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Overview

A Review of Pain Assessment Methods in Laboratory Rodents

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Ensuring that laboratory rodent pain is well managed underpins the ethical acceptability of working with these animals in research. Appropriate treatment of pain in laboratory rodents requires accurate assessments of the presence or absence of pain to the extent possible. This can be challenging some situations because laboratory rodents are prey species that may show subtle signs of pain. Although a number of standard algesiometry assays have been used to assess evoked pain responses in rodents for many decades, these methods likely represent an oversimplification of pain assessment and many require animal handling during testing, which can result in stress-induced analgesia. More recent pain assessment methods, such as the use of ethograms, facial grimace scoring, burrowing, and nest-building, focus on evaluating changes in spontaneous behaviors or activities of rodents in their home environments. Many of these assessment methods are time-consuming to conduct. While many of these newer tests show promise for providing a more accurate assessment of pain, most require more study to determine their reliability and sensitivity across a broad range of experimental conditions, as well as between species and strains of animals. Regular observation of laboratory rodents before and after painful procedures with consistent use of 2 or more assessment methods is likely to improve pain detection and lead to improved treatment and care—a primary goal for improving overall animal welfare.

Abbreviations: CFA, complete Freund adjuvant; CPP, conditioned place preference; CPA, conditioned place avoidance; HPA, hypothalamic-pituitary-adrenal gland; MGS, mouse grimace scale; RGS, rat grimace scale; TINT, time to integrate to nest; USV, ultrasonic vocalization

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The scientific study of pain in humans and animals and its underlying mechanisms have been the subject of extensive research for over 200 y.[109] Despite this, theories underpinning our current understanding about pain signal transduction and related mechanisms were developed only 50 y ago, with Melzack and Wall's proposal of a gate control theory to describe the mechanism of spinal cord transmission and regulation of pain signaling (reviewed by[82,89]). With improved understanding about the underlying mechanisms of pain came improved recognition of pain as a disorder in its own right,[113] and a desire for human clinicians to improve assessment and treatment of pain in their patients.[82] Development of assessment methods for pain in domestic animals mirrored those in human species, although it was not until the late 20th century that pain management of veterinary species and research animals were prioritized by veterinarians and regulatory agencies. Today, it is recognized that accurate identification and assessment of pain are essential for refining care of research animals undergoing painful procedures and improving the validity of translational pain research.[90,108] Animal ethics committees and regulatory authorities require researchers and laboratory animal veterinarians to assess and manage pain in animal subjects. However, even today, this is seldom a straightforward task—particularly for research rodents.[138]

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Defining Pain and Nociception

Pain and nociception are often confusingly described and poorly defined, leading to their incorrect usage or the assumption that they are synonyms. For clarity throughout this paper, the definitions of the International Association for the Study of Pain[54] are used:

Pain: "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage."

Nociception: "The neural process of encoding noxious stimuli." This process may have autonomic (for example elevations of heart rate or blood pressure) or behavioral (for example withdrawal reflex or more complex nocifensive behavior, such as licking or rubbing) consequences. Importantly, nociception can occur without the sensation of pain."

Challenges to Observing and Monitoring Pain in Laboratory Rodents

Because animals are nonverbal, assessment of pain involves observing surrogate measures of pain and signs of animal well-being and then making a judgment about the animal's condition based on the interaction between these 2 sets of data. Assessing the presence or severity of pain from only 2 subjective measures provides only an approximation of a painful state.

Many commonly used measures of pain are indirect, including activity, heart rate, blood pressure, and body weight, all of which can be altered by factors other than pain, thus confounding their interpretation. Furthermore, many measures require a sound knowledge of species-typical behavior for accurate interpretation. Even in humans, the primary factors driving the experience of pain; that is, motor, sensory, and psychologic, were rarely studied or assessed together until recently.[13] This means that approaches used for the assessment of pain in laboratory rodents may be suboptimal, and thus multiple measures of pain assessment should be used together to improve accuracy.[153]

To further complicate monitoring pain in laboratory rodents, a significant confounding variable may be the sex of the individual who is assessing the animal. Direct exposure of mice and rats to men or male scent increased defecation rates in control animals of both sexes, as well as decreasing perceived pain intensity, as detected by various standard assays, such as tail and paw withdrawal tests and von Frey fiber thresholds (see Figure 1).[131] This sex-based stress-induced analgesia may be responsible for conflicting opinions about animal wellbeing in studies with painful outcomes.

Finally, because rodents are nocturnal, more accurate pain assessments would be likely if animals were monitored for pain during the most active times in their circadian cycle. Nociception is most acute in the dark phase of their day/night cycle,[20,86] concurrent with the time of their lowest responsiveness to opiate analgesics,[105] and peak activity of inflammatory signaling pathways, as controlled by their internal molecular clocks.[16] Animal monitoring and assessment during the dark phase would require widespread changes in practice, including housing management, and there are practical difficulties in achieving this.

Pain Assessment and Monitoring Methods in Laboratory Rodents

Many methods have been proposed for assessing and monitoring pain in mice and rats (see Figure 1 for an overview of methods), including examining the patient directly and making a subjective assessment of animal comfort based upon hair coat, posture, general activity level, and degree of alertness.[1,65] Unfortunately, very simple assessment methods may fail to detect subtle changes in animal behavior and comfort, and a nonstandardized approach to pain assessment is likely to result in wide variations of opinion between individuals looking at the same group of animals. This has been demonstrated to occur for larger animals, such as cattle and sheep, and is not expected to be any different for much smaller animals, such as mice and rats, which are harder to observe.[64,140] Handling and restraint increase the potential for stress-induced analgesia, so pain assessments that can be conducted with unrestrained animals are preferred. However, the question remains: how should the clinician and researcher evaluate pain monitoring methods in the face of such a wide array of pain assessment techniques?

Validation of Assessment Methods

Authors of papers that include a pain assessment scale often use the term 'validated' to claim that the scale can be relied upon to measure the outcome of interest. Unfortunately, this is often not the case. The scale may have never been formally validated, the context (for example, the specific pain model) in which it was originally developed may differ from the subsequent clinical use, or the scale may have been modified in some way by other users, such that the effect(s) of this change on scale validity are unknown. In general, pain scale development and validation should be viewed as a continuum rather than a discrete process. For example, it is common to assume that a scale developed in one strain of mice or rats under specific conditions and applied by a particular group of users will perform equally well under different circumstances. This assumption that scale performance is a fixed property is an oversimplification and fails to account for the multitude of factors affecting scale function, and therefore, makes it difficult to compare studies.

An overview of the key steps in scale validation are briefly presented as a background to the discussion of different pain assessment methods. The key concepts of scale development are validity and reliability. For more in-depth coverage of this topic, interested readers are referred to the review by Streiner and colleagues.[136]

A simple definition of validity is: does a scale measure what it claims to measure?[103,136] It is important to know if the items making up a scale are all necessary and important (face and content validity), how a scale compares to a gold standard should one exist (criterion validity), and whether the scale can identify meaningful changes (construct validity).

Content validity is concerned with the scale items and whether they fully capture the measure of interest. Content validity is frequently established through face validity, which uses the opinions of experts to determine the scale items. Initially, it is often useful, to begin with, a large number of potential items, reducing the list based on discussion and consensus. More formal methods to assess utility of potential items include evaluating the relationships between items and the effect of item omission on scale performance.

Criterion validity is the comparison of a scale with an accepted standard. In adult humans capable of verbal communication, the self-report is usually considered the 'gold standard'. In contrast, where self-reporting is impossible, such as in animals and neonates, it is often argued that criterion validity cannot be performed. Therefore, it is common to employ construct validity as a substitute for criterion validity in pain scale development in animals.Construct validity is experimental testing of a hypothesis based on what is known (or assumed) about a construct like pain, anxiety or depression. An example of this is the assumption that giving an analgesic will reduce the pain scale score or that pain scale scores will increase immediately after surgery and return to baseline as inflammation subsides.[90] Because construct validity is only limited by the number of hypotheses that can be generated, it should be viewed as an ongoing rather than static process. Additional useful components of validity are sensitivity and responsiveness. These terms describe the ability of a scale to detect a change, and although they are often used interchangeably, sensitivity can be considered detection of any change whereas responsiveness is detection of an important or relevant change.[75]

Reliability is the amount of error associated with a measurement scale; that is, the reproducibility of the results.[143] For a scale to be useful, this statistical error should be appropriate for the encountered range of observations. For example, a weight scale for use with cats with accuracy reported to ± 0.1 kg would be acceptable but would be of no use for weighing rats. Similarly, the measurement error of a pain assessment scale should be smaller than the range of scores reflecting an important change in pain level. This error can be assessed for various situations, including differences between different users (interrater reliability), and differences within the same user (intrarater reliability). Commonly employed measures of reliability are the intraclass correlation coefficient (ICC) and κ coefficient. For both measures, the higher the level of correlation observed the better the reliability. As reliability decreases, the harder it becomes to detect small

Test	Stimulus	Method	Measurement
Tail-flick [8,69]	Radiant heat	Apply thermal radiation to tail	Reaction time of tail movement. Typically, does not exceed 2–10s
	Hot water immersion	Submerse tail in hot water	
Hot plate test [8,69]	Thermal heat, 50–55°C	Place animal on hot plate	Reaction time to paw licking and/or jumping. Baseline latency 5–10s
Paw withdrawal test [8,69]	Thermal and inflammatory	Carrageenin-induced inflammation followed by response to radiant heat	Reaction time to paw withdrawal from heat source
von Frey test [8,69]	Mechanical allodynia	Apply filaments to inflamed area that bend at calibrated pressure	Paw withdrawal associated with force of filament bending
Randall–Selitto test [8,69]	Mechanical allodynia	Application of a fixed element with linear increasing mechanical force in grams	Appearance of pain behavior such as paw withdrawal, struggling or vocalization
Formalin injection test [8,69]	Chemical	0.5–15% formalin injected into plantar surface of paw	Biphasic response: a) initial response within first few minutes is acute pain, b) secondary response at 20–30 min representing inflammatory pain. Analgesia often assessed using response to mechanical stimulus such as von Frey or Randall–Selitto
Complete Freund adjuvant test [8,69]	Inflammatory	Injection of CFA into hind paw	Response to thermal or mechanical stimuli
Neuropathic pain [8,69]	Thermal and mechanical	Ligation of the sciatic nerve. Often referred to as chronic constriction injury.	Response to thermal or mechanical stimuli
Writhing test [69]	Chemical	Intraperitoneal administration of acetic acid	Abdominal contractions, reduced motor activity, and incoordination
Facial Grimace Scale [65,130]	Various	Assess facial expression to noxious stimuli	Scoring based on orbital tightening, and position of nose, ears, cheeks and whiskers
Ultrasonic vocalization[8]	Various	Assess ultrasonic vocalizations—evoked or spontaneous	Emit ultrasonic vocalizations with acute pain
Nesting behavior [58,114]	Various	Assess nest building complexity	Animal in pain will be less inclined to build a nest or maintain them
		Time to integrate nest material (TINT)	
Burrowing behavior [57]	Various	Assess burrowing	Animal in pain will be less inclined to burrow
Ethograms [1,63,100,119]	Various	Observation of behavioral changes associated with pain	Animal in pain will demonstrate altered behaviors

Figure 1. Common methods to assess analgesic efficacy in rodents.

differences (the user must decide if these differences are worth trying to detect). Therefore, reliability sets the limit for validity, and if measurement error is large, it will not be possible to detect important differences. Importantly, reliability does not refer to the scale, as in the reliability of the scale, but rather, to the results obtained with the scale when applied in a given situation.

As this discussion indicates, describing a scale as validated is both misleading and an oversimplification. Validity and reliability are all limited by the population and context in which the testing was performed. Therefore, the best that can be said is that a scale is valid for the population studied within a defined context.[136]

Use of Algesiometry Assays for Assessment of Pain

Algesiometry assays are standardized tests of evoked response reflexes that can be used as models of nociception in rodents and other species and to determine the potency of a given analgesic agent or regimen.[8,71] Examples include the tail-flick, hot plate, acetic acid writhing, and von Frey tests (see Figure 1). During research, these tests are widely used because the assays are relatively simple to perform, inexpensive to run, and many can be automated.[94,107] While they have been used for decades to

assess aspects of nociception,[36] these assays likely represent an oversimplification of the condition of pain as it is understood today. For example, classic algesiometry assays all require the observer to remove the mouse or rat from its home enclosure and manually restrain them prior to initiating the test which can result in stress-induced analgesia. The results from these tests are sex- and strain-dependent, and are only effective in reflecting changes in a limited number of chronic pain models, for example, paw swelling but not chronic back pain or migraines. Likewise, interpretation of the results can be confounded by many parameters, such as impaired animal locomotor ability, rapid learning of the expected test response by the animal, observer sex and its influence on animal behavior,[131] and social housing conditions and interanimal cues.[68]

More recently developed tests for assessing spontaneous pain in mice and rats are likely to be of increased utility, and many of these are discussed in subsequent sections below. These include behavioral assessments of nonspecific or specific activities (ethograms), ultrasonic vocalizations, facial expressions of pain (that is, pain grimacing), burrowing, open field tests (in which more anxiety and less exploration are seen in a number of animal models of pain), free-choice thermal preference assays, voluntary wheel running tests, conditioned place preference tests, and conditioned place avoidance tests (for a review, see reference 138). While more difficult to design, and interpret, these tests are potentially more sensitive and specific measures for detecting pain in mice and rats. As novel methods for assessing pain are developed, it will be necessary to characterize their relationship with traditional (evoked) measures to determine their utility.

Behavioral Assessments of Pain

When implementing any metric for assessing pain in rodents, it is imperative to first understand the baseline for the particular patient population, inclusive of species, strain, sex, age, and health status of the animals. Also, factors that could potentially confound the assessments chosen should be considered, such as the presence of a human observer. Additional confounders may stem from the experiment itself, such as residual effects of anesthetics or debilitation to motor function or cognition as a component of the model. Because of the long list of variables that can impact pain assessments, a triangulation approach, using multiple methods that assess various components of the pain experience, such as, evoked, nonevoked, physiologic, clinical assessments may be needed.

Ethogram Assessments

The presence of an observer is an often underappreciated confounder to animal welfare assessments. Prey species have a natural drive to suppress pain behaviors in the presence of another animal, especially if it is perceived that it could pose a threat. Mice demonstrate the ability to suppress grimacing and guinea pigs have been shown to suppress pain-specific behaviors in the presence of a human observer.[85,102] Therefore, methods that can be conducted indirectly, without the presence of a human, such as via remote video, can be particularly helpful for pain assessments in rodents.

Understanding the impact of anesthetics and analgesics on pain assessments is also critical for accurate identification of unalleviated pain. Depending on the species of the patient, anesthetic and analgesic drugs may suppress or enhance measurements of postprocedural pain. For example, buprenorphine can cause sedation resulting in a decrease in ambulation in guinea pigs, and hyperactivity in mice; in both instances behaviors observed in pain-free mice, such as rearing and grooming, are reduced.[23,49,79,84,102,119,151] Interestingly, buprenorphine does not influence mouse grimace scores, whereas isoflurane can increase grimace scores in both mice and rats, due to unknown mechanisms.[84,85] Given the varying direct effects of anesthesia and analgesia on pain assessments, it is helpful to understand the half-life of administered drugs and what effects they will have on the assessments used. Comparing known effects of anesthetics and analgesics on the postprocedural condition can highlight pain-specific changes in behaviors. If evaluating change from baseline or change from an anesthesia/analgesia condition, a smaller change indicates fewer differences between the nonpainful and potentially painful conditions and therefore better pain control.

Ethograms capture 2 major types of nonevoked responses useful for pain assessment: loss of normal behaviors, such as rearing and ambulation, and presence of new pain-specific behaviors, such as back-arching (Figure 2 A), writhing (Figure 2 B), weight shifting and staggering.[120-123,150,151] General ethograms are available for use, or custom ethograms can be created for a particular patient population or model system by crafting clear, detailed descriptions for all behaviors for inclusion in the assessment.[35,41] Normal behaviors generally occur with greater frequency and may be easier to score using automated software.[151] However, new pain-specific changes in behavior, such as increased paw licking in certain cancer pain models in mice,[5] may be subtle, performed quickly, and occur more fleetingly, lending themselves more to manual scoring.[35,123]

In some cases, a surrogate indicator can be used to assess animal wellbeing or pain. Nesting behavior and burrowing are examples of surrogate behaviors that can be used when assessing pain in mice. Grooming is another surrogate behavior that can be used to indirectly assess painful states. This can be achieved through the Grooming Transfer Test (Figure 3).[104] The Grooming Transfer Test takes advantage of the fastidious nature of mice and their highly patterned grooming behavior. A nontoxic, inert powder that fluoresces under black light is suspended in mineral oil and applied to the top of a mouse's head. As the mouse grooms, they transfer the fluorescent signal to additional body locations, the cage environment, and nesting material. Eventually, the mouse's normal grooming behavior will completely remove the oil/powder suspension. This behavior is conserved across inbred and outbred mice, is performed by both males and females, and allows for individual assessment of mice, even when socially housed. The Grooming Transfer Test exhibits construct validity as grooming behavior is delayed after abdominal surgery and is restored with appropriate analgesia.[104]

While general activity evaluations and formal behavioral scoring have significant potential to uncover both known and novel expressions of pain in mice and rats, these techniques are laborious to conduct and require training of observers to ensure intra- and interrater consistency. Observations must be conducted and acted upon in real-time to be useful for effective analgesia administration. For these reasons, open field tests (OFT) have also been explored for assessing analgesia efficacy in mice[18] and rats.[106] OFT can examine aspects of mobility, anxiety and exploration behaviors in a relatively short period of time, and observation collection and processing can be automated. This test has only been studied for a limited number of painful conditions in rodents, and thus is currently of limited utility for widespread clinical assessment of pain.

Conditioned Place Preference Test

The conditioned place preference test (CPP) is unlikely to be used for spontaneous pain assessment in rodents on a

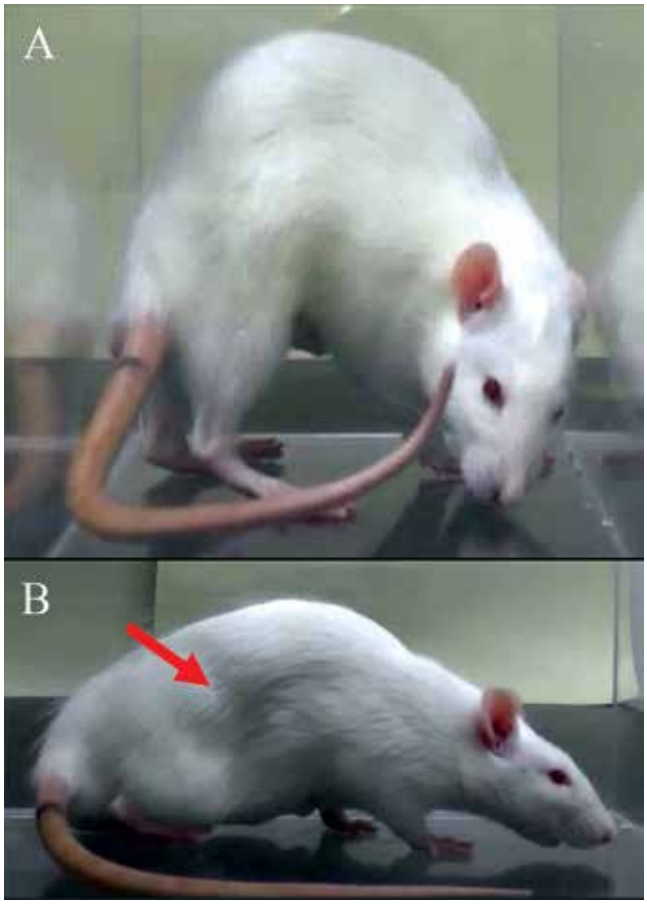


Figure 2. Examples of pain-associated behaviors demonstrated after a laparotomy in an adult male Sprague–Dawley rat. (A) Back arch—this behavior is described as a vertical cat-like stretch upward. (B) Writhe - this behavior is described as the contraction of the abdominal muscles (arrow).

day-to-day basis within vivaria; however, it has been used to demonstrate analgesic efficacy in models of rodent pain. In this assay, 2 outer chambers that are distinguishable by visual or olfactory cues are linked by a neutral middle chamber. Animals are preconditioned with free access to all 3 chambers, and then provision of an analgesic is paired with a particular chamber. In pain models using both mice and rats animals, strong preference can be seen for the chamber in which the analgesia was administered.[50,66,137] This model is based on positive reinforcement, a system that requires that the animal have intact contextual memory of the reward.

Conditioned Place Aversion Test

The conditioned place aversion test (CPA) is similar to the CPP test, but uses a neghative stimulus rather than a reward. This test has been used for models of pedal inflammation in rodents.[156] The test paradigm consists of a similar 3-chamber setup with visual cues except for the floor of the chambers consists of mesh. At regular intervals, von Frey fibers of a diameter to invoke a noxious stimulus in an animal with pedal inflammation, are poked through the mesh into the animal's paw in one of the outer chambers. Over time, the animal learns to avoid the chamber in which they receive the stimulation to the inflamed paw.[9] This mechanical hypersensitivity and chamber aversion are partially or completely abolished after administration of an efficacious analgesic agent. As with the CPP Test, this assay requires an intact memory, and the test is only useful for very specific models of pain in rodents.

Burrowing Behaviors

The vast majority of pain assessment assays in laboratory animals continue to rely on evoked responses; therefore, exploring the relationship between these assays and burrowing behavior, a nonevoked response, is valuable in terms of characterizing what different endpoints may be measuring and providing an indication ofthe validity of an endpoint compared with the human experience, and their temporal relationship. Unfortunately, few studies have directly compared these measures.[52,69,95,126,128]

An important appeal of burrowing as an outcome measure of pain is that it requires simple equipment and is relatively easy to implement. Observations are easily performed in the home cage, reducing potential interference from stress, and methods employing latency to begin burrowing, burrowing duration and total volume displaced have been described.[3,29,52,58-60] Of these, the measurement of the total volume displaced is the most common. Burrowing represents a goal-directed behavior that laboratory mice and rats are motivated to perform, and the technique can be applied as a research tool, as well as for clinical assessment.[29,30,32,34,129,131] While it can be used to specifically assess pain,[3,12,46,52,59,61,69,95,111,124-126,128,130,145,147] reductions in burrowing activity can also identify the influence of a range of factors, not all of which may be associated with pain, such as cognitive dysfunction, anxiety, systemic bacterial and viral infections, and inflammation.[24,27,29,31-34,47,55,57,98,144] As such, burrowing may be viewed as a surrogate measure of pain in rodents, and also as a reflection of instrumental activities of daily living (IADL), an outcome used in humans to reflect the impact of disabilities such as pain on day-to-day activities (for example, general mobility, care of others, maintaining the living space).[88] Factors other than pain that result in a reduction in burrowing behavior have been better characterized in mice and these include neurodegenerative disease, anxiety, and systemic infection or inflammation, thus interpretation of changes in burrowing behavior needs to be case-specific.[24,27,29,32,33,39,47,60,78] In addition, the assessment of burrowing can be confounded by variations in housing, including type of flooring,[7] familiar surroundings,[60] presence of conspecifics,[58] diet,[70] and estrous cycle.[19] Further, strain differences exist in mice, with reduced burrowing behavior observed in CBA, 129-substrains and Egyptian spiny mice (*Acomys cahirinus*).[29] In rats, scoring of burrowing behavior has been employed successfully in Hooded Lister, Wistar and Sprague–Dawley strains.[3,29,52,69]

Burrowing is reduced in a wide range of pain models in rats and mice, including laparotomy,[4,58-60] colitis and mucositis,[57,74,144] neuropathic pain,[3,52,69,95,124] inflammation,[3,46,95,130,139,144,147] and arthritis.[12,125,126] In addition, many of these studies have confirmed responsiveness, showing either an improvement or return to baseline burrowing behavior following the administration of antiinflammatories and analgesics.

Detailed information regarding burrowing tube dimensions, construction, substrate, and test paradigm are readily available.[3,28,29,126,147] In rats, gravel is most commonly used, though sand has also been reported (Figure 4).[125,147] In mice, earth, sand, bedding, and food pellets have been used.[28,29] In both mice and rats, individual variability in burrowing behavior exists, and this is an important fact to consider in its evaluation.[3,28,29] Under experimental conditions, burrowing behavior can be encouraged with social facilitation, pairing good and poor burrowers to encourage burrowing behavior.[3,28,29,147] In tracking individual changes, animals could serve as their own controls.

While burrowing behavior has been useful for experimental studies of pain and analgesia efficacy in mice and rats, there are important considerations in applying it to a

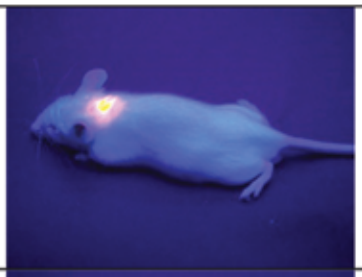
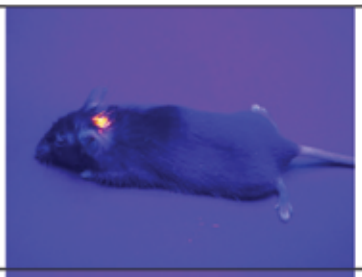
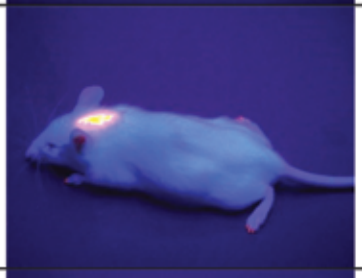
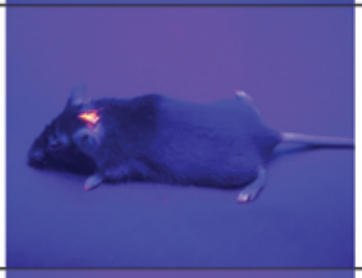
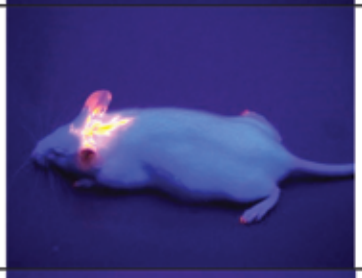
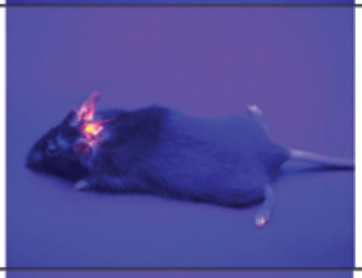
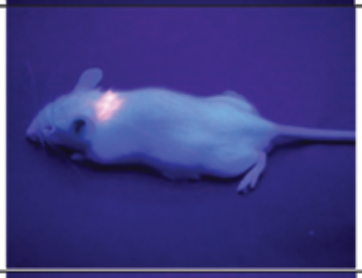
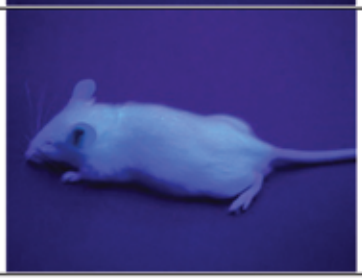
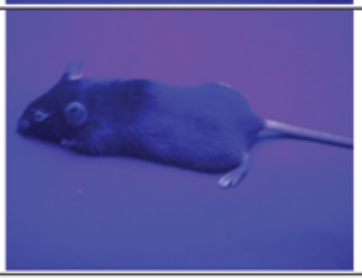
Score	Description	Example Image	
		CD1	C57BL6
1	A strong fluorescent signal is present at the application site on the forehead between the ears		
2	Fluorescence present at the application site as well as the front and/or rear nails		
3	Fluorescence present at the application site and the ears. Front and/or rear nails may also fluoresce		
4	Fluorescence is absent from the nails and ears but remains present in trace amounts at the application site		
5	Fluorescence is no longer detected		

Figure 3. The Grooming Transfer Test allows indirect assessment of a mouse's grooming behavior. If grooming well, the mouse will transfer the fluorescent signal from the top of their head to additional body locations, and in time, completely groom away from the signal. The latency to progressive grooming scores is increased in mice with unalleviated postlaparotomy pain. Reproduced with permission from AALAS.[51]

more clinical setting. These include individual variability in the propensity to burrow, the current reliance on individual testing, and occasional failure of the assay. In a recent international, multicenter randomized, blinded study (8 different laboratories across 4 countries) of burrowing in rats (adult Sprague–Dawley and Wistar strains) that used a well-characterized model of inflammation (Complete Freund adjuvant injection into a paw), the ability to reproduce the expected inflammation-induced suppression of burrowing varied considerably between centers.[147] The underlying reasons are unknown, but this suggests that more investigation of the technique is needed.

Figure 4. Burrowing behavior in an adult female Sprague–Dawley placed in a burrowing tube with 2-5 mm gravel.

Ultrasonic Vocalization

Ultrasonic vocalization (USV) has been suggested as a means of going beyond simple evoked reflex measures to reflect an integrative behavioral pain response. Rodents are capable of producing USV, defined as vocalizations at frequencies greater than 20 kHz. These vocalizations are used for communication, but are also triggered by drugs producing either a negative or a positive affective state.[10,11,149] These USV are typically categorized into 2 distinct frequency bands centered approximately at 22 kHz (generally associated with negative situations) and 50 kHz, which are associated with positive situations.[10,99,142,149] Specifically, noxious or painful stimuli have been associated with the 22 kHz band.[14,62,67]

Although initial promising results documented USV in response to electrical shocks to the tail in rats[62] and an arthritis model in which Freund adjuvant was injected into into tail,[14] recent studies have revealed several important concerns and potential limitations with USV as an outcome measure. These include variable correlation with traditional evoked reflex responses in rats and mice,[142] a lack of specificity and sensitivity for emission of USVs in mice,[146] a failure of USV emissions to identify likely chronic pain states[63,142] and a confound of the presence or absence of a conspecific on the expression of USVs.[14,63]

Employing 3 well-characterized models of inflammation (formalin injection in the hind paw), neuropathy (partial sciatic nerve ligation) and referred pain (bladder inflammation following instillation with turpentine and olive oil), Wallace and colleagues did not find any correlation between USV and withdrawal responses evoked by thermal (heat and cold) or touch (von Frey filament) stimuli.[142] These experiments were performed in male Wistar rats (partial sciatic nerve ligation), female Wistar rats (bladder inflammation) and male Wistar rats and C57BL/6 mice (formalin test). The expression of USV was limited to the initial presentation of the testing chamber during habituation, before model induction. After this, no USV were recorded either at baseline or following model induction despite predicted withdrawal responses occurring. These findings indicate failure to achieve adequate construct validity. In a study of weanling mice (male and female, 21 to 28 d old, B6;129S6-Stat5b) undergoing tail snipping or ear notching for DNA testing, Williams and colleagues found the incidence of USV to be highly variable, with 65% of animals not vocalizing in response to either procedure.[146] Furthermore, of the mice that did produce USV, audible vocalizations (less than 20 kHz) occurred concurrently in all but one animal. This suggests that during these potentially painful procedures, USV could not be reliably elicited and was not superior to monitoring audible vocalizations alone. Testing an acute (carrageenan injection into the hind paw) and 2 chronic (Freund adjuvant injection into the base of the tail and diabetic neuropathy induced with IP streptozotocin) pain models in male Sprague–Dawley rats, Jourdan and colleagues found a nonsignificant tendency to reduced USV in painful animals when in the presence of another rat, but no USV when animals were tested alone.[63] These findings highlight the critical role of experimental design in affecting results and study interpretation.

A possible explanation for some of the observed differences between studies is difficulty in separating responses to pain from distress or anxiety.[67,96] In studies that have reported USV, the numbers of animals that emitted vocalizations were low, suggesting, as that it is not a sensitive indicator of nociception or pain.[62,146] However, an important consideration when interpreting USV studies is the central role of vocalization in communication and the impact of single compared with group housing.[10] The tendency to vocalize may be linked to the presence of conspecifics, a situation that is seldom present during experimental testing but may occur during assessment of pain in colony conditions with social housing.[14,63]

Physiologic Assessments of Pain in Laboratory Mice and Rats

Body Weight Changes. Reductions in body weight and growth rate are commonly used as indicators of pain and distress and as humane endpoints in research rodent studies.[15,27,53,76,92,93,100,101,136] While loss of body weight may reflect behavioral changes associated with pain, it is a nonspecific indicator that can also reflect compromised wellbeing, malaise and adverse environmental or social conditions.[27,53,100,101,118,135] Weight loss due to reduced body mass (rather than dehydration) may also occur with chronic disease states that may or not be painful, such as cancer or infection.[101,115,118]

In experimental models when changes are slow and progressive, the number of consecutive days of ongoing weight loss or a weight loss maintained for several days may be a more sensitive measure of deterioration than absolute weight change alone.[115,148] An upper limit of 15% to 20% is often cited as an endpoint for weight loss;[91,148] however, a recent evaluation of 90 rat toxicity studies from 13 pharmaceutical companies and contract research organizations found that the maximum tolerated dose (defined as when dosing had to be stopped or animals were lost through death or euthanasia) was exceed in 12/13 studies in which a 20% weight loss was allowed. As a result, the authors suggested using a weight loss threshold of greater than 10% to trigger a decision regarding study continuation.[17]

An elegant study that combined several approaches to assess pain and wellbeing (conditioned place preference, automated behavior identification, evoked response [heat], weight loss) in a mouse model (female, C3H/HeN) of bladder cancer showed that weight loss of approximately 5% was closely and significantly associated with increasing preference for the morphine-associated CPP chamber.[118] Importantly, the link between weight loss and morphine seeking occurred well before animals approached the typical 15% to 20% weight loss range applied in many studies.

Thus, in consideration of this work, particularly where more specific and sensitive measures of pain are available, weight loss should not be used as the sole measure of pain, but may be used to complement other assessments as a global reflection of deterioration of animal welfare.[92] Furthermore, where weight loss is used, consideration should be given to the appropriate change for the model used.

Alterations in the Hypothalamic-Pituitary-Adrenal Gland Axis. With acute stress, including acute pain, the hypothalamic-pituitary-adrenal gland axis (HPA) is activated, releasing stress hormones that then induce increases in heart rate, respiratory rate blood pressure, and body temperature.[4,21,56,110,114] However, the HPA axis and associated physiologic changes are impacted by many other factors, which can make it challenging to use as a single measure for pain assessment. Stress from handling or restraint (when necessary for some forms of algesiometry testing) also significantly stimulates the HPA access and can either conceal or exacerbate changes attributable to pain.[118,121] Similarly, other variables that can cause significant and persistent perturbations in heart rate and blood pressure include routine cage changes, social housing, various experimental manipulations, and the provision of in-cage resources, such as running wheels.[1,43,65,127,133] Therefore, remote assessment of variables such as heart rate, respiratory rate, body temperature, and blood pressure is preferable, whenever possible. This can be achieved through telemetry, and some physiologic parameters, such as respiratory and heart rates, can even be measured by automated 'smart caging'.[44,77] Assessment of these physiologic variables should never be used alone when attempting to assess pain in mice and rats.

Facial Grimace Scales for Assessment of Pain in Rodents. The use of grimace scales in mice, subsequently described in numerous species, was introduced in 2010.[68] This work and the subsequent description of the Rat Grimace Scale (RGS) demonstrates many of the features expected in validation studies (Figure 5).[132]

Both the mouse grimace scale (MGS) and RGS show construct validity and reliability. Construct validity was most comprehensively demonstrated in the MGS using responses to analgesia, different levels of pain (by generating dose-response curves during induction of pain models) and following temporal changes as models progressed. For the RGS, construct validity was shown with temporal changes in RGS scores over time (predicted increase followed by decrease in scores as induced inflammation resolved) and an analgesic dose-response curve. Content (face) validity is grounded in the proposal by Darwin (first published in 1872) that facial expressions revealed emotions in humans and animals, with overlap across the species.[25] Furthermore, as grimace scales have been developed in other species, similar facial features have proved to be robust as signalers of pain.

Grimace scale interobserver reliability has been rated as 'good' to 'very good' by several research groups, indicating that error associated with the scale is small for differences between treatments typically examined in these studies.[68,103,118,132] Importantly, reliability is not uniform across features included in facial expression evaluation. For example, reliability associated with scoring whisker shape and position is frequently low.[103] It is unclear if this represents inherent difficulty in scoring whiskers or difficulty in collecting images of sufficient quality to be scored.[72] Linked to interobserver reliability, though seldom directly addressed, is the question of observer training. The overwhelming majority of MGS and RGS papers are from research groups with the time and personnel to invest in achieving proficiency in use of the MGS and RGS, though substantial variability between observers in this setting has been reported.[87] Therefore, while some studies have supported the use of naïve observers, it is largely unknown how these scales might perform in the hands of casual users, such as animal care staff or clinical veterinarians.[117] One study investigating the role of training has shown that the combination of practice scoring images alongside structured discussions is more effective than practice scoring alone.[155] This may explain why adoption of the MGS and RGS by the laboratory animal veterinary community has been limited.

A comparison between grimace scales and a standard/traditional assessment method has been evaluated with both the MGS and RGS.[26,68,98,134] In these cases, mechanical hypersensitivity testing with von Frey filaments has been performed with interesting results. With the MGS, in an inflammation model (zymosan injection into the hind paw or ankle joint), an analgesic effect of acetaminophen could be detected; however, the same dose of acetaminophen (300 mg/kg, SC) did not significantly reduce mechanical hypersensitivity.[68] In the case of the RGS, the duration of mechanical hypersensitivity resulting from inflammation (induced with intraplantar injections of CFA or carrageenan) was considerably longer-lasting than that of facial expression changes[26] Interestingly, the findings of the RGS study mirror a report of the experiences of a pain researcher who inadvertently self-injected CFA into a finger.[45] In this case, the pain experience was relatively short-lived (subsiding at 48 h) compared with the presence of mechanical hypersensitivity (42 d). Similarly, in a model of induced orofacial pain, mechanical hypersensitivity persisted beyond the changes in RGS.[2,134] Taken together, these findings raise important questions about the relevance of traditional hypersensitivity testing, particularly in light of the relative unimportance and infrequent occurrence of hypersensitivity in human chronic pain states.[90,108] There have been limited comparisons of facial grimace scales to behavior-based systems of pain assessment, with a study in mice undergoing vasectomy surgery showing strong correlation between the MGS and a suite of behaviors altered in the presence of pain.[72] In contrast, in a rat model of mucositis induced with IP 5-fluorouracil, an ethogram consisting of writhing, twitching, and back-arching showed increased frequencies of these behaviors in the presence of mucositis, without concurrent changes in the RGS.[145] This finding suggests that this that more research is needed for reliable conclusions to be drawn.

One of the major challenges in providing analgesia to laboratory rodents is the identification of efficacious doses of analgesic drugs.[37] Grimace scales have been applied to reevaluate commonly used analgesics.[81,132] These studies have shown that historic dosing strategies may not provide adequate analgesia in the models and strains studied. More research is needed in this area to gather perform confirmatory studies that show the effectiveness of grimace scales alongside other methods of pain assessment.

The application of MGS and RGS to chronic and neuropathis pain and the role of confounding factors during their application have not been resolved. After the development of the MGS, it was assumed that grimace scales could not accurately detect chronic pain states,[68] and this assumption has continued as scales were developed for other species. The basis for this assumption was the absence of changes in MGS scores in 2 well-characterized chronic pain models, spared nerve injury and chronic constriction injury. Subsequently, others have reported that chronic pain may be identified using grimace scales in other models, notably colitis (dextran sodium sulphate-induced), cervical radiculopathy (surgical compression model), neuralgia (chronic constriction injury of the infraorbital nerve), orofacial pain (movement or load-induced), spinal cord injury (cord impact model) and migraine (nitroglycerin-induced) in rats and mice.[2,6,48,74,80,112,134,152] These data suggest that limiting application of the MGS and RGS to acute pain may be premature.

Collection of images for grimace scoring and later assessment has limited use where a quick evaluation to guide clinical decision-making is desired. Two potential solutions to shorten the process while maintaining scoring integrity are real-time and automated scoring.[73,132,141] The feasibility of real-time scoring, where the observer assigns a score based on

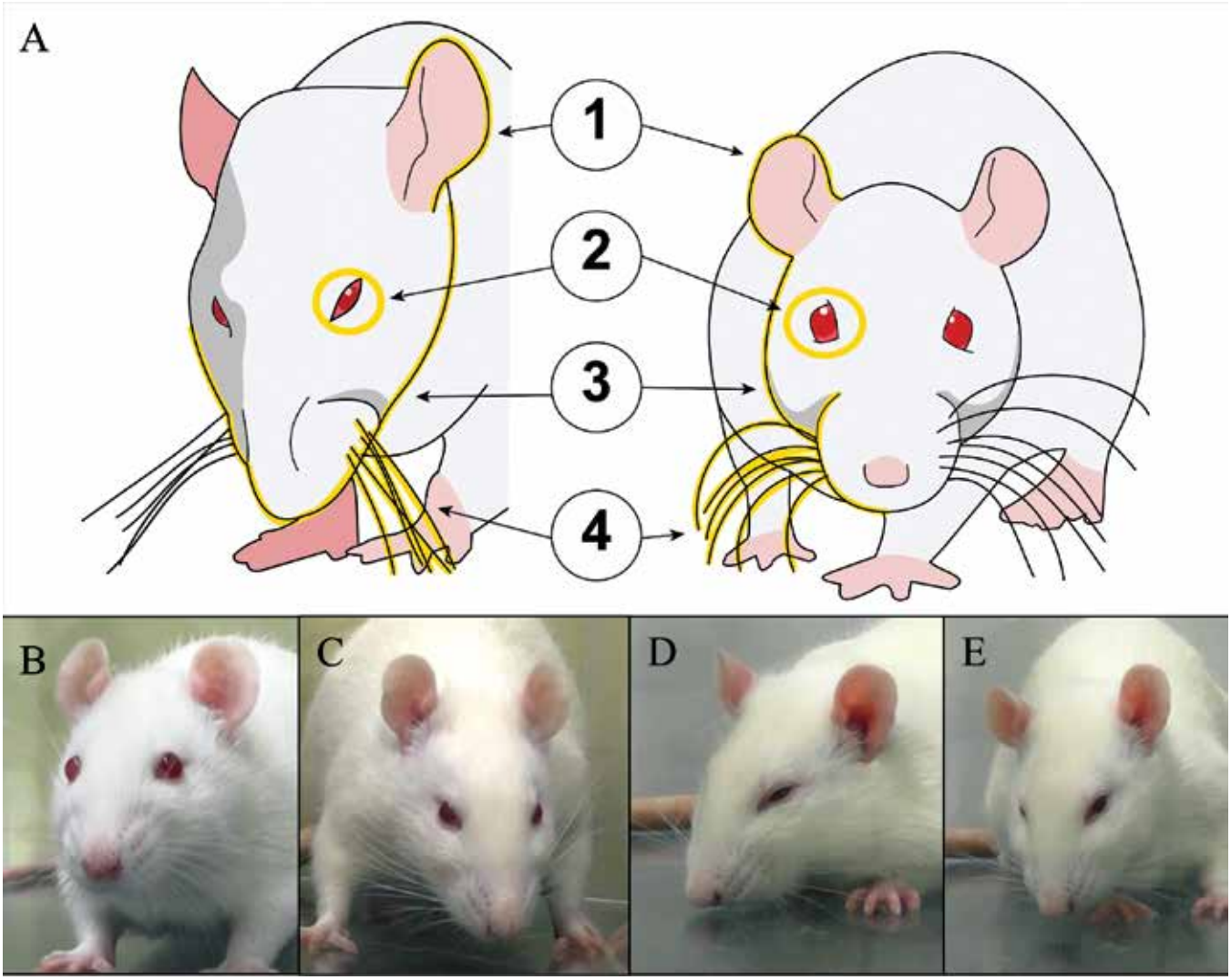


Figure 5. The Rat Grimace Scale (A) Rat depicted with 'pain' (left) and with 'no pain' (right). The 'pain' rat has 1) folded ears that are angled away from the front of the face, 2) partial eye closure, 3) a flattened and elongated nose and 4) whiskers that are bunched together and directed away from the face. The 'no pain' rat has 1) rounded ears that face forward, 2) no eye closure, 3) a rounded nose and cheeks and 4) whiskers that are fanned and droopy at ends. (B) Image depicts the face of a normal male Wistar rat with no pain. Its eyes are round and open. Its ears are rounded, facing forward and roughly perpendicular to the top of its head. Its nose and cheeks are rounded with an evident bulge and crease between the nose and cheeks. Lastly, the whiskers are spread apart and droop downward at the ends. (Action unit scores—Eyes: 0, Ears: 0, Nose/cheek: 0, Whiskers: 0). (C) Image depicts an adult male Wistar rat grimacing with orbital tightening, nose/cheek flattening with only a slight crease between the nose and cheeks and straightened whiskers that are pulled toward the cheeks. Its ears are curled and slightly rotated outwards. (Action unit scores—eyes: 1, ears: 1, nose/cheek: 1, whiskers: 1, overall score of 1 [from average of 4 action units]). (D) Image depicts an adult male Wistar rat grimacing with an overall score of 2. It has a tightly closed eyelid. Its nose and cheeks are flattened with the nose appearing elongated. The nose and cheek flatten with no crease evident between them. The whiskers are straightened, bunched together and horizontal to the cheeks. Its ears are rotated outwards and curled inwards. (Action unit scores—eyes: 2, ears: 2, nose/cheek: 2, whiskers: 2). (E) Image depicts an adult male Wistar rat grimacing with an overall score of 1.75. Its eyelids are tightly closed. The ears are curled and rotated away from the front of the rat's face. Its nose and cheeks are flattened with no crease evident between them. The whiskers are straight and pulled toward the cheeks. (Action unit scores—eyes: 2, ears: 2, nose/cheek: 2, whiskers: 1). Illustration by Dr Vivian SY Leung.

direct observation of the animal, has been shown with the RGS.[73] Scores based on continuous or discrete observations over periods as short as 2 min were able to identify predicted differences as a result of analgesic treatment. Importantly, the presence of the observer did not affect the RGS. Most recently, Tuttle and colleagues have shown machine learning to be a promising means of automated scoring.[141] Following training a neural net with close to 6000 images, automated scoring was able to identify pain and no-pain states with an accuracy (compared with human-generated scores) of 83 to 93%. Critically, confidence associated with scores (as determined by the neural net) was greatest with images representing the greatest changes in MGS, at either extremity of the scale. Further work is required to improve machine-based scoring across the encountered spectrum of images.

A key step in adoption of the MGS and RGS as tools for rapid evaluation of animals and facilitating decisions about care is development of an intervention threshold. One has been derived for the RGS (greater than 0.67/2, sensitivity; 85%, specificity; 89%).[103] An intervention threshold identifies the score above which a rat is more likely to be painful and the score should be viewed as a guide for rescue analgesic treatment rather than an absolute rule. Increasing the threshold will increase specificity at the expense of sensitivity and vice versa.[103]

Limitations in the use of the MGS and RGS are still being defined. In addition to their roles in identifying chronic pain (as described above), exposure to general inhalant anesthetics temporarily inflates RGS scores and should be considered when assessing animals in the early postoperative period.[83] Olfactory cues, such as exposure to clothing worn by men, can result in stress-induced analgesia and a consequent reduction in the MGS.[131]

Use of Nest-Building Behaviors to assess Pain in Laboratory Rodents. Nesting behavior is a major evolutionary driver for most rodents, particularly mice. Evaluations of mouse health and well-being through nest-building have been in continual evolution for at least a decade. The advantage of this approach is that it is easily incorporated into standard husbandry practices and takes advantage of an animal engaging in intrinsically motivated behavior. It may, therefore, be more sensitive to subtle aspects of the pain experience and may better reflect pain as it affects quality of life.[104] In its first iterations, evaluations of cage structure were used, assessing if the mice engaged in organizing their cage space to include a dedicated sleeping area separate from their toilet area.[4] Next the focus shifted to the complexity of the nest with scores ranging from zero, for an unmanipulated compressed cotton square, to a score of 5, if a bowl with well-defined walls was built.[60] The type and amount of nesting material which would optimally allow for the construction of a complete nest followed, facilitating a progression of nest scoring (Figure 6).[51] A complete nest has a full dome that encloses the center of the nest and allows the mouse to create a warm, dark, microenvironment that can be up to 10 °C degrees warmer than the rest of the cage environment.[42] Mice that underwent surgery had significantly lower nest scores than those that underwent sham procedures or had surgery with adequate pain management.[60]

Whereas all of these nesting and cage organization assessments were valuable steps in the evolution of cageside pain assessments in mice and added a quantitative component to otherwise qualitative measures, they were also relatively subjective. Therefore interobserver reliability could be problematic. In addition, while the mice may have had effective analgesia in the immediate postoperative period, subsequent changes in their pain state may go unrecognized because the nest complexity or cage structure score does not change once the nest is formed. Therefore, the next iterations of nest assessments corrected for these limitations by creating on-demand assessments of nest consolidation. In the time-to-integrate-to-nest or TINT test, mice are given a small piece of nesting material in the opposite end of the cage from their existing nest which they must retrieve, and then return the piece to their nest and integrate it. This was found to occur in 9 of 10 mouse strains within 10 min after the provision of the new nesting material. The likelihood of a negative TINT (taking greater than 10 min to retrieve) was significantly increased after having undergone a painful procedure.[116] The benefit of this approach is that the mice can be recovered in their home cage with an existing nest, supporting positive welfare in the postprocedural time period. In addition, TINT can be assessed on demand and in repetition, so the arc of recovery or absence of effective analgesia can be assessed over time. However, this method is assessed in a binary fashion, making it difficult to create a gradient with which to assess relative analgesic efficacy. So, while it is a good initial tool for identifying which cages require additional veterinary attention, it may not reveal smaller changes in pain severity or alleviation. The zone clearance test is a similar assessment of how quickly a mouse will retrieve pieces of nesting material around the cage.[97] Using this test, a mouse can retrieve 6 pieces of cotton nesting within 100 min at baseline, but likely fails this test after a painful procedure. However, normal retrieval and consolidation behavior is observed once analgesia is provided. The benefit of this assessment is that a greater range of scores are possible, allowing for a greater ability to tease apart differences in efficacy between analgesic regimens, as well as differences in the response to treatment. The challenge is that this method requires mice to recover from a procedure in a cage without an existing nest, and importantly, requires that the mouse chooses to build their nest in a corner such that they are clearing zones as they gather the nesting material. However, dependent on a number of caging and animal factors, mice may choose to build a nest in a location other than the corner, significantly reducing the number of zones cleared, even if the mouse is clearly exhibiting nest consolidation behavior. As published, this test would be used for a one-time assessment as it requires starting without an existing nest; this limits its use at multiple time points without significantly altering the home-cage environment.

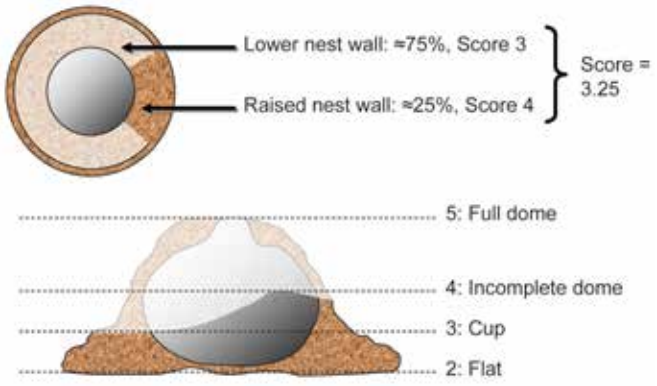


Figure 6. When given the appropriate amount and material, mice can build a full domed nest and a nest complexity score can be assigned. Reproduced with permission from AALAS.[51]

The most recent iteration of nest consolidation tests combines the strengths of the prior 2 assessments, TINT and zone clearance, by allowing mice to recover in a home cage with an existing nest and facilitates on-demand assessment that allows for minimal disturbance and longitudinal assessment while also providing more sensitivity in scoring to allow for gradations of scores.[104] In addition, this new assessment allows the nest to be built anywhere in the home cage, rather than only in corners. The Nest Consolidation Test requires mice be provided 4 pieces of cotton nesting material to be placed either in the 4 corners of the cage if no nest is already present in the home cage, or on the opposite end of the cage from an existing nest (Figure 7). The mice are then timed for how long it takes to retrieve the pieces of nesting material and consolidate by moving the pieces at least half of the cage length and/or width to be joined with another piece of nesting material or within one inch of the existing nest. This test was found to identify postprocedural pain in males, females, inbred and outbred mice, and was minimally impacted by anesthetics and analgesics. It not only identified unalleviated postoperative pain but could differentiate between different analgesic regimens allowing for better drug discrimination than traditional measures of mechanical threshold and clinical measures such as body weight loss. One important consideration for all nesting assessments for pain in mice is that to assess individual animals, they must be singly housed.[104] Single housing of animals that could otherwise be socially housed is not desirable as it can contribute to unnecessary stress in a recovering animal.

Recommendations for Ongoing Monitoring of Pain in Laboratory Rodents. If it is unclear whether pain or a confounder is underlying the pain assessment score, a tried and true approach

Score	Description	Example Image(s)
	Single or Pair Housed	
Start	Mice begin assessment with clean cage containing one-half square of cotton nesting material in each corner	
1	No cotton pieces grouped together	
2	Cotton pieces paired together in one or two pairs	
3	3 cotton pieces grouped together	
4	All cotton pieces grouped together	
5	All 4 cotton pieces grouped together and completely shredded	
	Single with Nest	
Start	Mice begin assessment with clean cage containing 4 half square cotton pieces placed at the lixit end of the cage and an Enviropak at the opposite end	
1	1 cotton piece is within a 1-inch perimeter of the Enviropak	
2	2 cotton pieces are within a 1-inch perimeter of the Enviropak	
3	3 cotton pieces are within a 1-inch perimeter of the Enviropak	
4	All 4 cotton pieces are within a 1-inch perimeter of the Enviropak	
5	All 4 cotton pieces are within a 1-inch perimeter of the Enviropak and have evidence of shredding and incorporation with crinkle paper	

Figure 7. The Nest Consolidation Test allows mice to retrieve one of 4 pieces of nesting material, either with or without an existing nest. The pieces must be consolidated to within a specific distance of one another or within the existing nest. The nest can be built anywhere in the home cage. Reproduced with permission from AALAS.[104]

in both human and veterinary medicine is to assess the response to analgesia (assuming that an effective dose of analgesic is administered).[40] Assessing animals before and after analgesic administration or comparing animals that received different agents or routes can often reveal whether the analgesic regimen is effective (Figure 8).[22] With this approach, it is important to understand the direction in which the chosen assessment parameter will change if pain is alleviated. For example, for paw withdrawal in response to mechanical pressure, one would expect latency to increase if the animal becomes more comfortable.

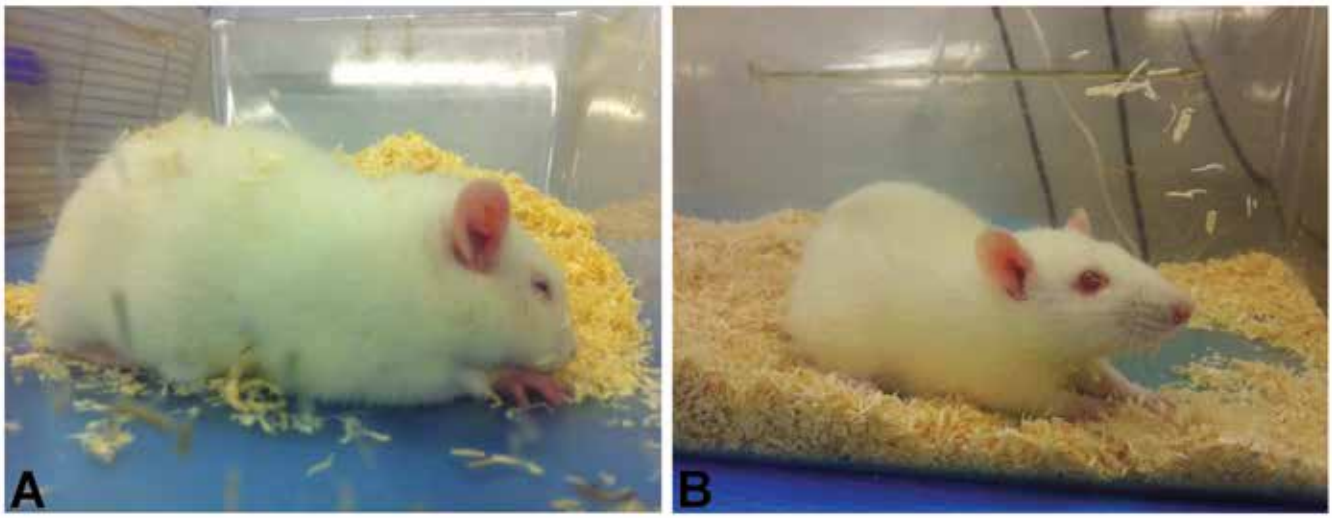


Figure 8. The postsurgical rat on the left (A) received a single analgesic, carprofen, whereas the rat on the right (B) that underwent the same procedure received multimodal analgesia: carprofen and tramadol. When comparing the 2 animals it is clear that the animal that received multimodal analgesia is more alert, paying attention to the observer, and in addition, its fur is lying flat, and its eyes are wide open, all indicating this animal is more comfortable than the rat that only received only carprofen. Reproduced with permission.[22]

In contrast, with nest consolidation scoring, alleviation of pain will be expected to shorten the latency to nest consolidation as the animal becomes more comfortable and regains normal behavior.

Assessments should be made starting from before pain is anticipated to begin, allowing the observer to identify when the pain starts. This facilitates early treatment and minimizes the period of untreated pain. Once pain is present, the observer should estimate the length of time that the pain is anticipated to continue – whether it is acute with a rapid recovery or the start of a long-term disease process, like arthritis or tumor induction and progression.

Once pain is present, the frequency of assessments should be tailored to match the expected duration of analgesic therapy. In human medicine, pain is expected to lessen within 30 min of analgesic drug administration.[38] In veterinary patients, this may not be likely in all situations because of analgesic pharmacokinetics,[40] but is a useful rule of thumb to consider when treating animals. Thus, assessments should be made before and after the provision of an analgesic to ensure that the agent is achieving the desired effect, and to provide additional analgesic if needed. Assessments should be repeated, based on the known pharmacokinetics of the drug to determine if pain has returned, and whether additional doses or different therapies are required. In human medicine, to be considered clinically useful, a minimal 33% change in an outcome measure is sought after treating patients with additional rescue medication for acutely painful conditions.[38] This clinical cut-off point was developed recognizing that there are no objective measures of painful experiences in human patients and individuals show wide variability in response to interventions. While a worthy goal, the utility of a clinical cut-off point is untested in veterinary medicine.

Analgesic therapy for laboratory rodents must be performed with a clear goal in mind. Dynamic pain occurs only when the animal is engaged in a particular behavior or when it adopts a particular body posture. Dynamic pain is often less severe and may affect more of the nonevoked measures of pain, such as nest building and grooming. Less potent analgesics,[40] lower doses or shorter regimens of analgesic may be sufficient to allow an animal to be engage in these higher-level, spontaneous behaviors. Alternatively, static pain occurs when the animal is at rest. Static pain is likely to prevent even basic maintenance behaviors, such as eating and drinking. While it may not be possible to alleviate all pain, goals for analgesic therapy should be to prevent static pain at a minimum, while helping the animal to return normal spontaneous behavior. This allows them to create and maintain a microenvironment that further supports recovery, such as normal nesting, burrowing and social behaviors with cagemates. Static pain conditions may require more potent analgesics, higher doses, and a longer course of therapy to maintain the animal in a comfortable state.

Conclusions

The search for novel measures to assess pain in laboratory rodents that do not rely on traditional evoked-response reflex testing is important for the evolution of translational pain research and for enhancing laboratory animal welfare. Many advances have occurred in rodent pain assessment techniques, but additional work is needed to understand the range of circumstances for which each test is useful. Future work should focus on the development of additional nonevoked cageside measures of pain that do not require handling or even the presence of an observer to aid in accurate identification of rodents in need of veterinary care. For mice, facial grimacing, nest building, and grooming have served this need under some experimental conditions. However, these evaluations should be more broadly implemented in formal clinical pain assessments, through institutional training programs for animal ethics committees, research groups, and technical personnel. Unfortunately, these approaches have not been widely tested for other laboratory rodents, although alternate assays, such as analysis of burrowing behavior, may be appropriate. Identifying behaviors that these species will readily engage in, that are significantly altered by a painful stimulus, and that can be restored by analgesia must be developed. Advancements in technology, such as home cage ethogram analysis, automated facial grimace analysis, and smart cage read-outs of animal physiology and activity may also assist with discovery of new or more efficacious analgesic treatment regimens for different rodent conditions. However, even if better assessment tools can be developed, a major challenge remains: how to provide individualized animal

assessments and pain mitigation when large numbers of rodents are on study at any given time. This ethical issue merits further consideration by the community at large.

Acknowledgment

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