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Refinements to rodent head fixation and fluid/food control for neuroscience

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ABSTRACT

The use of head fixation in mice is increasingly common in research, its use having initially been restricted to the field of sensory neuroscience. Head restraint has often been combined with fluid control, rather than food restriction, to motivate behaviour, but this too is now in use for both restrained and non-restrained animals. Despite this, there is little guidance on how best to employ these techniques 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.

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 et al., 2020), brain atlases (Wang et al., 2020), advances in neural measurement (Dana et al., 2019; Jun et al., 2017; Peron et al., 2015; Steinmetz et al., 2021), and growing awareness of their sensory (Seabrook et al., 2017) and

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learning capabilities (Nakajima and 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. For example, many different stimulus types or presentations (e.g. in different locations and/or combinations) may be needed for a task, 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. The use of head fixation is also broadening beyond its original use in these high-yield studies, including the increasing use of mice over rats in awake functional magnetic resonance imaging (fMRI) studies (X. Chen et al., 2020; Gutierrez-Barragan et al., 2022; Han et al., 2019), making this approach increasingly common. Establishing best practice for head fixation therefore represents a timely refinement opportunity.

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 fMRI, two-photon calcium imaging (Dombeck et al., 2007) and patch-clamp recording (Margrie et al., 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 and Sigg, 1976; Pare and 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 et al., 2013) and repeated restraint, for as little as 10 min 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 et al., 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 and Dombeck, 2018; Williams et al., 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 and 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 and 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 and Dombeck, 2018; Sato et al., 2017; Sofroniew et al., 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 et al., 2018; Han et al., 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 and Dombeck, 2018; Sanders and 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 et al., 2017; Pinto et al., 2018; Poort et al., 2015). Fluid control is now used to motivate behaviour in non-restrained animals, meaning its possible applications are on the rise and its use has displaced that of food restriction. That food control has been more commonly used in rodents in the past means greater expertise is often available for this approach than for water control. 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 behavioural neuroscience 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 studies employing head fixation and water control, 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 these 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 awake rodent neuroscience 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. Materials and 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 online in the supplementary material to this article. 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 similar 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 online in the supplementary material to this article. 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 $\pm$ 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 and Berdoy, 2017), as well as the support offered locally. An example surgical standard operating procedure (SOP) is available online in the supplementary

material to this article for a more detailed account of a typical approach to these surgeries.

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 and 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 et al., 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 et al., 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 and 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.

The head-post implant is one of the key elements of this procedure. Implants are typically designed to the experimental requirements as well as spatial constraints. For illustration, Fig. 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, biocompatibility and intended use 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 be used, for example if MRI will form part of the work to be conducted. Another alternative is to use 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 recommended to have a number (three to four) of sterile backups ready for use before the surgery, since these small parts can easily get lost or contaminated during the procedure. In addition to the detailed instructions online in the supplementary material to this article, further information 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).

3.2.2. Anaesthesia and analgesia

Implantation of a head-post is an invasive procedure that must be

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.

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)

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).

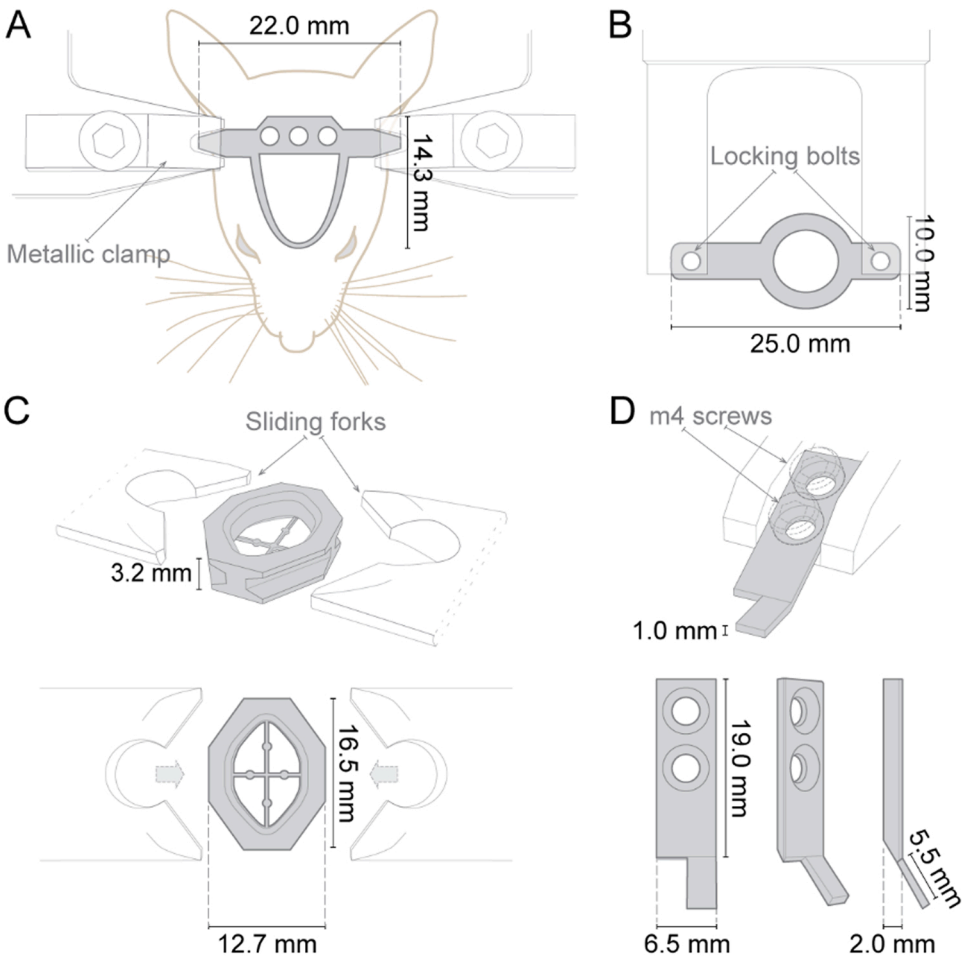


Fig. 1. Examples of different head bar designs (in grey) with their corresponding locking system (in white).
A "Bar and arc" shaped head bar designed to provide access to the dorsal part of the cerebral cortex (Galinanes et al., 2018). The 430 mg head bar is machined from 1.2 mm thick titanium sheets 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 et al., 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 and 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 calcium 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 et al., 2012). These represent a small number of example systems of which more are available, including some specially adapted for, for example, imaging within an MRI scanner (Gutierrez-Barragan et al., 2022), many of which are 3D-printed by researchers to allow for customisation to fit individual animals and study needs.

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

Agent	Administration	Effect
Buprenorphine (opioid)	Pre-anaesthetic (injectable, 20 min 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 min before skin incision)	+ Local anaesthesia; lidocaine fast onset / short duration (10 min / 1 h); bupivacaine slow onset / long duration (30 min / > 4 h)
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

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.

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,

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.

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)

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).

Alfieri, 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. Dosing regimens for anaesthesia and analgesia should be established in conjunction with the local veterinary staff, taking into consideration not only the day of surgery but also subsequent post-operative care.

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 et al., 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 min, 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 1 ml injection should be sufficient. For surgeries lasting longer than an hour, a second 1 ml 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 min, ranging from 45 to 300 min, 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.

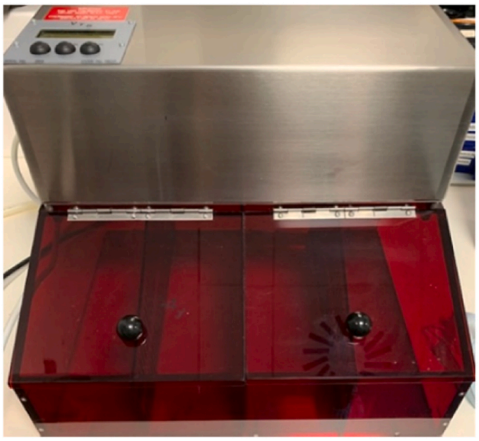
3.3. Post-operative care

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 et al., 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 et al., 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 et al., 2015), whereas highly palatable substances, such as

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

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.

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.

chocolate-hazelnut spread, are readily consumed without habituation (Kalliokoski et al., 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.

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.

The initiation or return to diet control and behavioural testing should be informed by the monitoring of the animal's health during this time.

From our survey data, it seems that whilst some groups do not wait to begin/resume testing or diet control following surgery, most allow animals to recover for a number of days (Fig. 2). Diet control is typically resumed around one-week post-surgery, with testing beginning a few days after this. However, these values represent common practice and we recommend that these decisions are made on a case-by-case based on the welfare of the animals, for example once weight has stabilised following surgery and no other signs of distress are evident.

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 et al., 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 et al. (2019).

3.3.3. *Monitoring of post-operative animals*

Practices such as providing additional palatable food are already commonplace (Fig. 3) 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.

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 and 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

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

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)

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).

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.

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)*

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).

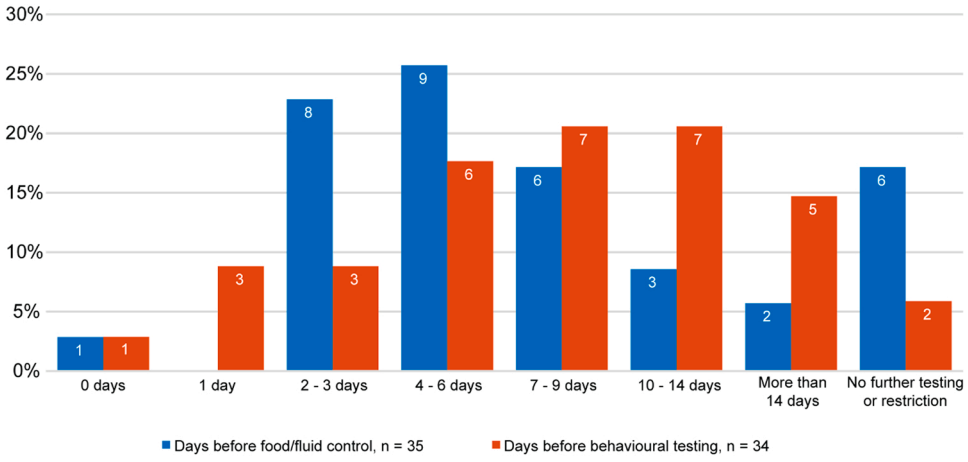


Fig. 2. Responses to the survey questions, "What period of time are the animals typically given to recover from surgery before food/fluid restriction is started/resumed?" and "What period of time are the animals typically given to recover from surgery before the first behavioural test?" for respondents 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.

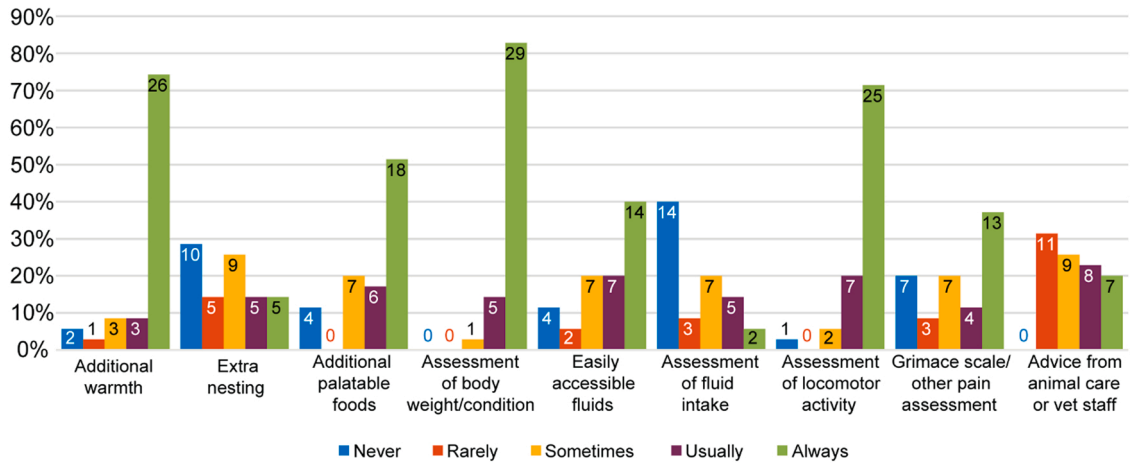


Fig. 3. Responses to the survey question, "In addition to the drugs detailed above, which of the following additional steps form a part of post-operative care immediately after surgery (i.e. for the first day or several days post-surgery)? Select all that apply" from respondents employing head fixation, 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.

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). This continued monitoring is important as welfare concerns may arise at any time during a study, for example those in Table 6. Clear intervention points based on this monitoring should be established through discussions with local animal care staff, colleagues and the local ethical review committee and adhere to local and national expectations. The duration for which an implanted animal can be maintained will be determined by the requirements of the study but a longer period with an implant will increase the chances of an adverse outcome. If a limit is therefore set on how long such animals can be maintained these considerations need to be balanced against each other and the time needed for the study minimised by other means (see also Section 4.6).

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 et al. (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 an example scoresheet that can be found in online in the supplementary material to this article. This scoresheet covers the major indicators of health and provide space for recording whether signs of concern are present in either a mild or more severe form. It is 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 online in the supplementary material to this article.

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; for example, mice that have been ordered in from a supplier should be given time to habituate to the new facility, then handled regularly by the experimenter and also habituated 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, for example assessing the wound for signs of infection and the animal for behavioural changes that may indicate poor recovery.
- 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 behavioural tasks. This immediately raises animal welfare concerns as food or fluid control are aversive and can be stressful (Rowland, 2007; Toth and 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 et al., 2012), locomotion (Darmohray et al., 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.

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.

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 5.2).

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 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 et al., 2021), although this is likely due to the weight loss rather than the method used to achieve this.

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 but is increasingly being used to motivate a wide variety of tasks. 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 h water deprivation has been found to decrease plasma volume and alter blood composition, and increase plasma corticosterone and renin activity (Bekkevold et al., 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 behavioural studies (Table 8). In male C57BL/6 J mice, plasma markers of metabolism were also altered after 24 h water deprivation (Cui et al., 2015). C57BL/6 mice also showed increased urine osmolality following 12 h water deprivation, this increase differing between male and female mice (28% versus 59%, respectively; Nair et al., 2019).

A study investigating the effect a lack of oxytocin has on stress responses also found that 18 h 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 et al., 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

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.

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)

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).

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 behavioural studies employing fluid control, 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 h 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 25 g, 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 et al., 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/30 g 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).

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.

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 and Edmonds, 1969). Our survey suggests that the onset of fluid control typically precedes the start of testing by two – three days (Fig. 2).

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

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.

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)

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).

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.

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)

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).

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.

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)

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).

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.

4.2. Health indicators for mice under chronic 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 summary of the most commonly used intervention points when used over a prolonged period (days, weeks, up to months). 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 (available online in the supplementary material to this article) 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 online in the supplementary material to this article.

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 h, 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 et al., 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 and Hall, 1962; Tucci et al., 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 5.2), 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 (Fig. 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.

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 the online supplementary material to this article). 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 et al., 2019). This high-throughput approach may even be combined with voluntary head-fixation (Aoki et al., 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 (Fig. 5). 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

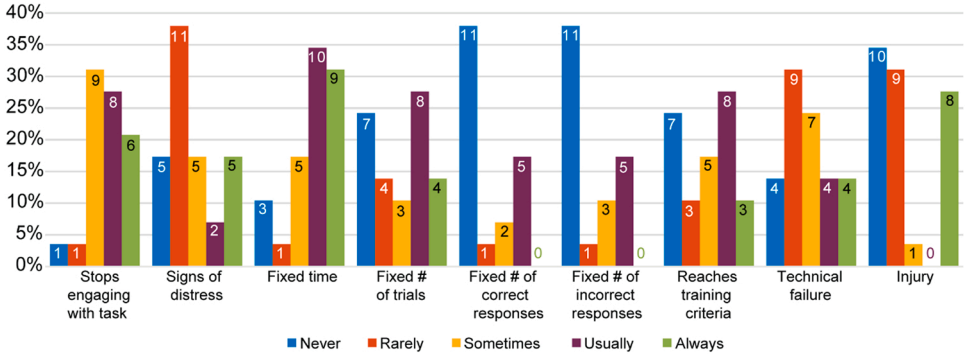


Fig. 4. Responses to the survey question, "Which of the following may terminate a behavioural session? How commonly is this the reason for ending a session?" from respondents employing head fixation, n = 29.
A variety of different events may lead to a session to be terminated, ranging from measures relating 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.

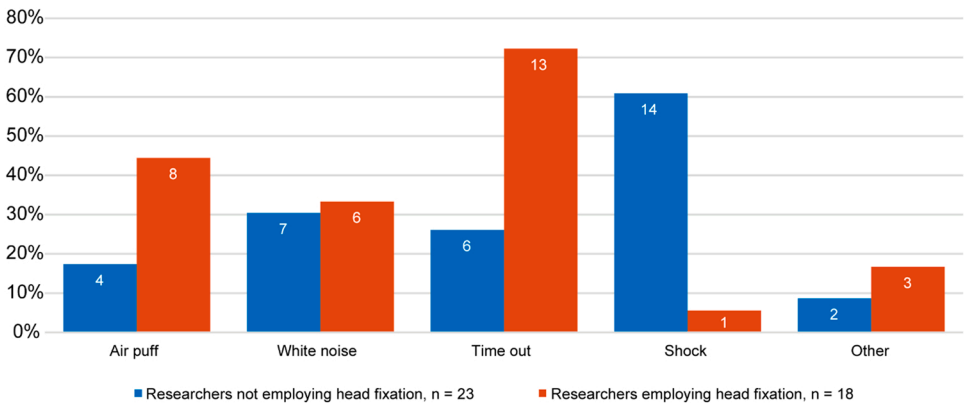


Fig. 5. Responses to the survey question "Are any of the following aversive training methods used? Select all that apply." from those employing and not employing head fixation. The use of different negative reinforcers differed between those employing head-fixation versus those that do not. The use of 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.

sparingly and only when justified to the regulatory authority and research institution's ethical review board. Additionally, the use of these approaches is likely to increase the cumulative stress the animals experience which may consequently affect performance and limit the length of session and overall length of study that can reasonably be achieved.

4.7. Recommendations to refine motivation

4.7.1. 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; for example, easing restriction once mice are performing reliably after initial training and habituation.
- 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 5.2).
- The overall welfare of each animal must be monitored daily using a range of welfare measures and clearly defined intervention points; for example, mice losing weight more rapidly than expected may need to be returned to free dietary access for a period of time, whereas changes to behaviour or other signs for concern may trigger more regular and in-depth monitoring.
- A rigorous documentation system must be maintained for monitoring the welfare of each animal (see templates online in the supplementary material to this article).
- 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. For example, a blocked reward port may have disrupted a behavioural session giving apparently poor performance.
- 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 h before the procedure, and a few days following the procedure.
- Negative reinforcers should be used sparingly, prioritising time-outs over more aversive stimuli.

4.7.2. 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).

4.7.3. 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 (~5 mm 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 behavioural response required from the animal may be minimal or more complex and is often paired with some form of neuronal recording or imaging.

5.1. Experimental design considerations

An important consideration beyond the practical concerns discussed here in detail is the number of animals to be used in a study. This should be well justified and calculated before a study commences using

approaches such as a power calculation. It should also be made clear what the experimental unit ("n"), of a study is; whilst this is true for all study types, it is of particular importance in longitudinal and electrophysiological studies where the issue of pseudoreplication needs to be avoided (Lazic, 2010). This issue can be addressed in part by clear reporting (Percie du Sert et al., 2020; "Recommendations for the Design and Analysis of In Vivo Electrophysiology Studies," 2018) and appropriate analytical approaches (Lazic et al., 2020; "Recommendations for the Design and Analysis of In Vivo Electrophysiology Studies," 2018), but in general studies need to be powered sufficiently to reliably detect the effects of interest.

Once the experimental unit has been identified, a power calculation typically requires, in addition to the power and significance level desired, the use of an estimated effect size as well as an estimate of the expected variance. These estimates can be based on previous experiments from your own laboratory or the published literature, what is important is that they are as relevant and as accurate as possible for the proposed work to ensure that the number of animals to be used is sufficient for the scientific goals of the study without being excessive. Choice of an appropriate effect size is particularly important to ensure that the minimum difference between groups that can reliably be detected would be of interest scientifically. These values can then be used in power calculators such as G*Power (https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeits-psychologie/gpower.html; Faul et al., 2007) or the NC3Rs' EDA, a free online tool to assist researchers with the design of their experiments (Experimental Design Assistant: RRID:SCR_017019, https://eda.nc3rs.org.uk; Cressey, 2016; du Sert et al., 2017; Percie du Sert, Bamsey, et al., 2017) which can also be consulted for further discussions of these issues (e.g. https://eda.nc3rs.org.uk/experimental-design-group#effectsize).

5.2. 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 time spent under restraint as reported in our survey ranged from 15 to 150 min from 27 respondents, with a median value of 60 min. 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.

The term "habituation" is defined as the diminishing of an innate response to a frequently repeated stimulus (Leussis and Bolivar, 2006). In the context of head fixation 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. In the case of studies involving the use of fMRI, habituation to the noise of the imaging system will also be required (e.g. Chen et al., 2020). 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 et al., 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.

5.2.1. 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 and Sigg, 1976; King et al., 2005; Pare and Glavin, 1986; Russo et al., 2021; 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. This has been shown to decrease measures of stress in rats in imaging studies (Russo et al., 2021). 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).

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. During imaging, for example, lower levels of stress may lead to steadier breathing rates and other movement being

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.

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

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).

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.

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)

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).

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.

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)

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).

reduced, minimising artefacts (Russo et al., 2021). 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 et al., 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, et al., 1979; Shanks and Anisman, 1993; see Section 5.2.2). Note also that responses to habituation may differ between male and female animals (Lindhardt et al., 2022).

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).

5.2.2. *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 et al., 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, et al., 1979; Shanks and 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.

5.2.3. *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 5.2.1), 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 et al., 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 et al., 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 and West, 2010). The choice of handling method has also been shown to have an impact on habituation (Gouveia and Hurst, 2017) and, perhaps crucially, reward processing (Clarkson et al., 2018). Further guidance and resources are available from the NC3Rs: https://nc3rs.org.uk/how-to-pick-up-a-mouse.

5.3. 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.

5.3.1. 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 et al., 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

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

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. seeHarris et al., 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.

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.

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.

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 et al., 2019), reaching (Galinanes et al., 2018), manipulation (Barrett et al., 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 and Redgrave, 2020; Vale et al., 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 et al., 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 (G. Chen et al., 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 and 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.

5.3.2. *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 5.3.4, 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 and Kerr, 2019), configurations should be optimised to minimise stress and maximise welfare, and an appropriate habituation regimen established (see Section 5.2).

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 5.3). 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 5.3.4). 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 et al., 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.

5.3.3. *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 et al., 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 and Kanold, 2017; Maor et al., 2019; Reinert et al., 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 et al., 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 (G. Chen et al., 2019; Ghosh et al., 2011; Voigts and 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 et al., 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?

5.3.4. *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 et al., 2019; Salkoff et al., 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 et al., 2015). Furthermore, differing set-ups can promote different postures, which, in turn, can impact both welfare and the willingness of the mouse to engage with the task; if a more natural posture can be achieved, this is likely to reduce the stress of the animal in the head-fixed set-up and thus improve its engagement 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 et al., 2019; Dennis et al., 2021; Mathis et al., 2018; Mathis and Schneider, Lauer, et al., 2020; Mathis and Mathis, 2020; Wiltschko et al., 2015). See also Sections 5.2.2 and 5.2.3, in particular Table 15, for further discussion and suggestion of methods to assess welfare during these studies.

5.3.5. *Further suggested refinements to procedures for restraint and training*

As described in the Section 5.2, 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.

5.4. 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; for example, restrain mice in the set-up for an increasing length of time before formal testing, pairing this with positive reinforcement.
- 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, approaches increasingly being used more broadly in the neuroscience field. Both methods 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.

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.

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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.

Competing interests

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Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jneumeth.2022.109705.

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