The search for translational pain outcomes to refine analgesic development: where did we come from and where are we going?

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ABSTRACT

Pain measures traditionally used in rodents record mere reflexes evoked by sensory stimuli; the results thus may not fully reflect the human pain phenotype. Alterations in physical and emotional functioning, pain-depressed behaviors and facial pain expressions were recently proposed as additional pain outcomes to provide a more accurate measure of clinical pain in rodents, and hence to potentially enhance analgesic drug development. We aimed to review how preclinical pain assessment has evolved since the development of the tail flick test in 1941, with a particular focus on a critical analysis of some nonstandard pain outcomes, and a consideration of how sex differences may affect the performance of these pain surrogates. We tracked original research articles in Medline for the following periods: 1973-1977, 1983-1987, 1993-1997, 2003-2007, and 2014-2018. We identified 606 research articles about alternative surrogate pain measures, 473 of which were published between 2014 and 2018. This indicates that preclinical pain assessment is moving toward the use of these measures, which may soon become standard procedures in preclinical pain laboratories.

Keywords: analgesia; pain; behavior; animal model; grip strength; postural changes; wheel running; burrowing; nesting; home cage activity; intracranial self-stimulation; exploratory activity; facial expressions.

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1. INTRODUCTION

Pain is undoubtedly an important global health problem, with millions of people suffering from chronic pain (Goldberg and McGee, 2011; Pina et al., 2017), and an estimated 18% of individuals in developed countries with chronic pain conditions (Sá et al., 2019). Current analgesics (including opioids, nonsteroidal anti-inflammatory drugs [NSAIDs] and gabapentinoids) show limited efficacy in many pain conditions, or a number of side effects which limit their use (Yaksh et al., 2015; Phillips et al., 2017). Therefore, the urgent need for novel analgesics is clear. However, despite major advances in our understanding of the somatosensory system in recent decades, derived substantially from research in rodent models of experimental and pathological pain, the translation of new analgesics from bench to bedside has been limited. The relatively slow progress in this area is exemplified by the discontinuation of clinical trials of several analgesic drugs, including NK1 antagonists, kappa opioid agonists, cannabinoid agonists and TRPV1 antagonists, among others (Villanueva, 2000; Rivière, 2004; Huggins et al., 2012; Yekkirala et al., 2017; Fallon et al., 2017). Consequently, most "new" pain medications consist of refined delivery methods for known analgesic drugs, or combination therapies comprising well-known analgesics (Kissin, 2010; Mao et al., 2011; Gilron et al., 2013; Dale and Stacey, 2016; Wolkerstorfer et al., 2016).

For the purposes of translation, animal testing should mimic routine clinical practice and clinical trials as closely as possible. Nociception is the neural process encoding noxious stimuli, and this term does not necessarily imply pain sensation. Pain is much more complex than nociception, as it is by definition a subjective experience (Yezierski and Hansson, 2018). This obvious distinction between pain and nociception in humans is largely underestimated in preclinical pain research, because the classical pain-like responses in experimental animals reflect almost exclusively reflex responses

based in turn on responses evoked by sensory stimuli (as we will summarize below), and therefore signal nociception rather than pain.

It can be argued that effective bench to bedside translation requires not only research models that successfully mimic the pathological condition in humans, but also measurements of translational pain outcomes. Achieving both aims, however, is challenging, since the absence of verbal communication in animals is an obstacle to the evaluation of pain in preclinical studies. In recent years the use of novel pain outcomes not limited to reflex responses has gained the attention of the preclinical research community (e.g. Negus et al., 2006; Cobos and Portillo-Salido, 2013; Negus, 2013; Percie and Rice, 2014; Yezierski and Hansson, 2018). However, it is unclear whether these new measures are becoming widely established or not, given that to date there have been no quantitative analyses of their use in preclinical pain research.

Our primary goal in this article is to provide the reader with a broad sense of how preclinical pain research has evolved through successive attempts to approximate the human pain phenotype in experimental animal models of pain, with a particular focus on the evolution of pain measures in rodents. As a proxy for the use of pain outcomes in preclinical pain research, we tracked the time-course of the number of research articles reporting work based on the initial and newer pain outcomes. Our analysis included the most classical measures of acute heat nociception (hot plate and tail flick tests). Among the models of pain induced by chemical irritants, we focused on the writhing and formalin tests, which – as discussed below – played a pivotal role in the transition of preclinical research from models of nociceptive to tonic pain. We also considered measures of sensory hypersensitivity (mechanical and heat hyperalgesia, and mechanical and cold allodynia), given their current use as standard measures of pain in preclinical research. Finally, we also tracked research reports that used some of the most

recent surrogate pain measures in rodents to investigate different aspects of the pain phenotype, such as interference in physical functioning (postural changes and grip strength deficits), pain-depressed behaviors (exploratory locomotion, home cage activity, borrowing and nesting behaviors, intracranial self-stimulation, and wheel running), and facial expressions in pain conditions. Readers should note here that in addition to these behavioral measures, there are others which are not reviewed here, including (but not limited to) conditioned place aversion and preference induced by pain and analgesia, and specific tests to evaluate depression-like behaviors in rodents; these measures can also yield useful data regarding the rodent pain phenotype (Cobos and Portillo-Salido, 2013; Navratilova et al., 2013; Yalcin et al., 2014; de la Puente et al., 2017). With the measures covered in the present review we aim to provide a sense of how preclinical pain assessment has evolved from earlier decades to the present time.

Our secondary goal is to perform a critical analysis of some of these methodologies to facilitate the selection of behavioral tests that can be used by preclinical pain researchers to expand the repertoire of behavioral phenotyping tools in current and future research. Finally, since there is cumulative evidence of sex differences in pain responses and mechanisms (e.g. Mogil et al., 2000; Rosen et al., 2017; Sorge and Totsch, 2017), we also discuss whether the sex of the experimental animals might influence the results obtained with some of the newer pain outcomes.

2. LITERATURE SURVEY

As a proxy for the use of pain outcomes in preclinical pain research, we used Medline to track original pain research articles published in English from 1973 to 2018. The numbers of articles published in each 5-year period were recorded to obtain information on publication trends in the years 1973-1977, 1983-1987, 1993-1997, 2003-

2007, and 2014-2018. The terms used for the Medline searches are shown in Table 1. Searches were performed from December 2019 to January 2020. Because some of the most recent surrogate pain measures in rodents, such as the interference of pain in physical functioning, pain-depressed behaviors, and facial expressions in pain conditions, were not developed specifically to measure pain (as explained below), these articles needed to be manually screened to ensure that the selected outcomes were used as surrogate pain measures. An initial reading of the abstract was used to determine whether the outcome of interest was used as a pain measure; if the outcome was not explicitly mentioned, the full text was read to decide whether the study should be included or excluded from the analysis. All articles were screened by independent pairs of investigators who were supervised by EJ Cobos. Of the 2565 research articles identified in these searches, 606 were included for analysis after manual curation.

We also searched for original research articles that used these novel pain outcomes in rodents of both sexes, by adding to the terms "sex" or "male and female" to the Medline searches specified in Table 1. We used no restrictions for the publication date, and considered all articles retrieved, including those published in 2019. As described above, research reports that used these methodologies were manually screened to ensure that the selected outcomes were used as surrogate pain measures. Of the 973 research articles identified in these searches, manual curation identified only 29 articles that reported physical functioning, pain-depressed behaviors or facial expressions in pain conditions as pain surrogates, and which explicitly mentioned that animals of both sexes were compared in any of these measures under a given pain condition.

3. THE EARLY TIMES OF PRECLINICAL PAIN RESEARCH: FROM ACUTE NOCICEPTION TO TONIC PAIN

In 1940 Hardy and coworkers published their results with a new device to reliably measure heat pain thresholds in humans, i.e. with a light beam controlled by a rheostat (Hardy et al., 1940). This was one of the first quantitative methods for measuring pain, and served as an inspiration for preclinical pain researchers who soon developed, only one year later, a similar device to study pain and analgesia in rodents (D'Amour and Smith, 1941). In this test, the animal is restrained and a light beam is directed against its tail until an abrupt movement of the tail occurs. The "tail flick" reaction time can be monitored as an index of pain or analgesia. A representative picture of a mouse in a tail-flick apparatus is shown in Figure 1A.

Soon after the first description of the tail flick test, the hot plate was developed (Woolfee and MacDonald, 1944). The hot plate test has the advantage over the tail flick test in that the animal is not restrained, but is able to move freely (so the experimental conditions are much less stressful) on a plate heated to a constant temperature and surrounded by an uncovered evaluation cage (so as not to prevent the animal from escaping). However, and in contrast to the tail flick test, heat stimulation with a hot plate is not restricted to a specific body area, as all four limbs, the tail, and even the genitals can be stimulated simultaneously. This stimulation of multiple body areas may, however, trigger diffuse inhibitory controls that are likely to affect the responses observed (Le Bars et al., 2001). The behavioral outcomes used most often in the hot plate test are paw licking or flinching, and the jumping response if exposure to the nociceptive stimulus is prolonged. A representative picture of a mouse performing a licking response on a hot plate is shown in Figure 1B. A recently developed alternative to the classical hot plate test is the so-called "unilateral hot plate test", in which the

animal is gently held by the skin of the interscapular region, and only one paw is placed on the hot surface (e.g. Menéndez et al., 2002; Montilla-García et al., 2018 and 2019), thus avoiding the likelihood that diffuse inhibitory pain controls will be triggered.

The use of these tests for acute heat nociception increased steadily until 2003-2007, but in 2014-2018 the number of articles reporting results obtained with these methods decreased (Fig. 2, Supplementary Table 1). Currently, the hot plate test appears to be preferred over the tail flick test, as the numbers of articles reporting results with the former has remained more or less constant whereas the number of research articles that used the tail flick test has decreased steadily since 1993-1997 (Fig. 2, Supplementary Table 1).

Some years later, the first models of tonic pain were developed. The first of these tests may have been the writhing test (also termed the abdominal constriction test), which was described in 1957 (Siegmund et al., 1957). The intraperitoneal injection of chemical irritants induces stereotyped behavior in rodents, characterized by abdominal stretching together with full extensions of the hindlimbs. A representative picture of a mouse performing the writhing response is shown in Figure 1C. Although it was originally based on 2-phenyl-1,4-benzoquinone as the irritant (Siegmund et al., 1957), several other irritants have subsequently been tried for the same purpose, including acetylcholine, dilute hydrochloric, acetic or lactic acid, bradykinin, adenosine triphosphate, potassium chloride, tryptamine, and oxytocin. All of these compounds induce this stereotyped behavior when administered intraperitoneally (reviewed by Le Bars et al., 2001).

Twenty years after the first description of the writhing test, formalin-induced pain was reported (Dubuisson and Dennis 1977). The intraplantar injection of diluted formalin (a mixture of formaldehyde, water and methanol) produces a biphasic

behavioral reaction (licking/biting or flinching of the paw), with an initial phase lasting 5 min or less and a quiescent period, followed by a second tonic pain phase lasting typically from 10 to 40 min (reviewed in Sawynok and Liu, 2003). A representative picture of a mouse licking/biting the formalin-injected hindpaw is shown in Figure 1D. Research with these models has increased steadily, accounting for 1709 research articles in 2014-2018 (Fig. 3 and Supplementary Table 1). Although the writhing test was developed 20 years earlier than the formalin test, the latter has been far more prevalent than the former in the past three decades, as shown in Figure 3 and Supplementary Table 1.

The sensitivity to analgesics of acute heat pain and tonic pain models was found to be strikingly different. Mild analgesics such as NSAIDs, which induced few if any analgesic effects on nociceptive heat pain, were found to induce robust analgesic-like effects in tonic pain models (e.g. Siegmund et al., 1957; Collier et al., 1968; Drower et al., 1987), and it was shown that tonic pain induced by formalin was more resistant to morphine analgesic tolerance than nociceptive heat stimulation in the tail flick test (Abbott et al., 1982). Furthermore, microinjections of local anesthetics in specific brain areas differentially affected the nociceptive responses induced by formalin and tail flick responses (reviewed by Melzack, 1996). These findings contributed clear evidence that nociceptive heat pain differed from the more sustained tonic pain. Because the clinically relevant type of pain is sustained, this probably influenced the increase in the use of these tonic pain models.

4. THE DEVELOPMENT OF PATHOLOGICAL PAIN MODELS AND THE ASSESSMENT OF SENSORY HYPERSENSITIVITY

Animal models of pathological pain conditions were also developed to further

preclinical pain research in clinically relevant human pain. Inflammatory pain was the first type of pathological pain to be modeled in rodent studies, by the administration of different inflammatory compounds such as brewer's yeast, which was used as early as 1957 (Randall and Selitto, 1957). Subsequently, several other agents were used to induce painful inflammation, and this was followed by the development of different animal models designed to cover a wide repertoire of clinically relevant pain conditions, including neuropathic, osteoarthritis, cancer or postoperative pain (Wang and Wang, 2003; Sisignano et al., 2014; Challa, 2015; Muley et al., 2016; Pogatzki-Zahn et al., 2018; O'Brien et al., 2017).

The search for suitable models of painful pathological conditions was accompanied by the search for appropriate pain outcomes. Because sensory hypersensitivity (i.e. allodynia and hyperalgesia) is an important feature of clinical pain, several behavioral tests attempted to quantify the enhanced sensitivity to sensory stimuli in models of pathological pain. The first to measure sensory hypersensitivity were Randall and Selitto, in 1957. They used a model of inflammatory pain (induced by brewer's yeast) to demonstrate mechanical hyperalgesia by applying a blunt mechanical stimulus, and quantifying the reduced paw pressure threshold (Randall and Selitto, 1957). This was termed the Randall–Selitto test, also known as the paw pressure test. Much later, in 1988, Hargreaves et al. complemented the Randall–Selitto test by measuring heat hyperalgesia during inflammation using a light beam directed to the inflamed paw in freely moving rats placed on a glass floor. Heat hyperalgesia is manifested as decreased latency to paw withdrawal during inflammation (Hargreaves et al., 1988). This test, named after the first author of the article that initially reported it, is also termed simply the plantar test. Other more complex procedures to measure heat hypersensitivity have recently appeared, such as the temperature preference test

(reviewed by Deuis et al., 2017). Measures of heat hyperalgesia have been used extensively in pain research, and since the 1990s they have grown steadily in popularity among preclinical pain researchers, as shown by the 983 research articles in 2014-2018 – a much higher number than the 155 research articles we identified in searches for the Randall–Selitto test (Fig. 4 and Supplementary Table 1).

In the early 1990s, inspired by quantitative sensory testing by neurologists to assess neuropathic pain in human patients, some researchers began to use von Frey filaments in rodents with neuropathies (Shir and Seltzer, 1990; Kupers and Gybels, 1993; Chaplan et al., 1994). This test is based on the sequential application of calibrated flexible filaments until the animal shows a pain-like response (fast withdrawal, licking or flinching of the stimulated paw), to determine the threshold of painful mechanical stimulation and hence the presence of mechanical allodynia. The use of von Frey filaments gained popularity not only for evaluating the mechanical threshold of neuropathic pain in rodents, but also for virtually all types of pathological pain models (reviewed in Mogil, 2009; Cobos and Portillo-Salido, 2013). Athough the von Frey filaments are by far the tool used most frequently to determine tactile allodynia in rodents, some devices are able to apply a punctate mechanical stimulus to the target area with a rigid filament driven by an electronically-controlled force (e.g. Entrena et al., 2009; Clark et al., 2012; Nieto et al., 2012 and 2014). Although both types of sensory stimulation consist of the application of a punctate mechanical stimulus, these measures can hardly be considered equivalent, since the results of comparisons (including work in our laboratory) show that the threshold force in mice when von Frey filaments are used is typically about 1 g (e.g. Montilla-García et al., 2019; Cobos et al., 2018), whereas an electronic device with a rigid filament yields a threshold of about 5–6 g (e.g. Nieto et al., 2012 and 2014).

Other forms of tactile allodynia are tested by applying a dynamic mechanical stimulus with a cotton bud (e.g. Field et al., 1999) or with soft brush (e.g. González et al., 2000; Cheng et al., 2017); this approach is mechanistically different from the more widely used punctate stimuli (Cheng et al., 2017). Dynamic mechanical stimuli are also used in human diagnosis and research in neuropathic pain (e.g. Reimer et al., 2014), and therefore they hold the potential to be useful tools for preclinical research, although this type of stimulation is rarely used in rodent studies. Measures of tactile allodynia rapidly became popular among preclinical pain researchers: the number of articles we found increased markedly from 117 in 1993-1997 to 787 in 2003-2007 (by which time these measures were already used more frequently than the paw pressure test or the Hargreaves test), with a further huge increase to 2071 research articles reporting work based on measures of tactile allodynia in 2014-2018 (Fig. 4 and Supplementary Table 1).

Finally, given that cold allodynia is also a feature in patients with neuropathies (Jensen and Finnerup, 2014), cold stimuli have also been used to study changes in cold sensitivity during pathological pain in rodents. The test used most widely in this modality is the acetone drop test, based on the sensation of cold produced by the evaporation of acetone, which may elicit pain-like responses recorded as brisk foot withdrawal or the time spent licking or shaking the stimulated paw under pathological conditions (reviewed in Sandkühler, 2009; Deuis et al., 2017). Since this test was first developed in the mid-1990s (Choi et al., 1994), more sophisticated methods to measure cold hypersensitivity have appeared, including the application of dry ice through a glass platform, the cold plate test, and the temperature preference test (Deuis et al., 2017). Although responses to cold stimuli in rodents have been used for more than 20 years, cold measures have not become as popular as the Hargreaves test or the von Frey

threshold in preclinical pain research. However, the number of research articles showed a clear increase in the most recent period we examined, with 301 articles that measured cold allodynia in 2014-2018 (Fig. 4 and Supplementary Table 1).

Taken together, the use of these measures of hypersensitivity has increased exponentially since the 1990s, particularly measures of tactile allodynia, which currently clearly outnumber all other measures of hypersensitivity in pain behavior research. Heat hyperalgesia was the second most frequently reported measure of sensory hypersensitivity in 2014-2018, followed by (much less widely used) cold allodynia measures, and finally, by the paw pressure test (Fig. 4 and Supplementary Table 1). The infrequent use of the paw pressure test in pain research is consistent with a previous analysis covering each year from 1970 to 1999 (Le Bars et al., 2001). This is interesting, since the paw pressure test is the oldest measure of hypersensitivity, but did not gain the popularity of the other tests. One possible reason might be that, in our experience, it is harder to train experimenters to perform the paw pressure test than any of the other measures of hypersensitivity.

5. "NEW" BEHAVIORAL OUTCOMES IN PAIN RESEARCH: IN SEARCH OF MEASURES CLOSER TO THE HUMAN PAIN PHENOTYPE

Although von Frey testing, currently the behavioral test used most widely in preclinical pain research, is viewed with something approaching veneration by neurologists and is undoubtedly useful to detect sensory alterations in patients with neuropathies (e.g. Bennett, 2001; Bouhassira et al., 2005; Moharić et al., 2012; Reimer et al., 2014), the use of these filaments in other human pain conditions such as rheumatic diseases or cancer pain is virtually absent. For example, we found only three published studies

which used von Frey filaments in patients with rheumatoid arthritis (Morris et al., 1997; Hendiani et al., 2003; van Laarhoven et al., 2013), one of the most worrisome painful conditions that occurs with joint inflammation (Scott, 2000; Lee, 2013). Moreover, we found only three research articles on the use of von Frey filaments in patients with cancer, excluding articles which studied neuropathic pain induced by antineoplastics (Boyette-Davis et al., 2012; Kosturakis et al 2014; Roldan et al., 2018). Therefore, although von Frey testing has been clearly established as the standard for preclinical pain testing for practical reasons, it should be remembered that it is not a widely used pain measure in patients with chronic painful diseases – a clear discrepancy between animal and clinical studies.

In this connection, it is worth noting that all pain responses typically evaluated in rodents with the standard methods described in the preceding section, including nociceptive withdrawal, licking or abdominal stretching are reflex measures which persist even in decerebrated animals (reviewed in Mogil, 2009), and therefore they clearly measure nociception rather than pain. Although sensory hypersensitivity is a feature of clinical pain, the human pain phenotype is far more complex than a simple reflex response. In fact, consensus-based recommendations for the main outcomes that should be measured in clinical trials of treatments for pain include not only pain itself, but also alterations in physical and emotional functioning (Dworkin et al., 2008) – characteristics which are far removed from the measures used in preclinical research methods summarized in the preceding sections. Although physical and emotional functioning in human patients are often assessed with questionnaires (e.g. Dworkin et al., 2008) – which are (for obvious reasons) not applicable to rodents – several different outcomes are now being used in preclinical research to investigate the rodent pain phenotype beyond standard measures of hypersensitivity, in an effort to approximate

preclinical pain research to the human pain phenotype. Some of these measures will be described below.

5.1 Pain-induced alterations in physical functioning: postural changes and grip strength deficits

Several of the nonstandard pain outcomes aim to provide a wider view of the rodent pain phenotype by measuring pain-induced alterations in physical functioning. The impact of pain on physical functioning can be assessed in rodents by measuring postural changes at rest or during movement (by recording weight-bearing asymmetry or gait alterations, respectively) and grip strength deficits.

5.1.1 Pain-induced postural changes: weight-bearing differences and gait alterations

The weight-bearing distribution in humans and rodents is approximately equal in each lower extremity in normal conditions. However, after unilateral hindlimb injury, body weight support is shifted to the noninjured side. Weight-bearing asymmetry has long been targeted in preclinical pain research since the 1980s, by using a simple scoring system based on the position of the animal at rest (e.g. Coderre and Wall, 1987). Schött and coworkers made the quantitative analysis of this parameter possible thanks to the development, in 1994, of a device consisting of two adjacent force plates able to measure weight bearing by the hindlimbs in rats (Schött et al., 1994), known as the incapacitance test. Recently, a new version of this test was developed in which rodents can move freely in a chamber with a floor covered by pressure sensors, thus making possible to simultaneously measure weight bearing by all four limbs (e.g. Tétreault, et al., 2011). Although the animal can move freely in the evaluation chamber, the system

only measures the weight supported by the limbs when the rodent is motionless long enough to make the recording feasible, and therefore it is not able to measure limb use during walking.

Alterations in weight bearing in different pain states, including but not restricted to inflammation, osteoarthritic pain or plantar incision-induced pain, have been repeatedly shown to be sensitive to analgesic treatment (partially reviewed by Cobos and Portillo-Salido, 2013; Chen et al., 2014; Kim et al 2015b; Peters et al., 2015; Philpott et al., 2017; Luk et al., 2018). In addition, weight-bearing differences are attenuated by analgesic treatment in several models of peripheral nerve injury. Most reports used chronic constriction injury to the sciatic nerve (e.g. King et al., 2006; Di Cesare Manelli et al., 2014; Kim et al., 2015b; Chen et al., 2014), but some used spinal nerve ligation (Dyuizen et al., 2014) or spared nerve injury (Mamet et al., 2014). Therefore, the assessment of weight-bearing differences in pathological pain conditions appears to be an appropriate outcome to measure in efforts to test the efficacy of candidate analgesics. Interestingly, it has been shown that weight-bearing asymmetry at rest is more sensitive to analgesic treatment than the standard measures of hypersensitivity (Munro et al., 2008; Huntjens et al., 2009; King et al., 2006; Luk et al., 2018).

Weight-bearing changes in rodents have been suggested as a measure of spontaneous pain or the pain evoked by pressure exerted on the injured limb when it makes contact with the floor. However, alterations in weight bearing might also be consistent with pain avoidance behavior, since it is known from humans studies that anticipated pain plays a role in guiding motor control, and can result in the avoidance of activities (such as supporting substantial weight on the injured limb) which might induce pain or aggravate existing pain (Cobos and Portillo-Salido, 2013). Therefore

weight bearing measures and von Frey testing may not yield equivalent results, and it should thus not be surprising that these two outcomes show differential sensitivity to analgesic drugs.

The usefulness of this outcome, however, is known to be limited in pain models which do not involve injury to any of the hindlimbs. Nonetheless, it has been shown that mice with visceral pain shifted their weight distribution toward their front paws (Laux-Biehlmann et al., 2016), suggesting that measures of weight-bearing differences might be useful in other pain models apart from those based on unilateral injury to a hindlimb, although further studies are needed to determine the usefulness of this outcome in models not involving this type of injury. In addition, several pain models involve systemic injuries, such as neuropathic pain induced by antineoplastics or diabetes, or systemic arthritis, and it is unclear whether these models induce any change in the weight distribution among all four limbs.

A summary of the characteristics of weight-bearing asymmetry as a pain measure is provided in Table 2.

Pain-induced postural changes in animals during movement can be tracked by gait analysis, in which the animal must move about in order to produce the test results (in contrast to typical measures of weight bearing). Like changes in weight bearing, pain-induced gait alterations have been targeted in preclinical pain research since the 1980s by simply observing how the animal walks, and using a scoring system (e.g. Higuchi et al., 1986; Coderre and Wall, 1987). More recently, and in particular after the development of more complex devices such as the CatWalk™ or Digigait™ systems, these methods have been used more often to measure adaptive dynamic postural changes during pain conditions. The outcomes measured include not only weight bearing during walking, but also other parameters such as the duration of stance (the

time that the paw is on the floor) and swing (the time that the paw is in the air) phases, and parameters indicative of interlimb coordination. Alterations in gait parameters have been reported in unilateral inflammation induced by the classical inflammatory agents CFA and carrageenan (e.g. Angeby-Möller et al., 2008; Piesla et al., 2009; Adams et al., 2016; Angeby-Möller et al., 2018), osteoarthritis (e.g. Jacobs et al., 2014; Ishikawa et al., 2014), peripheral nerve injury (e.g. Vrinten and Hamers, 2003; Piesla et al., 2009; Mogil et al., 2010; Kobayashi et al., 2015; Chiang et al., 2016; Matsuda et al., 2016; Sheahan et al., 2017), and discogenic back pain (Fukui et al., 2018), among other models.

Alterations in gait parameters in several pain models, including inflammatory or osteoarthritic pain, have repeatedly been shown to be sensitive to analgesic treatment (partially reviewed by Cobos and Portillo-Salido, 2013; Angeby-Möller et al., 2018; Ishikawa et al., 2014), and can therefore be considered appropriate surrogate measures of pain. Pain at rest needs to be differentiated from pain during movement, given that the latter may be more severe than the former, and this difference may directly impact the analgesic efficacy of the drugs being tested. In this connection, it is known that during osteoarthritis, the restoration of weight-bearing symmetry in resting animals requires lower doses of analgesics (such as morphine and tramadol) than those needed to normalize gait alterations (Ishikawa et al., 2014). In fact, although weight-bearing differences at rest appear to be more sensitive to analgesics than standard measures of sensory hypersensitivity (as noted above), analgesic treatment at doses with clear antiallodynic effects failed to alter gait changes induced by inflammation (Shepherd and Mohapatra, 2018). Therefore, analgesic drug potency differs for each of these outcome measures, and this difference needs to be taken into account in the process of experimental design. If measures of gait alterations are used to test drugs under

development, there is a risk that promising analgesic drugs might be discarded if they have no efficacy on gait disturbances at the same doses as those that show effects on other more sensitive outcomes.

Studies of the sensitivity of gait changes to drug-induced analgesia after peripheral neuropathic pain have yielded conflicting results, in contrast to the clearer effects on musculoskeletal pain as mentioned above. The sciatic nerve contains not only sensory but also motor axons, and the severity peripheral nerve injury can vary considerably depending on the model and possibly on the operator. For example, analgesic drug effects are clearly absent when gait alterations are used as an outcome measure after spared nerve injury (Piesla et al., 2009; Mogil et al., 2010; Sheahan et al., 2017; Shepherd et al., 2018). This may reflect the consistently severe axotomy produced in this model, since it involves the complete transection of two of the three branches of the sciatic nerve (Decosterd and Woolf, 2000). However, some studies reported drug-induced analgesia whereas others found no effect for analgesic drugs when gait parameters were used as a pain index during chronic constriction injury or partial sciatic nerve ligation (Piesla et al., 2009; Shibayama et al., 2014; Chiang et al., 2016; Matsuda et al., 2016; Kang et al., 2017), i.e., where nerve injury was milder than after spared nerve injury. Therefore, it might be hypothesized that if surgical damage to the motor axons is too severe, gait alterations might indicate irreversible motor deficits, rather than pain. In light of these considerations, the choice of neuropathic pain model based on motor impairment may be a determining factor in experiments designed to test the possible efficacy of putative analgesics in ameliorating gait alterations.

The assessment of postural changes as pain indicators is therefore not free from motor-related confounders. In fact, sedative drugs can have an impact on weight-bearing asymmetry and may result in false-positive analgesic-like effects (Munro et al.,

2008). In addition, several drugs are known to alter gait patterns (Authier et al., 2016), which might also influence the results of gait analysis when used as a surrogate pain measure. A summary of the strengths and limitations of gait alterations as a pain measure is provided in Table 2.

The use of postural changes (either weight-bearing or gait alterations) as a pain outcome increased slowly from 1983-1987 to 2003-2007, albeit with only 41 research articles in the latter period, but there was a marked increase to 184 in the number of research articles that reported this measure as a pain index in 2014-2018 (Fig. 5 and Supplementary Table 1). Therefore, although the earliest observations of postural changes after unilateral hindlimb injury were published in the 1980s, it was only recently that this measure came to the attention of larger numbers of researchers.

5.1.2 Grip strength deficits as a measure of pain-induced functional disability

Grip strength has been widely and routinely evaluated for decades in rheumatology as a functional measure in patients with joint inflammation, osteoarthritis or other musculoskeletal diseases (e.g. Bijlsma et al., 1987; Pincus and Callahan, 1992; Spacek et al., 2004; Lee et al., 2013a; Donato et al., 2018; Beaudart et al., 2019), and remarkably, it has long been known to correlate with pain (Callahan et al., 1987; Fraser et al., 1999; Overend et al., 1999; Lee et al., 2013b; LoRusso et al., 2018). Grip strength assessment in experimental animals, however, was developed for a very different purpose: to assess the effects of muscle relaxants and to test drug-induced toxicity (Tilson, 1990; Nevins et al., 1993). Although grip strength in rodents is usually measured in the forelimbs, it can be studied in the hindlimbs as well (as shown in Fig. 6A). This technique was recently adapted to study pain resulting from a variety of etiologies mostly affecting the limbs, such as muscle inflammation (Kehl et al., 2000

and 2003; Souza et al., 2018), muscle pain induced by repeated exercise (Fujiwara et al., 2017), musculoskeletal hyperalgesia induced by stress (Goudie-DeAngelis et al., 2016), bone cancer (Kehl et al., 2003; Wacnik et al., 2003), osteoarthritis (e.g. Chandran et al., 2009; Honore et al., 2009; Lee et al., 2011; Hinata et al., 2018), inflammatory joint pain (Montilla-García et al., 2017; Dutta et al., 2018; Montilla-García et al., 2019), osteoporosis (Suzuki et al., 2018), and a model of sickle cell disease (Calhoun et al., 2015), among others. Grip strength deficits have also been observed during discogenic back pain (Millecamps et al., 2015; Millecamps and Stone, 2018; Yang et al., 2018), indicating that this outcome may be useful to detect pain even if the injury is not located in the limbs.

Decreased grip strength under painful conditions is known to be reversed by analgesics, and therefore can be used as a measure of the impact of pain on physical functioning in rodents with a painful condition (partially reviewed by Cobos and Portillo-Salido, 2013; Montilla-García et al., 2017 and 2019), and to test candidate analgesics. It is worth noting that substances producing significant sedation or muscle weakness, although able to attenuate reflex responses in standard measures of hypersensitivity and thereby induce false analgesic-like effects (reviewed by Le Bars et al., 2001; Cobos and Portillo-Salido et al., 2013; Gregory et al., 2013; Deuis et al., 2017), will exacerbate rather than ameliorate grip strength deficits (e.g. Chandran et al., 2009; Montilla-García et al., 2017). Therefore, grip strength assessment is not prone to the same confounders as standard pain measures – an advantage of this outcome as a pain measure.

We recently found that the *in vivo* ablation of TRPV1+ neurons in mice with CFA-induced inflammation completely reversed tactile allodynia without altering grip strength deficits at all (Montilla-García et al., 2017). Therefore, the neurobiological

mechanisms of tactile allodynia and grip strength deficits during inflammation are probably not identical.

A summary of the characteristics of grip strength as a pain measure is provided in Table 2.

Because grip strength was first used as a pain measure relatively recently (Kehl et al., 2000), few research articles to date have reported this outcome as a pain index, although its use has increased steadily from 12 research articles in 2003-2007 to 32 in 2014-2018 (Fig. 5 and Supplementary Table 1). Therefore, grip strength is still in the process of being established as part of the repertoire of outcomes for preclinical pain research.

It is worth noting that grip strength is also used to monitor functionality after recoverable sciatic nerve injury (crash injury) (e.g. Martins et al., 2018; De França Almeida et al., 2018), and although these studies are not usually designed to use this measure as a pain index (and were therefore not included in our analysis), in view of the variety of pain models which are sensitive to decreased in grip strength, researchers aiming to investigate nerve regeneration should be aware of the influence of pain on this outcome since it may negatively influence the apparent functional recovery. Conversely, grip strength cannot be used as a pain outcome in most standard models of neuropathic pain which involve surgical injury to the sciatic nerve, because the motor nerve lesions prevent the animals from gripping.

5.2 Pain-depressed behaviors

"Pain-depressed behaviors" are among those normally displayed by rodents which decrease in frequency or intensity during painful conditions (Negus et al., 2006). Although these behaviors (exploratory locomotion, home cage activity, burrowing behavior, nest building, intracranial self-stimulation and wheel running) require correct physical functioning to perform, they can also be influenced by the motivation of the animal to engage in them. Therefore, the interference of pain in these behaviors can be considered a more accurate reflection of the animal's general state and not exclusively a measure of physical function. Importantly, all pain-depressed behaviors, like the assessment of grip strength, have the potential to distinguish between substances producing significant sedation and those with true analgesic effects, since sedative drugs are expected to decrease the target behavior further, not to restore it (as opposed to drug-induced analgesia) (Negus et al., 2006; Cobos and Portillo-Salido, 2013; Negus, 2018).

A further consideration is that the pain-depressed behaviors reviewed here require only minimal intervention by the experimenters, since in most cases they do not need to be in the same room as the animal during the measurement process. This is obviously advantageous, as it is known that the experimenter's presence can alter the results of behavioral tests (Sorge et al., 2014).

These measures will be summarized and discussed below.

5.2.1 "Natural" behaviors as pain indicators: exploratory locomotion in a novel environment, home cage activity, burrowing behavior and nest building

Instinct is a powerful force in nature, and instinctive behaviors can be displayed in

response to an appropriate cue even in animals raised in isolation (Gould, 2004). This group comprises ethologically relevant rodent behaviors such as exploratory locomotion in a novel environment, burrowing behavior and nest building.

5.2.1.1 Pain interference in exploratory locomotion in a novel environment

Exploratory locomotion is an innate behavior in rodents, and its driving force is environmental novelty. The simplest assessment of this behavior consists of recording the distance traveled by the rodent in an evaluation chamber (ambulatory locomotion), but it can be complemented with additional measures such as vertical activity (rearing). The most economical version of this test consists of placing the animal in an open field arena divided into equal-sized sections, and counting the number of lines the animal crosses between sections during the evaluation period. Currently, it is far more common to use electronically-controlled devices often equipped with infrared detectors, such as the one shown in Figure 6B.

To our knowledge, depressed locomotor activity was first proposed as a pain index in 1979 (Harada et al., 1979). These researchers showed that the intraperitoneal injection of acetic acid induced locomotor activity depression in mice, which was ameliorated by analgesic treatment with the NSAIDs acetyl-salicylic acid, aminopyrine and mefenamic acid, and the opioid drugs morphine, pethidine and codeine; therefore the decreased motor activity was attributed to pain (Harada et al., 1979). This surrogate pain measure was later used in a model of arthritis in rats (e.g. Larsen and Arnt, 1985), and further studies also detected deficits in exploratory locomotion in a large number of pain models, including inflammation in either the paw or the orofacial region, hindlimb osteoarthritis, postsurgical pain, peripheral or central neuropathic pain, and visceral pain, among others (partially reviewed in Cobos and Portillo-Salido, 2013; de la Puente

et al., 2015; Zhu et al., 2015; Buvanendran et al., 2016; Zychowska et al., 2016; Das et al., 2017; Zhang et al., 2018, among others). The decrease in exploratory behavior has been pharmacologically validated as a pain outcome in most models mentioned above (see references for further details).

It is worth noting that although several studies used this measure of behavioral depression induced by pain, other reports showed that ambulatory locomotion in rodents did not decrease (or did so only minimally) in a variety of pain models such as CFA-induced inflammatory pain, prostatitis, ovariectomy-induced osteoporosis, diabetic neuropathy, or spared nerve injury, even in animals that displayed marked sensory hypersensitivity (e.g. Urban et al., 2011; Tékus et al., 2014; Schwartz et al., 2015; Zhou et al, 2015; Sałat et al., 2016; Montilla-García et al., 2017; Suzuki et al., 2018). The quantification of vertical activity may be more sensitive than ambulatory locomotion in detecting pain-induced alterations, in particular in pain models affecting the hindlimbs; this is exemplified in studies of monoiodoacetate-induced osteoarthritis or plantar incision (e.g. Nagase et al., 2012; Ewan and Martin, 2014).

It is also important to note that some studies compared two of the "new" measures summarized in this review, and found that under different circumstances, exploratory activity was less sensitive in detecting pain-induced alterations than grip strength (Montilla-García et al., 2017; Suzuki et al., 2018), decreased intracranial self-stimulation (Ewan and Martin, 2014), or facial pain expressions (Herrera et al., 2018; Kawano et al., 2014). These results suggest that exploratory activity is not a particularly sensitive method to measure pain-induced behavioral depression under all circumstances.

A further issue with using exploratory locomotion as a pain measure is that the return towards baseline activity associated with nonanalgesic drugs that increase

locomotion (such as caffeine) might induce a false analgesia-like effect, although this effect can be readily detected by testing drug effects in noninjured control animals (e.g. Stevenson et al., 2009). Finally, exploratory locomotion tests are not suitable for repeated measures, as repeated exposure to the test chamber decreases novelty and the consequent exploratory activity (e.g. Montilla-García et al., 2017).

The analysis of rodent exploratory activity in an open field can be complemented by measuring the time spent near the walls of the evaluation chamber (thigmotaxis) versus time spent in the innermost area. This is not exclusively a measure of locomotor activity in mice, since it reflects the natural conflict between the drive to explore a novel environment and the avoidance of open spaces as potentially dangerous areas. Therefore, the avoidance of inner areas is thought to be indicative of anxiety in rodents (Ramos, 2008). Since anxiety is a well-known component of the human pain phenotype (Gatchel et al., 2007; Yeh et al., 2018), the open field test has been used to explore the anxiogenic properties of pain in rodents. The findings reported to date are contradictory: some studies found pain-induced anxiety-like behavior, whereas others found no apparent changes in this behavior (partially reviewed by Cobos and Portillo-Salido, 2013; Liu et al., 2015; Shang et al., 2015; Wang et al., 2015; Sheahan et al., 2017). In is worth noting that nonanalgesic anxiolytic drugs such as diazepam may also ameliorate anxiety-like behavior in rodents during pain conditions (e.g. Wallace et al., 2008); therefore this particular outcome lacks specificity for detecting drug-induced analgesia.

A summary of the characteristics of exploratory locomotion as a pain measure is provided in Table 3.

Although exploratory locomotion has not always been shown to be affected by by pain in rodents, this outcome measure is currently widely used in preclinical pain

research, and the number of studies which used it as a pain measure has increased steadily, particularly in the most recent period examined here: 132 relevant research articles were published in 2014-2018 (Fig. 5 and Supplementary Table 1). One possible reason for the popularity of exploratory behavior measures is that measures of locomotor activity are used in (almost) any behavioral phenotyping laboratory, and have been used classically (and remain in frequent use) in pain-free animals to study the influence of analgesic drugs on locomotor activity (e.g. Sánchez-Fernández et al., 2013; Mansouri et al., 20014; Birmann et al., 2018; Scapinello et al., 2019). Therefore, actimeters and open fields are available to many pain researchers who can readily use them to test whether their animal pain models reveal deficits in exploratory activity as a complementary outcome.

5.2.1.2 Home cage activity alterations during pain conditions

Home cage behavior can be used to measure locomotion in a familiar environment; this approach, however, is not equivalent to measuring exploratory locomotion given that the driver of this latter behavior is the novelty of the environment, as noted above. Alterations in home cage locomotion have been explored with the aim of detecting overall behavioral changes in daily living activities of rodents during chronic pain. As noted previously, rats and mice are nocturnal animals, but behavioral tests are almost always performed during the light phase of the daily cycle. Monitoring activity throughout 24 h in the home cage has the clear advantage that activity is recorded when natural nocturnal activity peaks, in an environment that is completely familiar to the animals, thus minimizing stress conditions which are known confounding factors in animal studies.

To our knowledge, the first reports of decreased home cage activity in rodents during pain conditions date from the year 1997: rats with polyarthritis (Philippe et al.,

1997) or bilateral osteoarthritis (Guingamp et al., 1997) showed decreased home cage activity as measured by telemetry. However, experiments with unilateral inflammation yielded mixed results: some authors detected a decrease in home cage locomotion (Millecamps et al., 2005) whereas others did not (Urban et al., 2011), or found only minimal changes (Pitzer et al., 2016b), suggesting that bilateral injuries may be necessary to induce robust impairment. In fact, unilateral nerve injury was shown repeatedly to have little or no effect on home cage activity (Pitzer et al., 2016a; Rácz et al., 2015; Urban et al., 2011). These conflicting results may be due to the relative ease with which these quadruped animals use their three noninjured limbs to compensate for motor deficits after injury to a single limb.

Experiments with pain states not involving the limbs showed more robust results, e.g. in models of postsurgical pain induced by mock ova implantation (Goecke et al., 2005), laparotomy (Sharp et al., 2003; Arras et al., 2007), or inguinal hernia repair (Bree et al., 2015 and 2016). We found only two studies on visceral pain, specifically in chemically-induced pancreatitis (Houghton et al., 1997) and gastritis (Painsipp et al., 2007), and both showed a significant decrease in home cage activity. A summary of the characteristics of home cage activity as a pain measure is provided in Table 4.

In the two most recent publication periods examined (2003-2007 and 2014-2018), we identified only 4 or 5 research articles that used home cage activity in rodents during a pain condition (Fig. 5 and Supplementary Table 1). Taking into account that the first description of home cage activity as a pain index dates from more than 20 years ago (1997), the assessment of this parameter appears not to have gained the popularity of other behavioral tests described here. Recording home cage activity requires expensive equipment that limits the number of animals to be tested, and this factor, together with the difficulties in detecting pain phenotypes in the rodent pain models

used most commonly in preclinical research (as described above), may contribute to the lower uptake of this measure by the pain research community. This is not to say that preclinical researchers should disregard this measure, given that it may provide valuable information about the animal's general state. Recently developed devices are able to measure not only home cage activity but also much more complex parameters even in groups of mice – a notable potential advantage since social isolation is a known stress factor in rodents. These additional parameters cover social interaction as well as several cognitive outcomes, so these new devices are expected to considerably improve our understanding of rodent pain behavior in the near future (Tappe-Theodor et al., 2019).

5.2.1.3 Burrowing behavior during pain states

Burrowing, another example of an ethologically relevant rodent behavior, is performed vigorously in the wild not only by rats and mice, but also by hamsters and gerbils, so it is a highly conserved behavior in this group of mammals (Deacon, 2009). Decreased burrowing has been used as an indicator of distress and suffering in several disease models (Deacon, 2006b). Typically, a long tube filled with a suitable substrate, sealed at one end and raised at the open end, is used (Deacon, 2006b) although this test can also be performed with a regular water bottle (as used in animal facilities) filled with the substrate and placed on the floor of the cage (Jirkof, 2014). Since burrows are used in nature to hide from predators, a dark-colored tube motivates rodents to displace the substrate more vigorously than a transparent one (Shepherd et al., 2018). Different substrates have been used in animal research, e.g. gravel, clay balls, sand, food pellets, or even the bedding used for housing laboratory animals (Deacon, 2006b). Species differ in their preferred substrate. For example, rats do not usually burrow efficiently in food pellets, but prefer earth-like substrates such as sand or gravel, whereas mice

burrow actively in food pellets (Deacon et al., 2006b). The type of substrate has repercussions on the time needed for the rodent to empty the test burrow. For example, when tubes are filled with a high-density substrate such as sand, the animals typically need 1–2 h for the task (e.g. Bryden et al., 2015; Gould et al., 2016; Smith et al., 2016; Rutten et al., 2018), but need between 30 min and 1 h when the substrate is gravel or food pellets (Andrews et al., 2012; Muralidharan et al., 2016; Morris et al., 2018), and as little as 10–15 min when low-density materials such as corncob or other bedding substrates are used (Shepherd et al., 2018; Shi et al., 2018a). Therefore, the effort expended by the rodent to empty the test burrow is proportional to substrate density. After an appropriate time the amount of substrate displaced from the test burrow is weighed as an index of the intensity of the behavior (see references above). The latency to burrowing can also be used as a pain index (e.g. Jirkof et al., 2010; Huang et al., 2013). When rodents are unwell, the amount of material removed from the test burrow is decreased, and the latency to burrowing is increased. A representative picture of a mouse inside a test burrow previously filled with food pellets is shown in Figure 6C.

Decreased burrowing as a pain indicator was first described in 2010 in mice after laparotomy (Jirkof et al., 2010). This test was rapidly adopted by pain researchers as a surrogate measure of pain, in particular after it was shown to be sensitive to CFA-induced inflammation and several common models of peripheral neuropathic pain targeting the sciatic nerve (Andrews et al., 2012). It has also been used for rodents with several other pain conditions such as osteoarthritis, neuropathic pain in the orofacial region, discogenic low back pain, neuropathy induced chemically by stavudine or paclitaxel, a model of complex regional syndrome, visceral pain, and radiation-induced oral mucositis, among others (e.g. Andrews et al., 2012; Huang et al., 2013; Jirkof et al., 2013b; Lau et al., 2013; Rutten et al., 2014; Bryden et al., 2015; Muralidharan et al.,

2016; Wodarski et al., 2016; Nolan et al., 2017; Shi et al., 2018a; Deseure and Hans, 2018; Griffiths et al., 2018; Rutten et al., 2018; Shepherd et al., 2018). This outcome measure has been repeatedly shown to be sensitive to analgesic treatment in most pain models, validating this outcome as a suitable pain measure for detecting drug-induced analgesia (see references above).

It is worth noting that as for other measures, CFA-induced paw inflammation induces a self-limited depression of burrowing behavior (e.g. Muralidharan et al., 2016), whereas other chronic painful situations such as chronic constriction injury or osteoarthritis models induce much longer-lasting depression of burrowing behavior (Rutten et al., 2018; Bryden et al., 2015).

One published study showed no decrease in burrowing in mice after CFA-induced inflammation in the hindpaw or after nitroglycerin administration as a model of migraine, but found robust depression of this behavior after spared nerve injury (Shepherd et al., 2018). In this particular study, the substrate used for the burrow was corncob, which is less dense than other substrates typically used in these experiments. The dependence of pain-induced impairment on substrate density was clearly exemplified in a recent report showing that after chronic constriction injury, burrowing behavior in rats was depressed to a much greater extent when gravel was used as the substrate compared to sand (Rutten et al., 2018). Therefore, the absence of effect of CFA-induced inflammation or nitroglycerin-induced migraine on burrowing behavior when corncob is the substrate may be due to the permissive conditions used, and indicates that it may be useful to test several substrates to determine which one is optimal for measuring how different pain conditions affect burrowing. A summary of the characteristics of burrowing as a pain measure is provided in Table 3.

Although the first description of burrowing as a pain measure dates from 2010

(we found no research articles based on this measure in 2003-2007), 19 research articles had been published by 2014-2018 (Fig. 5 and Supplementary Table 1), indicating, as for the other pain outcomes described in the preceding sections, a clear tendency toward increased use in preclinical studies.

5.2.1.4 Pain interference in nest building

Like burrowing behavior, nest building is also a naturally occurring behavior in both rats and mice (Smith and Corrow, 2005; Deacon, 2006a), and decreased nest building has been used as an indicator of distress and suffering in several disease models (Deacon, 2006a). Nests are important not only for reproduction and shelter, but also for heat conservation, and are built by rodents of both sexes (Deacon, 2006a; Jirkof, 2014). Nest building has recently been used as a measure of postsurgical pain (Arras et al., 2007; Jirkof et al., 2012 and 2013a; Rock et al., 2014; Moore et al., 2017; Oliver et al., 2018), pain induced by the intraperitoneal administration of chemical irritants (Negus et al., 2015; Lewter et al., 2017), carrageenan-induced inflammation (Beninson et al., 2018), inflammatory arthritis (Dutta et al., 2018), and bone cancer-induced pain (Forte et al., 2016). Most studies showed that the depression of this behavioral task by pain states was reversed by known analgesics, so it is therefore suitable for testing drug-induced analgesia. Every study to date on pain-induced depression of nest building has been done in mice, possibly because they are more vulnerable to heat loss than rats due to their small size, and consequently make nests rapidly (Deacon, 2006a), which is an advantage when this particular behavior needs to be studied.

Experimental animals can make nests from different materials, but the one used most widely in research is the standard “nestlet” made of pressed cotton, which is often used in animal facilities for environmental enrichment (Deacon, 2006a). Because nest

building has been used for decades as a rodent monitoring tool in several scientific fields (Jirkof, 2014), a variety of protocols are available to assess nest building. Therefore, there is as yet no clear consensus regarding the methodology to assess this behavior in pain studies. For example, some authors score the quality of the nest (ranging from no shredding of the nestlets to a perfect nest with a crater inside and high walls) (e.g. Gaskill et al., 2013; Dutta et al., 2018), whereas others measure the integration of new pieces of nestlet into a preexisting, fully finished nest (Rock et al., 2014; Kendall et al., 2016), the time spent on nesting (Jirkof et al., 2012), or the number of cleared zones after placing several pieces of nestlets at distant sites in the home cage (e.g. Negus et al., 2015; Forte et al., 2016; Lewter et al., 2017).

The way nest building is assessed has a direct impact on the time needed to perform the task. For example, when the outcome of interest is a fully completed, perfect nest, protocols often use evaluation periods as long as 12–24 h (e.g. Gaskill et al., 2013; Dutta et al., 2018), whereas when the outcome is only the collection of pieces of neslets, a 60–100 min observation period is considered long enough (e.g. Negus et al., 2015; Forte et al., 2016). Figure 6D illustrates the appearance of the home cage at the start and the end of this latter protocol.

The time of day when this test is used is important, because nesting behavior in mice is usually most intense just before dawn, with few additional bouts during the dark phase of the 24-h cycle (Jirkof, 2014). Therefore, nestlets should be available to the mice immediately before the dark phase, at the latest, if the aim is to test nest building after nighttime (e.g. Gaskill et al., 2013; Dutta et al., 2018), or very early in the morning when a shorter protocol is used (e.g. Negus et al., 2015). A summary of the characteristics of nest building behavior as a pain measure is provided in Table 3.

Although only 10 research articles reported nest building as a pain outcome in

2014-2018 (Fig. 5 and Supplementary Table 1), taking into account that the use of this behavior as a pain indicator was first described as recently as 2007, that it is highly preserved in mice, and that it can be tested at almost zero cost, the number of researchers using this methodology can be expected to increase in the near future to clarify the potential usefulness of nesting behavior in pain research.

5.2.2 Other pain-depressed behaviors: intracranial self-stimulation and wheel running

Other behaviors depressed by pain are wheel running and intracranial self-stimulation (ICSS). The potential usefulness of these behaviors for pain assessment in experimental animals is discussed below.

5.2.2.1 Intracranial self-stimulation and pain

ICSS, a method developed in the early 1950s (Olds and Milner, 1954), is a behavioral procedure in which operant responding is maintained by pulses of electrical brain stimulation to a brain-reward area. In this type of assay, intracranial electrodes that target specific brain regions are implanted in the experimental animal, and performance of the operant response (such as lever pressing) results in the delivery of an electrical stimulus to the target area (Negus and Miller, 2014). Once the animal has been trained to emit reliable baseline responses, the effect of experimental manipulations on the operant response can be evaluated objectively. Drug-induced increases in ICSS are interpreted as an abuse-related effect, and these experiments yield results similar to drug self-administration procedures (Negus and Miller, 2014). Therefore, this method has been widely used to study brain circuits likely to play key roles in driving the development of compulsive drug-seeking behaviors (Negus and Miller, 2014; Kenny et

al., 2018).

Recent studies have aimed to test whether operant responses are decreased by pain states. The earliest evidence that ICSS was depressed by intraperitoneal injections of lactic acid dates from 2009 (Do Carmo et al., 2009), and this work was followed by several additional studies that mostly used the same chemical irritant (Negus et al., 2010; Negus et al., 2012a and 2012b; Rosenberg et al., 2013; Kwilasz, et al., 2014; Altarifi and Negus, 2015; Freitas et al., 2015; Miller et al., 2015a and 2015b; Hillhouse and Negus, 2016; Lazenka et al 2018). The NSAID ketoprofen reversed both acid-induced ICSS depression and abdominal constrictions (Hillhouse and Negus, 2016; Negus et al., 2012a), indicating that successful analgesia was able to revert the pain-induced depression in these behaviors.

As noted previously, drug-induced ICSS facilitation (in pain-free animals) is typically used as an index of the rewarding properties of drugs. Pain is fundamentally an aversive sensation, whereas drug-induced analgesia is rewarding (Navratilova and Porreca, 2014). Therefore, it can be hypothesized that the amelioration of pain-induced ICSS depression by analgesic drugs reflects the rewarding properties of analgesia. The intrinsic rewarding properties of some drugs such as opioids might thus be a confounder when ICSS is used as a pain outcome measure. However, this is also a notable potential advantage of this parameter in pharmacological studies, because it makes it possible to study analgesia (reversal of ICSS depression in animals with pain) and the rewarding properties of drugs (facilitation of ICSS in pain-free animals) in the same experimental setting, and thus to obtain comparable data to evaluate whether the drugs tested show the (desirable) preferential effects of analgesia or not. Morphine and other known μ-opioid analgesics reversed both abdominal constriction and ICSS depression at doses which did not alter ICSS in pain-free animals (Altarifi and Negus, 2015; Negus et al.,

2010), indicating that they are able to induce analgesia in a dose range which does not appear to induce obvious rewarding effects. Interestingly, although μ-opioid drugs reversed both pain-induced effects, it was shown that ICSS depression was more resistant to morphine analgesic tolerance than abdominal constriction (Altarifi and Negus, 2015). In a sense, these findings parallel previously reported differences in sensitivity to morphine tolerance between classic standard pain outcomes such as the tail flick versus the formalin test (Abbott et al., 1982). It is thus not unreasonable to posit that because different pain outcomes or stimuli may reflect different aspects of the pain experience, there may also be differences in the development of opioid tolerance. More studies of pain outcomes other than standard reflex-based outcomes are needed to improve our understanding of the mechanisms that underlie opioid tolerance development.

Several additional pharmacological experiments have compared drug effects on acid-induced ICSS depression and abdominal constriction, in order to test whether putative analgesics not only inhibit the reflex response but also recover the depressed behavior (as morphine and ketoprofen do). For example, kappa agonism failed to block acid-induced ICSS depression (Negus et al., 2010), a result that mirrored the poor performance of kappa agonists as clinically useful analgesics (Rivière, 2004). However, it blocked acid-stimulated writhing (Negus et al., 2010), suggesting a low predictive value of the reflex response for these particular drugs. These results clearly show the importance of testing putative analgesics in different types of outcome measures.

Other studies of ICSS have used pain stimuli other than lactic acid injection, e.g. plantar incision (Ewan and Martin, 2014), CFA-induced paw inflammation (Leitl et al., 2014), intraplantar formalin administration (Leitl et al., 2014; Leitl and Negus, 2016), spinal nerve ligation (Ewan and Martin, 2011a and b, 2014), and paclitaxel-induced

neuropathic pain (Legakis et al., 2018). Spinal nerve ligation and paclitaxel-induced neuropathic pain did not lead to decreases in ICSS, whereas they did result in marked mechanical allodynia (Ewan and Martin, 2011a and b, 2014; Legakis et al., 2018). Both spinal nerve ligation and paclitaxel-induced neuropathic pain are among the standard chronic pain models, and they would both be expected to induce sustained ICSS depression indicating sustained pain. However, intraplantar formalin induced long-lasting ICSS depression lasting at least 14 days (Leitl and Negus, 2016). Therefore, pain-induced ICSS depression, although susceptible to long-lasting alterations, cannot be predicted by hindpaw mechanical allodynia, further suggesting that these types of measures reflect different aspects of the pain phenotype. The characteristics of ICSS as a pain measure are summarized in Table 3.

Although the first description of ICSS depression as a pain indicator dates from only around 10 years ago (2009), this outcome measure was used in 14 separate studies published in 2014-2018, most of which were from two research groups (S.S. Negus and E.E. Ewan). The difficulties with electrode implantation and the lack of experience with conditioned responses in most pain research groups may limit the use of this methodology despite the highly informative data it provides.

5.2.2.2 Wheel running depression during pain conditions

Running wheels are obviously not present in rodents' natural habitat, so the use of these devices by experimental animals in research may be considered an artificial behavior performed only in captivity, in contrast to burrowing or nest building behaviors, which are performed by rodents in the wild. However, since the first report in the 1950s (Skinner and Morse, 1957) thousands of articles have been published based on research with running wheels in experiments with rodents, and spontaneous wheel running was

also reported in wild rodents (particularly mice) when a wheel was present in their natural habitat, indicating that wheel running is an elective behavior rather than an stereotypic behavior shown only by rodents in captivity (Meijer and Robbers, 2014). Figure 6E shows a mouse running in a wheel.

Since the first description of wheel running depression associated with postsurgical pain induced by splenectomy (Clark et al., 2004), activity wheels have been tested successfully in several pain models. Known examples of these models to date include (among others) postsurgical pain after laparotomy (Kendall et al., 2016), CFA-induced hindlimb inflammation (Cobos et al., 2012; Kandasamy et al., 2016; Pitzer et al., 2016b; Kandasamy et al., 2017a; Sheahan et al., 2017), hindlimb osteoarthritis (Stevenson et al., 2011), chronic constriction injury of the sciatic nerve (Whitehead et al., 2017), paclitaxel-induced neuropathic pain (Griffiths et al., 2018), migraine induced by microinjection of allyl-isothiocyanate in the dura (Kandasamy et al., 2017b and 2018a and 2018b), bone cancer pain in the femur (Tang et al., 2016), a model of osteogenesis imperfecta (Abdelaziz et al., 2015), and visceral pain induced by sodium dodecyl sulfate as a model of colitis (Häger et al., 2018) or by the intraperitoneal injection of acetic acid (Miller et al., 2011). These results indicate that wheel running is sensitive enough to detect several pain conditions.

Two independent reports found that after spared nerve injury in mice, there was no decrease in wheel activity (Pitzer et al 2016a; Sheahan et al., 2017). In addition, neuropathic pain induced by the antineoplastic bortezomib or by the antiretroviral drug stavudine, as well as pain induced by intraplantar formalin, all induced marked mechanical allodynia but did not decrease wheel running behavior (Weber et al., 2007; Grace et al., 2014; Duggett and Flatters, 2017). These results could not be ascribed to use of the noninjured limbs to compensate for decreased use of the injured one, given

that bortezomib and stavudine produced systemic neuropathy in all four limbs, and formalin was injected bilaterally in both hindpaws. Under these circumstances the rodents inevitably use their hypersensitive limbs for wheel running. These results demonstrate that changes in wheel running cannot be predicted by hindpaw mechanical allodynia. In fact, we and others have reported that the recovery of wheel running after hindpaw inflammation is much more sensitive to drug-induced analgesia than recovery of the baseline mechanical pain threshold, to the point that drug doses unable to significantly ameliorate tactile allodynia induced a clear recovery of wheel running (Cobos et al., 2012; Kandasamy et al., 2017a). Taken together, these results indicate that mechanical allodynia in the paws is not the major driver of wheel running depression, even in models involving hindlimb injury.

Both rats and mice are nocturnal animals; thus they are much more active during the night. In fact, when an activity wheel was placed in their home cage, activity during the light phase is almost negligible (e.g. Luna-Sánchez et al., 2015). Some of the research cited above was done with a running wheel in the home cage to monitor activity throughout the 24-h cycle (e.g. Stevenson et al., 2011; Pitzer et al., 2016a and b; Kandasamy et al., 2016; Duggett and Flatters, 2017; Kandasamy et al., 2017a and 2017b, 2018a and 2018b). Wheel running is a well-known self-rewarding behavior for rodents (Novak et al., 2012). Therefore, if rodents are exposed to the wheel for a limited time (30 min to 2 h) during the day, their behavior will increase and they will run distances travelled that are long enough to detect possible pain-induced wheel running depression during the light phase (e.g. Miller et al., 2011; Cobos et al., 2012; Abdelaziz et al., 2015; Benson et al., 2015; Tang et al., 2016; Whitehead et al., 2017). In our experience, C57BL/6J mice can run an average of about 0.5 km in just 1 h of exposure to the wheel during the light phase (Cobos et al., 2012).

Of relevance in this connection are several reports showing that regular physical exercise can ameliorate pain hypersensitivity in several pain models, through activation of the endogenous opioid system (e.g. Brito et al., 2017; Lima et al., 2017; Shi et al., 2018b), and therefore may be a potentially confounding factor in experiments with wheel running as a pain surrogate, since the target behavior for pain assessment may simultaneously ameliorate the animal's pain state. To our knowledge, all studies showing pain amelioration by physical exercise used home cage running wheels, and it has been shown that access to the wheel for 1 h per day does not affect either the neuropathic or inflammatory mechanical threshold (Cobos et al., 2012; Whitehead et al., 2017). Therefore the use of short protocols might minimize this confounder.

A summary of the characteristics of wheel running behavior as a pain measure is shown in Table 3.

Wheel running is making its way into the repertoire of pain assessment tools in preclinical research. In 2003-2007 there were 3 published research articles exploring whether animals showed decreased wheel running during pain conditions, yet by 2014-2018 the number of research articles had grown to 18 (Fig. 5 and Supplementary Table 1). All articles that focused on the use of physical exercise to alleviate sensory hypersensitivity were excluded from the present analysis, since they were not designed to use a running wheel to measure pain in rodents, but instead used it as a therapeutic strategy. Concerning the other outcome measures detailed here, and in view of the increase in the use of wheel running in pain research, the number of researchers using this methodology is expected to increase in the near future.

5.3 Facial expressions as a pain indicator

In humans, painful situations trigger characteristics facial patterns (Ekman and Friesen,

1978; Grunau and Craig, 1987). Similarly, painful insult in rodents can be scored by their facial expressions based on orbital tightening, ear position, whisker change, and nose and cheek bulge, which together constitute the so-called grimace scale (Langford et al., 2010; Sotocinal et al., 2011). Figure 6F illustrates the expressions in a pain-free mouse (left panel) and an animal with cyclophosphamide-induced cystitis (right panel).

In humans, facial expressions are well-known social signals that convey emotional states. Similarly, it was recently shown that facial expressions of pain can be recognized as such by other rodents (Nakashima et al., 2015), indicating that they are evolutionarily preserved emotional signals with a communicative function, and therefore reflect an affective aspect of pain. Support for this notion comes from the finding that a lesion in the rostral anterior insula (which is known to play a pivotal role in pain-related emotion in humans) decreases facial pain expressions in mice after intraperitoneal acid injection, without altering abdominal stretching (Langford et al., 2010).

Facial expressions were found to be reliable pain indicators under several circumstances, including pain induced by chemical irritants in somatic tissues or visceral organs (Langford et al., 2010; Sotocinal et al., 2011; Kim et al., 2015a; Hassan et al., 2017; Herrera et al., 2018), inflammation (e.g. Langford et al., 2010; Sotocinal et al., 2011; De Rantere et al., 2016; Wang et al., 2017 and 2018), migraine models (Harris et al., 2017; Rea et al., 2018;), spinal cord injury (Schneider et al., 2017; Chaves et al., 2018), and different types of postsurgical pain, e.g. after laparotomy (Langford et al., 2010; Sotocinal et al., 2011; Matsumiya et al., 2012; Kendall et al., 2016; Tuttle et al., 2018), laminectomy (Chaves et al., 2018), castration, or vasectomy (Miller et al., 2016; Dalla Costa et al., 2018). In some of these pain states the grimace scale has been validated for analgesic testing with opioid drugs and NSAIDs during inflammation

(Langford et al., 2010; Sotocinal et al., 2011; Leung et al., 2016), postoperative pain (Matsumiya et al., 2012; Kawano et al., 2014 and 2016), and other painful situations (e.g. Saine et al., 2016; Herrera et al., 2018; Rea et al., 2018).

However, this measure does not appear to be sensitive enough to detect pain induced by peripheral nerve injury, since standard models of chronic injury to the sciatic nerve (i.e. chronic constriction or spared nerve injury) did not elicit obvious pain-related facial expressions (Langford et al., 2010; Sotocinal et al., 2011). In contrast, infraorbital nerve constriction induced long-lasting (10 days or more) pain-related facial expressions, and this effect was reversed by fentanyl, indicating that the phenotype was driven by pain rather than by nonspecific changes in the face caused by the surgical procedure (Akintola et al., 2017). These latter results may reflect the marked emotional component of orofacial pain often seen in humans (Cole and Carlson, 2018). This latter study on orofacial pain is however the report showing the pain faces for longer durations, since in studies of postsurgical pain or CFA-induced inflammation they lasted for a much shorter time (typically less than 12 h) (Sotocinal et al., 2011; De Rantere et al., 2016). These finding again mirror the discordance described in this review between mechanical allodynia and pain-induced alterations in several additional outcomes, which are often much shorter-lasting than mechanical allodynia. Therefore, further research is needed to elucidate whether facial pain expressions are able to provide robust information on chronic pain.

The basic set-up for assessing facial expressions of pain in rodents consists of a video camera and a computer. However, analysis by the experimenter can be time-consuming. Specialized software has been developed to increase the usability of the grimace scale in preclinical research (Sotocinal et al., 2011), and more recently a computerized convoluted neural network has been tested for this purpose (Tuttle et al.,

2018). Both strategies aim not only to decrease the time needed to score facial expressions, but also to decrease experimenter bias by eliminating the need for human observers to score the animals. Modifications in the grimace scale have been proposed, and some studies have used only orbital tightening as a simplified surrogate for the full scale in order make assessments more straightforward and faster (albeit at the cost of losing information) (Kendall et al., 2016; Rea et al., 2018). A summary of the characteristics of facial expressions for pain assessment is provided in Table 4.

Although the first description of facial expressions as a pain outcome in rodents dates from 2010, this measure has rapidly gained popularity among preclinical pain researchers, who published 61 articles mentioning this test in 2014-2018 (Fig. 5 and Supplementary Table 1). By comparison, grip strength was proposed as a pain indicator 10 years earlier than facial expressions (in 2000), yet it was used in only 32 research articles as of 2014-2018 (Fig. 5 and Supplementary Table 1). As an index of the overall impact of facial expression assessment in animals, it is worth noting that this measure is increasingly used in veterinary care and animal welfare studies in rabbits (Hampshire and Robertson 2015), ferrets (Reijgwart et al., 2017), lambs (Guesgen et al., 2016), horses (Dalla Costa et al., 2014), sheep (Häger et al., 2017), and piglets (Viscardi et al., 2017). Therefore, studies which use this measure are not limited to the species used in preclinical research (mice and rats), but we note that studies reporting its use in domesticated and pet species were not included in Figure 5 or Supplementary Table 1, and that only studies in rodents were considered here.

6. ARE THESE NOVEL PAIN OUTCOMES SUITABLE TO STUDY SEX DIFFERENCES IN PAIN?

Studies of female rodents have long been neglected in biomedical research, particularly in the neuroscience field (Beery and Zucker, 2011). This is worrisome because sex-related differences are known to exist in the responses to clinical and experimental pain in humans (Fillingim et al., 2009; Rosen et al., 2017), and in the efficacy of analgesic treatment (Aubrun et al., 2005; Fillingim et al., 2009). In addition, it is well known that in general terms, the prevalence of pain-related disorders is higher in women than in men (Tsang et al., 2008; Greenspan et al., 2007). Accordingly, there is a need to include female animals in preclinical pain research – a need that has only started to be addressed in recent years.

Sex differences were described in nociceptive sensitivity in both rats and mice, with female animals generally tending to be more sensitive to painful stimuli (Mogil et al., 2000; Sorge and Totsch, 2017). However, the magnitude of this greater sensitivity in female animals is often small and statistical significance is not always found (Sorge and Totsch, 2017). In fact, a myriad of studies have considered values from female and male rodents to be equivalent, and the results for both sexes were averaged together (Zimmermann et al., 2007; Lagerström et al., 2010; Murthy et al., 2018).

Some of the behaviors described here that might be used as novel pain surrogates show marked sexual dimorphism in uninjured animals. Running wheel activity, for example, is known to be a sexually dimorphic behavior, since female rats and mice run further and faster than their male counterparts (e.g. Luna-Sánchez et al., 2015; Kandasamy et al., 2016 and 2017a; Basso and Morrell, 2017). Similarly, exploratory locomotion and home cage activity are typically much higher in female rodents (Malfait et al., 2010; Negrigo et al., 2011; Craft et al., 2013). Therefore, when

both sexes are tested with these methodologies, their data should not be averaged, but should be recorded and analyzed separately in single-sex experimental groups, or the effects of experimental manipulations (such as pain or analgesia) should be analyzed as a percentage of the baseline or control group values. A potential drawback with percentages, however, is that they might mask the fact that the actual amount of locomotion is much greater in female animals compared to males.

Sex differences in other endpoints are usually not obvious in the literature. For example, grip strength is typically greater in male rats and mice than in their female counterparts, although these differences can be camouflaged (or may even slightly favor females) if they are relativized to body weight, since male rats and mice are larger than females (e.g. Griffiths et al., 2012; Carmo et al., 2016; Sheth et al., 2018; Hernandez et al., 2019). Gait-related parameters such as stride width or length might also be influenced by the size of the experimental animals and hence by their sex. These sex differences appear to be more prominent in rats (Parker and Clarke, 1990) yet often absent in mice (Clarke and Still, 1999), which may be related to the smaller difference in size between female and male mice. Other behaviors such as nesting or burrowing do not appear to be overtly more prominently in either sex (Deacon et al., 2009; Jirkof et al., 2013a; Rock et al., 2014; Schwabe et al., 2019). Therefore the sexual dimorphism of these latter measures in baseline recordings is much smaller than for wheel running, exploratory locomotion or home cage activity, and this makes comparisons between the two sexes easier. In any case, data from each sex need to be carefully analyzed separately before considering whether the values from both sexes can justifiably be averaged together if appropriate for a particular study.

Although few studies to date have used the behavioral tests noted above as pain surrogates in rodents of both sexes, we found some studies based on each of these

methodologies in both female and male rodents. As shown in Supplementary Table 2, even considering that the number of published studies is limited, a substantial proportion of them found sex differences in these behaviors in a variety of pain states. In some studies the authors also tested a conventional pain measure (tactile allodynia in most cases), so the results with these novel methodologies can be compared to standard pain assessment methods. There is good overall agreement between the results with both types of assay, such that when sex differences are found with the standard methods, they are also often reported in alternative pain surrogates (e.g. Kohli et al., 2010; Burke et al., 2013; Ro et al., 2019). However, there are also reported discrepancies between attempts to detect sex differences in both standard pain outcomes and novel pain surrogates (Perissin et al., 2003; Freeman et al., 2008; Kandasamy et al., 2016; Shepherd et al., 2018) (see Supplementary Table 2). Taking into account the limited number of published studies, it is unclear whether this is simply a matter of differences in the sensitivity of the tests used in these studies, or whether the discrepancies are due to distinct mechanisms underlying the production of different aspects of pain in each sex. Additional research is needed with these methods in animals of both sexes to further clarify this issue.

Although sex differences are often expressed as a difference in pain sensitivity, this is not always the case. Differences in the mechanisms underlying pain transmission or modulation have been observed despite similar sensitivities to the painful stimulus in both sexes, and the evidence to date points to the differential involvement of neurotransmitter receptors, cytokines, and even immune cells during pain states in each sex (Sorge and Totsch, 2017; Megat et al., 2018). Although most of these mechanistic studies have been based on standard pain responses, differences between sexes were also seen in some of the novel outcomes presented in Supplementary Table 2. For

example, although both female and male rats exhibited similar gait alterations (specifically weight-bearing differences during motion) after carrageenan-induced knee joint inflammation, female animals were much more sensitive than their male counterparts to P2X7 antagonism-induced analgesia, and this correlated with differential decreases in the level of cytokines and number immune cells at the inflamed site (Teixeira et al., 2017). These findings suggest that the role of P2X7 in the control of joint pain is more prominent in female than in male rats.

In summary, despite the limited number of studies that report sex differences found with these methodologies for pain assessment, the information available to date is sufficient to consider these outcomes suitable to investigate this aspect of the pain phenotype.

7. WHICH METHOD SHOULD I USE?

Each method for observing and analyzing pain outcomes has its strengths and weaknesses. Each of the behavioral outcomes reviewed here has peculiarities that need to be considered before choosing the ones best suited to the needs and aims of each particular research project, as it will be described below.

7.1 The integrative nature of pain-depressed behaviors: a double-edged sword

Pain-depressed behaviors are much more complex than other measures covered here, such as postural changes or the standard reflex withdrawal response, and may reflect the overall effect of pain in rodents under painful conditions (i.e., a combination of hypersensitivity, and spontaneous or ongoing pain). During pain conditions there is a conflict between pain and the performance of these behaviors. Some of them are self-rewarding activities such as wheel running and ICSS (and maybe burrowing behavior),

and decreases in these particular behaviors might also be due to emotional aspects of pain such as anhedonia.

Although the integrative nature of these measures is one of their most notable advantages, this is also a double-edged sword, since they are not pain-specific at all; thus the results can be confounded by other factors that affect well-being apart from pain. This is exemplified by a recent experiment showing that in rodent diabetes models, standard analgesics were unable to restore normal burrowing behavior (Rutten et al., 2018). These findings suggest that sickness behavior due to sustained hyperglycemia, rather than pain per se, may drive the decrease in burrowing. Interestingly, sickness alone was not enough to decrease standard reflex responses in the animals, which showed obvious hypersensitivity denoting the presence of neuropathy (Rutten et al., 2018).

Not only burrowing behavior but also nest building, wheel running, exploratory locomotion and home cage behavior are clear examples of measures that can be altered by a variety of factors such as central nervous system lesions or sickness behavior (induced by lipopolysaccharide administration or infection), among others (Deacon, 2006a and b; Harden et al., 2011; Novak et al., 2012; Jirkof, 2014; Poon et al., 2015; Ahloy-Dallaire et al., 2019; Vichaya et al., 2019). Such factors are potentially important confounders in these types of assay – more so than they may be in standard measures of hypersensitivity. Therefore, validation of each outcome is needed with analgesics (preferably of different pharmacological classes) in the experimental pain model of interest, to ensure that the target measure can be reversed by the analgesic treatment and actually reflects pain rather than other alterations in well-being.

7.2 Are these “new” outcomes suitable for use in all pain models?

The present review and discussion suggest that not all measures are equally adaptable to all pain models. For example, pain-induced ICSS, like changes in wheel running behavior or facial pain expression, appear to be unsuitable for testing neuropathic pain. Gait analysis is probably not the best choice for studying neuropathic pain either, because it has the disadvantage of motor confounders inherent in standard surgically-induced peripheral neuropathic pain models (particularly spared nerve injury) which can mask pain or analgesia. However, changes in burrowing behavior and weight bearing appear to be sensitive to neuropathic pain.

Interestingly, the published time-courses of pain-induced alterations after CFA in several of the newer outcomes were consistently short-term, in contrast to sustained mechanical allodynia. This finding was seen for weight bearing (Huntjens et al., 2009; Cobos et al., 2012), gait alterations (Adams et al., 2016), grip strength deficits (Montilla-García et al., 2017), burrowing behavior (Muralidharan et al., 2016), ICSS (Leitl et al., 2014), wheel running depression (Cobos et al., 2012), and facial expressions of pain (De Rantere et al., 2016). In the case of ICSS and facial expressions of pain, it could be argued that these responses may be self-limited and thus more suitable for testing tonic rather than chronic pain. However, several reports showed that changes in weight bearing, gait alterations, grip strength deficits, and burrowing behavior during other pathological models of pain such as osteoarthritis were maintained for relatively long periods (Chandran et al., 2009; Bryden et al., 2015; Philpott et al., 2017; Carcolé et al., 2019). Therefore, the often-seen recovery of normal values for these outcomes at much earlier time-points than mechanical allodynia may be due to a peculiarity of CFA as a pain model, which does not exert long-lasting functional impairment, rather than to the intrinsic characteristics of these outcomes.

Regardless of the exact reasons for the discrepancies across studies, researchers should be aware that when inflammatory pain is studied with these measures, CFA-induced inflammation rarely induces chronic behavioral alterations apart from reflex-based sensory hypersensitivity. Further research in needed to test these new pain measures in more translational models of inflammatory pain such as collagen-induced arthritis (e.g. Inglis et al., 2007; Nieto et al., 2016), and in mutant mice which spontaneously develop arthritis (e.g. Auger et al., 2016).

7.3 Some considerations for drug testing

As previously commented in their respective sections, all pain-depressed behaviors, together to the assessment of grip strength deficits, share the potential to distinguish between substances producing sedation or motor impairment and true analgesic effects, since drugs that cause these effects are expected to further depress the target behavior rather than to restore it, and therefore will not induce analgesic-like effects – in contrast to the effects of these drugs on reflex-based outcomes. Therefore, the assessment of pain-depressed behaviors and grip strength can provide valuable information that complements the findings obtained with standard sensory tests.

It is worth noting that most of the novel methods for pain assessment described here, e.g. all pain-depressed behaviors and facial pain expressions, are not suitable for determining the precise time-course of drug effects, since these methods require the target behavior to be recorded for a relatively long period to obtain reliable data. This is a disadvantage over traditional, standard measures of hypersensitivity, in which recordings typically last a few seconds and therefore allow measurements to be recorded at very specific time-points. The only exceptions may be measurements of weight-bearing differences and grip strength deficits, which can be readily recorded in

short intervals.

Home cage wheel running provides a continuous recording of the animal's behavior. However, since this is an elective behavior, when mice are given unrestrictedly access to a running wheel each animal shows peaks of activity at random time-points during the night period (when rodents exhibit most of their activity). This makes it challenging to use home cage wheel running for analgesic drug testing, because there is no certainty that the animal's activity will parallel the drug effects. Some methods used to record nesting behavior, in particular those which allow the animals to build a complex nest overnight, are potentially problematic for drug testing, since the effects of putative analgesics might wear off before the end of prolonged experimental protocols, and the results might consequently be negative or inconclusive despite the successful induction of analgesia. As described in the preceding sections, wheel running and nesting behaviors can be observed with considerably shorter protocols, and this option can clearly facilitate drug testing.

8. WILL THE NEW PAIN OUTCOMES REPLACE STANDARD MEASURES OF SENSORY HYPERSENSITIVITY?

Methods to study pain-related behaviors have been evolving since the beginning of preclinical pain research in a continuous effort to approximate the evaluation of pain in experimental animals to the human pain phenotype. A timeline of the initial development of key behavioral pain measures is shown in Figure 7, and the number of published articles that have reported different types of outcomes since the early 1980s up until 2018 is shown in Figure 8. This information gives us an idea of the time needed in the past for new pain measures to become accepted and established in preclinical laboratories.

We believe pain researchers would generally agree that the development of models of tonic nociception was a significant conceptual advance in pain research. The tonic pain models used most widely were developed in 1957 (the writhing test) and in 1977 (the formalin test) (Fig. 7), yet in 1993-1997 these models were still used much less often than the classic tests for nociceptive heat pain (Fig. 8 and Supplementary Table 1), which were developed in the 1940s (e.g. the tail flick test in 1941, and the hot plate test in 1944), as shown in Figure 7. This observation is consistent with a previous study covering each year from 1970 to 1999 (Le Bars et al., 2001). However, the use of these models of tonic pain increased steadily, and by 2003-2007 (roughly 45–50 years after the description of the writhing test, and 25–30 years after the original description of the formalin test) their frequency of use was similar to tests for acute heat nociception. In the most recent period covered here (2014-2018) tonic nociception overtook nociceptive heat pain in terms of the numbers of articles based on each type of measure (Fig. 8 and Supplementary Table 1).

Another important conceptual change in pain behavior was the development of measures of hypersensitivity, which paralleled the development of several pathological pain models. With the exception of the paw pressure test, described in 1957 (the same year as the writhing test), the other most popular measures of hypersensitivity were developed much later (Hargreaves test in 1988, von Frey test in 1990 and acetone test in 1994) (Fig. 7). These methods were apparently being adopted by the preclinical pain research community at a faster pace than tonic pain models, but one decade ago (in 2003-2007) they were still being used at a frequency similar to nociceptive heat pain or tonic pain models (Fig. 8 and Supplementary Table 1). It was only recently, in 2014-2018, when hypersensitivity measures clearly dominated preclinical pain research over any other measure, to the point of slightly exceeding the sum of studies that used

nociceptive heat pain or tonic pain models (Fig. 8 and Supplementary Table 1). The current use of hypersensitivity measures clearly indicates a consensus among preclinical researchers regarding the closer proximity of these measures to the human pain phenotype in pathological pain models compared to acute heat or tonic nociception. Therefore, although historical shifts are evident in the experimental paradigms used by the preclinical pain research community, change has occurred at a slow, more or less steady pace more akin to a marathon than to a high-speed sprint.

Although some non-reflex pain measures were initially tested decades ago (e.g. exploratory locomotion or weight-bearing changes) (Fig. 7), these measures appear to have remained in an almost quiescent state for decades. In recent years since 2000, and probably motivated by the lack of translation of new analgesic drugs to clinical practice, the use of additional behavioral pain outcomes has gathered momentum (Fig. 7). Although the number of studies using these measures is currently small in comparison to any of the most common behavioral tests (Fig. 8 and Supplementary Table 1), the use of these additional measures is gaining popularity. In fact, in 2003-2007 we found only 96 research articles using any of these nonstandard measures, whereas in 2014-2018 the number had increased to 473 studies – a roughly 5-fold increase (Fig. 8 and Supplementary Table 1). Another aspect that should be taken into account is the fact that negative results tend not to be published (Fanelli, 2012), although some such studies might find that these novel outcomes are not significantly altered by a pain state. The present analysis may thus have underestimated the real use of these pain outcomes by the preclinical pain research community. Although the increasing trend in the number of published reports based on these outcomes is only a proxy for their real use, this increase parallels what we observed for measures of hypersensitivity in 1993-1997, and is expected to continue in the future.

None of the behavioral tests examined here has disappeared from the repertoire of pain research methods, not even measures of nociceptive heat pain – which have been used for more than 70 years (Fig. 8 and Supplementary Table 1). Therefore, these new measures are not expected to replace current standard tests, but instead will be slowly added to the set of standard pain measures in a process that, in view of the slow pace at which new behavioral tests have historically become established, will likely take decades and much effort.

Also, it is important to note that most standard measures of hypersensitivity have well-known advantages for drug development. All known analgesic drugs used clinically in humans are able to decrease sensory hypersensitivity in rodents. Therefore, the ability to decrease these standard rodent pain outcomes is probably a necessary quality for a drug to be considered clinically useful. However, failure to explore whether a novel putative analgesic drug is able to improve other relevant aspects of the pain phenotype is undoubtedly risky, since it might lead to the selection of drugs that, although able to affect hypersensitivity, have a limited analgesic profile and no effects on other important components of pain. In addition, drugs with a prominent effect on the these additional components of pain but with lower effects on sensory hypersensitivity might be discarded if drug testing is based exclusively on standard pain measures, even though at least some of these drugs might merit further testing.

9. CONCLUSIONS

In summary, we have examined trends in the incorporation of some novel behavioral pain outcomes to the repertoire of pain assessment methods in preclinical research. We also reviewed the evidence for the usefulness and limitations of these new approaches, with the ultimate aim of facilitating the selection of the most suitable methods by

preclinical laboratories. These measures are gaining popularity, and their use is expected to increase. We believe that these pain measures have the potential to profoundly benefit pain research and analgesic development by providing nonoverlapping information that extends and complements the findings standard pain measures are able to provide. These new methodologies not only complement stimulus-induced reflex measures, but are also critical to gain a more complete understanding of pain in rodents and the effects of newly developed analgesics within a more holistic approach.

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12. FIGURES

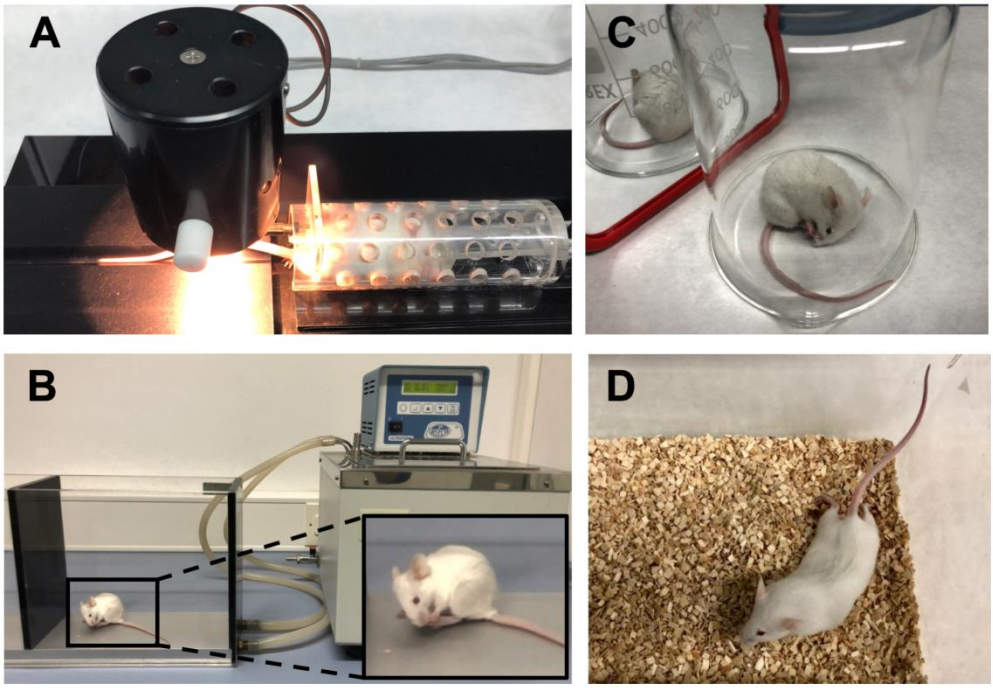

Fig. 1. Representative pictures of standard nociceptive heat pain tests (tail flick and hot plate tests) and the classic models of tonic nociception (writhing and formalin tests). (A) A mouse restrained in a plexiglass tube inside a tail-flick apparatus ((LI 7100, Letica, S.A., Spain). (B) A mouse licking its hindpaw on a hot plate set at 50°C (thermal analgesiometer Model PE34, Series 8, IITC Life Science Inc., Los Angeles, CA, USA). (C) A mouse performing a typical writhing response after the intraperitoneal administration of diluted acetic acid. (D) A mouse inside a glass cylinder (1L beaker) licking its hindpaw after the intraplantar injection of formalin.

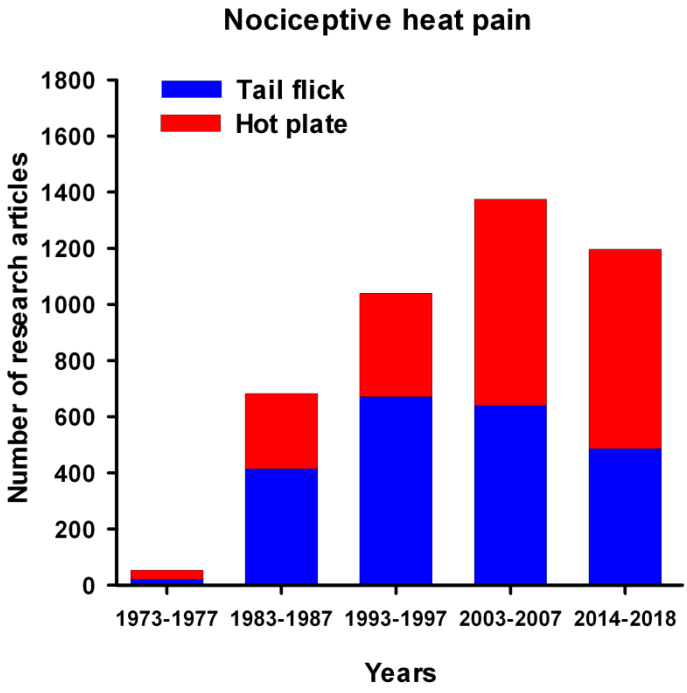

Fig. 2. Time-course of the number of original articles in English that used nociceptive heat pain (tail flick and hot plate) tests.

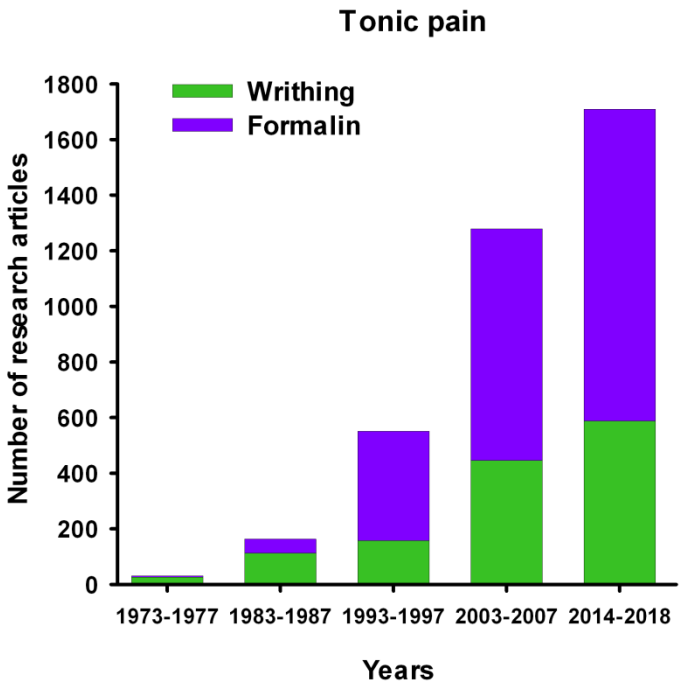

Fig. 3. Time-course of the number of original articles in English that used tonic pain models (writhing and formalin tests).

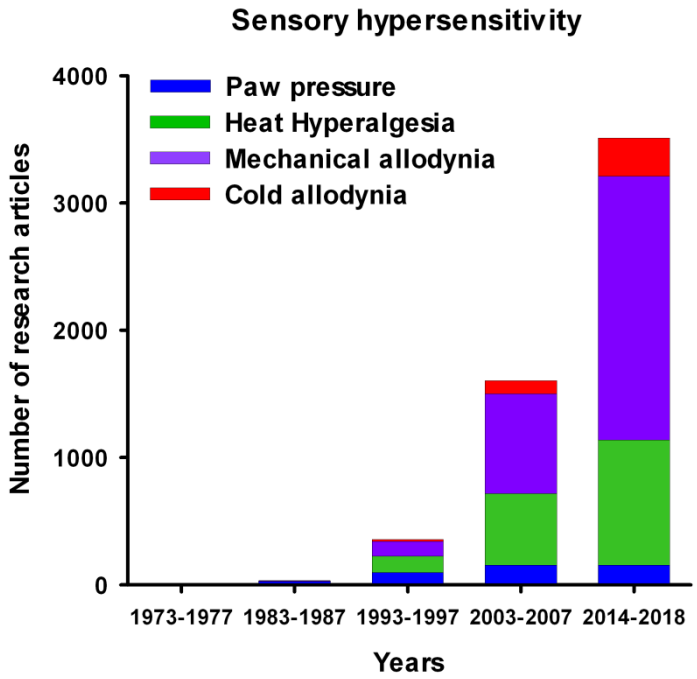

Fig. 4 Time-course of the number of original articles in English that used measures of sensory hypersensitivity including mechanical allodynia, heat hyperalgesia, cold allodynia and the paw pressure (Randall–Selitto) test.

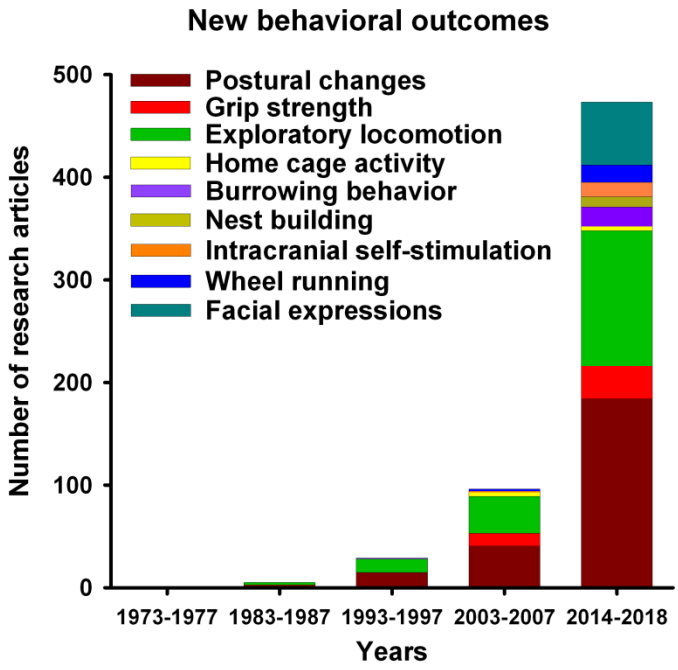

Fig. 5. Time-course of the number of original articles in English that used new behavioral outcomes in pain research.

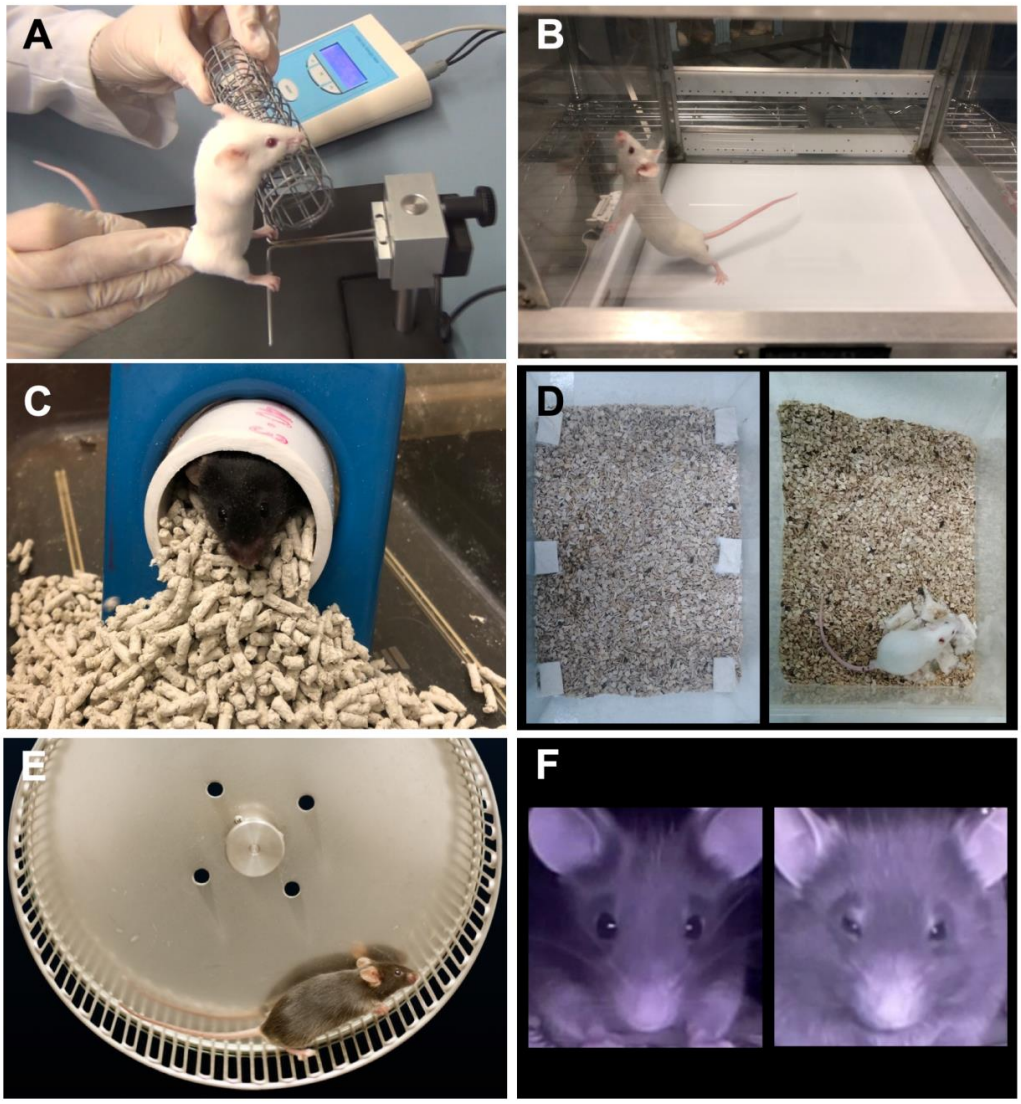

Fig. 6. Some examples of novel behavioral tests that can be used as pain indicators in rodents. (A) Grip strength (grip strength meter, Model 47200, Ugo-Basile, Varese, Italy), (B) exploratory activity in an electronically-controlled actimeter equipped with infrared detectors (activity cage, Med Associated Inc., St Albans, VT, USA), (C) burrowing behavior (home-made device), (D) nesting behavior (left panel: six pieces of nestlet placed at distant sites in the home cage; right panel: all nestlet pieces grouped in a corner after 100 min), (E) wheel running behavior (activity wheel, Bioseb, Boulogne, France), and (F) facial expressions of pain (left panel: naive mouse; right panel: mouse after intraperitoneal administration of cyclophosphamide). (Images were obtained with a Kuman infrared camera, Shenzhen, China).

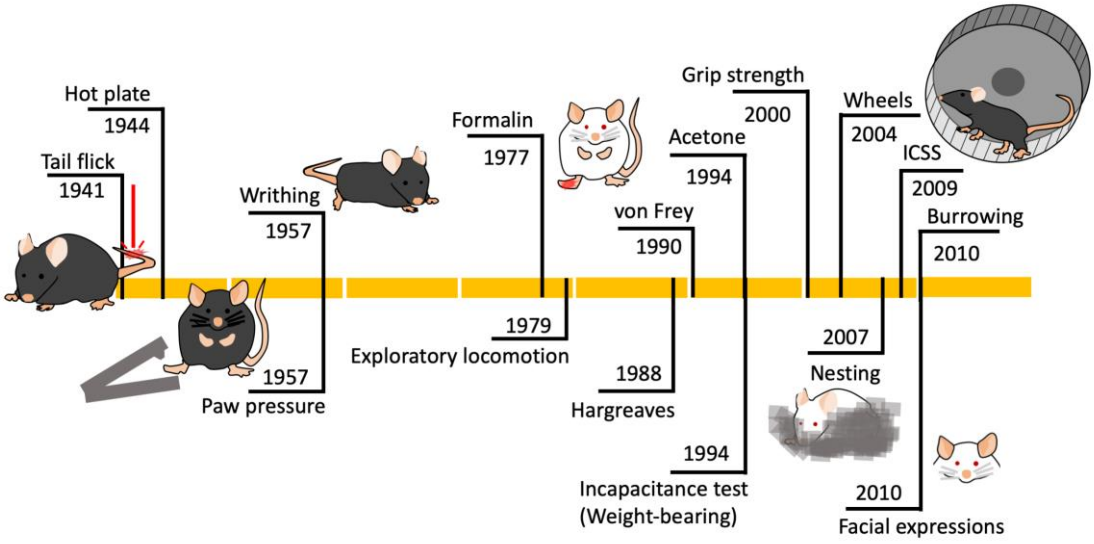

Fig. 7. Timeline of the initial use of different pain measures. The publication year of the first study describing each measure is highlighted.

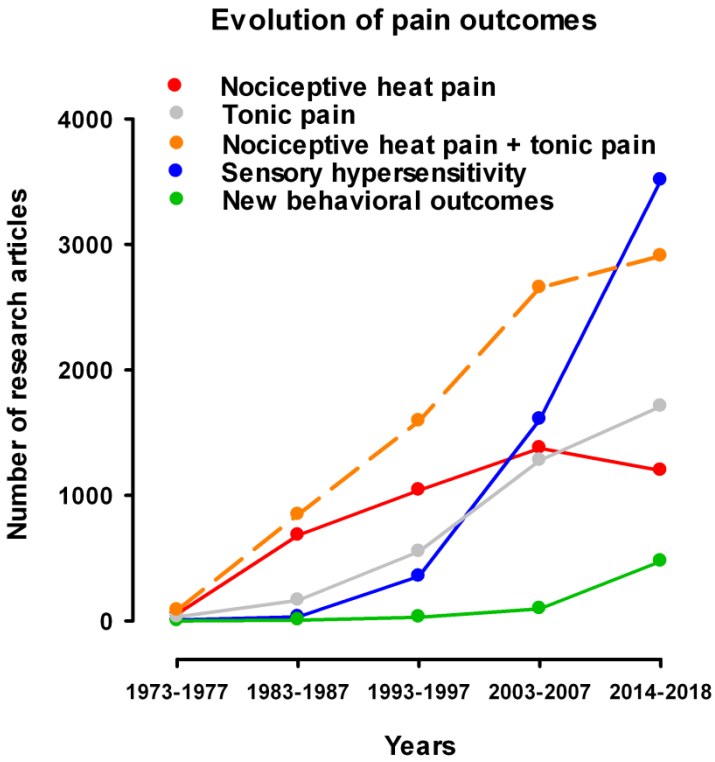

Fig. 8. Summary time course of the number of original articles in English that used all behavioral pain outcomes discussed in this article.

13. TABLES

Table 1. Terms used in the Medline searches of pain studies. These terms were used together with terms to identify pain studies (pain, nociceptive, nociception or analgesia), terms to retrieve preclinical studies (mouse, mice, rat or rodent), and the selected years for each period.

Type of assay	Outcome measures	Terms used in the Medline searches
Heat nociception	Tail flick	Tail-flick; tail flick
	Hot plate	Hot plate
Tonic pain	Formalin test	Formalin
	Writhing test	Writhing; abdominal constriction; abdominal stretching
Sensory hypersensitivity	Mechanical hyperalgesia	Paw pressure; Randall–Selitto
	Heat hyperalgesia	Hargreaves test; plantar test; heat hyperalgesia; thermal hyperalgesia
	Mechanical allodynia	von Frey; tactile allodynia; mechanical allodynia; dynamic allodynia
	Cold allodynia	Acetone; cold allodynia
Physical function	Postural changes	Gait; walk; postural changes; weight bearing; weight asymmetry; incapacitance
	Grip strength	Grip strength; grip force
Pain-depressed behaviors	Exploratory locomotion	Locomotion; exploration; exploratory behavior; locomotor behavior; locomotor activity; rearing; horizontal activity; vertical activity; open field
	Home cage activity	Home cage
	Burrowing	Burrowing; burrow
	Nesting	Nesting; nest
	Intracranial self-stimulation	Intracranial self-stimulation
	Wheel running	Wheel running; running wheel; activity wheel; wheel behavior
Facial expressions	Facial expressions	Grimace; facial expression; orbital tightening

Table 2. Characteristics of the most common rodent behavioral tests used to measure pain-induced alterations in physical function.

Type of assay	Outcome	Advantages	Limitations
Postural changes	**Weight-bearing asymmetry**	Validated as a pain outcome in many pain conditions	Drug-induced sedation or motor impairment might be erroneously interpreted as analgesia
		Appears to be highly sensitive in detecting drug-induced analgesia	Usefulness unclear in pain models not involving unilateral hindlimb injury
	Gait alterations	Validated as a pain outcome in many pain conditions	Less sensitive to drug-induced analgesia than weight-bearing asymmetry or von Frey testing
			Its use as a pain-related outcome is questioned in peripheral nerve injury (unsuitable as a pain index after spared nerve injury due to neuropathy-induced motor confounders)
			Drugs might alter gait patterns
Grip strength	**Grip strength deficits**	Widely used in clinical research and clinical practice	Cannot be used in surgically-induced peripheral neuropathic pain
		Sedative drugs will not induce analgesic-like effects	
		Particularly useful in musculoskeletal disorders	

Table 3. Characteristics of the most common rodent behavioral tests used to measure pain-depressed behaviors.

Type of assay	Outcome	Advantages	Limitations
"Natural" behaviors	**Exploratory locomotion**	Sedative drugs will not induce analgesic-like effects	Drug-induced stimulation of motor activity can result in false analgesic-like effects Anxiolytic (nonanalgesic) drugs can improve thigmotactic behavior
		Device available in many laboratories for testing drug-induced toxicity (or other effects unrelated to pain)	Limited sensitivity in detecting pain-induced alterations
			Not suitable for repeated measures
	Home cage activity	Measure of "daily living" in experimental animals	Limited sensitivity in detecting pain-induced alterations
			Requires costly equipment
	Burrowing behavior	Has been validated as a pain outcome in many pain conditions	Results can vary considerably depending on the substrate used
		Sedative drugs will not induce analgesic-like effects	
		Easy and cheap to build a home-made burrow	
	Nest building	Sedative drugs will not induce analgesic-like effects	No clear consensus on the best method
		No specific device needed	Time of day influences the behavior
			More experience in different pain models is needed
Other pain-depressed behaviors	**Intracranial self-stimulation**	Sedative drugs will not induce analgesic-like effects	The standard models of surgical sciatic nerve injury do not depress this measure
		Study of the analgesic and rewarding properties of drugs in the same experimental setting	
		Detailed pharmacological characterization	Complex methodology
	Wheel running depression	Sedative drugs will not induce analgesic-like effects	No alterations in several models of chemically- or surgically-induced peripheral neuropathic pain
		Validated as a pain outcome in many pain conditions	Careful assessment of whether a short or long evaluation period should be used
		Appears to be highly sensitive in detecting drug-induced analgesia	

Table 4. Characteristics of facial expression changes as a pain measure in rodents.

Type of assay	Outcome	Advantages	Limitations
Facial expressions	**Grimace scale**	Highly evolutionarily conserved response (in humans and other mammals)	No alterations in standard models of surgically-induced nerve injury (an exception is infraorbital nerve constriction)
		Set-up easy; low cost	Time-consuming analysis (although it can be sped up with specific software)