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Innovations and advances in modelling and measuring pain in animals

Katelyn E. Sadler[1], **Jeffrey S. Mogil**[2,3], **Cheryl L. Stucky**[1,✉]

[1]Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, WI, USA.

[2]Department of Psychology, McGill University, Montreal, QC, Canada.

[3]Department of Anesthesia, McGill University, Montreal, QC, Canada.

Abstract

Best practices in preclinical algesiometry (pain behaviour testing) have shifted over the past decade as a result of technological advancements, the continued dearth of translational progress and the emphasis that funding institutions and journals have placed on rigour and reproducibility. Here we describe the changing trends in research methods by analysing the methods reported in preclinical pain publications from the past 40 years, with a focus on the last 5 years. We also discuss how the status quo may be hampering translational success. This discussion is centred on four fundamental decisions that apply to every pain behaviour experiment: choice of subject (model organism), choice of assay (pain-inducing injury), laboratory environment and choice of outcome measures. Finally, we discuss how human tissues, which are increasingly accessible, can be used to validate the translatability of targets and mechanisms identified in animal pain models.

Pain remains a leading global health care problem[1]. Analgesic drugs are one of the primary tools used in clinical management of both acute pain and chronic pain. However, many of the current options in the pharmacological toolbox are riddled with unwanted side effects, high misuse potential and limited efficacy[2]. In recent years, many analgesic candidates directed against high-profile targets have failed in their translation to the clinic, with their failure routinely blamed on drug pharmacokinetics, clinical trial design or recruitment, on-target drug side effects or toxicity, and poor preclinical modelling[2,3]. With the last point in mind here, we review the status quo in preclinical pain modelling as revealed by our analysis of methods reported in articles recently published in the journal *Pain*, and discuss how current practices could be improved to increase translational success in our field.

✉ cstucky@mcw.edu .

Author contributions

J.S.M. researched data for the article. All authors contributed substantially to discussion of the content and reviewed and/or edited the manuscript before submission. K.E.S wrote the article.

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Competing interests

The authors declare no competing interests.

Since the previous review of this topic by one of the current authors[4], the only unambiguous pain drug development success story is that of calcitonin gene-related peptide (CGRP) antagonist use in migraine[5]. Although the anti-nerve growth factor drug tanezumab has shown efficacy in phase III clinical trials, it did not succeed into the clinic. As preclinical researchers, we might wish that improved animal models were a primary reason for the translational success of CGRP antagonists, but this is not the case. CGRP was originally identified as a key molecule in migraine pathology more than 30 years ago when elevated CGRP levels were measured in blood samples from patients with migraine[6]. It was later shown that injection of CGRP triggers headache in patients[7]. Parallel preclinical assessments of CGRP's causative role in migraine were originally not as conclusive, as application of CGRP to rodent meningeal afferents, the peripheral nerve fibres presumed to underlie noxious sensory transmission in migraine, had very little effect on neuronal activity[8], and the behavioural effects of CGRP administration varied widely across preclinical studies (reviewed in REF.[9]). Notably, these initial animal studies were performed exclusively in male rats; experimenters have only recently repeated these behavioural assessments in female mice and found that extremely low doses of CGRP induced robust migraine behaviours in them[10]. This case study is an example of how more appropriate animal modelling of clinical conditions (in this case, using female rodents to model a condition that predominantly affects women) could have potentially accelerated the identification, validation and implementation of a new pain target.

We believe that animal models can provide unparalleled mechanistic insight into complex biological phenomena, such as chronic pain, and should continue to be used in pain research in combination with human tissues. In this Review, we describe the decisions regarding model organism (subject), injury model (assay), testing environment and pain measurement that must be made before beginning a preclinical algesiometric experiment. The status quo of each of these factors is presented and compared with observations made in clinical patient populations with pain. Recognizing that human chronic pain is a complex, heterogeneous disease state, we end this Review by providing recommendations to assist in the design, execution and analysis of preclinical pain behavioural testing, so as to increase the translatability of every experiment.

Choice of subject (model organism)

When assessed in a 2009 review[4], young adult male Sprague Dawley rats were the most commonly used model organism in preclinical pain research, although the typical patient with chronic pain was (and still is) a middle-aged woman[11]. The 2009 review also revealed that mice were being increasingly used in pain research[4], a trend that began in the mid-1990s and was accelerated by the ever-expanding list of technologies that require the use of transgenic animals, such as optogenetics and chemogenetics. In our current analysis, we examined primary research articles published in the journal *Pain* between 2016 and 2020 (see Supplementary methods and Supplementary data 1 for details of the analysis) and found that in the past 5 years, the prevalence of mice and rats used in preclinical pain studies has been relatively similar; each species was used in approximately 50% of studies (FIG. 1a). Larger mammals, including dogs and pigs, are very rarely used in preclinical pain research, likely due to the size, cost and maintenance associated with these animals.

Although increased use of large mammals was advocated for previously[12,13], rodents are used far more frequently in preclinical pain research, and therefore will be the focus of this Review.

Until we have a more comprehensive understanding of the critical gene expression and electrophysiological differences between human tissues and mouse or rat tissues (see BOX 1), behavioural and anatomical differences will likely dictate whether mice or rats are used in a given experiment. Notable benefits exist for both species. Rats require shorter behaviour habituation periods and, although we are unaware of a research article directly comparing them with mice, rats are anecdotally easier to train in conditioning tasks. Similarly, although a direct species comparison has not been reported to our knowledge, rats are also believed to experience less experimenter-induced and procedure-induced stress than mice; for example, the Randall–Selitto deep pressure test requires extended periods of restraint, and therefore is almost exclusively performed in rats. The larger size of rats makes surgical injuries (see later) easier to perform. Alternatively, the smaller size of mice makes them more cost-efficient and space-efficient to house, greatly reduces the amount of drug required for pharmacological experiments and makes it easier to deliver light to or perform imaging in deep neural structures that are critical sites of pain modulation. The utility of mice is further increased by the number of transgenic lines that are commercially available and the fact that mouse tissues have been used more frequently than rat tissues in comparisons with human gene expression[14].

Habituation periods

Time spent in the testing room/apparatus before commencement of testing procedures.

Randall-Selitto deep pressure test

A behaviour test that measures deep tissue mechanical sensitivity via the application of calipers or a weight to a part of the animal’s body (generally the hind paw of a rat).

In addition to species, rodent strain has significant effects on both baseline and injury-induced pain behaviours. In our analysis, less than 10% of mouse studies published in the journal *Pain* between 2016 and 2020 used outbred mice (such as the CD-1 strain; FIG. 1b). This is in stark contrast to rat studies published in *Pain*, of which more than 80% used outbred strains (mostly Sprague Dawley; FIG. 1c). C57BL/6 inbred mice were used in more than half of the studies published in *Pain* between 2016 and 2020, despite being a phenotypically outlying strain[15,16]; compared to other inbred strains, C57BL/6 animals exhibit heightened sensitivity to thermal stimuli at the baseline and resilience to injury-induced hypersensitivity[15]. One reason for continued use of C57BL/6 and other inbred mouse strains is the expectation of reduced response variability, and subsequently smaller required sample sizes. However, this argument was recently negated by a meta-analysis of biomedical studies in which inbred and outbred mice were directly compared; no differences in phenotypic variability were observed[17]. The argument that reduced variability in an animal model is desirable is also antithetical to the idea of modelling chronic pain; wide

phenotypic variability is observed in patients with chronic pain receiving identical disease diagnoses or undergoing standardized pain tests. Therefore, pain in patient populations may be better modelled by outbred strains because of their genetic heterogeneity and phenotypic stability.

Outbred strains

Strains in which direct brother–sister mating is avoided to minimize inbreeding.

In our analysis of research articles published in *Pain* from 2016 to 2020, approximately 24% of preclinical pain researchers incorporated both male and female subjects in their studies (FIG. 1d). More detailed, temporal analyses regarding the use of both sexes in pain research were described in a recent review of this topic[18]. Inclusion of both sexes follows mandates from major research funding agencies (including the US National Institutes of Health, effective as of 2016 (REF.[19])), and has resulted in reports of robust, and often qualitative, sex differences in neural and immune system pain mechanisms[18]. This is an extremely important development in the pain field, as female subjects were, for many years, rarely included in preclinical chronic pain studies despite the fact that women disproportionately receive a diagnosis of chronic pain, a phenomenon that is perhaps due in part to influences of sex and gender on clinical pain reporting[20].

Experiences of acute and chronic pain vary greatly across the lifespan. In humans, the prevalence of chronic pain typically increases with age[11]. However, for certain disorders, such as migraine, pain prevalence declines later in life[21]. Although the average life expectancy of captive rodents is approximately 1/37 of the average human life expectancy, age-related effects on nociceptive processing have been grossly understudied by preclinical pain scientists[22]. Our analysis of articles published in *Pain* between 2016 and 2020 suggests that currently, most preclinical pain research is performed in rodents aged 2–3 months (Supplementary Fig. 1), an age range that roughly coincides to human years 15–20 (REF.[23]). The rare use of aged animals is likely due to the steep financial costs associated with housing animals for an extended period or purchasing aged animals; at the time of writing, 22-month-old C57BL/6 mice cost approximately 14 times as much as 3-month-old C57BL/6 mice on The Jackson Laboratory's website. Notably, middle-aged and old-aged transgenic mice are often not commercially available. When aged animals are studied, one factor that should be considered is the homogeneous life history of captive-bred animals. The intentional standardization of laboratory rodent life history fails to appropriately model the complex life history of patients with chronic pain, and may contribute to the limited translational success in our field. In rodent pain models, for example, prior injury (even if seemingly resolved) often affects the susceptibility to and intensity of pain that results from subsequent injury (reviewed in REF.[24]). This priming effect is not exclusive to noxious tissue damage as early life stress can have similar effects on subsequent pain responses[25–29]. Therefore, our current approach of studying naive, young animals may skew therapeutic development towards acute pain targets but poorly model the complex, cumulative life history of patients with chronic pain.

In summary, our analysis suggests that young, naive, inbred C57BL/6 male mice and young, naive, outbred Sprague Dawley male rats are the current status quo for preclinical pain experimental subject selection. To increase the translatability of preclinical pain experiments, we provide the following recommendation regarding model organism selection: increase the heterogeneity of animal subjects to more accurately model the heterogeneity observed in patient populations with chronic pain. Although nociception is a critical biological process conserved in all animals, we are just beginning to understand the biology that allows variable perception and expression of nociceptive behaviours across species, sexes and an individual animal's lifespan. At this time, mice and rats appear to offer similar levels of homology to humans with respect to modelling pain sensation and perception, and because of their predominance in the preclinical space for the last 50 years or more, rodent research infrastructure far exceeds that which is available for larger mammals. Therefore, we support continued use of (albeit not exclusive reliance on) both mice and rats in pain research, but we encourage mouse laboratories to consider using outbred strains and we argue for the inclusion of older animals and animals of both sexes, unless explicitly justified.

Choice of assay (injury model)

One of the leading concerns regarding animal pain models is whether the injury models (or assays) accurately mirror the temporal, anatomical and pathophysiological characteristics of patient phenotypes. According to our analysis of articles published in the journal *Pain* between 1980 and 2020, experimentally induced nerve injuries that result in neuropathic pain are currently the most popular method for inducing pain in rodents; one or more such injuries were used in 42% of all preclinical studies published in *Pain* in the last 5 years (FIG. 2a; Supplementary data 1). However, recent reviews estimate that only approximately 10% of human pain conditions have neuropathic qualities[30,31]. Although nervous system damage is difficult to assess in chronic musculoskeletal, cancer or nociplastic conditions, the current preclinical status quo appears to disproportionately favour neuropathic injury models relative to the prevalence of these conditions in patient populations. Our analysis shows that spinal nerve ligation[32], spared nerve injury[33] and chronic constriction injury[34] were the three most commonly used mononeuropathic models (models in which damage is restricted to a single nerve) in articles published in *Pain* between 2016 and 2020 (FIG. 2b). These assays produce relatively localized pain in rodents, the intensity of which diminishes over time in the spinal nerve ligation and chronic constriction injury models, but curiously not in the spared nerve injury model[32–34], at least in male mice[35]. Additionally, while these assays model causalgia (also known as chronic regional pain syndrome type II), it is unclear whether their mechanistic underpinnings recapitulate the most common types of human neuropathic pain, such as radiculopathy, postherpetic neuralgia, painful diabetic neuropathy and neuropathic low back pain. Thus, animal models likely oversimplify the spatial, temporal and biological complexity of human neuropathic pain conditions.

Neuropathic pain

Pain caused by a lesion or disease of the somatosensory nervous system.

Our analysis suggests that another popular means of inducing neuropathic injury is through use of chemotherapeutic drugs (reviewed in REF.[36]). Chemotherapy-induced peripheral neuropathy (CIPN) models have high face validity, because the compounds used in animals are the same as those that induce the dose-limiting side effect of (sometimes painful) neuropathy in patients with cancer. However, the vast majority of CIPN rodent studies are performed in cancer-free animals. The presence of a tumour, the induction of which is routinely modelled by those interested in cancer pain (reviewed in REF.[37]), should be considered when one is attempting to more accurately model CIPN pain.

Chemotherapy-induced peripheral neuropathy (CIPN) assays

Neuropathic pain assays in which nerve damage is caused by the administration of chemotherapeutic drugs.

Face validity

A subjective assessment of whether an assay replicates the patient population that it is attempting to model.

Inflammatory injuries are induced in rodents by introducing a chemical inflammogen or by mechanically or thermally damaging tissue. Our analysis of articles published in *Pain* between 2016 and 2020 suggests that the most widely used inflammatory assay in this period involves intraplantar injection of complete Freund's adjuvant (CFA) (FIG. 2c). Two of the primary advantages of this method are the ease with which the injury can be induced and the concentration-dependent, extended period of hypersensitivity that follows injection[22,38]. As discussed later, the temporal duration of high-dose CFA pain may position CFA as one of the few inflammatory assays that actually allow investigation of the 'chronic' phase of pain. The primary concern with CFA use, however, is the clinical relevance of the associated injury. Accidental laboratory exposure[39] notwithstanding, subcutaneous accumulation of dead bacteria is not a leading cause of chronic inflammatory pain in patients. Similar concerns exist with carrageenan[40], a seaweed extract. The underlying immune and inflammatory processes in these models may or may not accurately reflect inflammatory mechanisms of pain in patients. To this end, the use of more-face-valid models of cutaneous inflammatory pain, such as the increasingly popular plantar incision model of post-operative pain[41–43], in which both skin and muscle are incised, is recommended.

Many specific disease states are also modelled using direct infusion of inflammatory compounds into a target tissue or mechanical tissue damage that results in a robust immune response. Generic musculosketal pain is frequently induced by injecting CFA or carrageenan[44] directly into a muscle or into a joint to model arthritis. More-face-valid osteoarthritis models, however, rely on either surgical manipulation of soft tissues in the joint[45,46] or intra-articular injection of inflammatory compounds such as monosodium iodoacetate[47]. Similarly, gold standard rheumatoid arthritis models involve infusion of collagen into the joint to trigger an autoimmune response[48]; this response closely models

that which is observed in patients with rheumatoid arthritis, making this a model with high face validity (reviewed in REF.[49]).

Musculosketal pain

Pain affecting bones, joints, ligaments, tendons or muscles.

Osteoarthritis

A form of arthritis involving the degeneration of joint cartilage and the underlying bone.

Rheumatoid arthritis

A form of arthritis featuring inflammation in the joints and resulting in painful deformity and immobility.

Inflammatory approaches have also been used, albeit more controversially, to model low back pain and headache, the two leading causes of disability worldwide[50]. One common approach for inducing migraine in rodents is direct application of inflammatory agents onto the dura mater[51,52]. Other popular approaches include systemic administration of nitroglycerine[53], a compound that also induces headache in patients, or triptans[54], a class of migraine therapeutics that paradoxically induce headaches when overused. Similar to mononeuropathic assays, these models likely oversimplify the pathophysiology that leads to migraine in patients; experimental addition of stress paradigms or other common migraine triggers experienced by patients may increase the translational relevance of these models. In addition, assay-specific behavioural measures should be used for migraine studies to take into account the relatively unique set of symptoms experienced by patients: these could include photophobic, osmophobic and phonophobic behavioural tests.

Despite the obvious difference in bipedality versus quadrupedality between patients and rodents, rodent models are widely used to study low back pain. A number of inflammatory lumbar disc degeneration and soft tissue injuries have been developed for use in rodents (reviewed in REFS[55,56]). Clinically relevant outcomes such as decreased mobility or gait changes are not uniformly observed across these injuries. Therefore, a combination of different pathophysiological models or incorporation of additional behavioural measures should be considered. More successful modelling of low back pain in animals is one of the most pressing needs of the preclinical pain community given the high prevalence and associated disability in humans worldwide.

Most human chronic pain conditions do not adhere to the inflammatory versus neuropathic categorical dichotomy. A separate category of nociplastic pain conditions (previously called ‘functional pain disorders’, ‘dysfunctional pain’ or ‘idiopathic pain’) exists in humans and is defined as pain that arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing activation of peripheral nociceptors or disease/lesion of the somatosensory nervous system[57]. While this term cannot be directly applied to any existing

injury model in animals — by definition experimenters are inducing nociceptor activity or damage in the nervous system — many have attempted to model nociplastic conditions in rodents. Fibromyalgia is the most prevalent nociplastic clinical condition, affecting approximately 4% of women and 1% of men worldwide[58]. The most commonly used models of fibromyalgia[59] employ various combinations of targeted muscle injections[60,61] and fatigue[62], reserpine-induced depletion of central monoaminergic signalling[63] and a variety of stressors. A recently developed model that may have higher face validity is one in which antibodies are transferred from patients with fibromyalgia into naive rodents[64].

Nociplastic pain

Pain that arises form altered nociception despite no clear evidence of actual or threatened tissue damage.

Visceral nociplastic conditions, including irritable bowel syndrome, interstitial cystitis/bladder pain syndrome and chronic prostatitis/chronic pelvic pain syndrome, are another class of highly prevalent diseases[65–67] that receive little preclinical attention; only 2% of studies published in *Pain* between 2016 and 2020 involved noxious stimuli directed to visceral organs (Supplementary data 1). Visceral hypersensitivity is most frequently induced through mechanical or chemical means. High fluid or air pressure can be used to distend any hollow organ to a level that is considered painful[68,69]; recordings of the reflexive abdominal contractions that accompany this distension (visceromotor responses) are commonly used visceral pain measurements in rodents[70–72]. Organ distension is frequently coupled with the instillation of a pro-inflammatory chemical or biological agent such as trinitrobenzenesulfonic acid[73], dextran sulfate sodium[74], cyclophosphamide[75] or zymosan[76], the combination of which often leads to prolonged visceral hypersensitivity.

Complex regional pain syndrome is another nociplastic condition that frequently develops following limb trauma[77]. Therefore, a rodent tibia fracture/cast model was developed[78] that recapitulates many of the sex differences observed in patient populations with complex regional pain syndrome[79]. Additional assays of complex regional pain syndrome include chronic post-ischaemia pain[80] and distal nerve injury[81]. Although all of these nociplastic models involve peripheral tissue hypersensitivity at one or more time points in the course of pain development, it is unclear how many are also associated with emotional distress or functional disability, two additional diagnostic criteria for chronic primary pain disorders listed in the most recent edition of the International Classification of Diseases (ICD-11)[82]. Since we do not understand the biological basis of many of these conditions, we deem our best nociplastic assays to be those that induce rodent behaviours that are similar to behaviours observed in patients. However, as described later, this anthropomorphization may be a leading reason for failed translational success.

In addition to the pathophysiological cause, injury models can be classified by their temporal duration. According to the International Association for the Study of Pain, chronic pain is defined as pain that persists or recurs for more than 3 months[82]. Only 3% of the preclinical pain studies considered in our analysis of articles published in *Pain* between 2016 and 2020 would meet this inclusion criterion; 86% of studies were terminated at 1 month or less

following injury (Supplementary Fig. 2). Short data collection periods likely result from temporal, financial and external incentive (for example, publication or ‘scooping’) pressures, or the notion that biological processes happen on a more rapid timescale in rodents than in humans. Although it is true that rodent ageing happens more quickly than human ageing, it is unlikely that the same species-specific acceleration exists in pain-related processes. For instance, an 8-week-old mouse matures 45 times faster than a human at any point in their life[23]; with this time conversion, rodents would enter the chronic phase of pain only 2 days following injury. However, the basic biological processes associated with nociception do not happen this rapidly; injury-related immune cell recruitment, neuronal conduction velocity and synaptic transmission are not 45 times faster in rodents than in humans. And although wound healing and axon regeneration take less time in mice than in humans, this likely results from the smaller size or shorter distance over which these processes must occur, not from a fundamental difference in enzymatic time courses. Further complicating this issue is the fact that many acute pain processes occur on similar timescales in rodents and humans; capsaicin-induced pain occurs on similar timescales in humans[83] and mice[84], as does CFA-related mechanical allodynia[22,39]. Therefore, it is possible that the same temporal definition used for chronic pain in humans may also be appropriate for chronic pain in rodents. To the extent that this is true, very little extant preclinical research has actually studied chronic pain.

Mechanical allodynia

Mechanical pain due to a stimulus that does not usually provoke pain.

Alternatively, the historical definition of chronic pain — that is, pain that persists past tissue healing[85]— could be used in rodent studies. However, concerns about this definition exist when considering pain in humans; residual tissue damage is difficult to assess in deep tissue or visceral injuries and many nociplastic conditions[57], such as fibromyalgia, never present with obvious tissue damage. This criterion may be easier to assess in rodents; by definition, all injuries require some type of initial tissue insult and typical healing times could be assessed. However, as most of the injury models that we currently use have not been characterized in this manner, we are unsure how many models would meet this inclusion criterion.

In summary, our analysis suggests that intraplantar CFA injection and mononeuropathic surgical injury are the current status quo for inducing inflammatory and neuropathic pain, respectively, in rodents. These assays are highly reproducible between laboratories, but are relatively simplistic and not necessarily representative of the anatomical damage or underlying disease processes that occur in chronic inflammatory and neuropathic pain conditions in patients. To better model patient populations with chronic pain, we provide the following recommendation regarding pain assay selection: use multiple, face-valid, long-lasting pain assays in a given study. For example, if you are studying migraine, consider performing your initial experiments with the nitroglycerine assay and then confirm your results in a dural application priming model that has long-lasting behavioural effects. Regardless of outcome, these parallel assessments will provide insight into the generalizability of your findings; if a similar result is obtained in both assays, your

manipulation may have broad applicability as a migraine therapy. Alternatively, if 'positive' results are obtained only in one assay, this may reveal important pathophysiology that distinguishes the injury models from one another. We also encourage further development of more valid nociplastic, low back pain (and other musculoskeletal) and headache models as these are the most prevalent, yet least studied, chronic pain conditions in patients. Lastly, researchers are encouraged to extend the temporal duration of their studies to better understand the mechanisms of chronic pain over a time course that better recapitulates that of chronic pain in patients.

Laboratory environmental factors

Whereas organism and assay selection are routinely described in pain research and widely recognized to affect behavioural results, variations in husbandry and testing environments are less frequently discussed as potential sources of behavioural outcome variability, despite their ability to impact results to a similar extent. In this section, we highlight important environmental variables (FIG. 3), most of which influence pain behaviours by modulating rodent stress systems. Stress can have opposing influences on nociceptive processing, sometimes manifesting itself as increases in pain (stress-induced hyperalgesia) and other times resulting in stress-induced analgesia (SIA) (reviewed in REFS[86,87]). The differential effects of stress are difficult to predict but may be related to the severity or predictability of a stressor or the length of stress exposure[88,89]. Unless the intention is to specifically add stress as an experimental variable, care should be taken to mitigate this factor to the greatest extent possible before and during behavioural testing.

Stress-induced hyperalgesia

Higher sensitivity to pain due to stress-induced activation of descending pain modulatory circuitry.

Stress-induced analgesia

(SIA). Reduced sensitivity to pain due to stress-induced activation of descending pain modulatory circuitry.

Stress is encountered with essentially every environmental change made in the course of a behavioural experiment, beginning with the delivery of animals to the housing facility[90]. Our analysis of articles published in *Pain* between 2016 and 2020 shows that only 10% of mouse studies and 11% of rat studies published in *Pain* used animals bred in-house (Supplementary Figs. 3, 4). Thus, the vast majority of animals are likely to experience stress associated with commercial vendor delivery, the physiological effects of which typically normalize within 48 hours[90]. Various factors within an animal's home cage also influence pain behaviours (FIG. 3a); cage bedding can alter mechanical sensitivity[91,92], the addition of items intended to enrich the animals' environment such as igloos or tunnels can decrease periods of hypersensitivity following injury[93–95] and different dietary compositions can affect behaviour[96]. In addition to these inanimate factors, an animal's cage mates (or lack thereof)

have perhaps the largest impact on nociception. SIA has been repeatedly demonstrated in socially isolated rodents[97–99]. Group-housed animals' pain can also be bidirectly modulated by their conspecifics. Naive rodents will not only groom conspecifics exhibiting injury-evoked pain behaviours[100] but will also develop hypersensitivity themselves in the absence of injury[100–103]. Likewise, injured mice exhibit more pain in the presence of a similarly injured cage mate[104], less pain when observing analgesic-mediated reductions in cage mate pain behaviours[102] and less pain in the presence of an injured stranger rodent[105,106]. This stranger-evoked analgesia is thought to be mediated by canonical stress hormone signalling, whereas the social transfer of pain between cage mates is mediated through olfactory and visual cues[104,107]. Notably, 'social' pain transfer has also been reported between mice housed in separate cages in the same room[107]. While these studies did not incorporate standard filter cage tops[107] (the point of the studies was to increase olfactory cue dispersion), these data and other data[108] suggest that — at a minimum — naive and injured rodents will influence observable injury phenotypes in one another if housed in the same cage. To mitigate stress in the home-cage environment, we therefore suggest housing animals with at least one additional phenotypically similar conspecific (that is, an injured animal with another injured animal or a naive animal with another naive animal), adding one or more inanimate sources of environmental enrichment to the cage and maintaining consistent food, water and bedding suppliers throughout an experiment. One caveat to this approach is the increased risk of cage or cohort effects, or changes in behaviour induced by environmental similarity in groups of mice that are bred, housed and/or transported together. To mitigate this problem, we recommend ensuring that cages are randomized for experimental group assignment.

The reduction of all stress-inducing stimuli is most critical shortly before and during behavioural testing. We acknowledge that stress also influences clinical pain and its reporting in patients, and one could therefore argue that minimization of stress in rodent pain testing is unnecessary. However, the magnitude of stress experienced by rodents in these situations likely far exceeds that experienced by patients — imagine how you might feel if a human predator, such as a grizzly bear, approached you with a von Frey fibre. Ideally, behaviour testing should occur in a dedicated humidity-controlled, light-controlled and temperature-controlled room[109] (FIG. 3b). Performing euthanasia, tissue collection and/or blood draw procedures in the same room as behavioural tests is also strongly discouraged, considering the stress response that these procedures evoke in rodent bystanders[110,111]. Transport from the housing room to the testing room, whether by cart[112] or by hand[113], temporarily elevates circulating levels of the stress hormone corticosterone, as does removal from the home cage and placement into the testing apparatus[114]. Thus, it is critical that rodents be given proper time to acclimate to the testing apparatus before the start of testing. On average, rats require significantly less habituation time than mice; rats habituate to a wire mesh-floored surface within 10–15 minutes[115], whereas mice do not reach a similar behavioural state for approximately 2–4 hours depending on the strain[116]. Our analysis of articles published in *Pain* between 2016 and 2020 showed that habituation times were not reported in more than 50% of articles; however, in those that did report this variable, 30–59 minutes was the most common habituation time frame (Supplementary Fig. 5). While appropriate for rats, these times are likely too short for mice. Not only are animals not

reaching a steady behavioural state[116] but — if they are being tested by a male experimenter — they are also likely exhibiting SIA at the beginning of the testing period. Olfactory cues extruded by male experimenters initiate stress responses in male and female mice, thus reducing pain sensitivity for the first 60 minutes of experimenter exposure[117]. Circadian fluctuations have also been reported in both heat and mechanical sensitivity in naive rodents[109,118,119], so care should be taken to initiate habituation periods and complete behaviour tests (especially those within a sequential experiment) at the same time each day. In our analysis, only 4% of preclinical *Pain* studies reported housing animals on reverse light cycles (Supplementary Fig. 6), and we therefore assume that the vast majority of laboratories perform behaviour testing during the light (resting) phase of the cycle.

Variables inherent to the process of behavioural testing can also affect outcomes. As described in the next section, the vast majority of pain studies use stimulus-evoked reflexive behaviours as their primary outcome. While stimulus intensity and application site are obvious factors that should be controlled for in such tests[119], the identity of the experimenter is the most influential factor in reflexive behavioural outcomes[120]. Interexperimenter variability impacts reflexive behavioural test outcomes to a greater extent than genetic variability or other environmental factors[120] and, therefore, experimenters should not change from animal to animal or from day to day in sequential studies. Experimenter skill level can also affect the extent of stress induced by manipulations that are routinely used during behaviour testing. Handling, manual restraint, subcutaneous injections and oral gavage all induce measurable stress responses in rodents, the magnitude of which positively correlates with the length of time required by these activities (reviewed in REF.[121]). Specific details of testing apparatuses should also be reported in publications as these details can impact behavioural outcomes. For example, apparatus flooring can affect mechanical withdrawal thresholds. Higher, more variable thresholds were observed when rats were repeatedly tested on wire mesh as opposed to a plastic surface[115]. Furthermore, repeated testing in the same environment can also influence behavioural outcomes; male mice exhibit context-dependent hypersensitivity when behavioural measures are recorded in an environment associated with prior injury[122].

The presence of other animals and the order in which they are tested[120] can also influence pain behavioural test results. Similar to the phenomenon observed in home cages, rodents can transmit pain behaviours during the short periods associated with behaviour testing through visual and olfactory cues; male mice exhibit more or less stimulus-evoked pain when watching responses to the same stimulus in a familiar or stranger mouse, respectively[104–106]. Lingering stress-related odours prompt SIA in rats[123], but the effects of other odours (such as pheromones from opposite-sex animals) are unknown. While it is difficult to minimize olfactory cue transfer during behaviour testing because many animals are typically acclimated on the same platform, it is advised to limit visual access between animals to at least prevent this means of pain transfer between familiar animals.

In summary, the current status quo regarding housing and testing environments in preclinical pain studies is unclear because these variables are often omitted or described in very little detail in article methods sections. We recommend the following guiding principal when one is considering environmental changes in a preclinical pain experiment: unless you are

purposefully inducing stress as part of a pain model, great care should be taken to mitigate sources of animal stress, particularly during behavioural testing. Appropriate, and often lengthy, acclimation periods are critical for stress response normalization following any manipulation of the animal or its environment.

Choice of measures

Significant difficulties arise when one is attempting to measure pain (a sensation that is predominantly measured in humans with verbal ratings and/or descriptors) in non-verbal rodents. For this reason, measures of pain-associated symptoms, such as hypersensitivity, are the primary outcome measures in most animal behaviour tests (FIG. 4a). Currently, the most frequently used rodent pain measures are reflexive responses to experimenter-delivered stimuli (TABLE 1). Our analysis of all measurements reported in preclinical *Pain* articles between 2016 and 2020 showed that 53% were mechanical in nature (FIG. 4b). This current reliance on mechanical measures is a notable change from the historical use of heat-evoked responses, and may be attributed to the relatively large dynamic range of mechanically evoked behavioural outcomes or to the parallel rise in popularity of neuropathic injuries (FIG. 2a): in patient populations, these types of injury are more typically associated with mechanical allodynia or cold hypersensitivity than with heat hypersensitivity[124]. Our analysis showed that mechanical sensitivity is most frequently measured by stimulating the glabrous (bottom) surface of the rodent hind paw with von Frey monofilaments (Supplementary Fig. 7). Many variations of filament application exist, but more than 50% of *Pain* studies from between 2016 and 2020 used the manual ‘up/down’ psychophysical approach[125,126] in which only the presence or absence of a withdrawal response is measured (Supplementary Fig. 8). Electronic von Frey tools were used in only 16% of *Pain* studies during this period.

Von Frey monofilaments

A set of calibrated nylon filaments used for measuring mechanical sensitivity.

Our analysis suggests that there is an extremely wide range of observed naive von Frey withdrawal thresholds (Supplementary data 1), which can probably be attributed to the diversity of responses that are exhibited by naive and injured animals, and the lack of positive response standardization between experimenters and laboratories (for example, is toe flaring considered a withdrawal?). To this end, significant effort has recently been put into the development of automated, multidimensional withdrawal evaluation methods. High-speed video recordings have allowed individual components of stimulus-evoked paw withdrawals (such as paw height and velocity) to be analysed on a millisecond timescale[127–129]. Coupled with machine learning, these analyses provide unbiased assessments of paw withdrawal dynamics[129,130] on a level of resolution that allows experimenters to simultaneously assess multiple behaviours such as withdrawal and shaking. This machine learning approach has also been used to assess behavioural responses to dynamic mechanical stimuli such as a brush or cotton swab[128]. The inclusion of both dynamic mechanical stimuli and innocuous and noxious static mechanical stimuli is encouraged in behaviour assessments, considering the divergent neuronal circuitry that

mediates behavioural responses to these stimuli (reviewed in REF.[131]) and the high frequency of dynamic mechanical stimulation that occurs in routine day-to-day activities. Another less frequently used form of mechanical stimulation is deep tissue pressure: the Randall–Selitto test[132] (performed on the hind paw) and the use of strain-gauged forceps on muscle[133] are examples of this stimulus modality. However, one disadvantage of both of these tests is the stress associated with the required animal restraint.

Machine learning

Computer systems that are able to learn without following explicit instructions, by using algorithms that analyse and draw inferences from patterns in data.

While not as prevalent as mechanical sensitivity assessments, heat and cold sensitivity assessments are still routinely used in pain research. Our analysis suggests that the use of the hot plate test[134] has declined over time as the paw withdrawal to radiant heat (Hargreaves test)[135], which allows separate determinations of heat sensitivity on each hind paw, has become the predominant method (Supplementary Fig. 7). Cold sensitivity is most commonly assessed via the acetone evaporation test[136] or the cold plate test[137]. The cold plantar assay[138] is a similar reflexive test that could be easily integrated into automated video analyses and, therefore, should be more widely adopted. Combined use of evoked mechanical and thermal behaviours accurately models the experience of clinical quantitative sensory testing. However, there is significant concern regarding reliance on these evoked response techniques as they do not reflect the most bothersome symptoms experienced by patients with chronic pain — which are, by far, spontaneous and movement-evoked pain[139] — and evoked stimulus measures were recently shown to be unable to differentiate patients with painful and painless neuropathy[140].

Hargreaves test

An assay of thermal pain, also known as the radiant-heat paw withdrawal test.

For the purposes of this Review, all other pain behavioural measures are broadly defined as non-evoked; that is, elicited without an exogenous stimulus being applied by an experimenter at the time of measurement (TABLE 1). This class of measurements is extremely broad and contains reflexive, survival, elective and conditioned behaviours, all of which can be modified by ongoing or ‘spontaneous’ pain[141]. As noted earlier herein, ongoing pain (together with movement-evoked pain) is a significantly greater and far more common clinical problem than mechanical or cold hypersensitivity. Despite this, our analysis showed that only 13% of measurements reported in recent *Pain* articles investigated ongoing pain (FIG. 4b). Therefore, we recommend use of non-evoked behaviour measures in addition to evoked behaviours to better assess the entirety of an animal’s ongoing pain experience.

Algogen administration into body parts such as the hind paw or abdomen is associated with various time-limited spontaneous behavioural responses that have been termed ‘nocifensive’. More popular in the 1980s and 1990s (FIG. 2a), pain assays including acetic acid-induced

writhing, the capsaicin test[85] and the first phase of the formalin test[142] measure reflexive behaviours that are maintained in decerebrate animals[143,144]. More relevant involuntary measures of ongoing pain include visual and audible assessments of animal well-being. Like humans, rodents are capable of exhibiting emotional states through facial expressions[145–147]. Similar to the automated paw withdrawal assessments described above, the mouse[146] and rat[147] grimace scales average individual facial features such as orbital tightening or ear position, to result in a cumulative pain score. Facial recognition software, automated scoring and machine learning platforms have recently been developed to standardize these measures between laboratories[148] and allow the assessment of additional emotions[145]. Pain may[149] (or may not[150]) also be visually relayed through anatomically specific protective behaviours, such as limb guarding and gait changes. Finally, audible and ultrasonic vocalizations have been used, albeit with mixed success, to measure ongoing pain[151,152].

Writhing

A measure of visceral pain, characterized by stereotypical abdominal constrictions.

Formalin test

An assay of chemical/inflammatory pain in which dilute formaldehyde is injected into the hind paw; subsequent recuperative behaviours are directed towards the injected paw.

Grimace scales

Scales to quantify animal pain levels on the basis of facial expressions.

A future goal of preclinical pain research should be the incorporation of more unbiased and long-lasting rodent behavioural assessments. Since the review of this topic by one of the current authors[4], rapid technological progress has accelerated the parallel assessment of in vivo neural activity and naturalistic behaviours (neuroethology) in rodents. The ability to simultaneously measure nuanced facets of rodent behaviour while recording and/or manipulating nervous system activity will provide new insight into the biological basis of pain expression in rodents. Previously, complex survival and elective tasks were selected for study in pain models on the basis of anthropomorphized views of rodent behaviour. As predicted[153], many of these behaviours are supressed following injury, including feeding[153], burrowing[154,155], wheel running[156–159], grooming, nest building, cage lid hanging[160] and reward seeking. However, suppression of these behaviours is not ubiquitously observed across injury modalities[160–162], and sometimes a reduction in these behaviours arises independently of pain[163]. Far exceeding the analysis power of individual behaviour scoring, the development of home-cage monitoring systems has allowed unprecedented observation of naturalistic rodent behaviours before and following injury. Early versions of these systems could record only the activity of socially isolated animals and were trained to detect a limited number of behaviours, thus revealing little more than the previously described manual observations[160,164–166]. Very recent advances in high-speed videography, animal tracking software (such as DeepLabCut[167] and two tools described in recent preprints —

AlphaTracker[168] and SLEAP[169]) and machine learning algorithms (such as MoSeq[170] and two algorithms described in recent preprints — SimBA[171] and DeepEthogram[172]), however, now allow the unbiased classification of subsecond behaviours from multiple rodents within the same cage. The power of this approach has already been capitalized to characterize unique rodent behaviours induced by psychoactive drug administration[173]; similar studies by pain researchers may result in the development of unique injury or analgesic behavioural profiles, the resolution of which far exceeds any manually scored behaviour. We warn, however, that just because it is increasingly possible to observe and quantify new, subtle behaviours, this does not mean they are specific to or selective for pain.

Operant and classical conditioning paradigms are also used in some pain research studies (Supplementary Fig. 9). Conditioned place aversion and analgesic conditioned place preference paradigms are two of the most commonly used tasks of this type. Relying on the principles of Pavlovian conditioning, conditioned place aversion and conditioned place preference paradigm design allows rodents to associate the affective state induced by a given manipulation with a distinct spatial environment; changes in manipulation-associated chamber residency time before and following conditioning trials depend on the appetitive[174,175] or aversive[176] nature of the manipulation. Conditioned place aversion is routinely performed in naive animals, using cell type-specific optogenetic and chemogenetic approaches to determine if activity (or lack thereof) of a certain brain region or cell population contributes to the negative affective state associated with pain[177,178]. Alternatively, conditioned place preference is primarily used in injured animals so that the analgesic or ‘relief of aversive state’ effects of pharmacological, optogenetic or chemogenetic manipulations can be ascertained[174,179]. Real-time versions of these approaches that do not rely on conditioning have also been used to directly assess the perception that arises as a result of a neuronal manipulation[180–183] or the perception associated with evoked stimuli[184–187]. In addition to use of classical conditioning paradigms, the use of operant conditioning assays such as the mechanical conflict avoidance assay[188], the operant plantar thermal assay[189] and the operant facial thermal assay[190] can be helpful for determining pain tolerance to complement the assessment of pain threshold which is routinely measured in evoked assays.

Conditioned place aversion

An indirect measure of pain based on an animal learning an association between an environment with distinct cues and pain; the animal will avoid the environment paired with pain.

Conditioned place preference

An indirect measure of pain based on an animal learning an association between an environment with distinct cues and pain relief via analgesic administration; the animal will spend more time in the environment paired with the analgesic.

Operant conditioning assays

Associative learning processes through which the strength of a behaviour is modified by reinforcement or punishment (for example, pain).

Similarly to heterogeneous reports from patients with chronic pain, it is exceedingly unlikely that any one measure will ever be able to accurately and reproducibly assess pain across the diversity of injuries and assays described in the previous section. The current use of uniform measures across various injury modalities has likely biased model development to select for injuries that elicit maximum hypersensitivity, hence a robust dynamic range for treatment, in every animal (such as von Frey fibre-measured allodynia after spared nerve injury) as opposed to injuries that have variable symptoms from subject to subject, which more accurately reflect the variability in patient injuries and symptoms. The recent popularity of plotting individual subject data points has allowed a more thorough assessment of animal pain model heterogeneity. However, the question of what to do with so-called outliers still remains. Ultimately, injury-specific batteries of non-evoked and evoked assays, in addition to tests of pain co-morbidities such as anxiety and depression, will likely allow the most thorough assessment of pain-manipulated behaviours in rodents.

In addition to measurement selection, decisions regarding the number, frequency and timing of measurement collection should be made before beginning an experiment. According to our analysis, close to 90% of preclinical publications in *Pain* between 2016 and 2020 reported baseline measurements. Accurate reporting of these values is critical for the proper identification and interpretation of subsequent behavioural outcomes. Twenty-seven percent of reports in *Pain* between 2016 and 2020 reported data from only one time point, and 22% of reports included data from the baseline and a second time point (Supplementary Fig. 10). Longitudinal testing and area under the curve analyses are advantageous in that they measure the magnitude of a given effect over time; whereas an analgesic effect of a given manipulation may not appear to be significantly different from the effect of a control manipulation at any given time point, the cumulative effect of the drug may prove notably greater. This inherent power of longitudinal testing is essentially nullified, however, when different cohorts of animals are used for each time point. Consistency in both the experimenter and the subject are key to successful interpretation of time course experiments.

Conclusions and recommendations

As noted in BOX 2, the use of human tissue in pain research has great potential benefits. However, by definition, the experience of pain cannot be measured in a dish, but rather can be measured only in a living animal.

Therefore, we are confident that animal models will continue to play a vital role in preclinical pain research. Nevertheless, the suboptimal status quo revealed by our analysis and described in the previous sections suggests that changes are needed to increase the translational relevance of animal pain models. With this in mind, we outline several recommendations (FIG. 5).

Use more heterogeneous animal models.

Genetic, life-history and injury-specific factors undoubtedly influence pain in human patients. We should therefore consider letting these factors have a larger influence in our rodent models. By using an extremely reductionistic approach (for example, injecting 20 μl of CFA obtained from Sigma into the hind paw of an 8-week-old male C57BL/6 mouse purchased from The Jackson Laboratory), we are all studying the biology of a unique and artificial process that may have little if anything to do with human pain. After characterization of a potential analgesic in one assay, there is value in learning whether the analgesic is similarly efficacious in additional injury models, in both sexes and at various time points in the rodents' life. Establishing the generalizability of a phenomenon (or the lack thereof) is just as important as the initial identification of that phenomenon in one defined context.

Increase use of non-neuropathic assays and related behavioural measures.

Back pain and migraine are the two leading causes of disability worldwide[50], but animal models of these conditions are infrequently used in comparison with neuropathic and cutaneous inflammatory assays. To better match the needs of patients with chronic pain, musculoskeletal pain assays, migraine assays and associated pain measures such as deep tissue pressure tests for musculoskeletal pain or light aversion for migraine should be prioritized by basic pain researchers and not viewed as niche subfields studied by only a handful of laboratories.

Perform behavioural tests in more controlled environments.

Although increased variability in organisms and assays or injuries is encouraged, reduced variability in the act of pain behaviour testing is just as important. The impacts of stress on rodent pain behaviours cannot be overstated. If experimenters do not acknowledge and attempt to remedy the undue stress that they or the testing environment is causing animals, they may be unknowingly examining the effects of a given manipulation on stress biology as opposed to pain biology.

Collect more measurements over the time course of injury and healing.

The experience of pain changes over time. Therefore, any manipulation should be implemented and assessed at multiple time points during the injury trajectory. Furthermore, if one is assessing the effects of a pharmacological manipulation, multiple doses and multiple time points following dosing should be assessed; pharmacokinetic issues (plasma drug levels, target coverage, off-target effects) are vastly underappreciated by academic preclinical pain scientists, despite being a leading reason for translational failure.

Increase the diversity and resolution of pain behaviour measurements.

Touch hypersensitivity is rarely the primary concern of a patient with pain. Our over-reliance on dichotomous responses to von Frey stimulation is almost certainly due to the ease of assessment and the robust dynamic range it generates, but at what cost? Behavioural batteries involving unbiased and multidimensional pain measures should be tailored to the

cause of pain. Ongoing pain measures may be more complicated and nuanced, but their increased use could lead to higher translation of pain targets.

Conclusions.

We recognize that these recommendations will require more time, money, effort and creativity on the part of experimenters. However, we and others[191] believe that failed translational efforts are more costly in the long term. A specific recommendation that we believe will result in increased translational success is to increase collaborations between research teams with access to animal models and those with access to human tissues so as to bidirectionally validate new pain targets. Lastly, we encourage journals and funding agencies to incentivize and reward meticulously performed behavioural research in the same way that they reward and promote studies of novel molecular mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The data supporting the findings of the analysis described in this Review are available in the files in Supplementary information.

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Box 1 |

Which rodent (mouse or rat) is better for pain research?

Our analysis of articles published in the journal *Pain* between 1980 and 2020 shows that in the last decade the popularity of rats in pain research has markedly declined (FIG. 1a); rats were used in 67% of studies published in *Pain* in 2010, but in only 43% of studies published in 2020. Increased use of mice is completely responsible for this shift; mice were used in only 32% of studies published in *Pain* in 2010 but in almost twice as many studies (57% of publications) published in 2020. With the ultimate goal of clinical success in mind, this change begs the question of which rodent is better suited for modelling human pain. In the past decade, technical advances and increased affordability of gene expression technologies have allowed comprehensive comparisons of rodent and human tissues (reviewed in REF.[14]). Using these tools, multiple laboratories have demonstrated that classifiers traditionally used to delineate populations of sensory neurons in the mouse are not applicable in humans[192–194]. Expression differences in key therapeutic targets have also been observed between mice and humans[194]. These gene-level and population-level expression differences between species may be responsible for some of the unexpected side effects and limited analgesic efficacy of select targets in human clinical trials. Notably, rat tissue is absent from many of these genetic examinations but is routinely used in cross-species functional assessments. Direct comparisons of basic action potential properties[193,195,196] and individual ion channel and G protein activities[197–201] have been made between rodent and human dorsal root ganglion neurons and spinal cord[202], but very rarely between rodent species. Exceptions to this include comparisons of peptidergic neuronal marker expression in dorsal root and trigeminal ganglia of both rodents[203], comparisons of sciatic nerve anatomy in both rodents[204], comparisons of cold responses in epidermal keratinocytes from both rodents and humans[205], meta-analyses of chronic pain-related gene expression changes in both rodents[206] and a small number of injury model and analgesic cross-species comparisons[75,207–209]. The magnitude of genetic and functional differences between human, rat and mouse tissues is currently unclear due to a lack of tri-species comparative analyses; before a definitive recommendation of which rodent tissue better models human tissue in vitro is provided, detailed gene expression analyses of rat tissue and tri-species electrophysiological experiments should be conducted.

Box 2 |

Pain modelling in human tissues

The use of primary or derived human tissues is steadily increasing in preclinical pain research. as described in BOX 1, primary peripheral tissues are now being more readily acquired from both living and deceased tissue donors, a trend that will hopefully continue in the future[210]. Despite problems inherent to studying tissue in vitro[211], primary human tissues still allow analgesic target expression validation in tissues of interest, before proceeding to clinical trials. Because acquisition of freshly harvested human neural tissue for functional analysis remains geographically, financially and temporally challenging, laboratories have also developed protocols for differentiating human induced pluripotent stem cells into primary sensory neurons[212–217] and central nervous system interneurons[218,219] as an alternative. One benefit of this system is the increased capacity to compare functional responses between tissue derived from patients with pain and tissue derived from patients without pain, a strategy that is exceedingly difficult (albeit not impossible) to achieve with primary human tissues[220]. Similarly, tissues from painful and non-painful sites within the same patient can be generated. Needing only a skin biopsy sample from patients, this approach was recently leveraged to investigate the effects of a rare genetic mutation in the *PIEZO2* gene on sensory neuron mechanical responsiveness[221]. The potential benefits of human tissue use in pain research are vast. In addition to the in vitro analysis of neural tissues, which will provide new insight into the mechanisms of human nociception, the use of plasma or other readily collectable materials may also aid in biomarker identification.

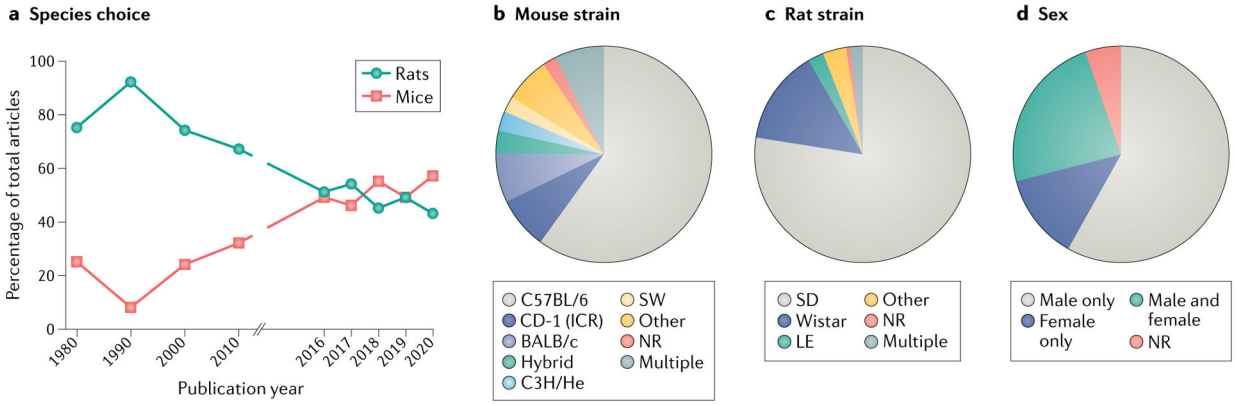

Fig. 1 |. Analysis of rodent use in pain studies from 1980 to 2020.

The methods and results sections of preclinical reports published in the journal *Pain* in the years 1980, 1990, 2000, 2010 and 2016–2020 (506 articles) were coded for a variety of terms, including ‘animal species’, ‘sex’ and ‘rodent strain’. *Pain* was selected as the representative journal for this analysis; however, it is recognized that the restriction of the analysis to a single journal may have implications for the generalizability of the findings to the wider pain literature. Inclusion was further restricted to studies that used inducible pain models and measured behaviours in awake non-human animals. Detailed coding methods and complete datasets are included in Supplementary methods and Supplementary data 1. **a** | Percentage of all studies published in *Pain* in the years listed that used rats or mice as the primary model organism. Between 2016 and 2020, only 4 of 320 studies analysed used non-rodent animals; dogs were used in 3 studies and pigs were used in 1 study. **b** | Mouse strain use in studies published in *Pain* between 2016 and 2020. The chart illustrates the percentage of mouse studies using each strain. **c** | Rat strain use in studies published in *Pain* between 2016 and 2020. The chart illustrates the percentage of rat studies using each strain. **d** | Use of male and female organisms in studies published in *Pain* studies between 2016 and 2020. The chart illustrates the percentage of all studies using male, female, or male and female organisms. LE, Long–Evans; NR, not reported; SD, Sprague Dawley; SW, Swiss Webster.

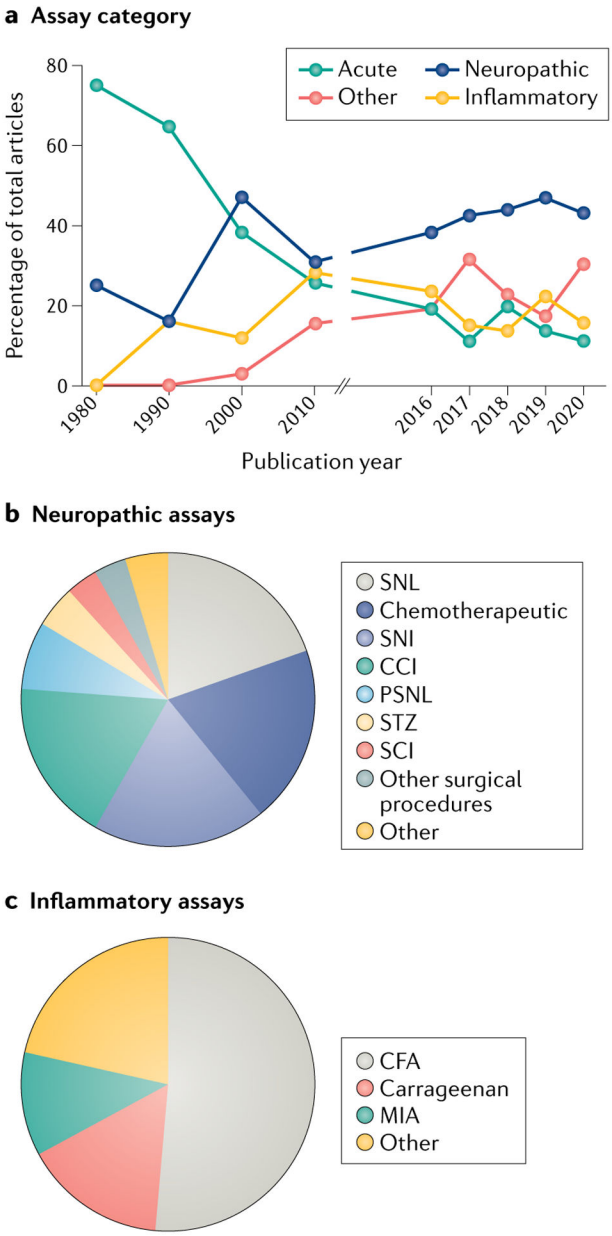

Fig. 2 |. Analysis of animal pain assays used from 1980 to 2020.

The methods and results sections of preclinical reports published in the journal *Pain* in the years 1980, 1990, 2000, 2010, and 2016–2020 (506 articles) were coded for a variety of terms, including 'assay category' and 'injury model.' *Pain* was selected as the representative journal for this analysis; however, it is recognized that the restriction of the analysis to a single journal may have implications for the generalizability of the findings to the wider pain literature. Assays were defined as belonging to one of 12 categories; detailed categorical definitions and complete datasets are included in Supplementary methods and Supplementary data 1. **a** | Percentage of all studies published in *Pain* in the years listed that included acute, neuropathic, inflammatory or one of the nine other remaining categories of assays. **b** | Comparison of different types of neuropathic assays used in studies published in

Pain between 2016 and 2020. The chart illustrates the percentage of total studies using each assay. **c** | Comparison of different types of inflammatory assays used in studies published in *Pain* between 2016 and 2020. The chart illustrates the percentage of total studies using each assay. CCI, chronic constriction injury; CFA, complete Freund's adjuvant; MIA, monosodium iodoacetate; PSNL, partial sciatic nerve ligation; SCI, spinal cord injury; SNI, spared nerve injury; SNL, spinal nerve ligation; STZ, streptozotocin.

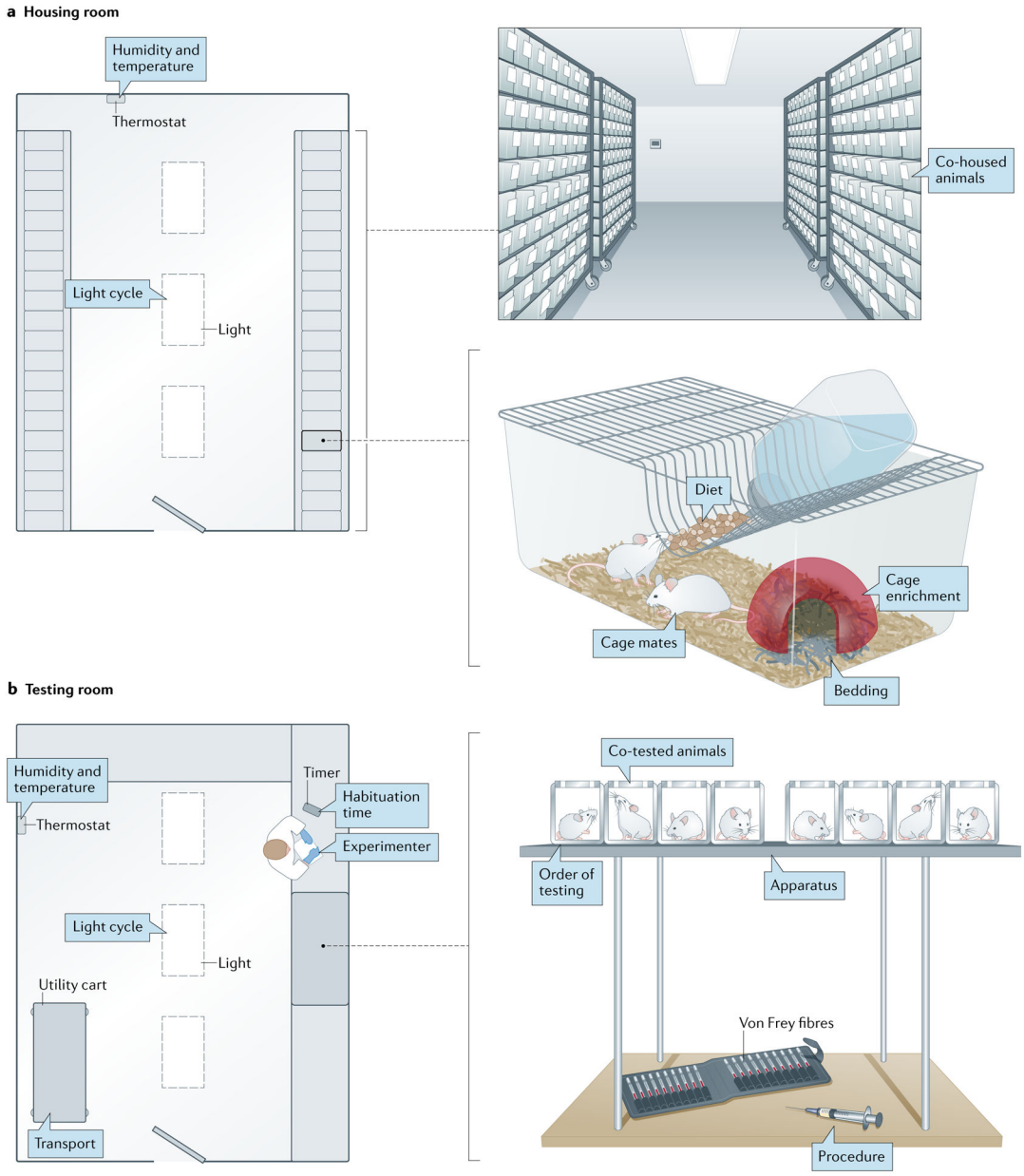

Fig. 3 |. Environmental considerations for pain testing.

a | Factors in a standard vivarium rodent housing room that may affect pain behaviours. Animal behaviour can be influenced by room characteristics such as humidity, temperature and light cycle or factors such as the pain status of other animals housed nearby. Similarly, factors within the home cage can affect pain behaviours even when animals are tested in a different environment. **b** | Factors in a standard behaviour testing room that may affect pain measurements. As in the housing room, humidity, temperature, lighting and the presence of injured animals may affect test outcomes in a behaviour room. Stress induced by transport to the testing room, novelty of the testing apparatus, order of testing and accompanying procedures such as manual restraint or injection may also affect pain testing

results. However, the presence, identity and experience level of an experimenter may be the largest factor that influences pain behaviours.

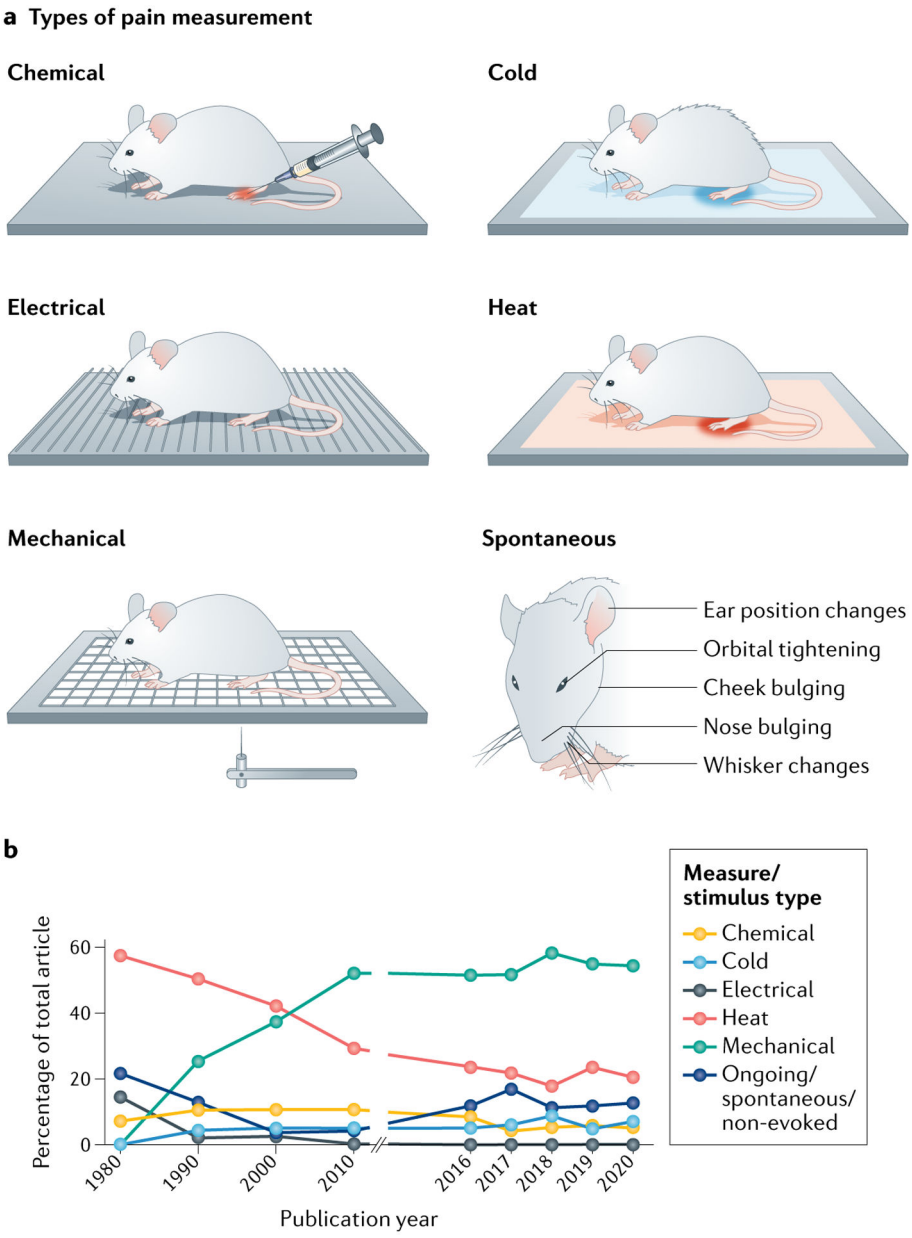

Fig. 4 |. Behavioural measures of pain and an analysis of their use in studies from 1980 to 2020.

a | Depictions of frequently used pain measurements. Chemical measures typically consist of an animal tending to the part of its body into which an inflammatory agent was recently injected. In common cold measures, the amount of time required for an animal to withdrawal its paw from a cold stimulus is assessed. Electrical measures determine the timing and intensity of behavioural responses to electrical stimulation. Heat measures commonly assess the amount of time required for an animal to withdrawal its paw from a hot stimulus. Mechanical measures characterize the manner in which an animal responds to mechanical probing of a body part, or assess the amount of force required to elicit a behavioural response. Spontaneous measurements assess the quality and intensity of non-evoked behaviours exhibited at various bodily locations. The Mouse Grimace Scale is one

such standardized tool that allows for measurement of facial expressions. **b** | The methods and results sections of preclinical reports published in the journal *Pain* in the years 1980, 1990, 2000, 2010 and 2016–2020 (506 articles) were coded for a variety of terms, including 'stimulus modality'. Detailed modality definitions and complete datasets are included in Supplementary methods and Supplementary data 2. The graph shows the percentage of all studies published in *Pain* in the years listed that measured reflexive responses to chemical, cold, electrical, heat or mechanical stimuli or used spontaneous behavioural measures.

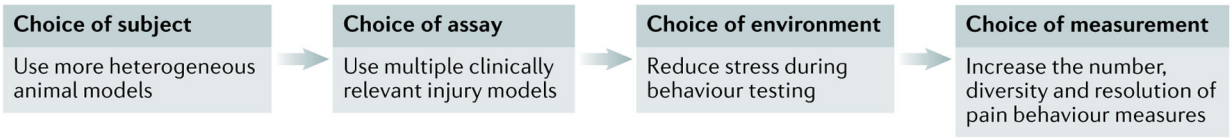

Fig. 5 |. Recommendations for increased translational relevance of animal pain models.
Before a preclinical pain experiment is begun, significant consideration should be given to the model organism, pain assay, holding and testing environments, and number and type of behavioural measures. Increased variability in model organism species, genetic background, age, sex and life history may increase the translatability of preclinical findings. To determine the generalizability of a new pain target or mechanism, experimenters should use multiple clinically relevant assays within a given study instead of a single, artificial injury. Specific care should be taken to minimize environmental stress before and during pain behaviour testing; appropriate habituation times are absolutely necessary to mitigate the effects of stress-induced analgesia or hyperalgesia on behavioural responses. Lastly, the number, type, and resolution of measures should be increased to allow more nuanced interpretations of pain behaviours during both the acute phase and the chronic phase of the injury.

Table 1 |

Classification of commonly used behavioural measures of pain

Category	Evoked or non-evoked?	Example behaviours	Pros	Cons
Elective	Non-evoked	Cage hanging, nest building and social interaction	Similar to human pain manifestations Can be tested in home cage	May not be specific to pain
Nocifensive	Non-evoked	Grimace, writhing, gait changes and ultrasonic vocalizations	Automated scoring possible	Infrequently observed in chronic models Stress associated with testing apparatus
Survival	Non evoked	Feeding and grooming	Easy to perform Can be tested in home cage	May not be specific to pain
Conditioned	Non-evoked and evoked	CPP, CPA and operant measures	Measures affective/motivational aspect of pain	Requires specialized equipment Time intensive Stress associated with testing apparatus
Reflexive	Evoked	von Frey, radiant heat withdrawal and the acetone test	Easy to perform Can control stimulus intensity	Not reflective of affective state Experimenter presence, skill and bias affect results May reflect motor deficits, not analgesia Stress associated with testing apparatus

CPA, conditioned place aversion; CPP, conditioned place preference.