a standard growth curve (e.g. Urai et al., 2021). These differing approaches will have distinct welfare implications which researchers should consider when planning restriction protocols and will be true whether fluid or food control is used. One specific case in which alternative approaches should strongly be considered is when testing begins when mice are still young; taking a fixed reference weight in young animals that is not periodically updated would not allow for normal growth. This has further implications for the welfare of the animals, as well as whether they are a representative sample due to this truncated growth. Further to this, members of the working group have observed that starting experiments in “young adult” rodents (8 – 12 weeks of age) has led to fastest and most robust training. Starting restriction below 8 weeks of age in mice would therefore require strong justification given that this would compound the issue of dietary restriction, potentially interfering with normal growth. If breaks in fluid or food control are incorporated into the design of the study, this provides opportunities to establish new *ad libitum* weights for the animals being studied and the reference weight updated. Otherwise, information from commercial growth curves or non-restricted animals in the same facility could be used to approximate a more appropriate reference value in these prolonged studies.

Table 10: Responses to the survey question, "Do you use a fixed value for the reference weight or adjust this throughout the study?" by respondents employing head fixation.

The reference weight used for animals under dietary control was typically based on a single value, although other approaches, such as using publically-available growth curves or correcting to new values periodically taken were also reported. Presented as percentage total responses (raw number of responses).

Response	Fluid control, n = 22	Food control, n = 14
Fixed value from one measure	50% (11)	50% (7)
Fixed value from several measures	18% (4)	14% (2)
Adjust to public growth curves	9% (2)	7% (1)
Adjust to own data	0% (0)	14% (2)
Adjust to control mice	0% (0)	7% (1)
Adjust with new measures	23% (5)	7% (1)

If the task used requires the highest levels of restriction, a number of days of restricted water may be required to establish the motivational state needed, likely due to the mouse being highly adapted to arid conditions (Fertig & Edmonds, 1969). Our survey suggests that the onset of fluid control typically precedes the start of testing by two – three days (Figure 3).

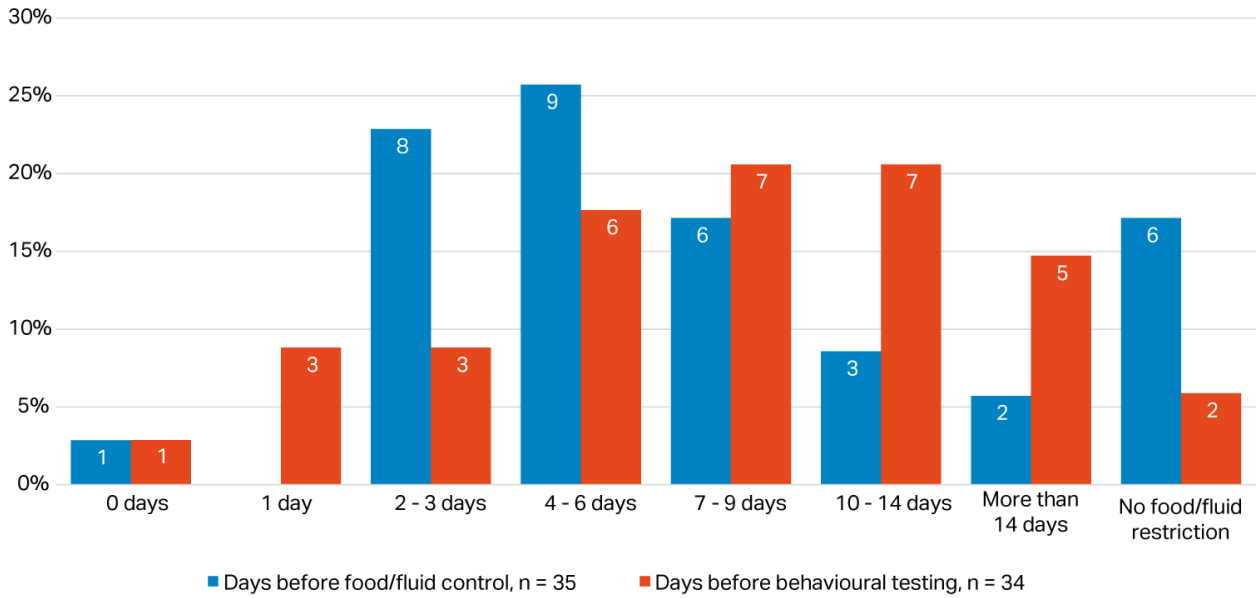


Figure 3: Responses to the survey questions, "What period of time are the animals typically given to recover from surgery before food/fluid restriction is started/resumed?" and "What period of time are the animals typically given to recover from surgery before the first behavioural test?" for respondents employing head fixation.

After surgery, dietary control was typically (re-)initiated in two to six days, with behavioural testing starting typically four to 14 days post-surgery. Plotted as percentages of responses with raw response numbers displayed.

Using fluid control often results in the use of water delivery as the reward and this was the most popular response in the survey from those employing head-fixation, but a variety of other rewards are also used (Table 11). More rewarding substances may improve motivation and require less fluid control, but conversely may lead to satiation at an earlier point, reducing the number of trials that can be completed by the test animals. If the substance is unfamiliar to the mouse, a period of habituation before and after dietary restriction is introduced may be necessary to avoid neophagia. For mice with restricted access to water, adding sucrose to the reward water (e.g. 10% solution) can lead to increased motivation and larger numbers of trials (Guo et al., 2014). It may be important to ensure that mice do receive some unadulterated water daily if another reward is being used to ensure adequate hydration.

Table 11: Responses to survey question "What type of reward is typically used to reinforce behavioural performance? Tick all that apply." from respondents employing head fixation, n = 30.

Responses are ranked by the number of positive responses, illustrating that water was the most commonly used reward amongst respondents, but sucrose solutions and soya milk were also popular. Presented as percentage of responses (number of positive responses).

Response	Percentage of responses (raw number of responses)
Water	60% (18)
Sucrose solution	33% (10)
Soya milk	27% (8)
Other	10% (3)
No reward	10% (3)
Sensory cue	17% (5)
Milkshake	13% (4)
Food pellet	13% (4)
Sucrose pellet	13% (4)
Flavoured pellet	10% (3)
Fruit juice	7% (2)
Optical stimulation	7% (2)
Electrical stimulation	3% (1)
Saccharine solution	3% (1)

4.2. Health indicators for mice under diet control

While the limits of severity of the fluid/food control should be determined for each project through a cost/benefit assessment (Rowland, 2007), the following pointers provide a useful reference for the vast majority of high-yield behavioural studies. In most cases, mice showing any of these signs should immediately receive fluids, be removed from restriction, and should be monitored closely:

- Weight reduction: below 80% reference weight
- Reduced activity in home cage: very sluggish, or only moves when touched
- Condition of fur: very shaggy, marked piloerection
- Body profile: highly hunched posture, emaciated look
- Skin turgor: skin stays pinched or tented after a brief pinch on coat, suggesting severe dehydration.

Following close observation of the animal and extent of the recovery, the fluid/food control may be resumed at a later date, or if the animal recovers promptly, on the same day. If the experimenter does not have prior experience or clarity on how to deal with a given animal welfare concern, local welfare advice should be sought from the veterinarian and/or animal welfare officer.

Notably, many of these indicators are identical to those used to monitor post-operative heath, with skin turgor taking the place of wound appearance. Modified example scoresheets are therefore supplied (Appendix 4: Example scoresheet and heath monitoring templates) following the same principles outlined above (Section 3.3.3). The rate of incidence of many of these outcomes may be assumed to be much rarer during fluid control than following surgery, although from 20 respondents reduced skin turgor (median of the weighted average 3 ± 1 IQR), altered behaviour (2.5 ± 1), a hunched posture (2 ± 1) or abnormal gait (2 ± 2) were seen at similar rates to deviation from the expected growth curve (2 ± 2), rapid weight loss (3 ± 1) or body condition deterioration (2.5 ± 1). Nonetheless, a more typical scoresheet is also provided, retaining a tick-box encompassing all of these welfare measures and prioritising the presentation of body weight measures. Daily monitoring is recommended, as is already widely practiced (18 of 22 respondents, 82%, employing fluid control, and 9 of 14 respondents, 64%, employing food control). Further details can be found in Appendix 4: Example scoresheet and heath monitoring templates.

4.3. Holidays/breaks from restriction

When animals are not required to perform a task for some days, researchers often provide *ad libitum* water and food during this “holiday” period. The choice of when and whether to give these holidays depends on several factors. For example, fluid control holiday over the weekend may result in unacceptable performance on the first day or two of the next week. A recent study in Sprague Dawley rats also found this sort of intermittent restriction produces greater levels of plasma corticosterone compared to continuous restriction, at least in the first few weeks of water control (Vasilev et al., 2021). This result suggests that intermittent designs where rodents receive controlled water during the workweek and free water during the weekend may actually interfere with renal adaptation and cause stress.

On non-test days, 46% of respondents (10 of 22) gave a fixed volume greater than what would normally be delivered, whereas one (5%) and two (9%) respondents allowed access to *ad libitum* water for 2 – 6 or over 12 hours, respectively. Despite this, 41% (9 of 22) gave an identical amount of water on non-test days to that given on test days, demonstrating that this additional access in place of water earnt as part of testing is far from universal. However, when scientifically feasible, training breaks of longer than 7-10 days should be treated as holidays and restriction should be removed. As noted above, these prolonged breaks also provide an opportunity to update the reference weight used to assess the health and restriction level of the animals during the study, ensuring age and growth are accounted for.

4.4. Fluid versus food control

In the absence of scientific reasons to choose fluid or food control, the question arises which is more refined from an animal welfare perspective. One study which directly compared fluid and food control in mice performing a visual discrimination task found that food restricted mice had lower discomfort scores than water restricted mice (Goltstein, Reinert, Glas, Bonhoeffer, & Hubener, 2018). This was true both when measured by the experimenters as well as by animal welfare officers, although the authors emphasise that in both cases the average scores remained mild. In addition, food restriction resulted in mice performing a significantly higher number of trials for the same degree of weight loss, though animals under fluid control reached criterion levels of performance more quickly. Lower discomfort and higher trial numbers when employing food restriction have also been observed in the laboratory of working group members who have used both fluid and food control.

Considering these findings, there is some limited evidence that food restriction could be considered a more refined approach over fluid control. Indeed, one member of the working group had run the same task using food or fluid control and found performance and welfare improvements using food control. However, further research is needed on this important point, particularly as this conclusion would contrast with the findings of earlier studies suggesting that mice tolerate water control better than comparable food control (e.g. Treichler & Hall, 1962; Tucci, Hardy, & Nolan, 2006). This would also need to be balanced against the hierarchy of dietary control (Table 7) and both welfare and performance measures considered. There exists the possibility of task-specific differences, so a comprehensive study across several common tasks may be necessary. Since liquid food and water can both be delivered by the same apparatus, transitioning between the two might not require major changes in experimental approach. Such a study would need to investigate welfare measures, such as stress and indicators of renal function, in addition to behavioural performance to fully assess which approach is more refined.

4.5. Considerations beyond dietary control

When animal performance is below expectation, a common assumption is that the degree of restriction is insufficient. In addition to initial steps to lessen the degree of restriction required, such as adequate habituation (see Section 5.1), other possibilities during the study should be considered before increasing the level of dietary control. These include potential illness, stress or discomfort, including possible infection at the surgical sites; the malfunctioning of equipment; errors in custom code; and raising of task criteria too rapidly. In addition to compromising performance if unnoticed, many of these possibilities would also lead to the premature ending of a given testing session once detected (Figure 4). Without due consideration of these alternatives before further restricting an animal's access to food or water, mice may begin to display signs of ill health due to over-restriction. The consequences of this state for behavioural performance are not well documented, but the experience of the working group is that too great a motivational state can lead to undirected rather than goal-directed responding, compromising the scientific goals of the study.

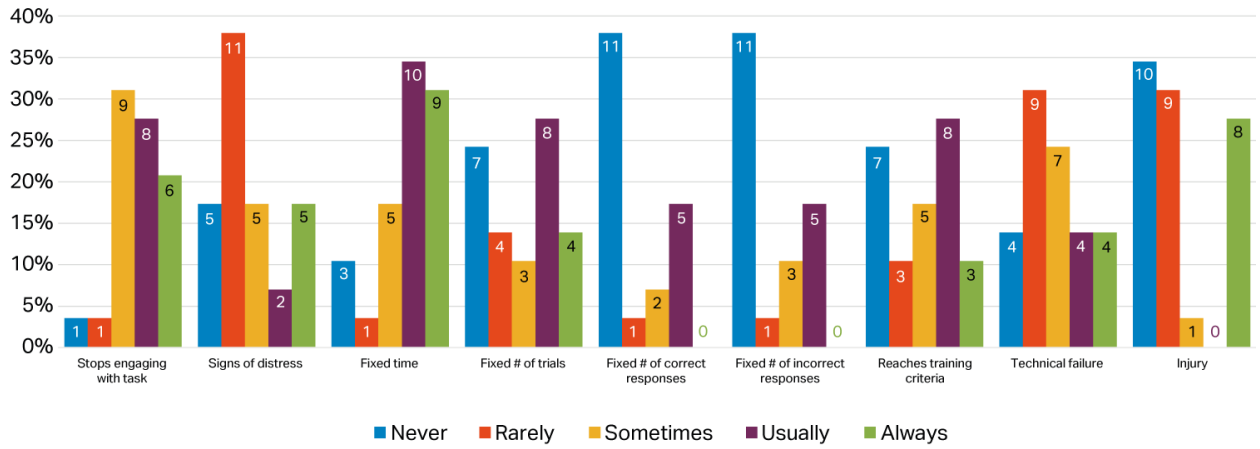


Figure 4: Responses to the survey question, "Which of the following may terminate a behavioural session? How commonly is this the reason for ending a session?" from respondents employing head fixation, n = 29.

A variety of different events may lead to a session to be terminated, ranging from measures relating to behavioural performance, a fixed period of time elapsing, or issues such as technical failures, an animal no longer engaging with the task, or showing overt signs of distress or injury.

Ill health as a cause of poor behavioural performance or as a consequence of dietary control can be avoided through careful monitoring of the animals under restriction (see Section 4.2 and Appendix 4: Example scoresheet and heath monitoring templates). Equipment and software should be tested before the experiment proper begins, including measuring the size of a drop of reward delivered in their apparatus and calibrating this carefully, especially if more than one spout is used in the task. This can be done by measuring the weight of 100 drops and dividing this value by 100. This should be checked throughout the study along with the functioning of other elements of the set-up. Regular maintenance such as cleaning of infrared beams used to detect responses, as well as any moving parts that may become unresponsive if left unattended, may be necessary to ensure the task continues to run as expected.

If animals are group housed, an established social hierarchy may lead to some mice receiving more or less food or water than expected. This in turn can complicate the maintenance of both good welfare and similar motivational levels across all animals in a study. With food restriction, breaking lab chow pellets into smaller pieces can ensure equal access with minimal conflict. However, separation of mice for short periods may be necessary with either food or water control if problems persist. This individual housing needs to only be brief (at most a few hours) to allow the measured amount of food or fluid to be consumed. All mice in a cage may need to be separated out or only the lightest, so this will need to be trialled.

4.6. Alternative approaches to motivation

Recent studies have explored methods to motivate mice without removing water or food, and yet obtain high numbers of trials in a task. One such approach involves adding citric acid to the water available freely in the home cage (Reinagel, 2018; Urai et al., 2021). The sour flavour of the 5% citric acid solution makes the mice drink less in their home cage, and then perform the behavioural task for plain or sweetened water reward. Another approach involves social housing multiple RFID-tagged mice in an autonomous behavioural environment where they receive all their fluids by performing self-initiated trials of the task, which is continuously available for them to do in their cages (Erskine, Bus, Herb, & Schaefer, 2019). This high-throughput approach may even be combined with voluntary head-fixation (Aoki, Tsubota, Goya, & Benucci, 2017; Bernhard et al., 2020; Murphy et al., 2016; Murphy et al., 2020). These approaches are very promising, although their welfare implications need more research. For example, a key study needed is to

investigate the physiological consequences of prolonged citric acid consumption and whether *ad libitum* access to citric acid truly represents a welfare refinement over more restricted access to water, including whether markers of dehydration are lowered by this constant access to a less palatable substance.

Negative reinforcers do feature as part of the behavioural paradigms used in some head-fixed set-ups, although these are in addition to the dietary control used; they are intended to shape behaviour, not motivate performance overall. The methods used are limited by the restricted set-up, with approaches such as small electric shocks being a lot more common in freely-moving behaviour than head-fixed studies (Figure 4). The most commonly used approach is a time-out, a period during which no action from the animal will elicit a reward, with air puffs and a short burst of white noise being the most common aversive stimuli used. Whilst negative reinforcers may be useful during behavioural training, they are not intended to be the sole motivator, so should be used sparingly and only when justified to the regulatory authority and research institution's ethical review board.

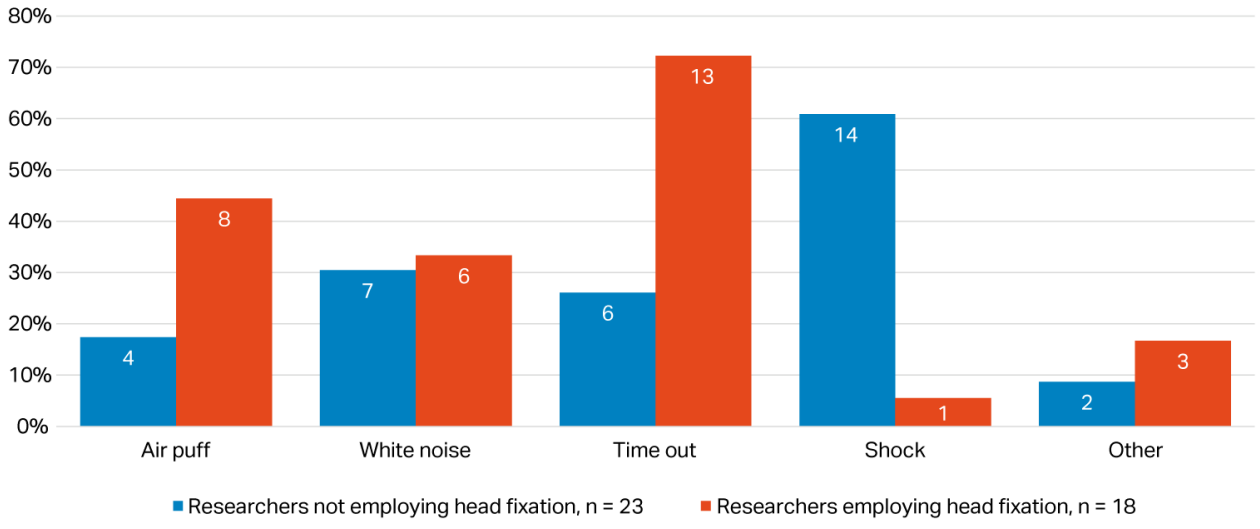


Figure 5: Responses to the survey question "Are any of the following aversive training methods used? Select all that apply." from those employing and not employing head fixation.

The use of different negative reinforcers differed between those employing head-fixation versus those that do not. The use of time-out was over-represented in the head-fixed group ($\chi^2(1) = 8.643$, $p = 0.003$) and shock under-represented ($\chi^2(1) = 13.320$, $p < 0.001$). There was a trend for air puff to be used more in head-fixed work than freely-moving behavior ($\chi^2(1) = 3.570$, $p = 0.059$), while the use of white-noise bursts ($\chi^2(1) = 0.039$, $p = 0.843$) and other negative reinforcers ($\chi^2(1) = 0.599$, $p = 0.439$) was equivalent in both groups. Plotted as percentages of responses with raw response numbers displayed.

4.7. Recommendations to refine motivation

General

- **The most refined approach to motivation should be used that is compatible with the scientific requirements of the study. This includes choosing whether restriction is needed at all, and the choice and method of food/fluid control.**
- **The degree of restriction should be the minimum required in order to obtain the necessary motivation levels and adjusted throughout the study to maintain this.**
- **Optimised habituation procedures should be used to reduce the level of aversion associated with restraint and the need to use high motivational states to overcome these (see Section 5.1).**
- **The overall welfare of each animal must be monitored daily using a range of welfare measures and clearly defined intervention points.**
- **A rigorous documentation system must be maintained for monitoring the welfare of each animal (see templates in Appendix 4: Example scoresheet and heath monitoring templates).**
- **Responses to dietary control may differ in mutant mouse lines compared to their wildtype counterparts. Food or water control should therefore be introduced gradually when using new lines to establish whether weight loss and other health indicators change in the expected manner or if adjustments to usual practice need to be made.**
- **The expected weight increase with age (i.e. normal growth) should be allowed for even under dietary control, for example by periodically updating the reference weight used in prolonged studies or by adjusting weights to established growth curves.**
- **If behavioural performance is poor, first consider possible technical failures or signs of ill health in the animal before restricting access to food or water further.**
- **Separating an animal for a short period to feed can address situations in which an individual mouse continues to lose weight while others in the cage remain stable, but the time apart should be minimised as reintroduction of an isolated mouse to its original cage after prolonged periods (i.e. multiple days) often results in aggressive behaviour, especially in males. Individual adjustments without a need to separate mice can be made using automated systems such as WaterR (https://github.com/DodsonLab/WaterR) when combined with RFID-tagged group-housed mice.**
- **Before any major or prolonged surgical procedure, animals should be removed from restriction for at least 24 hours before the procedure, and a few days following the procedure.**
- **Negative reinforcers should be used sparingly, prioritising time-outs over more aversive stimuli.**

Fluid control

- **Motivation to work should be optimised by identifying fluid rewards that are preferred over plain water (e.g. sucrose solution, Guo et al., 2014).**
- **When providing measured water to a cage with multiple fluid restricted animals, researchers should separate individuals temporarily into different cages or even better consider an automated system that allows for individual adjustment for RFID-tagged group-housed mice, such as the WaterR system (https://github.com/DodsonLab/WaterR).**

Food restriction

- **Before and after the first day of food restriction, animals should be familiarised to the taste of the liquid food reward by placing a petri dish with a few ml of the liquid (e.g. soy milk) in the cage.**
- **When providing measured food to a cage with multiple animals, pellets should be broken into small pieces (~5mm across), and the combined food for all the mice may be introduced into the cage in one go to reduce aggression around food consumption.**

5. Head-fixed behavioural set-ups

High-yield behavioural studies typically involve daily testing under restraint (21 of 30 respondents to the survey, 70%, test “five to seven times a week”), usually motivated by fluid control and liquid rewards (see Section 4). The time spent under restraint as reported in our survey ranged from 15 to 150 minutes from 27 respondents, with a median value of 60 minutes. This in itself raises welfare concerns that can be mitigated by habituation to the set-up; i.e., repeated exposure to elements of the study, such as restraint, in a way that does not elicit a stressful response, lessening the aversion to restraint in subsequent exposures to this potential stressor. Combined with a successful surgery, ensuring good habituation is crucial in maximising the data yield from every mouse used. Our survey revealed that groups experience up to 56% of mice failing to complete behavioural studies, with nine out of 29 respondents reporting loss rates at 30% or above. Encouragingly, 13 of the 29 respondents reported loss rates at or below 10%, with a median value of 15%; although this was still higher than the failure rate in those that do not use head fixation (5% median loss rate, standardised $U(1) = 3.914$, $p < 0.001$). Any steps that can minimise loss, such as more formal habituation to the testing set-up, should therefore be strongly considered.

5.1. Initial habitation to restraint

Head fixation is an essential part of many behavioural and physiological studies in rodents, but is highly aversive to them. Habituation provides an opportunity to reduce the stress response and associated affective state changes and may reduce the amount of food/water restriction required to initiate and maintain task engagement. Together these can improve welfare, but the extent to which this is achieved will likely depend on the methods employed.

The term “habituation” is defined as the diminishing of an innate response to a frequently repeated stimulus (Leussis & Bolivar, 2006). In the context of high-yield behavioural experiments, the use of habituation in published studies varies, with current best practice involving a graduated approach in which the animal is first accustomed to being held by the gloved hands of the researcher, then introduced to head fixation, and then the amount of time the animal experiences head fixation is increased over a period of days. Details of this habituation procedure vary between research groups and with the type of experiment being carried out.

Whilst habituation has welfare and scientific benefits, it is not known how best to achieve this reduced level of stress whilst also enabling the final experimental objectives to be achieved. Objective methods to assess the stress response are also limited, with most researchers relying on overt measures of distress to manage the initial habituation of animals. However, some studies have recorded levels of corticosterone and/or behavioural measures related to affective state and refined their habituation strategy accordingly (Goltstein et al., 2018; Juczewski, Koussa, Kesner, Lee, & Lovinger, 2020). In this section, we consider what is currently carried out as part of a habituation protocol and what evidence exists for the potential benefits of different habitation approaches.

5.1.1. Current status

A ubiquitous finding of restraint in rodents is that it causes a stress response with an increase in the stress hormone corticosterone and behaviours indicative of aversion and negative affect (Chiba et al., 2012; Keim & Sigg, 1976; Pare & Glavin, 1986; Stuart et al., 2013; Woo et al., 2018; Yun et al., 2010). Following an initial increase after the first exposure to restraint, corticosterone levels diminish over time, which has been suggested to reflect habituation (Juczewski et al., 2020). One complicating factor is that the nature of the restraint, and therefore the potential stress response, used in head-fixed procedures is quite variable between studies. Head fixation is sometimes combined with restraint of the torso, but many studies use linear or spherical treadmills to allow limb movement and some degree of locomotor behaviour.

Furthermore, the use of air-lifted platforms positioned under the animal allows a greater range of movements and more natural body posture.

Different perspectives exist as to the best approach to acclimatising animals to the head-fixed apparatus before starting experiments. These may include habituation to human handling only before a series of full-length head-fixed training and/or testing sessions, through to a graduated and tailored habituation protocol designed to gradually acclimatise the animal to both the apparatus and the head fixation. Our survey data revealed that researchers typically allow two or more days of habituation to restraint before behavioural testing begins (Table 12). However, it is also common for restraint to be introduced at the same time as the task, with 25% of respondents (8 of 32) reporting doing this (Table 13). Whilst some groups (11 of 30, 37%) allow for a habituation period at the start of every behavioural session, 63% (19 of 30) do not. An example of a gradual, 5-day habituation protocol is provided by the International Brain Laboratory (International Brain Laboratory, 2020b; International Brain Laboratory et al., 2021).

Table 12: Responses to the survey question "On average, how many habituation/acclimatisation sessions in total do animals normally receive (i.e. before formal testing begins)?" from respondents employing head fixation, n = 29.

Reporting the use of habituation sessions ahead of testing was widespread, although the length of this habituation period differed greatly across respondents. Presented as percentage of responses (number of positive responses).

Response	Percentage of responses (raw number of responses)
0	7% (2)
1	7% (2)
2	28% (8)
3	28% (8)
4+	31% (9)

Table 13: Responses to the survey question "Are the animals habituated to the behavioural procedure and the tethering/restraint method together or separately?" from respondents employing head fixation, n = 32.

Habituation to restraint alone was often done before formal testing began, although a quarter of respondents habituated animals to restraint and some form of the behavioural procedure together. Presented as percentage of responses (number of positive responses).

Response	Percentage of responses (raw number of responses)
Together	25% (8)
Restraint before behavioural testing	44% (14)
Behavioural training then restraint later	13% (4)
We do not habituate to either	3% (1)
We do not use restraint	6% (2)
Other	9% (3)

One argument might be that habituation protocols that take place over many days expose the animal to a longer period of restraint overall. However, a key consideration is that stress itself is very detrimental to the scientific objectives. In a recent study looking at an apparatus designed to reduce the impacts of restraint through the provision of a mobile home-cage, corticosterone levels in mice were initially ~9 times those of control-handled animals and did not significantly reduce until day 10 of the habituation protocol and remained elevated throughout the study (Juczewski et al., 2020). Kislin and colleagues (2014) also used a mobile homecage set-up and whilst they did not record corticosterone levels, they found that animals stopped showing freezing behaviour after the first day and that locomotor behaviour stabilised after 4 days of training. These behavioural and hormonal indicators, however, have limitations and may not accurately represent levels of stress. For example, corticosterone may show no changes after 5 days in a learned helplessness protocol despite there being a depression-like phenotype and neurochemical changes (Hellhammer, Rea, Bell, Belkien, & Ludwig, 1984). Animals exposed to repeated, inescapable stress also develop passive coping methods and thus may show a reduction in overt signs of distress, which may not in fact be due to a true habituation (Anisman, Remington, & Sklar, 1979; Shanks & Anisman, 1993; see Section 5.1.2).

When combining head restraint and fluid/food control, the level of restriction may need to be reviewed throughout the study. A high level of restriction may be necessary in the early stages of the task, but this may not be necessary following initial habituation to the testing set-up as well as acquisition of the task. In some designs, there is an automatic adjustment as mice perform trials more rapidly and perform more trials after learning, and thus obtain more daily fluids. This may even allow for movement up the hierarchy of restriction once behaviour is well established (Table 7), but equally a gradual lessening of restriction maybe more appropriate. Performance in the task or the number of trials completed can be used as a key metric to guide restriction. Indeed, both are used to guide levels of restriction, but working to a fixed percentage of the reference weight remains the most common approach, which may lead to the over-restriction of well-trained animals (Table 14).

Table 14: Response to the questions "How do you determine that your animals are at an appropriate level of fluid/food restriction? Select all that apply." from respondents employing head fixation.

Several measures are used to ensure animals are restricted to an appropriate level when using either fluid or food control. Working to a fixed percentage of baseline weight was the most popular response for both approaches, although task performance was also widely used. Presented as percentage of responses (number of positive responses).

Response	Fluid control, n = 23	Food control, n = 14
Task performance	44% (10)	50% (7)
Trials completed	39% (9)	36% (5)
Time engaged with task	35% (8)	21% (3)
We give a fixed amount of fluid/food	30% (7)	36% (5)
We work to a fixed percentage of baseline weight	57% (13)	79% (11)
Other	22% (5)	14% (2)

5.1.2. Objective methods to assess welfare

Much of how we assess the potential for negative consequences and the "cost" to a laboratory animal associated with a particular procedure or series of procedures is based on our subjective assessment. This poses challenges as our decisions about refinement may not be based on scientific evidence, but rather on our perceptions and possibly an anthropomorphic perspective of how the animal may experience our interventions. There are probably two main reasons for this: 1) it takes time and resources and dedicated experiments to assess the welfare impacts of different procedures, which is also often perceived as requiring the use of more animals; 2) There are limitations with current methods for quantifying objectively the negative welfare consequences of scientific procedures, particularly when considering the overall impact of a protocol on an animal's affective state. There are also different levels of suffering, and whilst we may be able to see and respond to overt signs of distress, the consequences of longer term, lower levels of suffering are much less easily quantified, but, overall, may have a greater burden on the animal. As an example, chronic mild stress is a known inducer of a depression-like state in laboratory mice and rats, but is composed of repeated mild interventions rather than a singular, highly aversive event (Moreau, Jenck, Martin, Mortas, & Haefely, 1992; Willner, 1997). Animals also respond to inescapable stress in different ways, which can include passive versus active coping and so animals may show reductions in overt signs of distress, but this may not be associated with a reduction in suffering (Anisman, Grimmer, Irwin, Remington, & Sklar, 1979; Shanks & Anisman, 1989, 1993). Alongside our need to understand and refine our methods from an animal welfare perspective, there are also very strong scientific arguments for refinement and hence using objective methods to recognise and improve scientific procedures. Animals experiencing stress (acute or chronic), do not represent normal subjects and hence their physiology and the resulting behavioural and neuroscientific readouts will be confounded. There is also a high degree of variability in animals' responses to stress and this will impact on the behavioural and neurophysiological readouts, statistical power and ultimately the reliability and reproducibility of the arising data.

5.1.3. Moving forward

Considering the current knowledge about the impacts of restraint on welfare and evidence that, even in the mobile home cage set-up, animals show elevated and sustained stress responses (Juczewski et al., 2020; Kislin et al., 2014; see Section 5.1.1), methods that improve the animal's ability to tolerate restraint will have obvious welfare and scientific benefits. Whilst repeated restraint has been used to induce models of depression, these protocols tend to be more severe than the restraint necessary for head fixation studies as restraint-induced stress is their primary objective, whereas here it could limit the value of the research being conducted. Stress can have profound effects on homeostatic mechanisms and impact the value of the resulting data. While operant tasks are widely carried out in restrained animals (most commonly using licking as the conditioned response), paradigms that allow a greater range of movement and more natural posture can increase the richness of the behavioural measurements while reducing stress (Yuzgec, Prsa, Zimmermann, & Huber, 2018).

There is also a trade-off between stress and arousal state/motivation; if methods can be developed that lead to less stress and aversion, then lower levels of fluid/food control would be required to motivate behaviour, as the animal would not need to be trained against an initial background of conditioned aversion. Animals will be in a more normal affective state and therefore provide more relevant neurophysiological data and with greater translational validity.

Table 15 provides a summary of measures that could be recorded to help quantify and compare the impact of different habituation methods, as well as different types of apparatus that may reduce the animal's experience of restraint. For most researchers, simple measures that do not require specialist training, such as recording faecal boli (Calvo-Torrent, Brain, & Martinez, 1999) and behavioural indices of distress, could be used to optimise habituation procedures for the specific experimental approach. These can also be used to monitor the progress of habituation and tailored to the individual animal's acclimatisation, rather than

applying a time-based strategy across the whole cohort. There is also an important knowledge gap in understanding the welfare impacts of head-fixation procedures, which warrants dedicated experiments where more specialist measures of affective state are used to guide future recommendations. Table 16 provides a summary of potential methods that could be piloted alongside such measures to investigate approaches for improving an animal's acceptance of restraint and reducing the impacts of stress on both welfare and scientific outcomes. Application of the 3Rs requires researchers to use the most refined methods and using a small number of animals to provide evidence to support best practice would achieve overall benefits for animal welfare as well as scientific outputs.

Several steps can be taken to reduce the stress of animals used in head-fixation studies (Table 16). This not only has welfare benefits but may also result in less variable, more reliable data due to better engagement with the task used. This includes the method of handling the animals, with use of a handling tunnel or cupped hands shown to decrease anxiety in mice as compared to handling by the tail (e.g. Hurst & West, 2010). The choice of handling method has also been shown to have an impact on habituation (Gouveia & Hurst, 2017) and, perhaps crucially, reward processing (Clarkson, Dwyer, Flecknell, Leach, & Rowe, 2018). Further guidance and resources are available from the NC3Rs: https://nc3rs.org.uk/how-to-pick-up-a-mouse.

Table 15: Methods to quantify animals' stress response and the welfare impacts of different head-fixed protocols including approaches to habituation.

This table expands on some of the measures that can most easily be integrated into head-fixed experiments yet still provide a measure of welfare.

Measure	Ease of use	Reliability	Recommendation
Faecal boli	Easy	Simple, reliable indicator of acute stress. Can be affected by fluid/food control.	Should be recorded in all studies and reported in publication.
Body weight and condition	Easy	Simple, reliable indicators of acute stress. Will be affected by fluid/food control.	Should be recorded in all studies and reported in publication.
Overt signs of distress e.g. struggling, vocalisation, freezing	Easy	Provides a gross measure of distress and important to monitor in initial stages to avoid injury. May indicate passive coping and learned helplessness and not a true habituation.	Should be recorded in the initial stages of habituation and used to intervene to avoid excessive distress. Key measures should be reported in publications e.g. freezing behaviour over time. Represents higher level of stress than measures such as faecal boli, so should not be used alone.
Task-dependent behavioural readouts (e.g. reward collection latency, learning rate, locomotor activity, grooming behaviours, etc)	Easy, but task-dependent	Can be compared with data from non-restrained animals in a similar environment or performing a similar operant task. Individual animals progress through graduated training schedules to provide a good indicator of individual variability.	Key measures should be reported in publications.
Corticosterone	Moderate	Reliable indicator of arousal and acute stress. Not a direct measure of habituation or negative affective states.	Useful method for studies comparing different types of set-up and as a gross measure of acute stress. Indicator of acute arousal, which may or may not be specifically associated with a negative affective state (e.g. see Harris, Baggott, Mendelson, Mendelson, & Jones, 2002), so should not be used alone.
Objective measures of affective state	Specialist	Good validity for quantifying stress-induced negative affective states e.g. sucrose preference test, novelty suppressed feeding.	Important measure for studies comparing different methods to provide an indication of chronic changes in affective state. Implement in animals exposed to different habituation procedures and/or apparatus.

Table 16: Methods which may reduce stress and improve habituation.

Opportunity	Rationale	How to implement
Initial handling and training	Improve the animal's association with human handling. Consider if pre-training in the task and apparatus before head fixation	Use standardised handling procedure to acclimatise to human contact. Consider including positive reinforcement to enhance positive affective experience. Pick up mice using non-aversive methods.
Controllability	Studies have consistently found that controllable versus uncontrollable stress have very different effects on the animal's affective state and long terms adaptive changes that arise from chronic stress. Increasing the control the animal has over restraint could reduce the negative impacts but will increase training times.	Provide animals with an initial period of self-fixation, i.e. they can enter and leave the fixation apparatus. Slowly increase the time of head fixation with monitoring to release animals when they show struggling.
Reduce the effects of conditioned aversion	If the initial experience of the apparatus is aversive then the animal will take longer and require a higher motivational state to overcome their association with the testing apparatus.	The time taken to train animals and initial performance measures could be used to indicate the success of a habituation protocol. Being able to reduce the level of restriction required to motivate animals would indicate improved habituation.
Apparatus modification	The impacts of restraint may be reduced if animals can move their bodies during head fixation.	Undertake comparison studies integrating scientific and welfare measures. Publish indices of stress alongside publications to complement scientific studies when new approaches are being used.
Integration with fluid/food control procedures	Animals experiencing stress are more likely to require higher levels of restriction to overcome the aversion of the set-up.	A potential indicator of a less stressful approach may be the ability to use less restrictive procedures to motivate animals' performance. As animals habituate to the set-up, they should also require lower levels of restriction. A well-habituated animal should ultimately be willing to perform the task unrestricted, albeit not necessarily with as high a number of trials as some studies may require. As such, a simple "test" of the success of habituation would be to run animals unrestricted and record and report performance measures.

5.2. Refinements to the testing set-up

In designing experiments that combine behavioural and neuronal data collection, one should consider the trade-off between increasing experimental data yield versus maintaining ethological relevance. High-yield mouse experimental configurations, particularly when involving head fixation, favour the former at the expense of the latter. A guiding principle for improving set-ups is therefore to balance data yield with ethological relevance as much as possible, in the interests of both experimental validity and animal welfare. Refinements in one element of set-up design may facilitate improvements in others. For example, changing the physical apparatus to make mice more comfortable and perform more natural movements may result in needing less fluid or food control to reach the same level of motivation. This section provides suggestions on refinements to ethological relevance, to the monitoring of animal state, and to procedures for restraint and training.

5.2.1. Considerations around ethological relevance

High-yield designs seek to achieve experimental power through the generation of large numbers of trials and, ideally, control of as many independent variables as possible and measurement of as many dependent variables. This limitation in the number of degrees of freedom is often key for allowing solid links to be established between behaviour and neural activity. A potential risk of this approach is, however, that the behavioural paradigm pushes the animal into a non-natural state, where the behavioural components are outside the animal's natural repertoire (Krakauer, Ghazanfar, Gomez-Marin, MacIver, & Poeppel, 2017). If the goal of the experiment is to broadly understand how the brain generates behaviours, studying such unnatural behavioural states might be of limited value, and a focus on ethologically relevant behaviours may, instead, be desirable. From an animal welfare perspective, forcing animals to execute behaviours that are distant from their natural repertoire often comes at the cost of extended fluid or food control and long training periods. A recommendation is to try to tap into natural behaviours when designing the behavioural paradigm, thereby minimising the amount of abstraction and learning that the animal must do. While this may not always be possible because of the nature of the problem being studied, possible design considerations to tap into natural behaviours include:

- using natural motor movements, e.g. digging (Deacon, 2006b), burrowing (Fink, Axel, & Schoonover, 2019), reaching (Galinanes et al., 2018), manipulation (Barrett, Raineri Tapies, & Shepherd, 2020) or obstacle avoidance (Warren et al., 2021).
- using sensory stimuli that emulate the animal's natural environment.
- exploiting major innate behaviours/motivations, such as foraging (Vertechi et al., 2020), exploration, sexual or defensive behaviours (Branco & Redgrave, 2020; Vale, Evans, & Branco, 2017), orienting towards stimuli of interest (Burgess et al., 2017; International Brain Laboratory et al., 2021), or sleep (Yuzgec et al., 2018).
- exploiting the natural aptitude of rodents to learn about space and report behavioural choices by moving through an environment (Dombeck et al., 2007; Holscher, Schnee, Dahmen, Setia, & Mallot, 2005) and using multisensory stimulation cues (Royer et al., 2012).

These recommendations can be applicable to head-fixed animals as much as to freely moving configurations. In head-fixed configurations, navigation is often accomplished by having the animal operate in a virtual reality (VR) environment with visual and sometimes tactile cues, coupled to movement on a floating ball or cylinder (Chen, King, Lu, Cacucci, & Burgess, 2018; Dombeck et al., 2007).

A significant challenge with moving towards natural behaviours is that, by their very nature, these might yield a lower number of trials. Achieving high yields requires sustained motivation and precise control of how that motivation is satisfied. While this is relatively easy to achieve for behaviours such as performing an action to obtain a small reward, a mainstay paradigm class in systems neuroscience (Carandini & Churchland, 2013), behaviours that rely on satisfying natural motivations (e.g. maternal, sexual or defensive) are often not repeated very frequently. For example, an animal that just has avoided a

threatening or painful situation will be less likely to again put itself through a similar situation in the near future and forcing it to do so might put the animals through stressful procedures or push them into unnatural states.

Another consideration is that when relying on highly trained animals, the large trial numbers that can be achieved often come from a small number of animals, typically the ones that reach some performance criterion early in the training process. This may also lead to a selection bias in which animals make it into many of the studies conducted in this manner. On the other hand, if training is fast or even not necessary, large trial numbers can in principle be achieved by studying larger animal cohorts. Both designs have statistical advantages of their own.

5.2.2. Degrees of freedom and the head-fixed configuration

The key principle to follow when choosing an experimental configuration is to ensure that it is consistent with the aim of the experimental design. If an experiment requires precise control of certain dimensions of behaviour, for example stimulation of a given sensory pathway or performing a certain motor action (e.g. reaching), the configuration should allow the animal comfort and a degree of free movement in other dimensions, for example by allowing locomotion, if appropriate, and by providing room for the animal to settle its spine into a natural posture (Yuzgec et al., 2018) and adopt a comfortable position of head relative to paws. These free dimensions should be carefully monitored in real time (see Section 5.2.4, below). There is no one-size-fits-all prescription for head-fixed versus freely moving designs; having chosen a design based on experimental need (Dombeck et al., 2007; Wallace & Kerr, 2019), configurations should be optimised to minimise stress and maximise welfare, and an appropriate habituation regimen established (see Section 5.1).

A recommendation based on our experience and that of many, though not all, other researchers is that even when rodent locomotion is not directly relevant to the task (e.g. the task does not involve VR navigation), the ability to locomote appears to enhance animal motivation and engagement during a session. Enabling an animal to run, particularly in as natural a fashion as possible, is an integral part of many experiments (e.g. facilitating navigation of virtual sensory environments), but is also thought to reduce the stress associated with head restraint (Juczewski et al., 2020). Unfortunately, there are currently no studies directly comparing the stress response or other indicators of the welfare benefits of more naturalistic set-ups (see Section 5.2). Findings differ on whether running improves task learning, and most likely this is task and context dependent. Locomotion does not require a floating ball or wheel and can be facilitated by a conventional treadmill allowing one-dimensional motion if the added degrees of freedom are not needed. Treadmill locomotion is readily adopted by mice and rats, and in any of these configurations, locomotion should be monitored (see Section 5.2.4). In sum, we recommend providing the opportunity for locomotion and formally testing whether this enhances performance and motivation. Of note, a potential issue in configurations involving running is that high performing animals often “like to run” and may need to be taken off the task temporarily or have their diet supplemented if their body weight drops below the thresholds used.

Given the considerations above, adjustability to an individual mouse’s preferred position should ideally be an integral part of the set-up, allowing monitoring and enhancements to posture and the relationship of head position relative to the paws. The spout/lick-port should not be too close (which facilitates impulsive licking) or too far away for comfort, and this balance will vary across individual animals and during training. In early stages, the lick-port can be placed slightly further away while the animal learns to avoid impulsive responses (Berditchevskaia, Caze, & Schultz, 2016; Guo et al., 2014).

We also encourage the use of designs where animals are given the opportunity to self-initiate trials. This can ensure that trials, and data collection, occur when the animal is motivated and may therefore avoid erroneous trials or latency measures by forcing a pace that the animal cannot maintain.

5.2.3. High-yield alternatives to conventional head-fixed configurations

In head-fixed configurations, trials can be configured to be relatively short and with comparatively little variability in duration, by carefully designing trial structure and titrating reward size, and this facilitates high trial counts (Guo et al., 2014). Freely moving set-ups typically involve a rodent moving with surgically attached headgear, such as a miniaturised widefield microscope, and tethered via an overhead optical fibre or electrical cable. Freely moving or tethered studies, however, usually lead to lower trial counts, with each trial taking longer to complete, and involve greater scope for variability in behaviour and duration because of this greater ethological relevance. Some recent designs have sought to combine the advantages of both approaches, and these are recommended if feasible.

One option involves training the animal to voluntarily poke its head into a head-fixing port. This is appropriate for task designs where the animal samples sensory stimuli at times when it is not engaged in locomotion (Scott, Brody, & Tank, 2013). This can be combined with home-cage training, where the animal voluntarily moves into a chamber accessible from the home cage, thus avoiding the need for the experimenter to move the animal and limiting the ensuing stress (Aoki et al., 2017; Bernhard et al., 2020; Murphy et al., 2016; Murphy et al., 2020). Limiting trainer contact also prevents biases in experimental outcome, which can arise, for example from differences in the animal's reaction to male and female experimenters (Sorge et al., 2014). Under voluntary head fixation, the animal performs the task when it is motivated to do so. Engagement and motivation are therefore improved and uninterrupted access to the operant chamber from the home cage can reduce the need for fluid or food access control. On the down-side, automated home cage set-ups can be complex to configure and maintain, and their suitability for controlled stimulus delivery depends on the sensory modality under investigation, with olfaction and hearing being particularly appropriate (Cruces-Solis et al., 2018; Erskine et al., 2019; Francis & Kanold, 2017; Maor et al., 2019; Reinert, Schaefer, & Kuner, 2019). In addition, home cage methods may not easily achieve the same degree of stable restraint as methods that rely on a dedicated setup.

A second option involves the use of an air-levitated platform for head-fixed mice to move on: this allows the animal to traverse a physical environment containing multisensory (visual, tactile, olfactory) cues (Kislin et al., 2014; Nashaat, Oraby, Sachdev, Winter, & Larkum, 2016). Such systems provide a more realistic environment than VR while still allowing high trial counts. They have been shown to allow place field mapping in the hippocampus (Go et al., 2021). Although animals can suffer from vestibular asynchrony, similar to that on VR platforms, the impairment of self-motion signals in head-fixed mice appears to have been largely addressed by recent systems (Chen, Lu, King, Cacucci, & Burgess, 2019; Ghosh et al., 2011; Voigts & Harnett, 2020).

Finally, approaches that have until now only been used in head-fixed setups are being applied in tethered systems. As an example, high-density electrophysiological recording have been performed in tethered animals (Juavinett, Bekheet, & Churchland, 2019), and a pre-print reports calcium imaging at cellular resolution in moving animals (Zong et al., 2021). Whilst this may present further options in the future, a case-by-case harm/benefit analysis is still required; can the scientific goals be achieved without the weight of the device on the animals' head presenting a further welfare concern, and do the benefits of performing studies with tethered animals outweigh the possible decrease in data quality and other complications due to the movement of the animal?

5.2.4.Monitoring behaviour

Regardless of configuration, a key aspect of experimental design is the need to monitor the animal's behavioural state. This is both for reasons of welfare, to help verify the actual severity of procedures and the animal's health, and also to ensure the validity and interpretability of neurobiological observations as it has become clear that spontaneous changes in motor state are a major driver of variation in brain activity, even in areas traditionally considered not to be involved in motor function (Musall, Kaufman, Juavinett, Gluf, & Churchland, 2019; Salkoff, Zagha, McCarthy, & McCormick, 2020; Stringer et al., 2019). For example, pupil size provides a measure of arousal during testing, and has been shown to be related to an animal's performance in sensory detection tasks (McGinley, David, & McCormick, 2015). Furthermore, differing set-ups can promote different postures (Figure 5), which, in turn, can impact both welfare and the willingness of the mouse to engage with the task (Yuzgec et al., 2018). Variables including pupil size, facial expression and posture can be readily tracked and captured and extremely effective software for this, based on deep learning algorithms, is freely available and is driving rapid improvements in standards for behavioural tracking (Datta, Anderson, Branson, Perona, & Leifer, 2019; Dennis et al., 2021; A. Mathis et al., 2018; A. Mathis, Schneider, Lauer, & Mathis, 2020; M. W. Mathis & Mathis, 2020; Wiltschko et al., 2015). See also Sections 5.1.2 and 5.1.3, in particular Table 15, for further discussion and suggestion of methods to assess welfare during these studies.

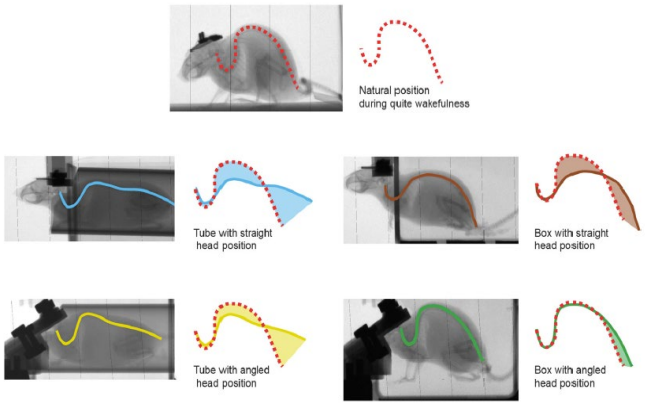


Figure 6: Different behavioural set-ups promote different postures in mice which may impact on their motivation to engage with behavioural tasks. Adapted from Yüzgeç and colleagues (2018).

If a more natural posture can be achieved, this is likely to reduce the stress of the animal in the head-fixed set-up, thus improving its engagement with the task. This not only has welfare benefits but may also improve the quality of the data obtained.

5.2.5.Further suggested refinements to procedures for restraint and training

As described in the Section 5.1, animals must be first habituated to the experimenter and to the training environment before training commences on accepting restraint and on the actual behaviour. This is key to reducing stress and facilitating engagement during training.

Our own experience and that of the researchers in the survey recommends against training based on aversive stimuli such as strong air puffs. Avoiding the integration of aversive elements into the experimental task design will help limit the animal's lifetime exposure to unpleasant experiences. Moreover, such stimuli may be ineffective and lead to a decrease in engagement and motivation. "Punishments" based on timeout have been shown to be effective, but are not as aversive (Guo et al., 2014).

Adding rewards or treats at the beginning and/or end of a session is often done to boost motivation. These may include, for example, sunflower seeds or chocolate cereals. When doing so, the effect of the specific treat on thirst and motivation should be considered. In addition, some treats have high fat content and can artificially increase weight, occluding weight losses. The timing of the delivery of these should be considered carefully, as discussed in Section 4.1 regarding top-up.

Indeed, when topping up an animal's daily fluid allocation once behavioural training has been completed, we recommend that this is done at variable times to limit the animal's expectation of further rewards on a fixed schedule, which could otherwise condition training and performance. Once initial learning has occurred, it may be possible to reduce fluid control whilst maintaining performance levels.

5.3. Recommendations to refine head-fixed behavioural set-ups

- **First, consider whether head-fixation is necessary or if your scientific goals could be achieved with less restraint. Check for advances in, for example, tethered recording techniques, which may allow for a shift away from using head-fixation in tasks where it was previously not possible.**
- **Habituation to restraint should be practiced before formal testing as this will reduce stress responses to head-fixation, improving task engagement and making the loss of headcaps less likely.**
- **Further steps to reduce stress throughout the task should also be taken, for example allowing for naturalistic behaviours as part of the required response, allowing for locomotion, and adjusting the set-up to account for an individual mouse's favoured position under restraint. Recent advances such as air-levitated platforms provide an integrated way to apply many of these refinements.**
- **Allowing for self-initiated head-fixation will improve on the above recommendation further and so should be strongly considered.**
- **Self-initiation of trials should be used where large numbers of omissions and/or high response latencies may confound the results, as these are more likely to occur when the task runs without requiring the subject to make a response to start a new trial.**
- **Monitoring factors such as pupil size and facial expressions via video, even when unrelated to the main task, provides useful metrics of welfare and engagement. Consider also other measures of welfare that can be incorporated into the set-up such as those in Table 15.**

6. Conclusions and areas of future focus

Rodent high-yield behavioural experiments often employ both head-fixation and fluid control. Both approaches raise welfare concerns and yet little guidance is available for what constitutes best practice. Refinements to these approaches are possible that prioritise the welfare of the animals used and, far from compromising the scientific outcomes of the study, are likely to improve the quality of the data obtained. Steps such as employing good aseptic surgical technique are now routine for many, but there are further refinements that could and should be implemented by all groups. We have recommended several such refinements in this report based on what we believe constitutes the current best practice that should be incorporated into research studies.

Many of our recommendations would be strengthened by further research. A major unanswered question is whether food or fluid control represents a more refined approach than the alternative, and whether both could be employed equally for all tasks used in the field. Another hindrance to assessing the best practices is a lack of objective measures of stress or affect that can be incorporated as a part of a head-fixation study (as opposed to requiring separate, dedicated welfare-focused experiments). Better empirical measures of

stress that are simple to obtain would therefore benefit this area, as well as behavioural neuroscience as a whole. Funding schemes from organisations such as the NC3Rs provide opportunity to address these unanswered questions (https://nc3rs.org.uk/funding).

We note also that techniques once only possible in head-fixed set-ups are now being used in mobile animals. Whilst the advantages and disadvantages of mobile set-ups over head-fixation still need to be considered on a case-by-case basis, they may in the future present a more refined alternative to head-fixation. Nonetheless, head-fixation is likely to still be employed by certain fields of study for some time to come and we hope that the recommendations from this study will be widely adopted by the community that helped shape them.

Acknowledgements

The authors would like to thank all the anonymous survey respondents and those who presented data to the working group during the course of this project.

Additional information

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Competing interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. The WaterR project received funding from the NC3Rs. Where NC3Rs resources are mentioned, this is made clear in the text of the manuscript.

Author contributions

All authors were involved in the conceptualisation of this work, defining the methodology used and writing the manuscript. Data were collected, curated, visualised and formally analysed by C Barkus who was also primarily responsible for project administration. Additional review and editing of the manuscript was principally performed by C Barkus and MJ Prescott with input from all other authors.

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Ethics

The research presented here did not comprise experimental work. Ethical approval for the survey was granted by the University of Oxford's Medical Sciences Interdivisional Research Ethics Committee (IDREC) Central University Research Ethics Committee (CUREC), reference R68817/RE001.

Appendices

Appendix 1: Search strategies for the systematic literature reviews

Head fixation literature search strategy

For the head fixation-focused search, the following searches were performed:

PubMed: ((head fix[All Fields] OR head fixation[All Fields] OR head fixed[All Fields] OR head fixing[All Fields]) OR (head restrained[All Fields] OR head restrainer[All Fields] OR head restraining[All Fields] OR head restraint[All Fields] OR head restraints[All Fields])) AND (("mice"[MeSH Terms] OR "mice"[All Fields] OR "mouse"[All Fields]) OR ("mice"[MeSH Terms] OR "mice"[All Fields]) OR ("rats"[MeSH Terms] OR "rats"[All Fields] OR "rat"[All Fields]) OR ("rats"[MeSH Terms] OR "rats"[All Fields]))

Web of Science: TS=(head NEAR/0 fix*) OR TS=(head NEAR/0 restrain*) AND TS=(mouse OR mice OR rat OR rats)

Scopus: TITLE-ABS-KEY ((head W/0 fix*) OR (head W/0 restrain*)) AND (LIMIT-TO (EXACTKEYWORD, "Mouse") OR LIMIT-TO (EXACTKEYWORD, "Rat") OR LIMIT-TO (EXACTKEYWORD, "Rats") OR LIMIT-TO (EXACTKEYWORD, "Mice"))

Ovid (Medline): "head adj restrain*".mp. or "head adj fix*".mp. limit to (mice or rats)

Ovid (Embase): "head restrain*".mp. or "head fix*".mp. limit to (mouse or rat)

Fluid control search strategy

For the fluid control-focused literature review, the following searches were performed:

PubMed: (water deprivation[MeSH Terms] OR water depriv* OR fluid depriv* OR water restrict* OR fluid restrict* OR water regulat* OR fluid regulat* OR water control* OR fluid control*) AND (dehydration OR thirst OR weight loss OR mortality OR reward OR refinement) AND (mouse OR mice OR rat OR rats)

Web of Science: (TS=(water NEAR/0 restrict*) OR TS=(water NEAR/0 restrict*) OR TS=(fluid NEAR/0 restrict*) OR TS=(water NEAR/0 regulat*) OR TS=(fluid NEAR/0 regulat*) OR TS=(water NEAR/0 control*) NOT TS="water (control)" OR TS=(fluid NEAR/0 control*) OR TS=(water NEAR/0 depriv*) OR TS=(fluid NEAR/0 depriv*) OR TS=dehydration OR TS=thirst OR TS="weight loss" OR TS=mortality OR TS=reward OR TS=refinement) AND (TS=(mouse OR mice OR rat OR rats))

Scopus: (TITLE-ABS-KEY (water W/0 restrict*) OR TITLE-ABS-KEY (fluid W/0 restrict*) OR TITLE-ABS-KEY (water W/0 regulat*) OR TITLE-ABS-KEY (fluid W/ regulat*) OR TITLE-ABS-KEY (water W/0 control*) OR TITLE-ABS-KEY (fluid W/0 control*) OR TITLE-ABS-KEY (fluid W/0 depriv*) OR TITLE-ABS-KEY (water W/ depriv*)) AND (TITLE-ABS-KEY (dehydration) OR TITLE-ABS-KEY (thirst) OR TITLE-ABS-KEY ("weight loss") OR TITLE-ABS-KEY (mortality) OR TITLE-ABS-KEY (reward) OR TITLE-ABS-KEY (refinement)) AND (LIMIT-TO (EXACTKEYWORD, "Rat") OR LIMIT-TO (EXACTKEYWORD, "Rats") OR LIMIT-TO (EXACTKEYWORD, "Mouse") OR LIMIT-TO (EXACTKEYWORD, "Mice"))

Ovid (Medline): (Water Deprivation/ or (water adj restrict*).mp. or (water adj depriv*).mp. or (water adj control*).mp. or (water adj regulat*).mp. or (fluid adj restrict*).mp. or (fluid adj depriv*).mp. or (fluid adj control*).mp. or (fluid adj regulat*).mp.) and (refinement.mp. or reward.mp. or mortality.mp. or thirst.mp. or dehydration.mp. or weight loss.mp.) limit to (mice or rats)

Ovid (Embase): (Water Deprivation/ or (water adj restrict*).mp. or (water adj depriv*).mp. or (water adj control*).mp. or (water adj regulat*).mp. or (fluid adj restrict*).mp. or (fluid adj depriv*).mp. or (fluid adj control*).mp. or (fluid adj regulat*).mp.) and (refinement.mp. or reward.mp. or mortality.mp. or thirst.mp. or dehydration.mp. or weight loss.mp.) limit to (mouse or rat)

Appendix 2: Survey questionnaire

(See overleaf)

Participant Information

General Information

You are invited to take part in a survey to collect information on rodent high-yield behavioural experiments. This survey is running for a limited period and will close on 10 July 2020 at 4pm BST.

The questions have been developed by an expert working group of the NC3Rs. The aim is to establish current practice in this field and to identify any refinements to improve animal welfare and scientific outcomes. We very much hope you will participate.

Please read through these terms before agreeing to participate by ticking "yes". You may ask any questions before taking part by contacting the principal researcher (details on next page).

The survey consists of 70 questions on chronic implant surgeries, how animals are monitored post-operatively, motivational tools and behavioural testing protocols. We estimate it will take 30 minutes to complete. We ask that you complete it based on your most commonly used procedures or the ones with which you have most experience. Multiple responses from a research facility are encouraged, but respondents should be the lead person responsible for carrying out the research or the person chiefly involved in the care of the animals involved.

The results will be reviewed by the NC3Rs working group and used to help identify opportunities to refine this area of work. The group's recommendations will be published in a peer-reviewed paper and promoted within the research community. To keep up to date with the progress of the working group, please visit the NC3Rs website.

Do I have to take part?

Your participation is voluntary, and all questions are optional. You may withdraw at any point during the questionnaire for any reason by closing the browser. However, once you have submitted your answers, they will be anonymous so you will not be able to withdraw them.

Data Management and Consent

How will your data be used?
All data collected in this survey will be anonymous, treated in strict confidence and held securely by the NC3Rs. The NC3Rs data management plan is available upon request (enquiries@nc3rs.org.uk).

Your data will be stored in a password-protected file and may be used in academic publications. Your IP address will not be stored. Research data will be stored for a minimum of three years after publication or public release.

Who will have access to your data?
The NC3Rs is the data controller with respect to your responses and these will be processed for the purpose of the research outlined above. Research is a task that we perform in the public interest.

The principal researcher is Dr Chris Barkus, NC3Rs, who is attached to the Department of Biomedical Services at the University of Oxford. Responsible members of the University of Oxford and funders may be given access to data for monitoring and/or audit of the study to ensure we are complying with guidelines, or as otherwise required by law. This project has been reviewed by, and received ethics clearance through, the University of Oxford Central University Research Ethics Committee (ethics approval reference R68817/RE001).

What if there is a problem?
If you have a concern about any aspect of this project, please contact chris.barkus@nc3rs.org.uk. Your concern will be acknowledged within 10 working days. If you remain unhappy or wish to make a formal complaint, please contact Dr Vicky Robinson, Chief Executive, NC3Rs (enquiries@nc3rs.org.uk).Address: The NC3Rs, Gibbs Building, 215 Euston Road, London, NW1 2BE, or the Chair of the Oxford Medical Sciences Inter-Divisional Research Ethics Committee (ethics@medsci.ox.ac.uk); Address: Research Services, University of Oxford, Wellington Square, Oxford OX1 2JD.

Please note that you may only participate in this survey if you are 18 years of age or over.

If you have read the information above and agree to participate with the understanding that the data (including any personal data) you submit will be processed accordingly, please check the relevant box below to get started. Thank you in advance for your participation.

* 1. Have you read and understood this information, can confirm that you are over 18, and consent to participate in this study?

- ◯ Yes
- ◯ No

General Questions

2. Please select which area best describes your current research.

3. Please select the option that best describes your primary role

4. Please select the country you work in.

5. Please select the species of animal you most commonly use in your research or support as part of your animal welfare role.

- ◯ Rat
- ◯ Mouse
- ◯ None of the above

PART A. Chronic implant surgery

6. Do you carry out surgery to implant cranial devices in rodents or are you responsible for their post-operative care, for example as a vet or part of the animal care staff?

- ◯ Yes
- ◯ No

Information about the implant

7. How many times is the animal typically anaesthetised for your most common surgical procedure?

- ◯ 1
- ◯ 2
- ◯ 3 or more

8. What permanent devices are typically implanted? *Select all that apply.*

- ☐ Head fixation device
- ☐ Chronic single electrodes
- ☐ Electrode arrays
- ☐ Ground and/or reference electrode(s)
- ☐ Ground screw(s)
- ☐ EMG electrode placement
- ☐ Optical fibres
- ☐ Miniscope
- ☐ Cranial window
- ☐ Intercranial cannula(e)
- ☐ Lenses / prisms
- ☐ Other (please specify)

9. Is this combined with other types of surgical intervention in the same surgery? *Select all that apply*.

- ☐ Yes, viral delivery of genetic material
- ☐ Yes, lesion of a particular brain area
- ☐ Yes, mini-pump implantation for osmotic diffusion delivery
- ☐ Other (please specify)
- ☐ Yes, mini-pump with intra-cranial cannula(e) implantation
- ☐ No

10. In your experience, how important are the following to the welfare of the animals over the course of the experiment?

	Not at all important	Slightly important	Moderately important	Very important	Extremely important
Weight of the implant	◯	◯	◯	◯	◯
Position of the implant	◯	◯	◯	◯	◯
Dimensions of the implant	◯	◯	◯	◯	◯
Depth of placement	◯	◯	◯	◯	◯

11. What is used to secure your device? *Select all that apply.*

- [ ] Skull screws (please give further details below)
- [ ] Bone cement
- [ ] Dental adhesive (e.g. superbond)
- [ ] Other (please specify). Please also add further details such as type of screw used (material, self-tapping or not, etc) and drill type (handheld, frame-mounted etc) used.

Peri-operative care

12. Which of the following form part of your standard surgical drug regimen?

	Pre-emptive (shortly before surgery)	During surgery (continuous or discrete)	Post-operative (within 12 hours of end of surgery)
Opioids, for example buprenorphine	☐	☐	☐
Sustained-release opioids, for example buprenorphine SR	☐	☐	☐
NSAIDs, for example meloxicam	☐	☐	☐
Steriods, for example prednisolone	☐	☐	☐
Other anti-inflammatories	☐	☐	☐
Local anaesthetic, for example lidocaine	☐	☐	☐
Inhalation anaesthesia, for example isoflurane	☐	☐	☐
Injectable anaesthesia, for example ketamine/medetomidine	☐	☐	☐
Fluids, for example saline or gluco-saline	☐	☐	☐
Oral/injected antibiotics (if given routinely for most surgeries)	☐	☐	☐

Other (please specify)

13. What steps are taken to ensure aseptic conditions? *Select all that apply.*

- ☐ Sterile equipment
- ☐ Sterile foil or similar for handling equipment that cannot be fully sterilised (e.g. hand drill)
- ☐ Sterile instruments
- ☐ Separate sterile instruments for each animal
- ☐ Sterile consumables (e.g. gloves, drapes, swabs etc)
- ☐ Sterile drapes/sterile tray/disposable sterile surface for instruments
- ☐ A trained assistant
- ☐ A trained anaesthetist
- ☐ Separate rooms/air compartments for preparation of the animal and the surgery itself
- ☐ Surgeon is scrubbing up with skin disinfectant, for example with a chlorhexidine-containing solution
- ☐ Mask

14. What is the average length of surgery?

0 Minutes 300

15. Are both the cranium and the dura typically removed?

- Yes
- No (please provide further information e.g. soften the dura chemically, thin the cranium)

16. Is brain tissue typically excised/lesioned to ease your implant?

- Always
- Usually
- Sometimes
- Rarely
- Never

Post-operative care

17. In addition to the drugs detailed above, which of the following additional steps form a part of post-operative care **immediately after surgery** (i.e. for the first day or several days post-surgery)? *Select all that apply.*

	Never	Rarely	Sometimes	Usually	Always
Provide additional warmth	○	○	○	○	○
Provide extra nesting material	○	○	○	○	○
Additional palatable foods	○	○	○	○	○
Assessment of body weight/condition	○	○	○	○	○
Easily accessible source of fluids (e.g. gel packs)	○	○	○	○	○
Assessment of fluid intake	○	○	○	○	○
Assessment of locomotor activity	○	○	○	○	○
Grimace scale or other pain assessment	○	○	○	○	○
Advice from animal care or veterinary staff	○	○	○	○	○

Other (please specify). Please also use this text box for any further information from the above options.

18. In addition to regular health checks, how frequently are "enhanced" post-operative health checks performed?

- ○ More than once a day
- ○ Daily
- ○ Several times a week
- ○ Other (please specify)
- ○ Weekly
- ○ We do not perform any checks other than usual husbandary checks

19. For how long do these checks continue?

- [ ] 1 day
- [ ] 2 - 4 days
- [ ] 5 - 7 days
- [ ] More than 7 days
- [ ] N/A
- [ ] Until defined criteria are met/Other (please specify)

20. Which of the following do you provide as part of more long-term monitoring of implanted animals (i.e. for the days and weeks after surgery)? (*Select all that apply*)

	Never	Rarely	Sometimes	Usually	Always
Provide additional warmth	○	○	○	○	○
Provide extra nesting material	○	○	○	○	○
Additional palatable foods	○	○	○	○	○
Assessment of body weight/condition	○	○	○	○	○
Easily accessible source of fluids (e.g. gel packs)	○	○	○	○	○
Assessment of fluid intake	○	○	○	○	○
Assessment of locomotor activity	○	○	○	○	○
Grimace scale or other pain assessment	○	○	○	○	○
Advice from animal care or veterinary staff	○	○	○	○	○
Administration of analgesia	○	○	○	○	○
Administration of steroids	○	○	○	○	○
Provide saline/glucosaline via injection	○	○	○	○	○
Routine administration of antibiotics (i.e. to every animal regardless of any evidence of infection)	○	○	○	○	○
Administration of anti-inflammatory agent to prevent brain swelling	○	○	○	○	○
Administration of steroids	○	○	○	○	○

Other (please specify)

21. Overall, how important do you think the following are for the long-term routine welfare of animals that have undergone surgery i.e. which make the biggest impact on their recovery and welfare throughout the rest of the study?

	Not at all important	Slightly important	Moderately important	Very important	Extremely important
Provide additional warmth	○	○	○	○	○
Provide extra nesting material	○	○	○	○	○
Additional palatable foods	○	○	○	○	○
Assessment of body weight/condition	○	○	○	○	○
Easily accessible source of fluids (e.g. gel packs)	○	○	○	○	○
Assessment of fluid intake	○	○	○	○	○
Assessment of locomotor activity	○	○	○	○	○
Grimace scale or other pain assessment	○	○	○	○	○
Administration of analgesia	○	○	○	○	○
Advice from animal care or veterinary staff	○	○	○	○	○
Administration of steroids	○	○	○	○	○
Provide saline/glucosaline via injection	○	○	○	○	○
Routine administration of antibiotics (i.e. to every animal regardless of any evidence of infection)	○	○	○	○	○
Administration of anti-inflammatory agent to prevent brain swelling	○	○	○	○	○
Administration of steroids	○	○	○	○	○

Other (please specify)

22. What period of time are the animals typically given to recover from surgery before food/fluid restriction is started/resumed?

- ◯ 0 days
- ◯ 1 day
- ◯ 2 - 3 days
- ◯ 4 - 6 days
- ◯ 7 - 9 days
- ◯ 10 - 14 days
- ◯ More than 14 days
- ◯ We do not use food or fluid restriction

23. What period of time are the animals typically given to recover from surgery before the first behavioural test?

- ◯ 0 days
- ◯ 1 day
- ◯ 2 - 3 days
- ◯ 4 - 6 days
- ◯ 7 - 9 days
- ◯ 10 - 14 days
- ◯ More than 14 days
- ◯ We do not perform behavioural tests on these animals

24. How are animals typically housed during an experiment?

	Singly-housed	Pair-housed	Group-housed
Before surgery (i.e. non-instrumented animals)	◯	◯	◯
Immediately after surgery (i.e. instrumented animals)	◯	◯	◯
Following recovery from surgery (i.e. instrumented animals)	◯	◯	◯

Adverse effects

25. How do you monitor for expected adverse effects and record these signs?

- [ ] We have a scoresheet used for all post-operative animals.
- [ ] We have a bespoke scoresheet specifically for these types of experiments.
- [ ] We keep detailed notes of each animal in a lab book.
- [ ] We keep detailed notes of each animal on their cage card/in our colony record system.
- [ ] We have no specific system but record concerns as they arise.
- [ ] We monitor but do not keep detailed records.
- [ ] Other (please specify)

26. How often do you see each of these adverse effects at any point post-surgery?

	Never	Rarely	Sometimes	Usually	Always
Scabbing/wounding around the head implant	○	○	○	○	○
Secondary infections	○	○	○	○	○
Wound rupturing/loss of stitches	○	○	○	○	○
Loss/repair needed of head implant	○	○	○	○	○
Bleeding	○	○	○	○	○
Loss of appetite	○	○	○	○	○
Piloerection	○	○	○	○	○
Shivering	○	○	○	○	○
Reluctance to move	○	○	○	○	○
Lack of alertness	○	○	○	○	○
Lack of grooming	○	○	○	○	○
Hunched posture	○	○	○	○	○
Vocalisation	○	○	○	○	○
Inflammation	○	○	○	○	○
Excessive weight loss (for example >20% over a few days)	○	○	○	○	○
Facial indicators of pain	○	○	○	○	○

27. Are there any other details of your surgical and peri-operative care procedure which you feel impact on the welfare of the animals? What could be done to acheive further improvements?

PART B. Fluid and food restriction

28. In your experience, what are the most important considerations when choosing how to motivate rodents in your studies, for example choosing between using food or fluid control?

	Not a consideration at all	Least important	Not very important	Important	Most important
Used by others in the field	○	○	○	○	○
Established lab practice	○	○	○	○	○
Better for scientific outcomes of the study (e.g. lower number of trials to criterion)	○	○	○	○	○
The alternative (food/fluid) has not worked in the past	○	○	○	○	○
The experimental set-up practically requires it/technical consideration	○	○	○	○	○
Animal welfare considerations	○	○	○	○	○
Regulatory advice	○	○	○	○	○

Please note here if you do not use either food or fluid control routinely.

29. Are the animals that you use or care for often under fluid control?

- ○ Yes
- ○ No

Fluid restriction protocol

30. How is fluid restriction initially introduced?

- ◯ We gradually reduce access to water to the level used during testing (i.e. starting with a large volume and decrease from there).
- ◯ We restrict to the level used during testing from the start (i.e. the volume used remains more-or-less constant throughout the procedure).
- ◯ We restrict at a level lower than is typically necessary and gradually increase this as weight and/or performance stabilises (i.e. starting with a low volume and increase from there).
- ◯ Other (please specify)

31. How do you determine that your animals are at an appropriate level of fluid restriction? *Select all that apply.*

- ☐ Task performance
- ☐ Number of trials completed
- ☐ Time engaged with task
- ☐ We give a fixed amount of food/fluid
- ☐ We work to a fixed percentage of baseline weight
- ☐ Other (please specify)

32. Is there a daily minimum amount of water that MUST be provided to each animal? If so, please specify this value with units.

Rats

Mice

33. What are the most important considerations for using this value?

	Not a consideration at all	Least important	Not very important	Important	Most important
Veterinary advice	◯	◯	◯	◯	◯
Values given in the literature	◯	◯	◯	◯	◯
Experience of collaborators	◯	◯	◯	◯	◯
Guidance from local ethical review process (e.g. from your IACUC or AWERB)	◯	◯	◯	◯	◯
Regulatory advice	◯	◯	◯	◯	◯
Established lab practice	◯	◯	◯	◯	◯

Please list any guidelines used in making this decision.

34. Do animals receive fluid on testing days outside of the behavioural training? (i.e. a "top-up")

- ◯ Yes, immediately after testing
- ◯ Yes, some time after testing
- ◯ Yes, at a fixed time of day
- ◯ No, animals only receive the fluid they gain in testing
- ◯ Other (please specify)

35. Do animals receive a different amount of fluid on non-testing days?

- ◯ Yes - 12 hours or longer/constant access
- ◯ Yes - 6 – 12 hours access
- ◯ Yes - 2 – 6 hours access
- ◯ Yes - 1 hour access
- ◯ Yes - less than 1 hour access
- ◯ Yes - until they have consumed a fixed amount of liquid
- ◯ No - animals receive the same amount of fluid access on testing and non-testing days
- ◯ N/A - we test seven days a week

36. How often do you see the following signs of dehydration?

	Never	Rarely	Sometimes	Usually	Always
Reduced skin turgor (e.g. skin tent test)	◯	◯	◯	◯	◯
Sunken eyes	◯	◯	◯	◯	◯
Marked variation in general behaviour (e.g. change in locomotor activity, increased/decreased activity)	◯	◯	◯	◯	◯
Change in faecal pellet consistency	◯	◯	◯	◯	◯
Dry, ‘tacky’ oral mucous membrane	◯	◯	◯	◯	◯
Reduced capillary refill time	◯	◯	◯	◯	◯
Hunched posture	◯	◯	◯	◯	◯
Abnormal gait	◯	◯	◯	◯	◯
Deviation from growth curve	◯	◯	◯	◯	◯
Rapid, undesirable weight loss	◯	◯	◯	◯	◯
Deterioration in body condition	◯	◯	◯	◯	◯

Other (please provide further information if you do not monitor dehydration)

37. How often do you monitor for signs of dehydration?

- ◯ More than once a day
- ◯ Daily
- ◯ Several times a week
- ◯ Other (please specify)
- ◯ Weekly
- ◯ Never

38. When do these checks take place? *Select all that apply.*

- ☐ At the start of each testing day
- ☐ Shortly before behavioural testing
- ☐ Shortly after behavioural testing
- ☐ Shortly before fluid access
- ☐ Other (please specify)
- ☐ Shortly after fluid access
- ☐ At the end of each testing day
- ☐ Around lights on in the animal house
- ☐ Around lights off in the animal house

39. What is the limit for intervention, for example increased monitoring or free access to water?

- ◯ Animals fall below 85% of reference weight
- ◯ Animals fall below 80% of reference weight
- ◯ Animals fall below 75% of reference weight
- ◯ Animals fall below another proportion of reference weight/other measure (please specify)

40. What is the limit for removing the animal from the study? (e.g euthanasia)

- ◯ Animals fall below 85% of reference weight acutely
- ◯ Animals remain below 85% of reference weight for an extended period of time (please specify)
- ◯ Animals fall below 80% of reference weight acutely
- ◯ Animals remain below 80% of reference weight for an extended period of time (please specify)
- ◯ Animals fall below 75% of reference weight acutely
- ◯ Animals remain below 75% of reference weight for an extended period of time (please specify)
- ◯ Animals fall below another proportion of reference weight (please specify)
- ◯ We do not remove animals from the study based on this measure (please specify)

Please specify the length of time considered if appropriate or if another measure is used.

41. Do you use a fixed value for the reference weight or adjust this throughout the study?

- ◯ We use a fixed value based on one measurement of free feeding weight.
- ◯ We use a fixed value based on several measurements of free feeding weight.
- ◯ We adjust based on publicly available growth curves (e.g. those available through JAX).
- ◯ We adjust based on data from within our lab/unit.
- ◯ We adjust based on control mice from the same cohort.
- ◯ We adjust by occasionally taking a new reference weight.

42. Are there other details of your fluid restriction protocol that you feel are important in maximising the welfare of the animals? Are there particular unanswered questions in this area that you would like see addressed?

Food control

43. Are the animals that you use or care for often under food restriction?

- ◯ Yes
- ◯ No

Food control protocol

44. How is food restriction initially introduced?

- ◯ We gradually reduce the food given to the level used during testing (i.e. starting with a large amount and decrease from there).
- ◯ We restrict to the level used during testing from the start (i.e. the amount given remains more-or-less constant throughout the procedure).
- ◯ We restrict at a level lower than is typically necessary and gradually increase this as weight and/or performance stabilises (i.e. starting with a small amount and increase from there).
- ◯ Other (please specify)

45. How do you determine that your animals are at an appropriate level of food restriction? *Select all that apply.*

- ☐ Task performance
- ☐ Number of trials completed
- ☐ Time engaged with task
- ☐ We give a fixed amount of food
- ☐ We work to a fixed percentage of baseline weight
- ☐ Other (please specify)

46. Do you typically provide a fixed amount of food or is it adjusted? What is your primary guide if you adjust?

- ◯ Adjusted to maintain good body weight
- ◯ Adjusted to maintain task performance
- ◯ Fixed (please specify)

47. Is there a daily minimum amount of food that MUST be provided to each animal? If so, please specify this value with units.

Rats

Mice

48. What are the most important considerations for using this value?

	Not a consideration at all	Least important	Not very important	Important	Most important
Veterinary advice	○	○	○	○	○
Values given in the literature	○	○	○	○	○
Experience of collaborators	○	○	○	○	○
Guidance from local ethical review process (e.g. from your IACUC or AWERB)	○	○	○	○	○
Regulatory advice	○	○	○	○	○
Established lab practice	○	○	○	○	○

Please list any guidelines used in making this decision.

49. On non-testing days is a larger amount of food given?

- ○ Yes - a variable amount based on the animal's body weight
- ○ Yes - a variable amount based on the animal's task performance
- ○ Yes - a fixed amount less than ad lib but greater than the typical amount on a test day
- ○ Yes - a fixed amount approximately equal to ad lib intake
- ○ No
- ○ N/A - animals are used in tests seven days a week.

50. How often are the animals under food control weighed?

- ○ More than once a day
- ○ Daily
- ○ Several times a week
- ○ Weekly
- ○ Other (please specify)

51. When do these weight checks occur? *Select all that apply.*

- [ ] At the start of each testing day
- [ ] Shortly before behavioural testing
- [ ] Shortly after behavioural testing
- [ ] Shortly before feeding
- [ ] Shortly after feeding
- [ ] At the end of each testing day
- [ ] Around lights on in the animal house
- [ ] Around lights off in the animal house
- [ ] Other (please specify)

52. What is the limit for intervention, for example increased monitoring or free access to food?

- ○ Animals fall below 85% of reference weight
- ○ Animals fall below 80% of reference weight
- ○ Animals fall below 75% of reference weight
- ○ Animals fall below another proportion of reference weight/other measurement (please specify)

53. What is the limit for removing the animal from the study? (e.g euthanasia)

- ○ Animals fall below 85% of reference weight acutely
- ○ Animals remain below 85% of reference weight for an extended period of time (please specify)
- ○ Animals fall below 80% of reference weight acutely
- ○ Animals remain below 80% of reference weight for an extended period of time (please specify)
- ○ Animals fall below 75% of reference weight acutely
- ○ Animals remain below 75% of reference weight for an extended period of time (please specify)
- ○ Animals fall below another proportion of reference weight (please specify)
- ○ We do not remove animals from the study based on this measure (please specify)

Please specify the length of time considered if appropriate or if another measure is used.

54. Do you use a fixed value for the reference weight or adjust this throughout the study?

- ○ We use a fixed value based on one measurement of free feeding weight.
- ○ We use a fixed value based on several measurements of free feeding weight.
- ○ We adjust based on publicly available growth curves (e.g. those available through JAX).
- ○ We adjust based on data from within our lab/unit.
- ○ We adjust based on control mice from the same cohort.
- ○ We adjust by occasionally taking a new reference weight.

55. Are any measures other than body weight assessed?

- [ ] Body condition
- [ ] Home cage activity
- [ ] Other (please specify)

56. Are there any other details of your food restriction protocol that you feel are important in maximising the welfare of the animals? Could anything further be done to improve welfare further?

PART C. Behavioural testing

57. Which of the following are routinely paired with the behavioural testing of your animals?

- [ ] Head fixation
- [ ] Tethered device
- [ ] Home cage testing
- [ ] Testing without restraint e.g. wireless recording or no recording device

58. Are the animals habituated to the behavioural procedure and the tethering/restraint method together or separately?

- ◯ Together, we acclimatise animals to being tethered/restrained whilst they also make basic responses.
- ◯ We acclimatise animals to being tethered/restrained first before adding any elements of the task, i.e. we have some sessions in which they are restrained but the task is not run.
- ◯ Animals receive some behavioural training before being tethered/restrained for the first time (or before surgery).
- ◯ Other (please specify)
- ◯ N/A - animals immediately enter testing using the full task and tethering/restraint method.
- ◯ N/A - We only perform wireless recordings or no recording so do not restrict the animals' movement in any way.

59. On average, how many habituation/acclimatisation sessions in total do animals normally receive (i.e. before formal testing begins)?

- ◯ 0
- ◯ 1
- ◯ 2
- ◯ 3
- ◯ 4+

60. Do the animals have a habituation/acclimatisation period at the start of each formal testing session?

- ◯ No
- ◯ Yes (please specify length of time and other details)

Protocol

61. What is the frequency of behavioural testing?

- ◯ More than twice a day
- ◯ Twice a day
- ◯ 5 – 7 times a week
- ◯ 2 – 4 times a week
- ◯ Weekly

62. What is the typical duration of a behavioural session?

0 Minutes 240

63. Which of the following may terminate a behavioural session? How commonly is this the reason for ending a session?

	Never	Rarely	Sometimes	Usually	Always	This measure would not end a behavioural session
Animal stops engaging in the task/fixed number of response omissions	◯	◯	◯	◯	◯	◯
Animal shows signs of distress/ill health	◯	◯	◯	◯	◯	◯
Fixed amount of time	◯	◯	◯	◯	◯	◯
Fixed number of trials	◯	◯	◯	◯	◯	◯
Fixed number of correct responses	◯	◯	◯	◯	◯	◯
Fixed number of incorrect responses	◯	◯	◯	◯	◯	◯
Animal reaches a training criterion	◯	◯	◯	◯	◯	◯
Technical failure	◯	◯	◯	◯	◯	◯
Injury	◯	◯	◯	◯	◯	◯

Other (please specify)

64. Are animals typically tested in the light or dark period of the animal's circadian cycle? Please also specify if you use reverse light or not.

- ◯ Light
- ◯ Dark
- ◯ It varies as part of our research (i.e. scientific reasons)
- ◯ It varies due to testing schedule (i.e. for non-scientific reasons)

Please provide further information (e.g. please specify if a reverse light cycle is used)

65. What approximate percentage of the initial cohort of animals typically **fail** to complete behavioural testing?

0 % 100

66. How frequently are the following a cause for an animal having to be removed from the study?

	Never	Rarely	Sometimes	Usually	Always
Animals persistently do not engage in the task	◯	◯	◯	◯	◯
Animals frequently show signs of distress during the task	◯	◯	◯	◯	◯
Removed due to ill health/implant complications	◯	◯	◯	◯	◯

Choice of reward

67. What type of reward is typically used to reinforce behavioural performance? *Tick all that apply.*

- [ ] Water
- [ ] Sucrose solution
- [ ] Saccharine solution
- [ ] Milkshake
- [ ] Soya milk
- [ ] Fruit juice
- [ ] Food pellet similar to standard lab chow.
- [ ] Sucrose pellet
- [ ] Flavoured food/sucrose pellet
- [ ] Electrical stimulation of, for example, the dopamine system
- [ ] Optical stimulation of, for example, the dopamine system
- [ ] A sensory cue (e.g. visual and/or auditory cue(s))

Please provide further information and specify any other rewards not listed above.

68. Has this choice of reward altered your food/fluid restriction regime?

- ( ) Yes, using this reward means less food/fluid restriction
- ( ) Yes, using this reward means more food/fluid restriction
- ( ) No
- ( ) N/A - We have only ever used this reward

69. Has this choice of reward altered behavioural performance in your animals?

- ( ) Yes - using this reward means animals complete a greater number of trials than previously or compared to other groups.
- ( ) Yes - using this reward means animals have greater behavioural performance than previously or compared to other groups.
- ( ) No - we have not seen any change/they respond as expected.
- ( ) We have only ever used this reward but would consider changing if it could improve behavioural performance or response rates.
- ( ) We have only ever used this reward and would not consider changing this.
- ( ) N/A - we do not use food/fluid restriction.

70. Are any of the following aversive training methods used? *Select all that apply.*

- [ ] Air puff
- [ ] White noise
- [ ] Time out
- [ ] Shock
- [ ] Other (please specify)

71. Finally, is there any other key information about your behavioural protocol that is important in ensuring the welfare of the animals? Can you identify any parts of the procedure where further improvements are possible?

Thank you for participating in this survey.

Appendix 3: Detailed surgical SOP

Preparing for surgery

- Animals new to the facility should be given an acclimatisation period before surgery, typically five days. Habituation to the experimenter and rooms that the animal will be exposed to as part of surgery will also ensure lower stress levels.
- Introduce before surgery new items in the cage that will be important post-operatively. This includes extra bedding and jelly to deliver analgesia. At this point, provide non-medicated jelly to acclimatise mice to this novel foodstuff.
- Check the animal is in good health in the days preceding the surgery.
- Autoclave all instruments and materials to be used during the surgery. Autoclaved foil or similar can be used to cover the surfaces of instruments that cannot be autoclaved, for example anaesthetic vaporisers and surgical microscopes.
- Weigh the animal and adjust injection volumes of the drugs to be used to this weight.
- Induce anaesthesia and administer pre-operative analgesia. If the surgery is likely to take last longer than 30 minutes, 1ml isotonic saline should also be administered. Use the most refined methods for delivery.

Preparing the scalp

- After anaesthetic induction, hair can be optionally removed with an electric shaver, scissors, or depilatory cream. Electric shaving should not be done near the operating table to avoid contamination. Alternatively, if hair is left in place (for additional anchoring of the skin with the cyanoacrylate primer and dental cement), it must be thoroughly disinfected, for example with a dilute chlorhexidine solution.
  - ⚠ When shaving, take care not to damage the whiskers around the eyes and nose; whiskers and facial fur can be protected with petroleum jelly.
- Once the scalp has been cleaned, administer local anaesthetic to the centre of the area to be excised during surgery. Local anaesthetics take several minutes to act, so this should be done as soon as the scalp is prepared, before moving the animal to the operating table.
- Rodents' eyelids stay open during anaesthesia leading to dryness and possible corneal damage. Eyes can be protected by applying sterile ophthalmic ointment or artificial eye drops. Alternatively, eyelids can be covered with petroleum jelly to keep the eyes closed.

Transferring the animal to the operating table

- If using gaseous anaesthesia, quickly apply the anaesthetic mask with an oxygen flow of ~1L/minute and the isoflurane concentration to ~3% for rats and ~1.5% for mice. Throughout the surgery, monitor the depth of anaesthesia and reduce the level of isoflurane accordingly, this may be as low as 1% towards the end or surgery. Note that every animal responds differently and the precise isoflurane level should be adjusted according to the apparent depth of anaesthesia.
- Any increase in heart rate, or movement of the limbs or whiskers should be taken as a sign to stop all procedures and increase the level of anaesthesia until the animal no longer responds to a firm foot-pinch.
  - ⚠ It is important not to confound respiratory distress from applying too much anaesthesia for under-anaesthesia! In some animals, if the level of isoflurane is too high you may notice the animal breathing in gasps or developing a hunched posture. If unaccompanied by foot-pinch response, this is a clear sign that the isoflurane should be reduced immediately. Airways also need to stay clear to allow optimal breathing.
- After reaching a stable level of deep anaesthesia in the induction box, the animal can be mounted on the stereotactic frame.

- Local anaesthetics (lidocaine or bupivacaine) are applied under the scalp. Bear in mind that bupivacaine has a slower onset than lidocaine (30 and 2 minutes, respectively) but longer duration of action (4-8 hours and <1 hour, respectively) .
- Some stereotactic surgeries require head immobilisation with ear bars. The depth of anaesthesia needs to be carefully stabilised since the insertion of the ear-bars can be a strong irritant even for animals that are unresponsive to other stimuli. The use of non-puncture ear bars is recommended. Evaluate the absence of withdrawal (tail or toe-pinch) or blink (gentle corneal touch) reflexes before any painful manipulation and adjust anaesthesia and analgesia levels accordingly.
- If using closed-loop controlled heating-blankets systems, insert the lubricated rectal thermometer and turn on the heating pad, fixing the temperature probe to the mat with a small strip of paper tape and make sure temperature of the mouse is stabilised to 37ºC.
  - ⚠ To prevent damage to the extremities, ensure that the temperature of the mat itself cannot reach excessively high temperatures, either by regulating the temperature of the mat (some systems may allow for this separately, or the probe would need to be positioned beneath the animal) rather than the core temperature of the mouse, or by using a system that restricts the maximum temperature of the heat source. Also ensure that the mouse is insulated by placing an absorbent pad between the animal and the heating mat and not in direct contact with the mat. The heating mat should also be insulated from the stereotactic frame.
- Cover the animal's body from the neck down with a surgical drape (additional insulation can be provided by using veterinary bedding and bubble wrap). This will help to maintain a constant body temperature. A transparent drape will ensure visibility of the animal's respiration at all times.

Scalp excision

- To break up fat and oily deposits in the fur of the animal which could interfere with the binding of the headcap in the long-term, apply a 2% ethanol solution to the scalp before excision if not shaved.
  - ⚠ Some groups use a greater concentration such as 70% ethanol. 2% ethanol is sufficient to break the superficial tension of the fur without acting as an irritant or risking drying the skin underneath, which may compromise the welfare of the animal.
- Thoroughly disinfect the area using a topical disinfectant such as betadine or a chlorhexidine solution with a cotton swab. The scrubbed area should be larger than the skin section to be excised. Avoid disinfectant contact with the eyes and respiratory airways.
- Scalp excision should be as large as necessary and as small as possible: it should expose the skull area necessary for subsequent implant. Ideally, skin excision should not go over the skull muscles. Unless the skin opening is very small, avoid performing a slit and pulling the skin over since skin will tend to adopt its natural position, risking implant damage, itching, scratching and inflammation.
- Scalp excision should be performed with sharp surgical scissors and a minimal number of cuts to avoid skin nicks. To perform a single-cut scalp excision, pinch the skin at the centre of the area to be excised with small toothed forceps and lift perpendicular to the operating table. Use large surgical scissors to cut the lifted skin by placing the open scissors parallel to the operating table. Before cutting adjust the vertical position of the pinched skin such that the blades of the scissors are at the level of the imaginary section of skin that will be excised. When cut, this should provide an oval shape excision with no nicks.
- Around the excised skin, protruding hairs can be trimmed with corneal scissors, using your fingers to keep the skin taut to avoid causing nicks. Wounded skin can be treated with topical anaesthetic ointments. At this stage, bleeding from the skin should be negligible.
- Keep in mind that the internal tissues of the animal are, in principle, free of pathogens. Therefore, it is not necessary to disinfect the tissues below the excision line. However, from this point on, any tool that contacts live tissue must be clean and sterile.

Preparing the skull

- After scalp excision, remove any loose hair with the help of sterile cotton swabs or small tweezers. Avoid exposing the skull to water peroxide, bleach solutions or other irritant products.
- The periosteum (connective tissue) is typically impregnated with local anaesthetic and thus becomes elastic and gelatinous. It must be removed to allow an adequate bonding of the skull with cementing materials. It can be cut out using corneal scissors and small forceps. When the periosteum becomes dry, it loses volume and becomes thin and fragile and can be easily removed by scraping.
- If using a stereotactic frame, roll, pitch and yaw of the skull should be adjusted at this point such that lambda and bregma are at the same height and their axis is perpendicular to the ear bars.
- Make any necessary landmark for future implants by using stereotactic tools, motorised systems or micro rulers. Long-term landmarks (e.g. bregma) can be made by carving the bone with a scalpel and filling the hollows with permanent markers (resistant to the solvents contained in the cementing materials to be used).
- Roughen the skull with the blade of a scalpel or with a dental drill at low speed in order to increase bone rugosity and cement adherence. Bleeding from the bone can occur if scraping is too deep.
  - ⚠ Avoid scraping around the sagittal sinus since piercing this sinus can lead to significant haemorrhage. The interparietal cancellous bone is thicker than the parietal and frontal bones and tends to bleed more profusely.
- Remove any tissue debris with the help of sterile cotton swabs or compressed sterile air cans. Use sterile saline or other physiological buffers to remove any blood clot on the bone. Make sure the skin and bone are dry before proceeding to the next step.

Skin protection

- It is crucial to secure the skin to the skull before head-post implantation or craniotomies. This will keep the skin in place throughout the surgery, protecting it from mechanical damage and preventing the open wound from coming into contact with liquids (e.g. dental cement solvent which is highly irritant) and other debris during the rest of the surgery.
- Put a few drops of tissue adhesive (e.g. cyanoacrylate) on top of the dried skull. Spread the adhesive around the edges of the open skin with the help of a thin wooden stick (broken cotton swab). Ideally a thin band (1-2mm) of tissue adhesive should also cover the surrounding skin and hair. This can provide an impermeable protective barrier and act as an interface for further cementing materials.
- Depending on the specific characteristics of the implants, head-post and craniotomy, the order of the following steps should be performed according to the experimental limitations.

Head-post implantation

- In mice, head-post implantation does not typically require the use of anchoring screws, but large implants and implants in larger animals like rats may be reinforced with small stainless steel anchoring screws.
- Anchoring screws should be inserted in bones that offer highest mechanical resistance. Screws should never traverse the skull and dura must remain intact. Suitable implantation points are the skull ridges due to their strength and thickness.
- Head-posts can be positioned on their definitive location with the help of stereotactic instruments. Alternatively, they can be transiently attached to the skull with cyanoacrylate glue. Once in place, the head-post can be covered with dental acrylic or dental cement. Wait enough time for the cement to cure.

Craniotomy

- Craniotomy is typically performed using dental drills. In cases where the skull is very thin (young mice or flattened skull), a scalpel can be used to cut the bone. Bone drilling should be ideally performed after delivering sterile saline or PBS solution at room temperature to the skull in order to soften the bone and reduce inflammation and bleeding. Thinned, soaked skulls become translucent (especially in mice) allowing visualisation of superficial blood vessels of the brain. Bone debris produced during the drilling process that occludes vision needs to be frequently removed with the help of a water suction system and sterile cotton swabs. Continuous buffer flow on top of the skull is most convenient. It can be implemented using water pumps or gravity systems to deliver the buffer into a silicone elastomer well on top of the skull. Overflowing is prevented by a suction pump.
- Always use sharp burrs. Keep them clean of bone residues and sterilise before use. Typical burrs used for craniotomy are FG ¼. For bone flattening it is preferable to use cylindrical burrs (typically 1 mm diameter and 3 mm long).
- Bleeding during craniotomy should be stopped rapidly. It can originate from blood vessels in the bone, sinuses, meninges or brain. Bleeding from the bone usually stops spontaneously if it originates in small vessels. Wetting the skull with saline usually helps clotting. Electric cauterisers can be used when bleeding fails to stop. Soaked gelfoam can be used to absorb blood and promote clotting. Bleeding from the sinuses is dangerous and usually leads to haemorrhagic death, thus drilling close over the sinuses should be performed with extreme care. Subdural bleeding can take place if drilling temperatures are too high and can damage brain cells. This can be prevented by keeping the bone moist with room temperature buffers.
- Dura mater is typically left intact in most chronic experiments to provide long-term protection of the brain. However, electrode insertion typically requires piercing the dura at electrode entry points or full dura removal. Micropipettes for viral delivery are typically bevelled to pierce the dura.

End of surgery care

- Once surgery is complete, ensure the animal's fur is clean of any eye ointment, cement or glue. Allow to come around from anaesthesia gradually.
- If the surgery has been over one hour, administer 1 ml isotonic saline. Move the animal to a heated recovery cage with access to sufficient water and food. Observe regularly until fully recovered from anaesthesia.
- If you are not using medicated jelly to deliver post-operative analgesia, injected analgesia may need to be delivered at this point or the day following surgery. Consult your local veterinary team for advice.
- Once fully recovered from anaesthesia, return to the homecage which should have additional bedding from the pre-operative period. If administering post-operative analgesia via jelly, remove the unmedicated jelly use dot habituate the animal and replace it with medicated jelly.

Appendix 4: Example scoresheet and heath monitoring templates

Post-operative monitoring

Across the working group, different styles of scoresheet were used, as discussed in Section 3.3.3. Using this experience, we have developed example scoresheets that are intended to be easy-to-use and adaptable to local practices, following discussions between researchers and their institutional ethics committee.

The example post-operative scoresheet below condenses the identified key health indicators into a series of tick box responses to show that either a mild or more significant form of each clinical sign is present. Any milder signs present would suggest that monitoring should continue. Any of the more significant variations being present would require a response as dictated by local procedure, typically seeking advice from veterinary staff, which may result in the administration of treatment or the culling of the animal (i.e. implementation of predetermined humane endpoint).

Indicators included cover the animal's weight and body condition (Ullman-Cullere & Foltz, 1999), signs of infection (beginning with reddening and signs of irritation around the wound before overt signs become apparent, such as discharge from the wound), changes to gross appearance (from slight dishevelment to hunched posture and piloerection) and alterations in locomotion, which should be assessed in the cage as well as during and following handling of the animal (ranging from small locomotor changes to reluctance to move or violent reactions).

For a simpler layout, but one which may obscure some of the information, each pair of tick boxes could be replaced by a single prompt and score of 0 – 2, or one or two marks made to indicate presence of mild or more significant signs. As this scheme does not give details of what is being looked for, this is more suited to experienced researchers working in animal houses used to this form of assessment.

In both cases, space to indicate that the checks have been made, any action that should be completed following this assessment, and if and when this follow-up activity has been completed, are all included. These should be prominent so that it is easy to confirm checks have occurred and any necessary actions taken.

Fluid restriction

Many of the health indicators that need to be monitored during fluid control are similar to those used post-operatively as discussed in Section 4.2. A modified version of the scoresheet is therefore presented, replacing the mild/severe items for infection with equivalents for skin turgor, assessed by lightly lifting the loose skin on the back of a rodent and assessing its elasticity. As measures of body weight and condition are often prioritised during fluid control, an alternative scoresheet is also presented, which concentrates on these measures, condensing the other indicators into a single tick box.

As with the post-operative scoresheet, these scoresheets are intended to be easy-to-use and easily adaptable, which should be first discussed by researchers and their institutional ethics committees and adjusted to suit local and national policies and legislation before being put into use.

Example post-operative welfare assessment scoresheet

[Details of experimenter and ethical approval]:									
Animal i.d.:									
Baseline weight:									
Date:									
Days post-op:									
Weight:									
% of baseline weight:									
Irritation at wound site?									
Signs of infection at wound site?									
Lack of grooming/rough coat? Vocalisation on handling									
Piloerection/hunched posture?									
Lethargic or slightly jumpy?									
Reluctant to move or reacts violently?									
Action required?									
Time checked:									
Initial:									
Action taken:									
Time performed:									
Initial:									

Example fluid control welfare assessment scoresheet

[Details of experimenter and ethical approval]:									
Animal i.d.:									
Baseline weight:									
Date:									
Days of fluid control:									
Weight:									
% baseline weight:									
Lack of grooming?									
Piloerection/hunched posture?									
Lethargic or slightly jumpy?									
Reluctant to move or reacts violently?									
Slightly reduced skin turgor?									
Skin remains tented									
Action required?									
Time checked:									
Initial:									
Action taken:									
Time performed:									
Initial:									

Example fluid control scoresheet prioritising body weight

[Details of experimenter and ethical approval]:						[date]			[date]			…		
i.d.:	Baseline weight	95%	90%	85%	80%	Weight	Body condition	Coat/ activity	Weight	Body condition	Coat/ activity	…	…	…
[mouse 1]														
[mouse 2]														
[mouse 3]														
[mouse 4]														
…														
…														
Action required?														
Time checked:														
Initial:														
Action taken:														
Time performed:														
Initial:														

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