Operant Assays for Assessing Pain in Preclinical Rodent Models: Highlights from an Orofacial Assay

Niall P. Murphy, Richard H. Mills, Robert M. Caudle and John K. Neubert

Abstract Despite an immense investment of resources, pain remains at epidemic proportions. Given this, there has been an increased effort toward appraising the process by which new painkillers are developed, focusing specifically on why so few analgesics make it from the benchside to the bedside. The use of behavioral assays and animal modeling for the preclinical stages of analgesic development is being reexamined to determine whether they are truly relevant, meaningful, and predictive. Consequently, there is a strengthening consensus that the traditional reflex-based assays upon which several decades of preclinical pain research has been based are inadequate. Thus, investigators have recently turned to the development of new preclinical assays with improved face, content, and predictive validity. In this regard, operant pain assays show considerable promise, as they are more sensitive, present better validity, and, importantly, better encompass the psychological and affective dimensions of pain that trouble human pain sufferers. Here, we briefly compare and contrast reflex assays with operant assays, and we introduce a particular operant orofacial pain assay used in a variety of experiments to emphasize how operant pain assays can be applied to preclinical studies of pain.

Keywords Operant · Pain · Orofacial · Testing · Assay · Preclinical

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1 Introduction

Pain is a deeply personal experience that virtually everyone has experienced at some point in their lives, and most people have benefited from its adaptive value. However, there are numerous situations, pathological and otherwise, where uncontrolled pain is counterproductive and debilitating. As such, chronic pain is an epidemic public health problem, costing individuals their well-being and costing society billions of dollars annually. Thus, the quest to find effective and safe analgesics stretches back millenia, and despite the time and resources dedicated to this quest, it is arguable that we still have a limited number of appropriate methods for safely, effectively, and permanently ridding ourselves of pain. Acute pain control is relatively straightforward with the use of nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, and local anesthetics; however, the management and relief of chronic pain is much more troublesome, as opiates, the gold standard for care in many chronic pain patients, are liable to inducing dependence, tolerance, and, to some extent, addiction.

So, why are we still without the perfect analgesic? Modern development of analgesics depends on a discovery process that ultimately relies on in vivo testing, which almost invariably assesses the effectiveness of painkillers in experimental animals. Some of these assay methods date back many decades, and while they have certainly been useful in the discovery process, there has recently been considerable interest in re-evaluating the utility of these assays, particularly in terms of their ability to model pain states relevant to the way pain is experienced by people (Vierck et al. 2008). Moreover, as time has passed, the realization that pain is not simply a reflex, but a complex psychological and emotional experience that can derive from a variety

of causes (i.e., nociceptive, inflammatory, neuropathic, or idiopathic) has called for more relevant assays and models that present more than face validity.

2 Pain Assays

The generation of animal models of neuropathic and inflammatory pain mimicking clinical pain symptoms has been steadily increasing. However, in vivo pain evaluation has been susceptible to problems that have hindered the development of models with strong content, face, and predictive validity. Until recently, most preclinical pain assays have consisted of exposing experimental animals to some type of painful stimulus, and then measuring the intensity, frequency, rate, or duration of a behavior believed to reflect the experience of pain. Noxious heat stimulation, such as exposure to a hot plate, has been used extensively, though a gamut of others have been applied over the years, including formalin or acetic acid injection and physical stimulations such as tail pinch and von Frey filaments. Generally speaking, the responses measured in these assays fall into one of four categories (1) simple withdrawal reflexes, such as paw withdrawal; (2) unlearned or innate behaviors, such as licking or guarding; (3) spontaneous behaviors, such as grooming; or (4) learned or operant behaviors, such as lever pressing to receive an analgesic (Chapman et al. 1985). While a brief discussion of the appropriateness of established animal models in pain is warranted here, the reader is also referred to recent reviews for a more in-depth discussion on this specific topic (Mogil 2009; Rice et al. 2008; Vierck 2006). In short, simple withdrawal reflexes and innate responses generally offer the advantage of being relatively reliable and open to objective scoring, but lacking in clinical face validity. This is largely due to an over-reliance on simple spinal reflexes; furthermore, these assays do not consider the complex central neural circuitry responsible for the affective experience, and executive control that animals must have to adapt to pain states. While reflex responses can be evoked in even decerebrate animals (Woolf 1984), their utility is exemplified by the efficacy of a few classes of established analgesics, particularly opioids such as morphine. Aside from the lack of clinical face validity, the one-dimensional nature of the outcome measures for reflex responses (e.g., latency to paw withdrawal) makes them susceptible to misinterpretation or overinterpretation. For instance, many drugs have sedative and/or psychomotor properties that can readily confound any outcome measure that relies solely on a motor response.

3 Operant Pain Assays

Here, we define “operant” as a voluntary behavior modified by the consequences of emitting that behavior, regardless of whether those consequences are positive or negative. For instance, a patient with chronic back pain may report their pain levels differently, if they know their spouse will support them differently (e.g., provide

massages). In this instance, alleviation of the painful state is the consequence of the voluntary report of higher pain. Another example is a person with a history of migraine headaches who is motivated to seek medication, as they have learned such behavior helps alleviate their pain. Importantly, characteristics of operant behaviors are that they are intentional, motivated, learned, and typically involve complex "higher" processing (Vierck 2006).

Conflict paradigms are an example of an instance where animals emit operant responses. For instance, Mauderli and colleagues (Mauderli et al. 2000) subjected rats to an avoidance/escape paradigm by providing them a choice between two compartments—one with a floor set to an aversive temperature (hot or cold), or an alternative escape compartment with a neutral floor temperature, but brightly lit. By determining the time spent in the heated versus the brightly lit compartment, Mauderli and colleagues inferred the amount of pain being experienced by the rats. Reward-conflict assays provide another type of operant testing paradigm and will be further highlighted in this chapter using the Orofacial Pain Assessment Device (OPAD). In a reward-conflict assay, an animal is given a choice between receiving a reward in the presence of an aversive stimulus or choosing not to pursue the reward and avoiding the stimulus.

In terms of pain testing, operant assays are characterized by their integration of the entire neuraxis. A stimulus sufficient to activate primary sensory afferents generates signals that propagate through spinothalamocortical projections leading to a subsequent cortically mediated influence over the behavioral response (Vierck 2006). The integration of these different levels of the nervous system better reflects human pain behavior because the outcomes depend on both nociceptive and motivated, learned processes (Vierck 2006). In this regard, operant assays differ markedly from reflex-mediated (e.g., tail flick) or unlearned behaviors (e.g., paw licking, guarding), in that spinalized and decerebrate animals cannot display these highly integrated pain outcomes (Woolf 1984). As pain is a complex sensation, one needs to utilize comprehensive assays to gain a thorough understanding of underlying mechanisms. Given this, interest in operant measures of pain is increasing, and recent years have seen the development of various approaches, including avoidance, conditioned place preference, escape, and drug self-administration. Some of the most common approaches are summarized in Table 1. As outlined in the table, operant measures typically monopolize on either the desire of animals to avoid or terminate pain (e.g., avoidance/escape, drug self-administration) or their willingness to endure pain for a reward (e.g., the OPAD system). Generally speaking, avoidance/escape paradigms are easier to execute experimentally, are arguably easier to interpret, and are considered by some to be the gold standard in preclinical operant pain testing (Vierck et al. 2008). However, reward-conflict paradigms can offer graded responses because the magnitude of the response can be carefully controlled by titrating the magnitude of the reward against the noxious stimulus conflicting with the reward.

Operant assays offer several advantages in addition to their ability to address the higher central pain circuits that underlie the complex learned and motivated behaviors used by humans to avoid pain. These assays have the ability to reveal

Table 1 Summary of major preclinical operant pain tests

Assay	Description	Outcome measures	Advantages	Disadvantages	Example references
Intracranial self-stimulation (ICSS)	Electrodes implanted into lateral hypothalamus; assess pain-depressed impairment of a motivated behavior	Intensity of applied current or frequency of stimulation	Pain-depressed behavior is representative of human clinical pain	Technically challenging implantation surgery; relatively new pain measure	Pereira Do Carmo et al. (2009) see Ewan and Martin (2013)
Escape test	Animals are given a choice between a non-noxious aversive stimulus (e.g., light) against an alternative noxious aversive stimulus (e.g., temperature)	Time spent in aversive light or on aversive thermal plate	Assessment of thermal pain versus innate behaviors	Observed effects could be due to effects on either of the two conflicting stimuli	Mauderli et al. (2000), Ding et al. (2005)
Dolognawmeter	Animals gnaw through polyethylene foam and ethylene vinyl acetate resin dowels	Time to gnaw through dowels	Use of innate behavior; minimal/no training; measure of deep pain (e.g., muscle, joint)	Vulnerable to drugs that induce gnawing; does not distinguish between pain and dysfunction	Dolan et al. (2010)
Orofacial Pain Assessment Device (OPAD)	Animals are given an opportunity to consume a palatable reward in the presence of a noxious stimulus (e.g., temperature) applied to their face	Reward licking number, face stimulus contact number, reward/stimulus pain ratio	Innate behavior, thermal and mechanical pain assessment	Specific to orofacial region; hairy animals must be shaved for thermal testing; observed effects could be due to effects on either of the two conflicting stimuli	Nolan et al. (2011), Anderson et al. (2013), Neubert et al. (2005, 2008), Rossi and Neubert (2009)

(continued)

Table 1 (continued)

Assay	Description	Outcome measures	Advantages	Disadvantages	Example references
Place conditioning	Animals physically avoid locations in which they experience pain, or gravitate to locations in which they have received relief from pain	Time spent in a physical locality where the stimulus was presented	Can assess both the rewarding and pain relieving properties of stimuli. Animals can be drug free during final testing	Can be confounded by simple reward or aversion effects independent of pain relief	Cahill et al. (2013) King et al. 2009), Sufka (1994) see Navratilova et al. (2013)
Lever pressing	Animals press levers to obtain analgesics	Number of presses made/ willingness to increase effort	Well-established paradigms. Strong content validity	Extensive training required. Easily confounded by motor impairments or psychomotor activation	See Ewan and Martin (2013), Martin and Ewan (2008)

ongoing and spontaneous pain states, such as inflammatory or neuropathic pain. For instance, a given drug may have no inherent motivating properties in an animal that is not in pain, and therefore animals will not seek out places where they have experienced this drug. In psychological parlance, they do not show a "conditioned place preference". In contrast, animals in pain will seek out such a place, thus revealing that they are in a pain state. Secondly, given the complex nature of operant measures, animals are afforded the opportunity to develop flexible response strategies for avoiding pain. For instance, animals may choose to expose themselves to pain in short frequent bursts, rather than in long occasional bursts to obtain the reward. This response flexibility more closely mimics the pain states of humans who also must develop novel strategies (e.g., frequently sitting down to avoid arthritic pain) to allow them to adapt and continue their lives. Thirdly, some pain-related responses consist of complex cognitive aspects that can potentially only be uncovered by operant assays in experimental animals. Examples of this could include social aspects, placebo effects or the ability of complex environmental sensory stimuli, such as noise or odor, to interfere with pain responding. Fourthly, as operant pain assays are often fully automated, they fully remove observer bias. In addition, in the context of studying orofacial pain and trigeminal neurobiology, these assays do not require restraining animals, which is necessary for some of the more traditional reflex tests, likely significantly reducing the contribution of stress and stress-induced analgesia. Finally, the dynamic nature of the assay may allow rapid and automated determination of otherwise laborious measures such as the temperature at which 50 % responding (i.e., an ET_{50}) occurs, as pain stimulus intensities can be controlled rapidly and titrated to instantaneous responding of animals.

However, operant assays are not without limitations. Firstly, their complex cognitive nature often requires special consideration of relevant and sometimes confounding psychological processes, such as learning, memory recall, anxiety, attention, salience, motivation, and reward efficacy. Particularly in the case of reward-conflict paradigms, two opposing processes are integrated, making it difficult to ascribe any change in behavioral responding to a specific process. To some degree, a well-designed experiment with adequate controls can tease these effects apart, but this may require a larger commitment of resources. Indeed, much of the central neural circuitry mediating affective states associated with pain, or the relief from pain, is shared with other affectively-laden stimuli, such as food and drugs. Secondly, the effect of drugs on motor systems requires consideration. Though, locomotor effects are generally more readily recognized in operant assays than traditional reflex assays, and can be more easily avoided by virtue of the higher sensitivity of the assay. Thirdly, relative to reflex-based assays that can be performed with equipment as simple as a water bath or a syringe and hypodermic needle, operant assays often require specialized equipment, such as Skinner boxes and computers, and more space. Fourthly, in comparison to reflex assays that measure innate unconditioned responses, operant assays rely on learned and conditioned behaviors that can require extensive training before reaching a stable baseline.

4 The Orofacial Pain Assessment Device (OPAD)—a Preclinical Operant Pain Assay

We have introduced preclinical operant pain assays and compared them with traditional reflex assays. The remainder of this review will highlight a new preclinical operant pain assay recently developed in our laboratory. This assay uses an Orofacial Pain Assessment Device (OPAD) and was conceived to address many of the concerns outlined above and, consequently, offers several advantages over reflex pain assays. We have completed a series of studies in both rats and mice using the OPAD (and previous prototypes) to demonstrate its utility.

At the heart of the OPAD is a reward-conflict assay whereby rodents express their willingness to withstand thermal pain applied to their face in order to gain access to a palatable liquid reward, such as sucrose solution or sweetened condensed milk. Thus, the primary outcome measure is the number of successful lick events. The device applies mild noninjurious temperatures (typically in the range 8–48 °C) using two "thermodes" against which the animal must place its muzzle to access the reward. The Peltier-based thermode can be adjusted in several ways, including width—to accommodate the size of the animal (e.g., from mouse to rat) and temperature—using a computer-controlled delivery of either static or dynamic stimulus temperatures. Additionally, we have added nickel titanium wires in front of the thermodes that provide sharp, punctate mechanical stimulation to assess mechanical allodynia and hyperalgesia (Nolan et al. 2011). This utilizes the same pain outcomes across different stimuli modalities (i.e., thermal vs. mechanical) and enables direct comparison of the relative impact of each stimulus on operant pain behavior. We recently published the detailed methodology and practical aspects of setting up, programing, and using the OPAD that includes a video reference (Anderson et al. 2013).

Aside from the above, the OPAD has several additional desirable features. In particular, the OPAD can detect failed access attempts to the reward by recording when thermode contacts are made without the animal successfully obtaining the reward. These failed attempts provide an indirect measure of motivation and pain sensitivity. Secondly, as outlined below, the OPAD produces a robust and smooth stimulus response curve, unlike reflex-based assays that depend largely on threshold behaviors. Thirdly, the assessment of pain-related outcomes can be exported in computer data files in a ready-for-analysis format.

5 Summary of Experiments Using OPAD

The breadth of studies completed using the OPAD demonstrates the range and utility of this system for assessing pain and analgesics. We have applied the OPAD to study the most commonly used pain models, including inflammatory pain (Neubert et al. 2005, 2006), neuropathic pain (Rossi et al. 2012), central pain

(Caudle et al. 2010), and chemotherapy-induced peripheral neuropathic pain (Mustafa et al. 2013). With these models, we have investigated several hypotheses related to physiology (Neubert et al. 2008), expectations (Nolan et al. 2012), environment (Rossi and Neubert 2008), and pharmacological treatments (Chapman et al. 1985; Neubert et al. 2005; Caudle et al. 2010; Mustafa et al. 2013; Kumada et al. 2012; Rossi et al. 2009). A summary of studies to date that have utilized the OPAD is provided in Table 2. Independent investigators have recently adopted OPAD-type methodology to complete studies relating to cold sensitivity via transient receptor potential (TRP) channel modulation and nerve injury, (Zuo et al. 2013; Cha et al. 2012) and we anticipate further increases in research employing the OPAD. The following five examples illustrate the many studies completed using this operant orofacial reward-conflict paradigm and highlight the breadth of research that can benefit from this type of behavioral assessment.

6 Study 1: Relating Behavior to Physiology—Characterization of Mouse Orofacial Pain and the Effects of Lesioning TRPV1-Expressing Neurons on Operant Behavior

A particularly important but difficult aspect of behavioral studies is relating the behavior of an animal back to the function of specific cells and proteins. As highlighted above, reflex assays generally depend on thresholds for initiating a response. Consequently, once the threshold is crossed, the difference between stimulus intensities is lost as they all evoke the expected motor response. Even if the precise force or temperature of the stimulus on the skin is known, there is little difference in behavioral response characteristics that can distinguish the stimuli. Thus, correlation of reflex behavior with cell or protein function, which can be measured with great precision, is handicapped by the floor effect of the behavioral assay. In essence, once the reflex is initiated the sensory stimulus is irrelevant. In reward/conflict operant assays, behavioral responses to the stimulus are modified in a more graded fashion by animals in response to the stimulus intensity, the stimulus unpleasantness, and the desire of the animal to acquire the reward. Furthermore, strategies to obtain the reward may change as the variables change and these strategies are easily and distinctly measured from the strategies used to evaluate the response to nonaversive stimuli. For example, as the OPAD thermode temperature moves into the aversive range, animals reduce the amount of time that they press their muzzle onto the thermodes, yet they increase the total number of times that they contact the thermodes. The new strategy results in consumption of a similar amount of the reward solution in the aversive conditions, but a clear difference in the duration and number of stimulus contacts.

Table 2 Examples of operant orofacial pain studies. Bolded references indicate additional information is provided in the text

Study design	Results	Reference
Substance P-Botox A conjugate; ICM; CIPN	Control animals developed thermal sensitivity post-paclitaxil treatment; SP-Botox A animals demonstrated analgesia	Mustafa et al. (2013)
N-INF; thermal ramping protocol	OPAD methodology, online video available	Anderson (2013)
High-fat or regular diet; C57Bl/6 J, SkH1-E mice	No effect of diet-induced obesity on acute thermal nociception in the absence of inflammation or injury	Rossi et al. (2013)
Trigeminal CCI; thermal, mechanical	Development of cold sensitivity and aversive mechanical behaviors after infraorbital nerve injury	Rossi et al. (2012)
Conditioning with morphine or PBS	Placebo effect produced in morphine-conditioned animals; naloxone reversed placebo response	**Nolan et al.** (2012)
Chronic morphine administration to induce tolerance	Chronic opioid use induced changes in NMDA receptor composition; differential pain sensitivity based on NMDA subunit change	Anderson et al. (2012)
Botox A; intramuscular; CCI	Botox A blocks development of CCI-induced heat hyperalgesia	Kumada et al. (2012)
N-INF; morphine; mechanical, thermal	Thermal versus mechanical stimuli with same outcome measures; demonstration of mechanical sensitivity using varying diameters of nickel titanium wire	Nolan et al. (2011)
Naïve; sucrose and sweetened condensed milk reward	Differing concentrations of noncaloric (sucrose) versus caloric (sweetened condensed milk) rewards can modify operant pain outcomes	Nolan et al. (2011)
SP-CTA; ICM; Naloxone; rats and mice	Central pain model (SP-CTA); naloxone sensitive; mu-opioid receptor knockout mice display sensitivity; endogenous opioid system implicated	Caudle et al. (2010)
Naïve; face place preference	Motivated behavior can be modulated based on hot/cold face preference	Rossi and Neubert (2009)
Icilin; ICM	Comparison of TRPA1/TRPM8 agonism on cold and heat sensitivity; low dose icilin (0.025 mg) induces cold sensitivity, but decreases heat sensitivity	Rossi et al. (2009)
RTX; TRPV1 KO and wild-type mice (C57BL/6 J, SKH1-)	Comparison of effects of pharmacologic and genetic TRPV1 manipulation on heat sensitivity; first mouse paper using orofacial operant assay	**Neubert et al.** (2008)
Sex differences; heat sensitivity	Males were hypersensitive to nociceptive heat	Vierck et al. (2008)

(continued)

Table 2 (continued)

Study design	Results	Reference
Environmental enrichment; general activity (rearing)	Environmental enrichment reduces exploratory behavior and increases pain thresholds (reduces sensitivity)	**Rossi and Neubert** (2008)
Mu and Kappa opioid agonists; N-INF; rearing	Comparison of reflex versus operant measures; demonstrated sensitivity of operant assay; GR89,696 ineffective for operant pain reduction	**Neubert et al.** (2007)
Naïve; menthol	Less robust stimulus response in the cold temperature range compared to heat; menthol can produce cold sensitivity	Rossi et al. (2006)
N-INF; morphine	Capsaicin-induced allodynia versus hyperalgesia demonstrated with varying temperature	Neubert et al. (2006)
INF; morphine	First paper describing the orofacial operant assay; inflammation produced thermal hyperalgesia reversed by morphine	Neubert et al. (2005)

Botox A Botulinum neurotoxin A
CCI Chronic constriction injury
CIPN Chemotherapy-induced peripheral neuropathy
ICM Intracisternal injection
INF Inflammation (carrageenan)
N-INF Neurogenic inflammation (capsaicin)
PBS Phosphate buffered saline
RTX Resiniferatoxin
SP-CTA Substance P-Cholera Toxin subunit A

An example of the above is shown in the previously unpublished data in Fig. 1, which illustrates the longest thermode contact as a function of thermode temperature (Neubert et al. 2008). The altered strategy produces a smooth sigmoidal stimulus response relationship. Figure 1 also shows the stimulus response relationship for the current evoked by heat through transient receptor potential vanilloid 1 (TRPV1) channels. The inward currents evoked in response to heating the buffer solution were measured by whole cell voltage clamp (−60 mV) in HEK293 cells that were transiently transfected with TRPV1. Previous work demonstrated that TRPV1 knockout mice exhibit a rightward shift in their stimulus response profiles indicating that the noxious range of 42–48 °C is likely mediated by TRPV1 receptors (Neubert et al. 2008; Mitchell et al. 2014). The effect of temperature on behavior and neurophysiological responses to heat are correlated (r^2 = 0.82, p = 0.036) suggesting that TRPV1 may transduce the stimulus that initiates the change in behavior. It is clear from Fig. 1 that the protein is activated at approximately the same temperature that animals start to reduce their contact time with the stimulus probes. As the temperature increases, the current flowing through the ion channel increases and the animals demonstrate a proportional decrease in their stimulus contact times. In thermal-evoked reflex assays, the temperature initiating the behavioral response is typically unknown and the behavioral response is

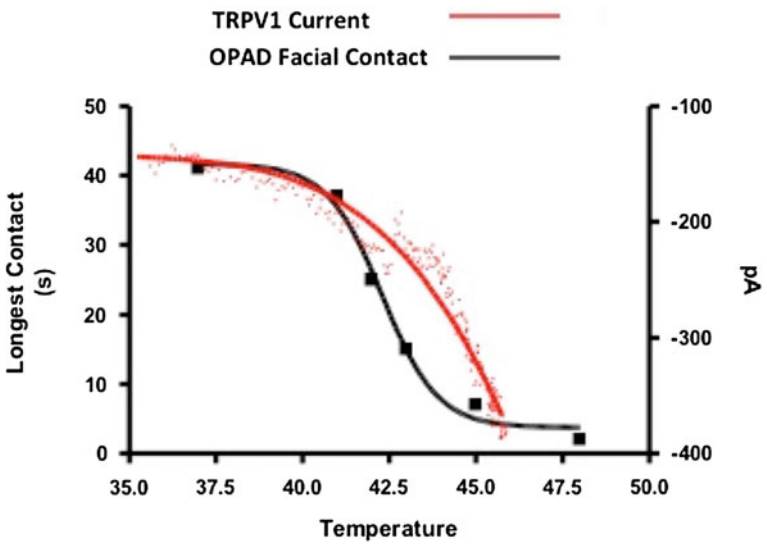


Fig. 1 Relationship between operant behavior and physiology. A representative temperature/current relationship for TRPV1 (*red*) was plotted with the temperature/response relationship for rats' longest contact with the thermal stimulus while performing in the OPAD (*black*). TRPV1 currents were obtained from whole cell voltage-clamped HEK293 cells that were transiently transfected with TRPV1. The bath solution was slowly raised as the current was monitored at −60 mV. The OPAD data were collected in separate experiments at multiple temperatures to evaluate pain tolerance (longest contact duration during a 10 min session)

measured only by response latency. In one of the few studies to examine skin temperature as it relates to reflex initiation, Hargreaves and colleagues found that hind paw stimulated reflexes in normal animals were evoked when skin temperatures reached approximately 45 °C (Hargreaves et al. 1988). As Fig. 1 illustrates, 45 °C is well above the temperature at which the TRPV1 ion channels are engaged and the animals begin to make behavioral adaptations in the operant assay. Thus, the graded responses and adaptive behaviors of rodents in operant assays provide more information with which to correlate behavior with protein or cell function than reflex-based assays.

To further evaluate the role of TRPV1 in operant pain, we used the OPAD system to evaluate thermal sensitivity after pharmacological intervention or gene deletion mutant mice (Neubert et al. 2008). Figure 2a shows that wild-type C57BL/6 J displays a typical thermal stimulus response, in that reward licking events decreased significantly as the stimulus temperature reached noxious temperatures (≥45 °C). TRPV1 KO mice (Fig. 2c) were insensitive to the thermal stimulus through the temperature range corresponding to TRPV1 activity, as displayed by the relatively flat response up to 52 °C (Neubert et al. 2008). Both genotypes showed a significant decrease in reward licking events at 55 °C (Fig. 2b, c), a temperature mediated by TRPV2 (Caterina et al. 1999). When wild-type C57BL/6 J animals were treated

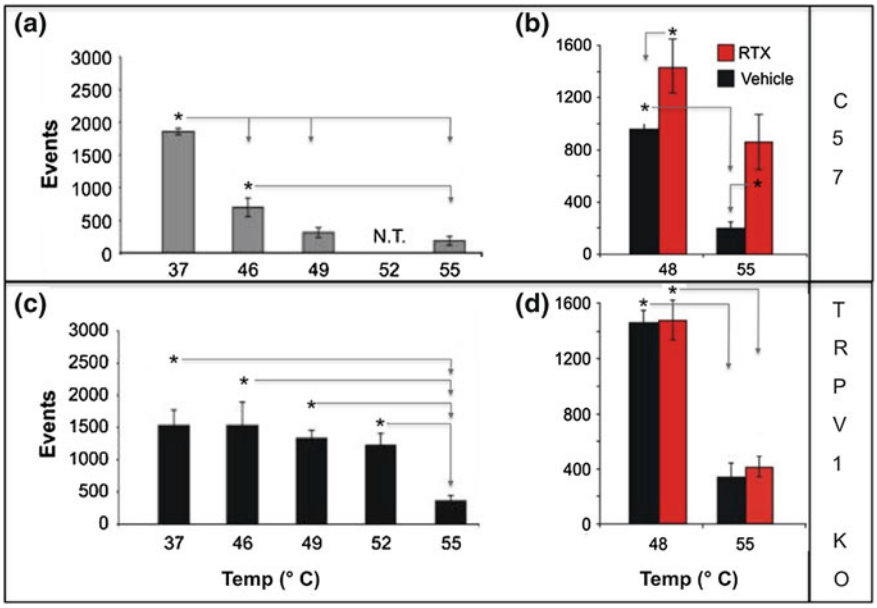


Fig. 2 Use of genetic and pharmacological manipulation of TRPV1 to assess the relationship between physiology and pain operant behavior in mice. **a** Operant reward licking events in naïve wild-type C57BL6 J mice decreased as stimulus temperature increased. *N.T.* not tested. **b** Wild-type C57BL6 J mice injected intracisternally with the TRPV1 agonist, RTX, had significantly higher licking events compared to vehicle-treated animals at both 48 and 55 °C. **c** TRPV1 KO mice were relatively insensitive to temperatures ≤52 °C, as their responses in the noxious heat range of 46–52 °C produced responses similar to baseline 37 °C testing conditions. **d** TRPV1 KO mice treated with RTX responded similarly to vehicle-treated KO mice at both 48 and 55 °C. Data are shown as mean ± S.E.M. * = $P < 0.05$. This figure was reproduced and modified from a previously published study (Neubert et al. 2008)

intracisternally with resiniferatoxin (RTX), a potent TRPV1 agonist used to molecularly lesion TRPV1-expressing neurons, the response increased significantly such that the C57BL/6 J animals resembled KO animals at 48 °C (Fig. 2b). As expected, RTX had no effect on TRPV1 KO animals (Fig. 2d). Interestingly, the C57BL/6 J RTX-treated animals also showed insensitivity at 55 °C, which we hypothesize is due to lesioning of TRPV2 neurons that coexpress TRPV1 (unpublished data).

These studies provide strong, direct evidence that the behavioral measures assessed using the OPAD are linked to pain processing. For example, setting the thermal stimulus at temperatures that are noxious (>42 °C) elicits the expected avoidance behavioral strategy. Notably, the ability to precisely control stimulus temperature with a ± 0.1 °C tolerance allows us to finely discern pain responses at 42 °C, the lower limit of TRPV1 activation.

7 Study 2: Action of an Established Analgesic (Morphine) on OPAD Operant Responses

Despite the immense cost involved with developing novel analgesics, only a few established classes of drugs—including antiepileptics, acetaminophen, opioids, and NSAIDs—remain staples for pain control. To validate the OPAD, we sought to demonstrate that morphine is an effective agent for increasing operant behavioral outcomes and is reflective of analgesic responses (Neubert et al. 2005, 2006, 2007; Nolan et al. 2012; Anderson 2012). Importantly, morphine at doses as low as 0.25 mg/kg can produce significant antihyperalgesic effects, and doses as low as 2 mg/kg can produce analgesia. We reasoned that the relatively high sensitivity of the OPAD allows for detection of responses in dose ranges that are clinically relevant (humans require approximately 0.15 mg/kg for pain relief) (Plone et al. 1996). The vast majority of reflex-based measures typically require 5- to 50-fold higher doses of morphine (5–50 mg/kg) to detect an "analgesic" response. At these high doses, confounding behavioral responses, such as sedation or hyperlocomotion, are likely to occur. This is important when considering the predictive value and translatability of these models and methods to human clinical care.

To illustrate the efficacy of low doses of morphine to reduce hyperalgesia, Fig. 3 shows results from two individual naïve animals given either phosphate buffered saline vehicle (PBS, Fig. 3a) or morphine (2 mg/kg, Fig. 3b). These are typical examples of OPAD output response tracings for nonanalgesic and analgesic treatments, respectively. The figure shows the use of a ramping protocol whereby animals are initially tested at 32 °C for 2 min, before the thermode is ramped to 43 °C, held there for 10 min, and then returned to 32 °C for 2 min. These data show that both animals completed the operant task when the thermodes were set to the neutral temperature (32 °C), but only the morphine-treated animal could maintain this behavior when the temperature reached nociceptive (43 °C) levels. Note that individual events (e.g., licks, and stimulus contacts) can be patterned over time to generate a complex behavior based on the external stimulus and internal processing of the animal as they form their response during the session. Nonetheless, the overall pattern quickly and simply distinguishes a painful from a nonpainful response.

8 Study 3: The Effect of Environmental Enrichment on Thermal Sensitivity in the OPAD Assay

The experience of pain is influenced by numerous factors, including molecular makeup (e.g., C- vs. A-delta nociceptors), genetics, sex, and epigenetics. Pain and stress are tightly related, and studies show that acute and chronic stress can modulate nociceptive responses (King et al. 2003, 2007; Gameiro and Gameiro 2006; Gameiro 2005; Khasar et al. 2005; Butler and Finn 2009; Olango and Finn 2014).

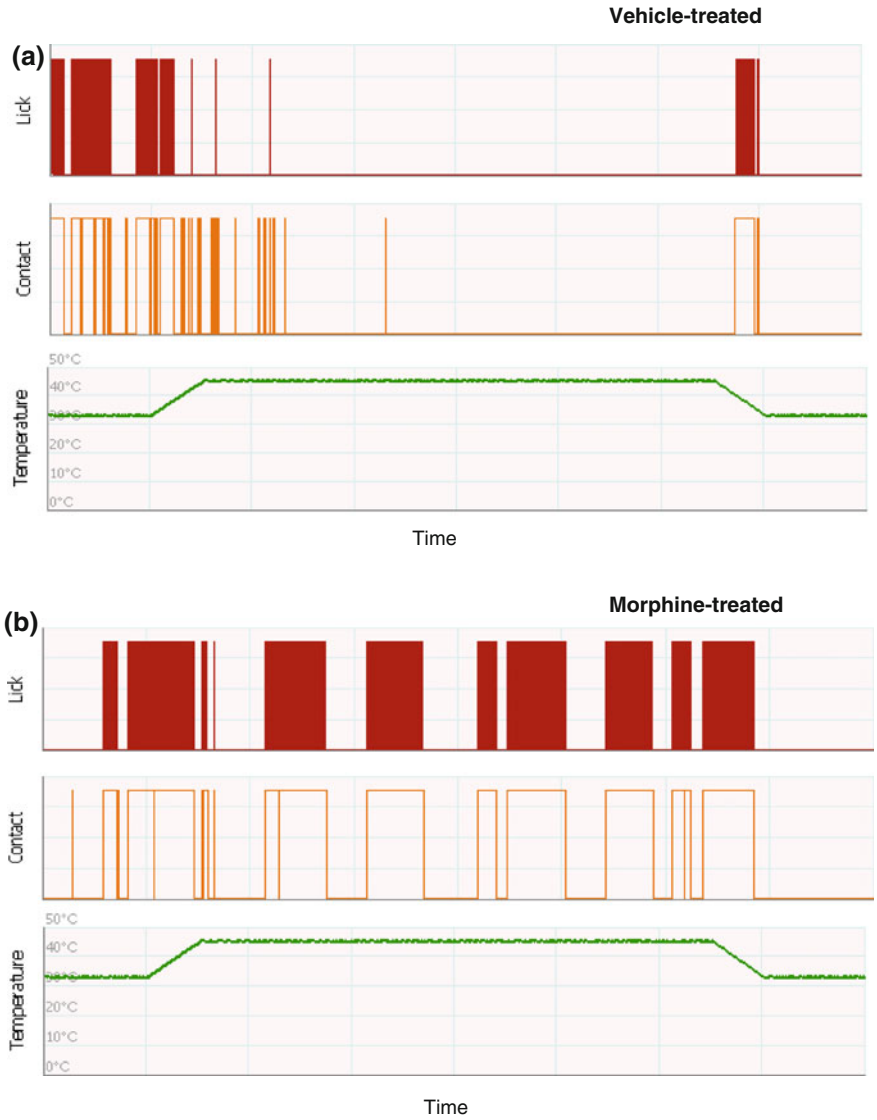


Fig. 3 An example of an analgesic versus painful response measured in the OPAD. The three traces in each panel display reward licking events (Lick), thermode contact events (Contact), and thermode temperature (Temperature). **a** Rat treated with vehicle (PBS) 30 min prior to testing. **b** Rat administered morphine (2 mg/kg, i.p.) 30 min prior to testing. These are typical traces for an animal that displays a normal "nonanalgesic" (**a**) and an "analgesic" (**b**) pain response. The stimulus was held at 32 °C for 2 min, ramped to 45 °C and held for 10 min, and then ramped back down to 32 °C. Note that both animals have similar responding during the lower neutral temperature period, characterized by long bouts of licking and few, but long, stimulus contacts. As the temperature becomes painful (45 °C), only the morphine-treated animal maintained responding

A straightforward approach to modify stress in laboratory animals is to change their housing to include environmental enrichment. Environmental enrichment allows for supplemental cognitive and physical tasks with additional sensorimotor activity (Duan et al. 2001) that can improve cognition and reduce anxiety (Benaroya-Milshtein et al. 2004; Nilsson et al. 1999). Conversely, isolation and environmental deprivation may increase or decrease pain, in the form of stress-induced hyperalgesia (Becker et al. 2006) or stress-induced analgesia (Coudereau et al. 1997; Puglisi-Allegra and Oliverio 1983), respectively. Given that operant assays depend on affective processes, we assessed the role of environmental enrichment on pain behavior. This was accomplished by evaluating two cohorts of Sprague Dawley rats, the first housed under standard conditions with two animals per cage and no enrichment, and the second housed in groups in an environment enriched with crawl spaces, chew toys, and an exercise wheel. The enriched group was also provided opportunities for increased social interactions that may be important for reducing stress and pain (Will et al. 2004; Pham et al. 2002). When evaluated for general exploratory (rearing) behavior (Fig. 4a, b), we found that rats in the enriched environment reared significantly less than the environmentally deprived animals (Fig. 4c). Over a range of temperatures (2–48 °C), environmentally enriched animals exhibited a significantly lower reward licking/stimulus contact ratio compared with the deprived rats (Fig. 4d).

A growing trend in pain management is the search for alternatives to pharmacotherapy for chronic pain control. Biofeedback, behavior modification, and stress relieving techniques are among these alternatives. Given the cognitive dependence of many of these techniques, it becomes important to utilize assays that depend on these cognitive processes. These data support the idea that environmentally enriched animals were both less stressed and displayed less pain than their deprived counterparts. Therefore, a change in living conditions may have an effect similar to a drug. Certainly, this is only one of many possible explanations regarding the role of environmental enrichment, but these results indicate that there may be alternatives to pharmacotherapy, such as cognitive-based techniques that rely on stress control that may be effective for pain control. Use of operant assays can better incorporate cognitive processes governing pain and allow for the evaluation of these pain management strategies.

9 Study 4: Effects of Mu- and Kappa-2 Opioid Receptor Agonists on Pain and Exploratory Behaviors

We have highlighted the differences between reflex and operant assays, but it is vital to address how these disparities may impact drug evaluation. We addressed this question by directly comparing the response in a thermal hindpaw withdrawal assay with the OPAD thermal operant assay. We used rearing as an index of exploratory activity to compare drug effects on general behavior. This also provided a secondary metric, in addition to analgesic potency, to assess dose selection in the pain assays.

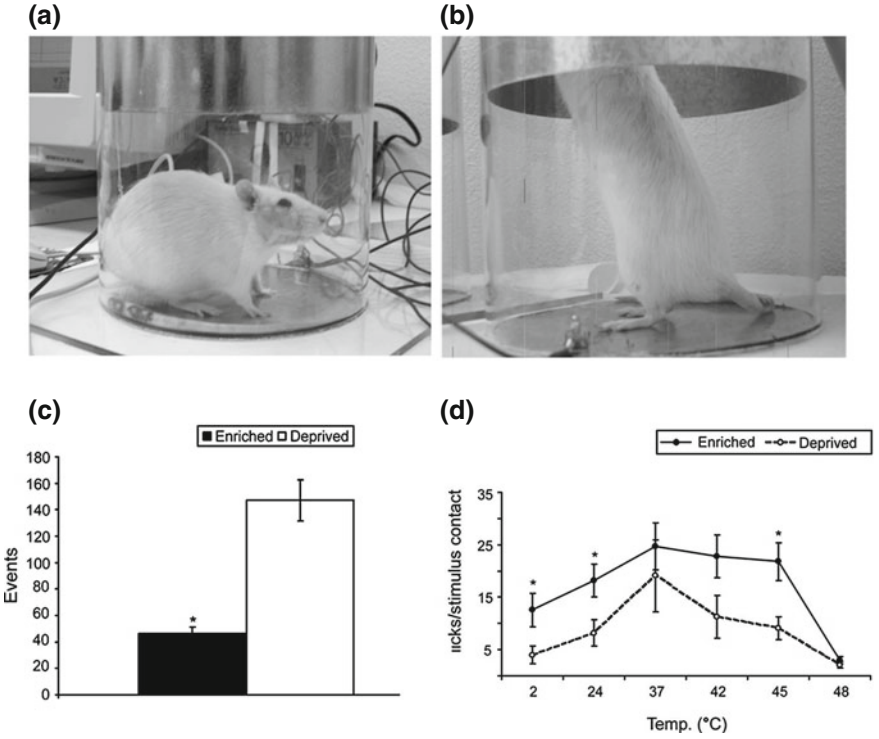


Fig. 4 The effect of environmental enrichment on general exploratory and operant pain behavior. Rats were housed either in standard Plexiglass cages (2/cage) or communal housing (3/cage) in an enriched open environment consisting of a metal cage containing cardboard boxes, two shelves, a hammock, PVC tubing, chew toys, and an exercise wheel. Vertical locomotion (rearing) behavior was automatically recorded and expressed as the number of reaching events in a 10 min session. **a** and **b** Representative images show a rat at rest and rearing in the cylinder. **c** Rats in enriched housing had significantly fewer rearing events compared with the deprived rats in standard housing. Data are shown as mean ± S.E.M., $*P < 0.05$. **d** When tested for orofacial pain sensitivity across a wide range of temperatures, there was a significant main effect of housing condition on the reward licking/stimulus contact ratio. Overall, the enriched animals had a higher reward licking/stimulus contact ratio, indicative of lower pain relative to the deprived group. Data are shown as mean ± S.E.M. $*P < 0.05$. This figure was reproduced from a previously published study (Rossi and Neubert 2008)

We compared the efficacy of the mu-opioid receptor agonist, morphine (0.5–5 mg/kg, s.c.), and the kappa-2 opioid receptor agonist, GR89,696 (0.000125–1 mg/kg, s.c.). We found that all doses of GR89,696 tested, except 0.000125 mg/kg, significantly reduced rearing (Fig. 5). At higher doses, catatonia was observed. Morphine at higher doses (≥2.5 mg/kg) also significantly reduced rearing, seemingly due to sedation.

For the reflex assay, the highest dose of each drug (1 mg/kg GR89,696 or 5 mg/kg morphine, s.c.) was required to observe an analgesic response in the hindpaw withdrawal test (Fig. 6a, b). In comparison to the rearing data shown in Fig. 5, the delayed response in the reflex pain assay may be due to factors other than pain relief,

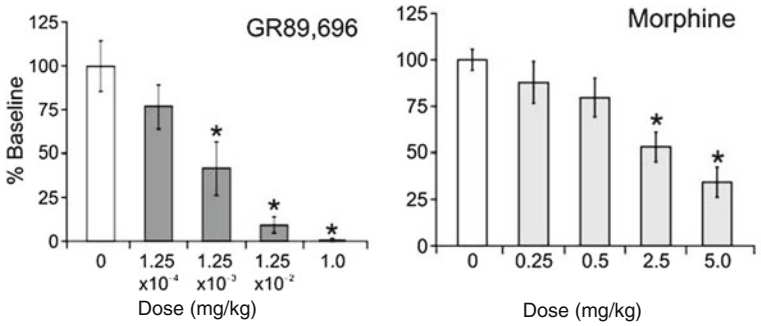


Fig. 5 The effects of the kappa opioid receptor agonist GR89,696 and mu-opioid receptor agonist morphine on rearing activity as a measure of general exploratory behavior. There was a significant dose-related decrease in the number of rears following GR89,696 or morphine administration compared with baseline values. Data are shown as mean ± S.E.M (*P < 0.05 versus vehicle [0] treated animals). We identified the decrease in locomotor activity as a potential confounding issue as animals are required to be mobile and motivated to complete operant testing. This figure was adapted from previously published data (Neubert et al. 2007)

such as general motor sedation, that leave the animals incapacitated. Conversely, a relatively low dose of morphine (0.5 mg/kg) can be antihyperalgesic against capsaicin-induced thermal hyperalgesia and sensitivity (Neubert et al. 2005, 2006). Based on the rearing behavior of animals dosed with GR89,696, and that animals would remain in whatever position they were placed in the OPAD after higher dose GR89,696 administration, we used only the lowest dose (0.000125 mg/kg) of GR89,696 to complete the operant task (Fig. 6c). As with the previous studies using morphine, the TRPV1 agonist capsaicin was used to produce a pain-depressed behavior by decreasing operant outcomes (e.g., licking reward/stimulus contact ratio). Pain-depressed behavior is characterized as a decreased response to a noxious stimulus (Negus and Bilsky 2010; Pereira Do Carmo et al. 2009). To be considered analgesic, GR89,696 should block this pain-depressed behavior as reflected by an increased lick/face ratio; however, we observed no such change, indicating a lack of analgesic effect at a dose that does not affect general behavior (Fig. 6c). These data emphasize the necessity of multifaceted approaches for drug evaluation and reveal how drugs and treatments can affect nonpain-related processes to alter the assay outcome itself and yield false positives.

10 Study 5: Placebo-Induced Analgesia in an Operant Pain Model in Rats

There has been much recent interest in psychosomatic effects and the role that expectancy states play in perceived experience and compliance (see for example, Wilson 2010; Horwitz and Horwitz 1993). In particular, the placebo effect has

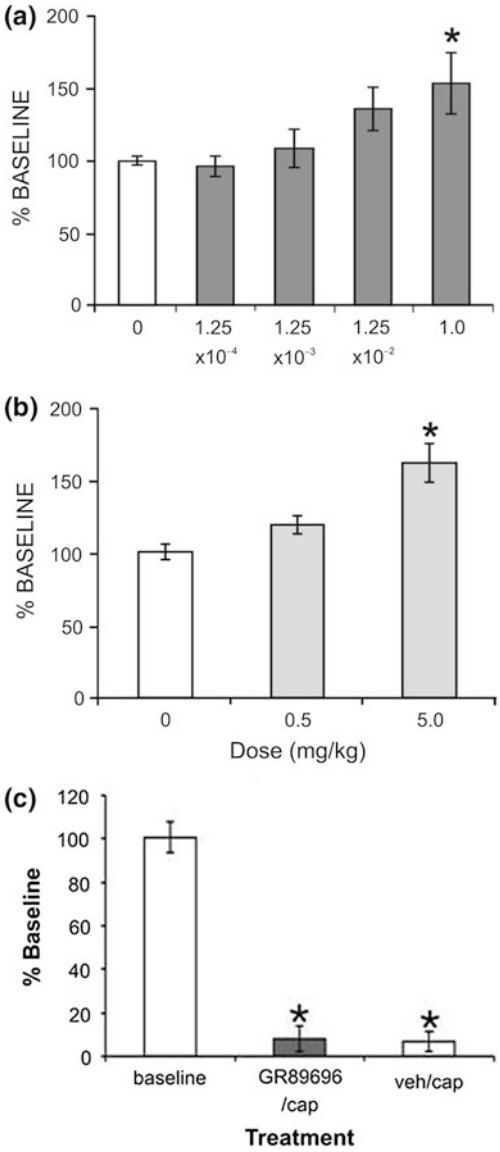
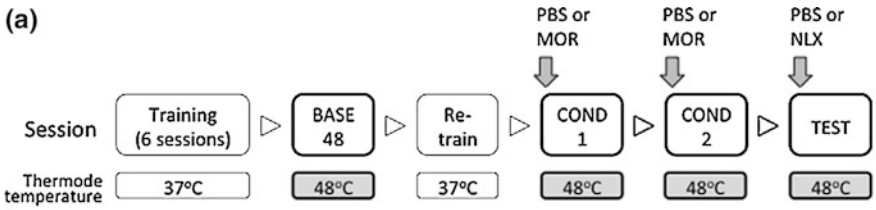


Fig. 6 Discordant responses between reflex- and operant-based pain behaviors following opioid receptor activation. To test reflex responses to thermal pain stimuli, we administered (**a**) GR89,696 or (**b**) morphine and then measured the hindpaw withdrawal latency of the animals. There was a significant increase in withdrawal latency only at the highest doses tested for GR89,696 and morphine. These doses severely impair general exploratory behavior, as shown in Fig. 5. **c** Animals treated with either GR89,696 (0.000125 mg/kg, subcutaneous) or PBS (veh, subcutaneous) prior to facial capsaicin (cap) application did not significantly differ from each other when evaluated using the reward licking/facial stimulus operant pain outcome measure. Note that a direct comparison of %baseline values between panels A/B and C is not possible, as different pain assays are used. Both groups were significantly lower (*$P < 0.05$) than baseline values. Data are shown as mean ± S.E.M. Figure adapted from previously published data (Neubert et al. 2007)

garnered much interest because pain is particularly susceptible to placebo-induced analgesia and likely makes significant contributions to genuine therapeutic effects in humans. Indeed, if such effects could be harnessed, they would be highly beneficial (Price et al. 2008). Expectancy states, such as the placebo effect, partially rely on higher levels of brain circuitry that underlie learning, prediction, and affective experience (see Price et al. 2008). The emotional components of pain are of particular interest here because they are one of the most debilitating aspects for chronic pain sufferers (Merskey 2007; Treede et al. 1999). Thus, we hypothesized that operant assays would be particularly effective at detecting placebo-induced analgesia. To test this hypothesis with the OPAD, we trained rats to expect morphine-induced analgesia, and then administered a placebo. Indeed, our results show that that rats exhibit placebo-induced analgesia (Fig. 7), and the characteristics of this analgesia bore many similarities with that seen in humans (Nolan et al. 2012). Namely, we found a strong inter-animal variability in the response and a significant positive predictive relationship between the genuine analgesic effect of morphine and the placebo effect; furthermore, this effect was suppressed by the opiate antagonist naloxone. These data suggest that operant assays are particularly well suited to probe higher brain circuits that underlie cognitive and affective functioning.

11 Do Results Using the OPAD Correspond with Reflex-Based Assays?

The search for better analgesics comes down to our belief and trust that preclinical assays truly reflect the human condition. One must be wary not to overly anthropomorphize findings in animal models and infer that an animal is feeling or experiencing those same emotions that encompass the affective aspect of pain experienced by humans. Even in humans it can be challenging to assess pain using standard assays, such as VAS given the large between-subject variability. Therefore, attempts have been made to rationalize designs that encompass as many pain pathways (i.e., primary sensory afferents, spinothalamic tract, cortex) as possible in preclinical models in the hope that this corresponds to the neurobiology of pain in humans. We and others (Vierck et al. 2008; Mogil 2009; Mogil et al. 2010; Negus et al. 2006) have argued that reflex-based assays generally fall short of this requirement and are inadequate for preclinical pain assessment compared with operant assays. Indeed, there are several examples of discordance when evaluating a drug using a reflex versus an operant assay. Previous studies in the field of social isolation demonstrated an increased pain threshold (i.e., lower pain) response in reflex tests that was hypothesized to be due to increased endogenous opioid levels (Coudereau et al. 1997; Tuboly et al. 2009). But, in our operant study, animals deprived of environmental enrichment showed relative increases in pain sensitivity (i.e., more pain) when tested across a range of temperatures. Additionally, previous preclinical studies of kappa opioid receptor agonists assessed by reflex-based measures were promising (Pasternak 1980); however when



(b)

Treatment group	Treatment prior to COND1 and COND 2	Treatment prior to TEST
PBS → PBS (n = 8)	PBS	PBS
MOR → PBS (n = 19)	MOR	PBS
MOR → NLX (n = 10)	MOR	NLX

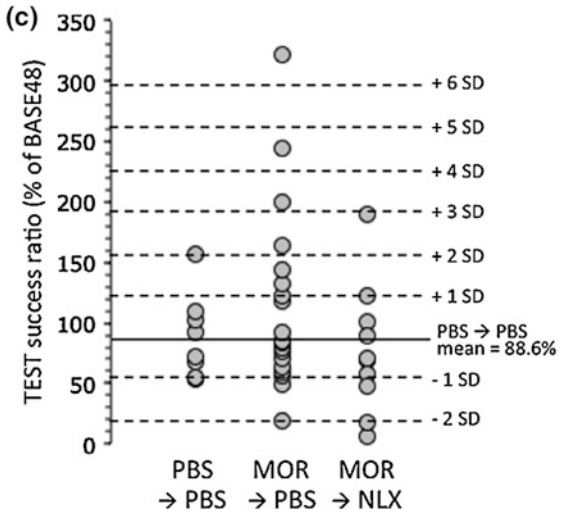


Fig. 7 Placebo-induced analgesia in an operant pain assay. **a** Schematic representation of the behavioral paradigm used for studying placebo effects. Boxes represent exposure to training in the OPAD. Variations of phosphate buffered saline (PBS) vehicle, morphine (MOR, 1 mg/kg), or naloxone (NLX, 5 mg/kg) were administered 30 min prior to each of the 3 conditioning [COND] sessions. **b** Summary of treatment groups studied. **c** Operant licking responses for palatable reward in the OPAD in individual rats during the TEST session. The solid horizontal line indicates the mean response in the PBS → PBS group. There were an increased number of animals with high responding in the MOR → PBS group, which was reversed by administration of naloxone. This figure was reproduced from a previously published study (Nolan et al. 2012). The figure has been reproduced with permission of the International Association for the Study of Pain® (IASP). The figure may NOT be reproduced for any other purpose without permission

tested in the OPAD operant assay, it was clear that these compounds had confounding locomotor properties, and ultimately clinical testing of these same compounds failed due to undesirable psychotomimetic and dysphoric side effects (Pfeiffer et al. 1986). Compared to a reflex assay, if a drug produces an adverse effect, the animal will simply be unable to perform the operant task and this is readily apparent. This highlights the importance of choosing an appropriate assay predictive of human pain.

12 Summary

Here, we have highlighted the utility and sensitivity of the OPAD, and its suitability for measuring pain-related behaviors in rodents. We do not suggest that the OPAD is the only method suitable for preclinical pain research. Rather, we wish to stress that operant assays might yield richer and more interpretable data sets that address the involvement of higher psychological processing in pain responding, such as the contribution of expectancy states. At its extreme, application of inappropriate pain assays may lead to incorrect interpretation of the effects of drugs, as exemplified by the case of GR89,696. However, we are mindful that operant assays do have their limitations, such as the possibility that neural circuits mediating orosensory reward may overlap with pain-related circuitry. Nonetheless, we suggest that operant pain assays should be at the vanguard of preclinical pain research, and we look forward to their contribution to the discovery of more effective and safer analgesics.

Conflicts of Interest Statement The authors are all employees of Velocity Laboratories, a company that provides fee-for-service behavioral testing using operant pain assays.

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