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Studying ongoing and spontaneous pain in rodents – challenges and opportunities

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Abstract

The measurement of spontaneous ongoing pain in rodents is a multiplex issue and a subject of extensive and longstanding debate. Considering the need to align available rodent models with clinically relevant forms of pain, it is of prime importance to thoroughly characterize behavioral outcomes in rodents using a portfolio of measurements that are not only stimulus-dependent but also encompass voluntary behavior in unrestrained animals. Moreover, the temporal course and duration of behavioral tests should be taken into consideration when we plan our studies to measure explicit chronic pain, with a particular emphasis on performing longitudinal studies in rodents. While using rodents as model organisms, it is also worth considering their circadian rhythm and the influence of the test conditions on the behavioral results, which are affected by social paradigms, stress and anxiety. In humans, general wellbeing is closely related to pain perception, which also makes it necessary in rodents to consider modulators as well as readouts of overall wellbeing. Optimizing the above parameters in study design and the new developments that are forthcoming to test the affective motivational components of pain hold promise in solving inconsistencies across studies and improving their broad applicability in translational research. In this review, we critically discuss a variety of behavioral tests that have been developed and reported in recent years, attempt to weigh their benefits and potential limitations, and discuss key requirements and challenges that lie ahead in measuring ongoing pain in rodent models.

Introduction

Despite major efforts and a large drive in preclinical research, a huge translational gap still exists between human and rodents. Chronic pain is not sufficiently understood in the currently available preclinical rodent models (Mogil, 2009; Berge, 2011) and the translation of insights observed to human chronic pain has remained largely problematic. Although this has several reasons, including redundancy between molecular mediators and pathways as well as side effects of developed therapeutics, a major limitation is given by the current deficiencies in addressing the full spectrum of spontaneous pain states that are difficult to study in rodents. Chronic pain in humans is very complex in nature and is not only represented by stimulus-evoked pain and changes in nociceptive thresholds, but also by ongoing pain conditions. In particular, the affective, emotional and cognitive components, which influence the daily quality of life, are difficult to assess in rodents.

While humans can address standardized questionnaires, and give defined ratings and detailed descriptions for pain episodes with their occurrence and quality, in rodent subjects we have to employ a series of surrogate measures and analyse components of their wellbeing. Unfortunately, this is far from straightforward and the currently available and recently developed measures for spontaneous and ongoing pain in rodents are indeed a topic of some debate. We still lack clearly defined paradigms, although there have been recent attempts to build up on the affective component of pain with measurable surrogate paradigms, such as voluntary wheel running (Cobos *et al.*, 2012), home-cage monitoring (Urban *et al.*, 2011), burrowing (Andrews *et al.*, 2012), passive avoidance (LaBuda & Fuchs, 2000), place preference (King *et al.*, 2009) and facial expression analysis (Langford *et al.*, 2010).

Another major factor to keep in mind is that limitations in measures of pain go hand-in-hand with limitations in animal models of chronic pain. Thus, the very large body of literature which has built up over decades relies nearly solely on common models of inflammatory and neuropathic pain. These models rely on measurements of the easily accessible hindpaw region of the animals but we should also consider other prevalent chronic pain conditions in humans that still lack appropriate corresponding preclinical animal models mimicking these conditions, such as diabetic neuropathic pain, trigeminal neuralgia, chronic back pain, phantom limb pain and headache.

In this review, we discuss key requirements from tests for spontaneous, ongoing pain in rodents and the inherent challenges. We then present recent developments and attempts to monitor behavioral changes in rodents and critically discuss the advantages, limitations and bottlenecks.

We will not discuss the use and effect of pharmacological interventions in the described rodent models, as many drugs in

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preclinical models are mostly given once and their effect on pain measurements is beyond the scope of this review to evaluate the behavioral approaches. Nonetheless, it would be important to consider potential general effects or side-effects of analgesics, e.g. sedation or motivational changes, which might influence the test paradigms and readouts with respect to spontaneous behavior.

Challenges in studying spontaneous pain in rodents and key requirements

Short tests with minimal effort to obtain significant changes in measurable behavioral parameters are most desirable as they permit analysis of many mice over short durations. Standard laboratory conditions include a non-inverted day–night cycle and behavioral measurements are performed during a short time over a limited time window during the day, when rodents are naturally more inactive. While this may suffice for measuring activation thresholds to nociceptive stimuli, given the fluctuations in spontaneous pain in humans, appropriate tests need to be designed to include observer-independent long-term observations of rodents. Thus, we need to consider long-term day and night measurements, particularly in the context of behavior related to the natural wellbeing of rodents in chronic pain states. Furthermore, to truly study chronic pain, it will be important to design longitudinal studies in rodents that span longer periods of time and involve multiple, phase-locked measurements.

There is a current need for standardized procedures, as the reproducibility of tests is often a problem, owing not only to insufficient methodological descriptions but also to differences in factors such as genetic background, sex, laboratory environmental factors and diet (Crabbe *et al.*, 1999; Shir & Seltzer, 2001; Noel *et al.*, 2009).

Not only should analyses in rodents closely mimic human conditions to aid translation, but they should also encompass pain-related emotional and affective states, such as anxiety, depression and changes in social interactions, which significantly impact on the quality of daily life in chronic pain patients. Importantly, anxiety and depression are key comorbidities in chronic pain patients, affecting about 20–40% of this population (Meyer-Rosberg *et al.*, 2001; Twillman, 2007). However, these factors are generally excluded from experimental planning in rodents.

Additionally, animals are either housed individually or in cohorts (to save cage space, mimic natural gathering, prevent social deprivation or prevent fighting and reciprocal wound biting), circumstances that involve stress conditions and have been shown to affect pain behavior, as social structures are very important in determining rodent behavior (Bravo *et al.*, 2013; Vachon *et al.*, 2013).

Moreover, because behavioral outcomes in diverse test systems have been observed to be very divergent, it has been suggested that each test may reflect different emotional aspects, thereby emphasizing the necessity of using multiple tests to study co-morbidities of chronic pain (Parent *et al.*, 2012). There appears to be a tug-of-war in deciding between the use of diverse tests on the same animal to comprehensively study various pain-related behaviors on the one hand and the need to protect the rodent subject from stress, which inevitably comes from continuous handling and divergence of tasks, on the other.

From the discussion above, it is evident that a combination of diverse behavioral tests addressing sensory thresholds, evoked pain, spontaneous and ongoing pain as well as diverse pain comorbidities and components is required to create an overall picture of the complex multidimensional state of chronic pain. However, this can impact on stress levels and the general wellbeing of the rodent subjects, and thereby further affect pain-related behavior. So, questions arise about how many and which tests can be combined in which order and what are the most appropriate time points of a particular behavior in an individual model.

Recently, many groups have started employing stimulus-independent, voluntary tests to assess pain behavior in rodent models, although these involve testing of different behavioral paradigms at different daily testing times and durations across different animal strains, thereby rendering overall interpretations and reproducibility difficult. It would be helpful to have standardized operational descriptions of how to use, when to apply and how many test combinations can be applied to the same rodent cohort without triggering stress and anxiety based upon over-handling the animals.

On the other hand, we should also consider that the behavioral tests discussed in this review use unrestrained, freely moving animals and can be performed within their home-cage environment or in a stress-reduced environment, which contributes to reducing suffering of laboratory animals.

New tests and measures – rationale, advantages, drawbacks and modifications

All measures and tests that are discussed in this review are summarized in Table 1.

Weight bearing

In rodent models involving induction of inflammatory, neuropathic or cancer pain in hindpaws/hindlimbs, there are different systems available for measuring the weight distribution of paws, with a view towards representing the change in hindpaw weight distribution between the affected and unaffected hindlimb or hindpaw. Older measurement devices such as static weight-bearing or the incapacitance tests require restraining of the animals, leading to false positive or negative results. Restraining the animals can elicit stress responses (Mogil, 2009) and thereby lead to either stress-induced analgesia (Terman *et al.*, 1984) or stress-induced hyperalgesia (Imbe *et al.*, 2006). The advantage of the static test is that it is an objective measure. Newer measures are subdivided into either dynamic weight-bearing (e.g. Dynamic Weight Bearing system, Bioseb, France) or systems for gait analysis (e.g. CatWalk; Noldus, Wageningen, the Netherlands; DigiGait; Mouse Specifics, Inc., Quincy, MA, USA; TreadScan; CleverSys Inc., Reston, VA, USA), which enable studying weight-bearing in freely moving rodents.

Static and dynamic weight-bearing has been used in diverse pain models; for example, significant differences in hindpaw weight distribution were observed up to 21 days following peripheral hindpaw inflammation with complete Freund's adjuvant (CFA) in mice (Cobos *et al.*, 2012; Robinson *et al.*, 2012) or in rats (Tetreault *et al.*, 2011), over 70 days in an osteoarthritis rat model (Combe *et al.*, 2004), in the chronic constriction nerve injury model (CCI) and femoral cancer model in mice for minimally 21 days (Tetreault *et al.*, 2011), in the hindlimb carrageenan model in mice (Lolignier *et al.*, 2011) and in different models of knee joint arthritis in rats (reviewed by Neugebauer *et al.*, 2007). However, most previous studies have employed static weight-bearing models, with the caveats outlined above, and new studies with dynamic weight-bearing are required, particularly in the context of longitudinal studies rather than single time-point measurements.

Gait analysis imaging systems have been used to analyse significant gait changes in arthritis models in rats (e.g. Otsuki *et al.*, 1986; Clarke *et al.*, 1997; Ferreira-Gomes *et al.*, 2008; Angeby Moller *et al.*, 2012). Recently, both the 'DigiGait' and 'TreadScan' systems

have been directly compared to analyse gait changes in a monoarthritis model in rats; however, neither system showed reproducibility or consistent results (Dorman *et al.*, 2014).

Mogil *et al.* (2010) showed significant gait changes in the spared nerve injury (SNI) model of neuropathic pain in 22 mouse strains using the 'CatWalk' system. These changes were detected and peaked just 1 day post-surgery and lasted shorter than the observed mechanical allodynia. This led to the interpretation that the mechanisms of spontaneous and evoked pain are distinct and that spontaneous neuropathic pain in mice cannot be assessed using locomotion and gait analysis (Mogil *et al.*, 2010). Similar results have been published by Lau *et al.* (2013), who could not detect any correlation between weight-bearing and allodynia in the SNI model in rats. Piesla *et al.* (2009) found no correlation between weight-bearing and nerve-injury related models in rats (SNI and CCI), unlike in an inflammatory Carrageenan model, using the DigiGait system, leading to the assumption that postural changes in SNI are based on motor dysfunction rather than sensory abnormalities. Thus, although dynamic weight-bearing holds considerable promise, it remains to be seen whether gait-analysis systems can be broadly put to use in determining ongoing pain over diverse models.

Interestingly, two recent studies have employed modifications of dynamic weight-bearing. One study combined weight-bearing with a tunnel system in which water-deprived rats were force to walk from a bright illuminated starting box through a weight sensor tunnel to reach an arrival box with a water bowl (Min *et al.*, 2001). One caveat of this system is that water-deprivation distresses the animals and affects their overall feeling of wellbeing, which, in turn, can affect pain-related behavior. A similar test set-up has been used very recently to force rats with spinal cord contusion to travel across an unpleasant surface in a tunnel, but without water or food deprivation (Lau *et al.*, 2012). This so-called 'Mechanical Conflict-Avoidance System' (Coy Labs, Grass Lake, MI, USA) could constitute a very useful, new measurement system to analyse pain behavior in freely moving rodents with partial freedom of decision and voluntary control and needs to be tested across diverse models and pain states as well as being validated in mice.

Activity measurements

Automated systems are available to monitor, analyse and register specific behaviors in an observer-independent manner. For example, the open field test can be used to measure horizontal and vertical activity as well as distance traveled to detect behavioral changes. Video camera-based tracking softwares are available to enable observer-independent and fully automated tracking of animal behavior, such as ANYMaze (Ugo Basile, Varese, Italy) and EthoVision-System (Noldus). Recently, automated systems have become available to record natural behavior of animals in their home-cage, not only including the above-mentioned activity parameters, but also behaviors such as grooming, drinking, eating and climbing. These include the 'Laboratory Animal Behavior, Observation, Registration and Analysis System' (LABORAS; Van de Weerd *et al.*, 2001) as well as custom-made, non-commercial devices (e.g. Goulding *et al.*, 2008).

The principal advantage of home-cage monitoring is given by the ability to perform long-term longitudinal studies in a familiar environment, one potential limitation being that animals have to be kept individually. It has been suggested that home-cage monitoring is more sensitive to detect pain-related behavior than acutely performed locomotor analyses (Urban *et al.*, 2011). This is particularly important because attempts to monitor spontaneous pain with signs of flinching, licking or guarding the paw acutely over short durations of time have either failed or not been entirely consistent across laboratories (Mogil & Crager, 2004; Mogil, 2009). Analysing modifications of changes in the spontaneous posture of the affected hindlimb (Attal *et al.*, 1990) or increased scratching behavior (Kupers *et al.*, 1992) have long been suggested as pain-related behavior in rat models of peripheral mononeuropathy. Since then, spontaneous paw lifting has been demonstrated in different nerve axotomy and nerve ligation models (Djouhri *et al.*, 2006), in the SNI model in rats (Lee *et al.*, 2012) and the spinal nerve ligation (SNL) model in rats (Kawasaki *et al.*, 2008) as well as in the CFA model in rats (Djouhri *et al.*, 2006) or in an inflammatory carrageenan and monoarthritis model in rats (Lolignier *et al.*, 2011). Other laboratories have also measured paw licking and paw flinching as signs of spontaneous pain in a mouse model of cancer pain (Asai *et al.*, 2005). On the other hand, no spontaneous paw lifting, licking or flinching has been found in a mouse model for CCI (Mogil *et al.*, 2010). It has been suggested that spontaneous foot lifting is driven by C-fiber firing rate (Djouhri *et al.*, 2006) and its presence might thereby depend on the injury model itself. Further evaluation and consistent analysis of comparable parameters with respect to spontaneous behavioral paw changes will help to evaluate this longstanding attempt to measure spontaneous pain. Importantly, most studies have been limited to a short span of close monitoring or are semiquantitative in nature. Therefore, exploiting the full potential of home-cage monitoring over the development and maintenance of chronic pain has yet to be performed.

The potential and promise of home-cage monitoring can be further improved if software systems and tracking options are better optimized to detect movements that are targeted specifically towards pain-related behaviors. Recently, a new instrument for measuring observer-independent behavior using video and vibration has been published (Brodkin *et al.*, 2014). The so-called 'behavioral spectrometer' can record detailed behavioral measurements for 23 unique behaviors related to diverse rearing, grooming, orienting and ambulatory parameters in mice. In the carrageenan model of inflammatory pain, a broad range of significant anatomically restricted behavioral differences were recorded compared with control mice (Brodkin *et al.*, 2014).

In many studies, the open field test has been employed to explore behavior of the animals in a novel environment with free access to a relatively large open field, which also represents a measure of anxiety-like behavior. A reduction in open field behavior, demonstrated as reduced number of rearings and distance traveled, has been shown in models of carrageenan-induced inflammatory pain, acetic acid-induced acute visceral pain and the streptozotocin (STZ) model of diabetic neuropathic pain in mice (Cho *et al.*, 2013), whereas no behavioral deviations have been detected in mice following SNI (Urban *et al.*, 2011; Cho *et al.*, 2013), CCI (Mogil *et al.*, 2010; Urban *et al.*, 2011) or CFA (Urban *et al.*, 2011). In a CFA rat model (Parent *et al.*, 2012) no differences were observed in vertical distance, but an increased anxiety-like behavior was detected, which is represented as reduced time in the central squares of the open field as compared with the borders (Parent *et al.*, 2012). Reduction in vertical and horizontal distance traveled in the open field test was reported in rats with knee joint inflammation (Matson *et al.*, 2007; Rutten *et al.*, 2013).

The advantage of the open field system is that a variety of behavioral parameters related to movement as well as exploration and anxiety are measurable within a short time frame, it neither requires habituation nor restrainment, is free of reflexive or evoked behaviors and relies entirely on objective behaviors. Furthermore, the open

field behavior can be used to test the analgesic effect of pharmacological components and thereby to exclude sedative effects. However, the method is not suitable for repetitive usage, which precludes longitudinal measurements, and has yet to be validated in a wide set of chronic pain models.

Free-choice thermal preference tests

To circumvent analysing and interpretation problems with the measurement of reflexive behaviors to heat or cold stimuli, observer-independent, free choice temperature tests have been established. Freely moving animals can choose their comfort temperature zone between two adjacent plates with different surface temperatures, a further test parameter being the measurement of escape latency from one to another plate. Commercially available systems (Thermal Place preference test, Bioseb) or various custom-made systems (e.g. AlgoTrack system, Baliki *et al.*, 2005), or double plate technique (Walczak & Beaulieu, 2006) have been reported to allow the investigation of cold allodynia or heat hyperalgesia in different neuropathic pain models in mice [SNI, CCI, partial sciatic nerve ligation (PSL) and chronic constriction of the saphenous nerve (CCS; Baliki *et al.*, 2005; Walczak & Beaulieu, 2006)]. Since then, the temperature preference test has mostly been used to characterize the temperature sensitivity in studies of knockout mice (e.g. Noel *et al.*, 2009; Mishra *et al.*, 2010).

The Rotterdam Advanced Multiple Plate (RAMP) method, a multiple plate assay, has recently been developed, based on four adjacent plates with different temperatures, to assess cold allodynia in SNI rats (Duraku *et al.*, 2013). Another difference between this and the other two plate preference tests is that the four test-plates are placed in a dark box with constant low background noise, whereas the other plate preference tests are performed under normal daylight in open transparent compartments. The RMAP test has been introduced as an alternative to diverse paw lift tests, such as the acetone test or the hot and cold plate test, which involve measurement of the latency to the paw lifting. Neuropathic mice (SNI) have been reported to show a transient peak in cold allodynia at 3 weeks and a slowly developing cold hyperalgesia at 7 weeks (Duraku *et al.*, 2013). This test truly holds tremendous promise, particularly because it is performed in a closed compartment so that test-influencing factors such as laboratory environmental factors (light, smell, noise) or the visual presence of the observer can be excluded. One important point to consider is that the test paradigm is very complex and might lead to increased stress levels in the animals based on the large choice of diverse temperatures.

Beside simple temperature choice test paradigms, attempts have been made to combine a learned operant and innate reflex response to a thermal stimulus. For example, an operant escape task in which animals could choose between a temperature-regulated floor-plate and a brightly-lit shelf in climbing distance with comfortable temperature has been reported (Mauderli *et al.*, 2000; Vierck *et al.*, 2003).

Thus, there are a number of tests available and numerous modifications are being reported to analyse operant free-choice behavior in rodents in conjunction with nociceptive sensitivity and pain.

Voluntary wheel running activity

Another attempt to objectively measure pain in freely moving animals is by voluntary wheel running, which has been shown an effective measure in mice or rats with CFA-induced hindpaw inflammation (Cobos *et al.*, 2012; Grace *et al.*, 2013), in an osteoarthritis rat model (Stevenson *et al.*, 2010) and in an inflammatory muscle pain model in mice (Pratt *et al.*, 2013), but not in the rat plantar Formalin model of early nociceptive hypersensitivity (Grace *et al.*, 2013). However, Cobos *et al.* (2012) only observed a significant reduction of voluntary wheel running when CFA was injected bilaterally over the first 2 days following CFA injection in a 1-h daily running attempt. The time course of reduced running distance was consistent with changes in the dynamic weight-bearing test with unilateral hindpaw inflammation but did not correlate with the time window of mechanical hypersensitivity, which lasted much longer (Cobos *et al.*, 2012). On the other hand, it is unclear why the intraplantar injection of Formalin leads to a long-term decrease in mechanical thresholds but no alteration in voluntary wheel running (Grace *et al.*, 2013).

It is unclear if decreased voluntary wheel running is based on decreased willingness to perform the task because of ongoing pain or by the mechanical hypersensitivity triggered by movement (Cobos *et al.*, 2012). Alternatively, changes in voluntary wheel running may constitute an indicator for daily wellbeing of the animal, which is undoubtedly also an important component in the overall picture of chronic pain. Unilateral hindpaw inflammation was observed to be insufficient in inducing changes in voluntary wheel running, which were only observed when bilateral paw inflammation was induced (Cobos *et al.*, 2012), a model for which clinical relevance has not been established. However, it is possible that an observation period longer than just a single hour per day would lead to different outcomes. In general, we should not underestimate behavioral changes associated with the circadian rhythm and it is likely that behaviors such as voluntary wheel running are highly influenced by the day–night cycle.

Conditioned place preference (CPP) test

The CPP test was elegantly combined with pain models by King *et al.* (2009) to test pain in combination with the reward system as a measure of ongoing pain. It was first established in rats, but has also been validated in mice (He *et al.*, 2012). The test utilizes a three-chamber system with a small middle neutral chamber and two outer chambers that are distinguishable by different visual, floor and odor cues (King *et al.*, 2009). After preconditioning of the animals with free access to all chambers, spinal administration of an analgesic is paired with a chamber. When animals are permitted free access to all chambers post-conditioning, a preference for the analgesic drug-paired chamber day is observed, which is indicative of the animal having ongoing pain. This test has been shown to be applicable to several models of chronic pain, including SNL and SNI in rats (King *et al.*, 2009), sciatic nerve axotomy in rats (Qu *et al.*, 2011), paw incisional pain in rats (Navratilova *et al.*, 2012), Osteoarthritis in rats (Liu *et al.*, 2011; Okun *et al.*, 2012) as well as SNL and CFA in mice (He *et al.*, 2012). Analgesia-associated reward behavior measured via this test has also been employed to analyse the contribution of particular anatomical regions in the brain as well as to test the efficacy of drugs (King *et al.*, 2011; Qu *et al.*, 2011; Navratilova *et al.*, 2012).

This test holds tremendous potential for applications in rats and mice. However, some points deserve discussion and further clarification. One, pairing of the CPP behavior with analgesia-associated reward and thereby its interpretation as a measure of spontaneous pain has been so far observed at very specific time points, which vary across models and days following surgery, for example at 24 h following paw incision or CFA injection, at 7 days post-SNI, at 7 or 10 days post-SNL, or at 14 and 28 days post-osteoarthritis

TABLE 1. Summary of behavioral tests for spontaneous chronic pain

Test/ Measurement	Short description	Measurable parameters	Model	Reference (s)
Non-reflexive tests				
Weight bearing	Unrestrained animals are placed for a short duration on a sensor plate	Weight distribution between all paws and/or gait analysis	CFA – mice	Robinson *et al.* (2012), Cobos *et al.* (2012)
			CFA – rats	Tetreault *et al.* (2011)
			Osteoarthritis – rats	Combe *et al.* (2004)
			CCI – mice	Tetreault *et al.* (2011), Mogil *et al.*(2010)
			CCI – rats	Piesla *et al.* (2009)
			Cancer – mice	Tetreault *et al.* (2011)
			Carrageenan – mice	Lolignier *et al.* (2011)
			Carrageenan – rats	Piesla *et al.* (2009)
			Arthritis (diverse models) – rats	Neugebauer *et al.* (2007), Angeby Moller *et al.* (2012), Clarke *et al.* (1997), Ferreira-Gomes *et al.* (2008), Min *et al.* (2001), Dorman *et al.* (2014)
			SNI – mice	Mogil *et al.* (2010)
			SNI – rats	Lau *et al.* (2012, 2013), Piesla *et al.* (2009)
			AXO – rats	Piesla *et al.* (2009)
			PSNL – rats	
			SNL – rats	
Home-cage monitoring	Natural behavior in a familiar environment for an unlimited duration with special detector plates under the cage and/or camera observation	Grooming, rearing, climbing, moving distance, drinking, eating	CCI – mice	Urban *et al.* (2011)
			SNI – mice	
			CFA – mice	
Open field	Short duration video monitored exploration behavior in a novel environment	Movement speed, distance traveled, rearing, grooming, anxiety-related behavior	Carrageenan – mice	Cho *et al.* (2013)
			Diabetic neuropathy – mice	Cho *et al.* (2013)
			SNI – mice	Cho *et al.* (2013), Urban *et al.* (2011)
			CCI – mice	Urban *et al.* (2011), Mogil *et al.* (2010)
			CFA – mice	Urban *et al.* (2011)
			CFA – rats	Parent *et al.* (2012)
			Knee joint inflammation – rats	Rutten *et al.* (2013), Matson *et al.* (2007)
Video-observation – paw	Short-term video observation selective for specific behavioral features	Spontaneous paw lifting, licking, flinching, guarding	CCI – rats	Attal *et al.* (1990)
			CCI – mice	Mogil *et al.* (2010)
				Djouhri *et al.* (2006)
			CFA – rats	Djouhri *et al.* (2006)
			Carrageenan – rats	Lolignier *et al.* (2011)
			Arthritis – rats	Lolignier *et al.* (2011)
			Cancer – mice	Asai *et al.* (2005)
Video-observation – facial expression		Characterizing facial features according to a scale that corresponds to the pain intensity; other emotional characterizations potentially possible	CCI – mice	Langford *et al.* (2010)
			SNI – mice	Langford *et al.* (2010)
			CFA – rats	Sotocinal *et al.* (2011)
			Arthritis – rats	Sotocinal *et al.* (2011)
			Postoperative (laparotomy) – mice	Langford *et al.* (2010)
			Postoperative (laparotomy) – rats	Sotocinal *et al.* (2011)
			Mustard oil – mice	Langford *et al.*(2010)
			Paw incision – mice	Langford *et al.* (2010)
			Post-vasectomy – mice	Leach *et al.* (2012)
			Arthritis – rats	Stevenson *et al.* (2010)
			Muscle pain – mice	Pratt *et al.* (2013)

TABLE 1. (continued)

Test/ Measurement	Short description	Measurable parameters	Model	Reference (s)
Ultrasound vocalization	Recording of vocalizations of animals in a unspesific sound-free environment	Specific frequency-dependent cries	PSNL – mice	Wallace *et al.* (2005)
			Diabetic neuropathy – rats	Jourdan *et al.* (2002)
			Carrageenan – rats	Jourdan *et al.* (2002)
			Arthritis – rats	Jourdan *et al.* (2002), Calvino *et al.* (1996), Han *et al.* (2005), Neugebauer *et al.* (2007)
			Arthritis – mice	Tappe-Theodor *et al.* (2011)
			SNI – mice	Kurejova *et al.* (2010)
			Cancer – mice	Kurejova *et al.* (2010)
Burrowing	Measuring the amount and or latency to burrow in gravel, pellets or others from a hollow tube	Wellbeing of animals	CFA – rats	Andrews *et al.* (2012)
			Nerve transection – rats	Andrews *et al.* (2012)
			PSNL – rats	Andrews *et al.* (2012)
			Knee-joint inflammation – rats	Rutten *et al.* (2013)
			SNI – rats	Lau *et al.* (2012, 2013)
			HIV-associated neuropathy – mice	Huang *et al.* (2013)
			Post-laparotomy – mice	Jirkof *et al.* (2010)
Free-choice tests				
Temperature preference	Rodents can freely move during a short time period between two (or four in the RAMP test) plates with different surface temperatures	Heat hyperalgesia, cold allodynia	SNI – rats	Baliki *et al.* (2005), Duraku *et al.* (2013)
			CCI – rats	Baliki *et al.* (2005)
			CCI – mice	Walczak & Beaulieu (2006)
			PSNL – mice	Walczak & Beaulieu (2006)
			CCS – mice	Walczak & Beaulieu (2006)
Conditioned place preference test	The test involves three phases – habituation, conditioning to an analgesic- paired or control-chamber and preference testing	Increased place preference in analgesic-paired chambers of animals with spontaneous ongoing pain	SNI – rats	King *et al.* (2009)
			SNL – rats	King *et al.* (2009, 2011)
			SNL – mice	He *et al.* (2012)
			Sciatic nerve auxotomy – rats	Qu *et al.* (2011)
			Paw incision – rats	Navratilova *et al.* (2012)
			Osteoarthritis – rats	Liu *et al.* (2011), Okun *et al.* (2012)
			CFA – mice	He *et al.* (2012)
Place escape/avoidance test	Unrestricted free movement of animals in a half white, half black painted compartment, receiving noxious stimulus at the affected paw in the black area and at the non-affected paw in the white area	Measuring escape behavior from a mechanical noxious stimuli	CFA – rats	LaBuda & Fuchs (2000), Boyce-Rustay *et al.* (2009)
			Nerve ligation (L5) – rats	LaBuda & Fuchs (2000)
			CCI – rats	Pedersen & Blackburn-Munro (2006)
			Spinal cord injury – rats	Baastrup *et al.* (2010a,b)
			Muscle pain – mice	Pratt *et al.* (2013)

Measures and tests that have been discussed in this review are summarized and classified into two groups, 'non-reflexive measures' and 'free-choice tests'. Short descriptions explain briefly the test paradigms, measurable parameters summarize the outcomes and the last two columns indicate the chronic pain models that have been tested with the corresponding reference. AXO, axotomy; CCI, chronic constriction injury; CCS, chronic constriction of the saphenous nerve; CFA, complete Freund's adjuvant; PSNL, partial sciatic nerve ligation; RAMP, Rotterdam Advanced Multiple Plate; SNA, spinal nerve axotomy; SNI, spared nerve injury; SNL, spinal nerve ligation.

induction. In most cases, this time frame does not quite match temporally with and is considerably shorter than the duration of allodynia. While this may reflect differences in the temporal course of ongoing pain and allodynia, it is puzzling why this is not seen along these lines in studies on humans. Secondly, the readout is dependent on contextual memory, i.e. the ability of the animal to remember the chamber that paired contextual and non-contextual cues with pain relief. Therefore, when a particular manipulation, e.g. lesion of a particular brain center or the application of a certain drug that plays a role in contextual and non-contextual memory, affects CPP, which will be interpreted in this test as a change in ongoing pain, in reality it may or may not play a role in spontaneous pain or reward associated with pain relief. A distinction between memory function and ongoing pain is not given by the existing protocols of CPP.

Place escape avoidance test (PEAP)

As with CPP, the PEAP incorporates the motivational and affective aspects of pain relief, its central readout being based on motivational escape from a noxious stimuli to avoid pain, which was introduced by LaBuda & Fuchs (2000). Rats were tested at 1 day following intraplantar CFA injection or at 2 weeks following L5 nerve ligation to provoke mechanically induced avoidance behavior (LaBuda & Fuchs, 2000). The principle of the test is to place animals on a mesh in a Plexiglas chamber where one half of the chamber is transparent (light area) and one half is black walled (dark area). Animals are allowed unrestricted movement throughout the chamber over a 30-min test period. Immediately after putting the animals in the test chamber they are stimulated with plantar von Frey hair application

at a nociceptive intensity every 15 s. The plantar stimulus is delivered either on the afflicted paw in the dark area or the contralateral, unafflicted paw in the lit area. Over time, this leads to the avoidance of the location in the test box where the stimulus was applied to the more aversive paw (Boyce-Rustay *et al.*, 2009).

The PEAP test has been proposed to address not only aversive but also initial escape-related behavior of the rats and unravel the complex interaction between sensory and affective pain processing (LaBuda & Fuchs, 2000), and has been further validated in the CCI model of neuropathic pain in rats (Pedersen & Blackburn-Munro, 2006). Interesting, the principle of the PEAP test has also been extended to test central neuropathic pain following spinal cord injury in rats to distinguish between at-level and below-level pain behavior by changing the region of von Frey hair stimulation (Baastrup *et al.*, 2010a,b). Recently, it has also been translated to mice with unilateral inflammatory muscle pain, using a 1-s von Frey hair stimulation interval, a smaller chamber and a lower stimulus intensity than for rats (Pratt *et al.*, 2013).

The principle as well as the data available on the PEAP test indicate that it would be a very valuable paradigm with wide applications, especially if it can be extended to low forces of mechanical application (allodynia) as well as thermal stimulation. Currently, the main drawback is that it is very laborious, as it requires frequent and regular von Frey hair stimulation over 30 min for each animal. In the studies published so far, the stimulus intensity was rather high and there is a concern that this frequent noxious stimulation elicits stress in the animals and might affect anxiety behavior. However, these have been addressed in part recently. Socially isolated neuropathic rats (CCI model) develop the same level of mechanical allodynia, showed no difference in anxiety-related behavior but showed an increased aversion to noxious stimuli in the PEAP tests compared with non-isolated rats (Bravo *et al.*, 2013). Furthermore, it has been shown that basal differences in inter-individual anxiety levels do not influence mechanical sensitivity and do not lead to differences in the PEAP test (Wilson *et al.*, 2007). Extending this paradigm to non-noxious intensities, particularly those representing allodynia, will help to address whether allodynia is also associated with an aversive avoidance response in chronic pain states. However, as with CPP, if the PEAP design is associated with memory function, similar constraints in interpretations to those described above for CPP would apply.

Facial expression of pain

As with humans, the facial expression of pain is hypothesized to be conserved in rodents and, accordingly, mouse- and rat-grimace scales (MGS, RGS) have been developed as a potential measure of spontaneous pain, in which either four (rat; Sotocinal *et al.*, 2011) or five (mouse; Langford *et al.*, 2010) facial features are classified on a three-point scale (Langford *et al.*, 2010; Sotocinal *et al.*, 2011). In initial analyses, mice that were tested in a nociceptive assay of moderate duration (10 min to 12 h; e.g. acetic acid abdominal constriction, late Formalin phase, laparotomy, paw incision) featured a 'pain face', but not animals in acute pain (e.g. tail clip, intraplantar capsaicin) or in chronic neuropathic pain conditions (SNI, CCI; Langford *et al.*, 2010). The method was subsequently tested in other rodent pain models and correlated with additional behavioral tests. For example, the MGS has been used in a model of post-vasectomy animals (Leach *et al.*, 2012), where the scale rating correlated with other pain related-behavioral changes that were detected using the home-cage monitoring.

However, the characterization of facial expressions appears to be easier in white coated animals with red eyes than in overall black animals and, indeed, so far there is no study published in which the MGS or RGS is used in non-white coated animals. As C57BL6 mice are still the most commonly used mice and indeed represent the primary strain in a vast majority of genetic studies, this test could make a substantial contribution, if the test paradigm can be successfully applied in these mice. Additionally, the issue of why facial pain scoring is not valid in several models of acute or chronic pain needs to be resolved, particularly in light of the proposed translational relevance.

Burrowing behavior

The natural burrowing behavior in rodents has been used to study the general wellbeing of animals (Deacon, 2006) and has been tested as a potential surrogate parameter for pain. Burrowing behavior is measured as the latency to burrow or the duration to burrow over a 2-h testing period or in overnight analyses. A reduction in burrowing behavior at different timepoints has been shown in rats following unilateral CFA hindpaw inflammation, tibial nerve transection, L5 spinal nerve transection and partial sciatic nerve ligation (Andrews *et al.*, 2012), knee-joint inflammation (Rutten *et al.*, 2013) as well as SNI (Lau *et al.*, 2013). However, in one study, changes in burrowing behavior did not correlate temporally with tactile allodynia (Lau *et al.*, 2013). While the test might constitute a valuable tool to monitor changes in the wellbeing of animals, affecting the motivation to burrow, it has some limitations. Most publications show significant burrowing differences only when they used animals that were housed together and only separated for the burrowing test, which seems to increase burrowing probability. Moreover, most burrowing-related publications so far are based on rat experiments and only a few are based on mouse models, e.g. HIV-associated neuropathy (Huang *et al.*, 2013) and post-laparotomy pain (Jirkof *et al.*, 2010). Therefore the test needs further validation, but holds promise.

Ultrasound vocalization

Vocalizations in rodents have been extensively studied with respect to several natural behavioral contexts. Audible (< 20 kHz frequency) and ultrasonic vocalizations (USVs; > 20 kHz frequency) are the main categories of rodent sounds (Roberts, 1975). There are commercial available systems to detect vocalizations (Ultravox; Noldus; Avisoft Ultrasound Gate; Avisoft Bioacoustics, Berlin, Germany; Sonotrack; Metris, Hoofddorp, the Netherlands; Ultravox; Tracksys, Nottingham, UK) but several groups have also reported custom-built systems.

While USVs are more consistently detected upon acute application of suprathreshold nociceptive stimuli (Jourdan *et al.*, 1995, 1998; Han *et al.*, 2005), there has been a longstanding discussion of whether intraspecies USV communication can be directly linked to chronic pain (Ko *et al.*, 2005; Oliveira & Barros, 2006) and many studies failed to measure USVs in chronic pain models. Negative results were reported in rats in the partial sciatic nerve ligation (PSNL) model, in a rat model for bladder inflammation and the Formalin model (Wallace *et al.*, 2005) as well as in an inflammatory Carrageenan, an arthritis and a diabetic model in rats (Jourdan *et al.*, 2002). On the other hand, diverse laboratories could successfully measure USVs for example in a variety of arthritis models in rats (Calvino *et al.*, 1996; Han *et al.*, 2005; Neugebauer *et al.*, 2007), and mice (Tappe-Theodor *et al.*, 2011), and in a tumor pain and

SNI model in mice (Kurejova *et al.*, 2010). Kurejova *et al.* (2010) developed an improved assay enabling the recording of USVs in freely moving mice repetitively over several weeks. This study is the first and only report measuring USVs on a longitudinal timescale and uses higher frequencies of 37 and 50 kHz, avoiding 22 kHz, which has been used in many of the past unsuccessful studies, because this frequency is known to be associated with alarm cries (Blanchard *et al.*, 1991) and is related to anxiety and freezing behavior (Borta *et al.*, 2006). Beside variations in the frequency of the analysed USVs, immobilization of the animals in the testing compartment (Jourdan *et al.*, 1998) induces stress and thereby leads to problems in the detection of pain-relevant USVs. Additionally, divergences in the USVs in the above described publications can also be based on the strain, sex and age of the animals that have been used. Additional variables include the measurement device and its sensitivity, the protocol of measurements including the acclimatization periods as well as the duration of analysis, the time of day of recording and finally the frequency of measurements ranging from 25 ± 4 kHz (Han *et al.*, 2005) to 50 kHz (Kurejova *et al.*, 2010). Moreover, the measurement of USVs is very sensitive to background noise, which has to be carefully filtered to avoid false positive results, and acclimatization (Kurejova *et al.*, 2010) and needs more validation before we can accept USVs as clear indicators for ongoing pain.

Pain-related changes in anxiety-, depression- and stress-related behavior

Very few animal studies discuss the component of anxiety in animal models of pain, which should not be underestimated. Rats with CFA-induced paw inflammation show a reduced central activity in the open field apparatus and reduced entries in the elevated-plus maze and the social interaction test (Parent *et al.*, 2012). Unpredicted chronic mild stress induces major depression, which leads to an increased intensity of ongoing pain in the CFA model in rats and increased spontaneous pain behavior in the formalin test in rats compared with non-depressed animals (Shi *et al.*, 2010; Wang *et al.*, 2013), indicating a correlation between pain and depression.

Other tests

A significant loss in body weight has been reported in various pain models (Chudler & Dong, 1983; Bennett & Xie, 1988; Medhurst *et al.*, 2002; Shi *et al.*, 2010; Urban *et al.*, 2011), although food intake has not been systematically quantified in all studies and the overall meaning of less food intake has still to be proven as a clear sign for non-wellbeing due to pain instead of simply representing post-surgery illness.

Following nerve injury, many animals chew on the area made anaesthetic by the lesion, a phenomenon which is called 'autotomy' (Wall *et al.*, 1979) and believed to reflect spontaneous dysesthesia and pain (Coderre & Melzack, 1986; Devor, 2007). Autotomy behavior has been shown in a variety of mouse and rat models following complete nerve transection (e.g. Zeltser *et al.*, 2000; Minert *et al.*, 2007; Koplovitch *et al.*, 2012) but is rare in partial nerve injury models (Minert *et al.*, 2007; Koplovitch *et al.*, 2012). The correlation between autotomy and spontaneous ongoing neuropathic pain has been discussed intensively (Koplovitch *et al.*, 2012) and it has been suggested that spontaneous pain is not expressed as autotomy in neuropathic models where the protective nociceptive sensory cover is partially maintained (Koplovitch *et al.*, 2012). Further studies linking autotomy behavior and its onset with other tests for spontaneous pain would be necessary to clearly correlate this behavior as an indicator for spontaneous pain or as purely dysesthesia-related behavior.

Concluding remarks and future perspectives

There is increasing evidence that we need better-characterized behavioral tests in unrestrained animals to be able to fully analyse diverse components of pain, wellbeing and pain-related disorders, such as fear, depression and negativity. While most studies continue to measure stimulus-evoked pain-related behaviors, there is an urgent need to develop and optimize free-choice and operant-based behavioral readouts.

On a positive note, there has been tremendous development in this field in recent years and the international research community is much closer to truly addressing chronic pain than it was just a few years ago. We also believe that a majority of apparent inconsistencies between studies could be resolved if methods could be standardized in a community-oriented transparent manner and, importantly, performed over a longitudinal manner taking circadian rhythms as well as the truly 'chronic' nature of changes into account.

There remains enormous potential in developing new paradigms and, in this regard, the pain community can learn from other fields, such as addiction and drug dependence, which are very advanced with respect to behavioral research in rodents. For example, self-stimulation paradigms using non-addictive drugs or *in vivo* optogenetics can be developed in chronic pain models in analogy to studies on reward and addiction.

Rodents undoubtedly constitute a highly validated, established and useful model system for studying pain. Recent developments as well as all of the above measures for further optimization hold promise in further facilitating the translational value of rodent models in chronic pain.

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Abbreviations

CCI, constriction nerve injury; CCS, chronic constriction of the saphenous nerve; CFA, complete Freund's adjuvant; CPP, conditioned place preference; MGS, mouse-grimace scale; PEAP, place escape avoidance test; PSL, partial sciatic nerve ligation; PSNL, partial sciatic nerve ligation; RAMP, Rotterdam Advanced Multiple Plate; RGS, rat-grimace scale; SNI, spared nerve injury; SNL, spinal nerve ligation; STZ, streptozotocin; USV, ultrasonic vocalization.

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