Journal of the American Association for Laboratory Animal Science
Copyright 2015
by the American Association for Laboratory Animal Science

Vol 54, No 4
July 2015
Pages 426–432

Assessment of an Orofacial Operant Pain Assay as a Preclinical Tool for Evaluating Analgesic Efficacy in Rodents

Harvey E Ramirez,[1,*] Timothy J Queeney,[2] Misha L Dunbar,[3] Michael C Eichner,[2] Dania I Del Castillo,[4] August H Battles,[1] and John K Neubert[5]

A model system capable of providing clinically relevant analgesic doses with minimal trauma has been elusive in laboratory animal medicine. Our laboratory has developed an orofacial operant pain system that effectively discriminates between nonnoxious and noxious thermal stimuli in rats and mice. Male and female rats (Crl:SD) and mice (Crl:SKR-HR^{hr}) were trained to perform a task (placing their face through an opening and having their cheeks stay in contact with thermodes) to receive a reward (a solution of sweetened condensed milk). Currently accepted doses of buprenorphine were tested by using a crossover design. Pain was induced in both species by sensitizing the depilated skin over both cheeks with capsaicin cream or by creating a surgical incision (rats only) and then allowing the animals to contact a temperature-regulated thermode while obtaining a reward. Optimal antinociceptive doses included 0.05 and 0.1 mg/kg in male mice but only 0.05 mg/kg in female mice. In rats, optimal antinociceptive doses included 0.03 and 0.05 mg/kg for male rats but only 0.03 mg/kg for female rats. The 2 pain-induction models in rats (capsaicin cream and surgical incision) did not differ. Our orofacial operant pain assay can determine clinically relevant analgesic doses for rodents in a preclinical assay. The automated, investigator-independent nature of the assay, in conjunction with its high sensitivity, makes this method an improvement over traditional noninvasive methods, providing better data for developing optimal analgesic recommendations for rats and mice.

Abbreviation: TRVP1, transient receptor vanilloid receptor potential 1.

The relief of postsurgical pain in rats and mice has been a major topic of discussion during the past decades. However, the availability of a model system capable of assessing clinically relevant analgesic doses in the context of minimal trauma has been elusive in laboratory animal medicine. Traditionally, the methods for assessing analgesic efficacy in rodents have included thermal (for example, hot-plate test, tail-flick test) and mechanical (for example, von Frey filament) assays. Albeit simple to perform and nontraumatic when performed correctly, these assays may not represent the best way to assess analgesic clinical efficacy, given that they rely on either reflex-derived outcomes (for example, tail-flick assay) or unlearned, brainstem-mediated responses (for example, grooming).[14,15]

Postoperative behavior-based pain assessment techniques, including the recently described facial grimace scale, have been used as a more comprehensive method for assessing clinical analgesic efficacy in rats and mice.[20-22] The basic premise in these assays consists of quantifying the relative magnitude of behavioral alterations after producing an injury, typically a laparotomy. Although these models have improved the assessment accuracy for recommended analgesic dosages, they can be time-consuming to perform.[9] In addition, these methods typically include a vehicle- or untreated control group, which introduces the ethical dilemma of uncontrolled pain in regard to some animals.

Received: 14 Apr 2014. Revision requested: 29 May 2014. Accepted: 24 Nov 2014.
[1]Animal Care Services, [2]College of Veterinary Medicine, and [5]Department of Orthodontics, University of Florida, Gainesville, Florida; [3]Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, Michigan; and [4]School of Veterinary Medicine, Tuskegee University, Tuskegee, Alabama.
[*]Corresponding author. Email: hramir@ufl.edu

The lack of clinical relevancy of the noninvasive methods on the one hand and the complexity of the behavior-based pain models on the other has limited the number of clinically effective analgesics or analgesic combinations that can be used effectively in rodents to minimize postoperative pain. As a consequence, several commonly available, centrally acting analgesics, such as tramadol, and multimodal analgesic combinations frequently used in human and other species remain underused in rodents. For this reason, there is still a need for a preclinical, minimally invasive pain model system that evaluates pain throughout the entire neuraxis, thus increasing the probability of providing clinically relevant analgesic doses.

Our laboratory has developed an orofacial operant pain system that effectively discriminates between nonnoxious and noxious thermal stimuli in rats and mice.[1,15,16] The system uses a reward-conflict paradigm in which animals choose between obtaining a reward in the presence of a thermal nociceptive stimulus and avoiding both the stimulus and the reward. The objective of the current study was to evaluate the analgesic effects of currently recommended doses of buprenorphine in our novel orofacial operant pain system. Our hypothesis was that this operant assay would reliably predict an analgesic effect of buprenorphine within the currently published dose range and would clarify optimal doses for male and female rats and mice.

Materials and Methods

Test subjects and housing conditions. Male (n = 12) and female (n = 12) Sprague–Dawley rats (Crl:SD; Charles River Laboratories, Wilmington, MA; initial weight, 175 to 200 g) were pair-housed in autoclaved polycarbonate ventilated microisolation cages (35 cm × 24 cm × 20.5 cm; Allentown Caging,

Allentown, NJ) containing corncob bedding (Harlan Teklad, Madison, WI). Male (n = 12) and female (n = 12) hairless mice (Crl:SKH1-*Hr*^{hr}l; Charles River Laboratories) were group-housed (4 or 5 mice per cage) in autoclaved polycarbonate ventilated microisolation cages (17 cm × 29 cm × 12 cm; Allentown Caging) containing corncob bedding (Harlan Teklad) with an added shelter (Shepherd Shack, Shepherd Specialty Papers, Milford, NJ). Both rats and mice were housed in a temperature-controlled (21 ± 2 °C) room on a 12:12-h light cycle (lights on, 0600). Standard rodent chow (Teklad Irradiated 7912 rat–mouse diet, Harlan Teklad) and reverse-osmosis–purified water supplied by an automatic watering system were available free choice. The rats were antibody-negative for coronavirus (sialodacryoadenitis virus, rat coronavirus), Kilham rat virus, lymphocytic choriomeningitis virus, mouse adenovirus, *Mycoplasma pulmonis*, pneumonia virus of mice, rat minute virus, rat parvovirus, reovirus type 3, Sendai virus, Theiler murine encephalomyelitis virus, and Toolan H1 virus. The mice were antibody-negative for ectromelia virus, epizootic diarrhea of infant mice virus, rotavirus, Hantaan virus, K virus, lactate dehydrogenase elevating virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse adenovirus types 1 and 2, mouse cytomegalovirus, mouse hepatitis virus, mouse parvovirus, mouse thymic virus, herpesvirus, pneumonia virus of mice, polyoma virus, reovirus 3, Sendai virus, and Theiler murine encephalomyelitis virus. Murine norovirus and *Helicobacter* spp were not in the pathogen-exclusion list and therefore were not tested at the time of the experiments. In addition, both rats and mice were free of external and internal parasites. The study was approved by the University of Florida IACUC. The facilities used for housing and testing the animals were AAALAC-accredited at the time of the study.

Fasting. For rats, food was removed in the morning (0800) of a planned training or test session. For mice, food was removed the night before (1700) a planned training or test session. Alternating training and testing sessions occurred 3 times each week at 1400 to 1600 for rats and at 0930 to 1130 for mice. Figure 1 depicts the chronologic events of the study for both rats and mice. Fasting never occurred on consecutive days in the mouse experiments. In rats, daily 6-h fasting occurred every morning during weekday acclimation and training sessions but never occurred on consecutive days once the experiment started.

Operant box and task training. Fasted, unrestrained animals were placed individually in the operant box for acclimation and training. The rat operant chamber consisted of an acrylic box (20.3 cm ×20.3 cm ×16.2 cm with an opening (4 × 6 cm) in one wall. The mouse operant chamber consisted of smaller acrylic box (5 cm ×5 cm ×5 cm) with an opening (2 × 2 cm) in one wall and placed within the rat box (Figure 2). The opening was lined with grounded aluminum tubing (thermode). Training consisted of allowing the animals to comfortably perform a task, which consisted of placing their faces through the opening and having their cheeks sustain contact with the thermodes; accomplishing this task earned them a reward of sweetened condensed milk (Nestle Carnation Company, room temperature) diluted with water.[1,2] A rodent-watering bottle containing the solution was placed outside the test box in such a way that the animal, when reaching toward it with its face, unavoidably maintained contact with the thermode. A detailed description of the orofacial operant system with diagrams can be found elsewhere.[16] A total of 5 (rats) to 7 (mice) training sessions (with thermode temperature set at 37 °C) were necessary for consistent completion of the task. Rats were deemed trained once they consistently achieved a minimum of 1000 lick counts during the 20-min session. Mice were deemed trained once they consistently achieved a minimum of 800 lick counts during the 20-min session. The test room temperature was maintained at 22 ± 1 °C for all behavioral tests. Test sessions were alternated with training sessions (no capsaicin, thermode temperature at 37 °C) throughout the experiment.

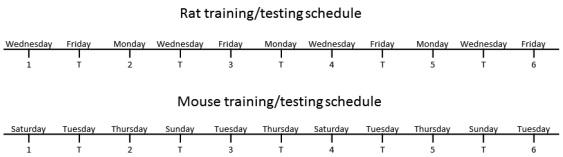

Figure 1. Chronologic order of events for the capsaicin dose–response studies. All animals were placed in the operant box 3 times a week; test sessions are numbered. Test and training sessions were alternated throughout the experimental timeline. Timeline does not include acclimation or training sessions, which occurred daily for rats (5 sessions) and 3 times each week for mice (7 sessions).

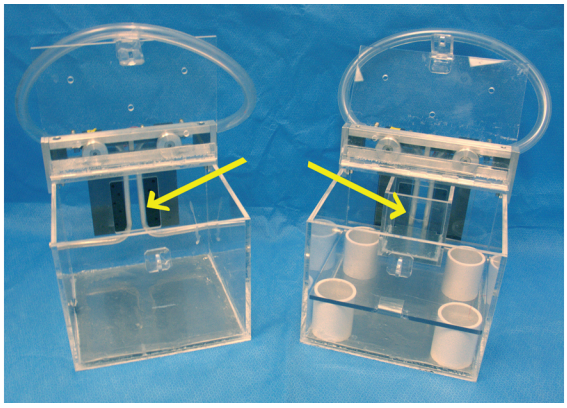

Figure 2. Rat operant box (left) and mouse operant box (right) used for testing. The openings are lined with metal tubing (thermode; arrows).

Preparation of skin and test area. Forty-eight hours prior to a test session, rats were anesthetized by placing them in an induction box and delivering isoflurane (5%) and oxygen via precision vaporizer until the righting reflex was abolished. Animals then were removed from the box and placed on a warm water-circulating blanket to maintain body temperature. Anesthesia was maintained with isoflurane (2% to 2.5%) and oxygen mixture delivered via nose cone. A bland ophthalmic ointment was placed liberally on both eyes to prevent corneal dryness. The hair over the rat's cheeks was removed by applying depilatory cream (Nair, Church and Dwight, Princeton, NJ) over the desired area and allowing a 2-min contact time. Care was taken to retain whiskers. Excess cream was removed with a wet facial cotton pad (Cotton Rounds, CVS Pharmacy, Woonsocket, RI) followed by a dry facial cotton pad. No skin preparation was necessary for hairless mice.

Description of test session. On test day, animals were brought to the room at least 15 min prior the start of each behavioral testing session. A schematic representation of the order of events during a test session for both rats and mice is depicted in Figure 3. Animals were weighed, and the selected dose of buprenorphine for the day was calculated. Buprenorphine (Buprenex Injectable, Reckitt Benckiser Pharmaceutical, Richmond, VA) was diluted with 0.9% saline as needed to obtain the desired injection volume of 1 mL/kg for rats and 0.1 mL for mice. All animals received all test doses in a crossover design. For rats, doses were tested in the following order: baseline (no injection), 0.01 mg/kg, 0.005mg/kg, saline, 0.05mg/kg, and 0.03 mg/kg. For mice, doses were tested in the following order:

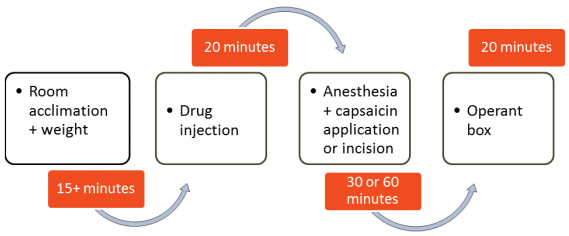

Figure 3. Order of events during a test session.

baseline (no injection), 0.05 mg/kg, 0.1 mg/kg, saline, 0.01 mg/kg, and 0.2 mg/kg. All injections were given 20 min prior to the induction of thermal sensitivity (capsaicin application) or incisional pain (rats only).

At the appropriate time after dose administration, animals were anesthetized as described earlier. Capsaicin cream (CapzasinHP 0.1%, Chattem,Chattanooga, TN) was applied liberally to the hairless side of the face, ensuring that the cream did not contact eyes or mouth. After 5 min of contact time, capsaicin cream was removed with alcohol-moistened wipes (BD Alcohol Swabs, Becton Dickinson, Franklin Lakes, NJ) and dried with facial cotton pads. Animals were allowed to recover from anesthesia for either 30 min (rats) or 60 min (mice) before being placed in the testing box as described. The thermode temperature during the testing period was set to 45 °C. Stimulus thermode temperatures were verified by using a contact thermometer (TC-324B Temperature Controller, Warner Instruments, Hamden, CT). The order in which animals were trained or tested remained unchanged throughout the entire study. A training session (no capsaicin, thermode temperature at 37 °C) was provided after each test session to create a wash-out period of at least 4 d and to maintain consistent task performance for each test session. The incisional pain test session (rats only) followed an adequate wash-out period after all capsaicin test sessions were finished.

Induction of incisional pain in rats. At the end of the capsaicin crossover study, male rats were divided in 2 groups (6 rats per group). On test day, rats were brought to the room according to the steps described earlier, except that a surgical incision was used to induce pain instead of capsaicin. Briefly, after anesthetic induction as described, the skin on the side of the face was cleaned with 3 preparations of 4% chlorhexidine scrub followed by sterile warm saline rinse. A skin incision (1.2 cm) was made bilaterally, starting 4 mm ventral to the medial canthus of the eye and extending inferiorly and posteriorly, ending 5 mm caudal to the commissure of the mouth. The incisions were closed with 2 simple interrupted sutures (5-0 Vycril, Ethicon, Omrix Pharmaceuticals, Somerville, NJ) on each side. One group of rats received 0.9% saline injections; the other received buprenorphine at 0.01 mg/kg SC as described.

Data collection and analysis. The metal spout on the watering bottle and the thermodes were connected via separate circuits to a 13-V DC power supply and, in series, to a multichannel data acquisition module (WinDaq Lite Data Acq DI-194, DATAQ Instruments, Akron, OH) as described previously.[16] The total number of events (licking, facial contacts) was recorded. The primary variable evaluated was the reward licks to facial contact event ratio, which was calculated by dividing the total number of licking events by the total number of facial contact events lasting more than 0.1 s. Each session lasted 20 min in both rats and mice. Data were analyzed by using Prism 5.01 (GraphPad Software, San Diego, CA) according to either repeated-measures ANOVA with a posthoc Bonferroni multiple-comparison test when normal distribution was achieved (female rat study) or the Kruskal–Wallis test with a posthoc Dunn multiple-comparison test (male rat and male and female mouse studies). One-way ANOVA with a posthoc Bonferroni multiple-comparison test was used to compare responses between capsaicin and incision with and without buprenorphine. *P* values less than 0.05 were considered significant. All data presented as the mean ± SEM. All data collected from animals that recorded no licks, thus giving a lick:face ratio of 0 in at least one test session, were not used in the statistical analysis; 2 male rats and 5 male mice were excluded from statistical analysis. All female rats and mice completed all testing sessions.

Results

The dose–response study involving male mice (Figure 4) revealed a statistically significant main effect (H_4=12.10, P = 0.0166) for the independent variable of dose on the lick:face ratio obtained among all groups and statistically significant differences between saline and buprenorphine at 0.05 mg/kg (P< 0.05) and 0.1 mg/kg (P< 0.001) only. Lick:face ratios(mean ± SEM) were 15.2 ± 2.4, 20.7 ± 5.8, 32.1 ± 4.7, 36.6 ± 4.1, and 22.2 ± 2.9 for buprenorphine doses of 0, 0.01 0.05, 0.1, 0.2 mg/kg, respectively. The lick:face ratio during training (37 °C, no capsaicin) was 30.6 ± 4.1.

Results of the dose–response curve for female mice (Figure 5) showed a statistically significant main effect (H_4=12.03, P = 0.0171) for the independent variable of dose on the lick:face ratio obtained among groups tested and a statistically significant difference between saline and buprenorphine at 0.05 mg/kg (P< 0.05) only. Lick:face ratios were 20.0 ± 2.7, 20.3 ± 2.1, 31.6 ± 2.9, 23.6 ± 3.4, and 19.8 ± 2.9 for buprenorphine doses of 0, 0.01 0.05, 0.1, and 0.2 mg/kg, respectively. The lick:face ratio during training (37 °C, no capsaicin) was 27.9 ± 3.7.

Results of the dose–response study for male rats (Figure 6) included a statistically significant main effect (H_4=35.34, P< 0.0001) for the independent variable of dose on lick:face ratio among all groups and a statistically significant difference between buprenorphine at 0.03 and 0.05 mg/kg when compared with saline (P< 0.001 and P< 0.001, respectively). Lick:face ratios were 7.2 ± 0.8, 9.5 ± 2.2, 11.5 ± 0.9, 32.6 ± 7.6, and 39.9 ± 4.2 for buprenorphine doses of 0, 0.005, 0.01, 0.03, and 0.05 mg/kg, respectively. The lick:face ratio during training (37 °C, no capsaicin) was 13.1 ± 3.3.

Results of the dose–response study for female rats (Figure 7) indicated a statistically significant main effect ($F_{5,59}$ = 5.785, P = 0.0008) for the independent variable of dose on the lick:face ratio among all groups and a statistically significant difference between saline and buprenorphine at 0.03 mg/kg (P< 0.01) only. Lick:face ratios were 6.5 ± 0.9, 6.9 ± 1.5, 10.8 ± 1.2, 17.0 ± 3.6, and 12.9 ± 2.4 for buprenorphine doses of 0, 0.005 0.01, 0.03, and 0.05 mg/kg, respectively. The lick:face ratio during training (37 °C, no capsaicin) was not recorded for female rats.

In this model, the hyperalgesic response of capsaicin (heat) was similar in intensity to the hyperalgesic effect produced when rats received bilateral facial incisions. In the absence of analgesics, responses did not differ between animals treated with capsaicin compared with facial incision (Figure 8). However, the 2 pain models differed significantly when the animals received buprenorphine at 0.01 mg/kg (P < 0.05),the lowest published recommended dose for rats. Lick:face ratios were 7.2 ± 0.7 and 11.3 ± 1.2 for capsaicin–saline and incision–saline, respectively, and 11.5 ± 0.8 and 32.0 ± 12.0 for capsaicin–buprenorphine and incision–buprenorphine, respectively.

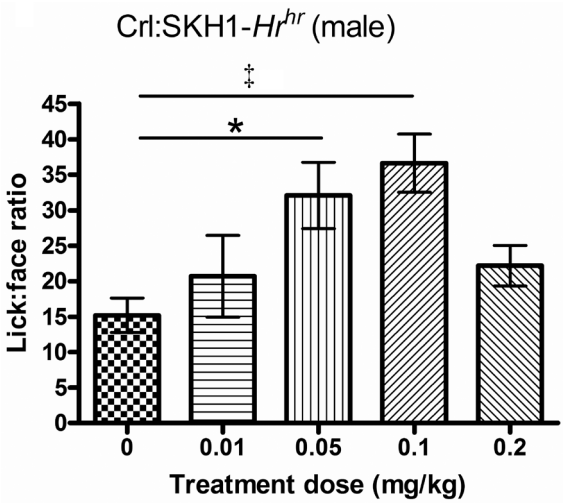

Figure 4. Effect of buprenorphine dose on the lick:face ratio in male mice. There was a significant ($P = 0.0166$) dose effect between groups. The Dunn multiple-comparison test showed a significant difference between saline and buprenorphine at 0.05 mg/kg (*, $P < 0.05$) and 0.1 mg/kg (‡, $P < 0.001$). Compared with saline (0), the mean increase in the lick:face ratio was 36%, 111%, 141%, and 46% for 0.01, 0.05, 0.10, and 0.2 mg/kg buprenorphine, respectively.

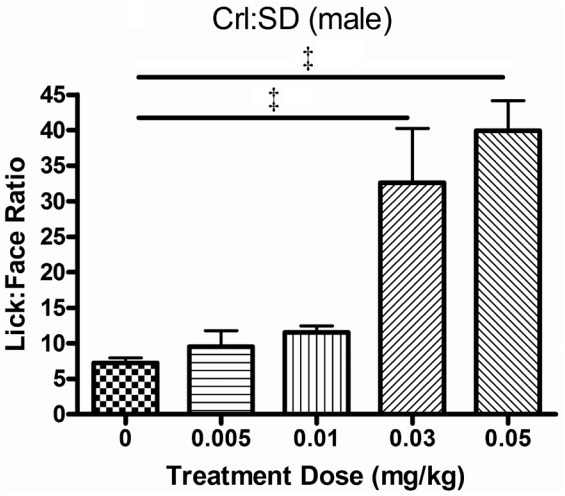

Figure 6. Effect of buprenorphine dose on the lick:face ratio in male rats. There was a significant ($P < 0.0001$) dose effect between groups. The Dunn multiple-comparison test showed a significant difference between saline and buprenorphine at 0.03 mg/kg (‡, $P < 0.001$) and 0.05 mg/kg (‡, $P < 0.001$). Compared with saline (0), the mean change in the lick:face ratio was 32%, 60%, 353%, and 454% for 0.005, 0.01, 0.03, and 0.05 mg/kg buprenorphine, respectively.

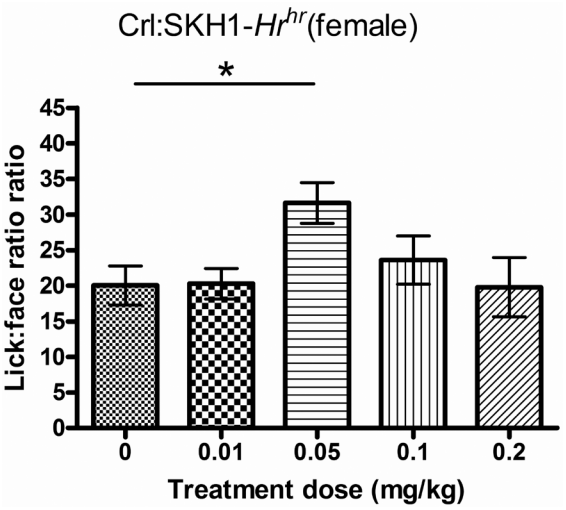

Figure 5. Effect of buprenorphine dose on the lick:face ratio in female mice. There was a significant ($P < 0.0171$) dose effect between groups. The Dunn multiple-comparison test showed a significant difference between saline and buprenorphine at 0.05 mg/kg only (*, $P < 0.05$). Compared with saline (0), the mean change in the lick:face ratio was 1%, 58%, 18%, and –1.3% for 0.01, 0.05, 0.10, and 0.2 mg/kg buprenorphine, respectively.

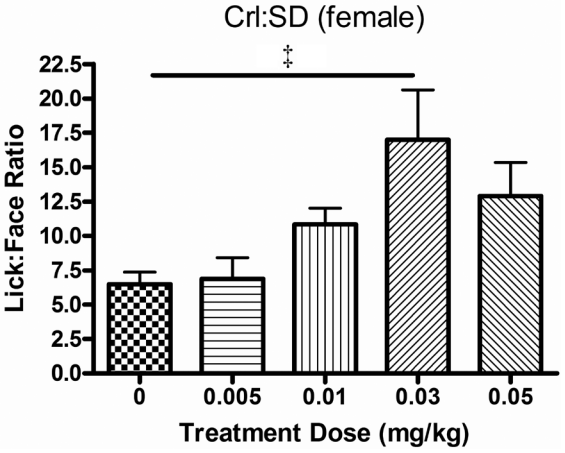

Figure 7. Effect of different doses of buprenorphine on the lick:face ratio in female rats. There was a significant ($P = 0.0008$) dose effect between groups. The Bonferroni multiple-comparison test showed a significant difference between saline and 0.03 mg/kg buprenorphine (‡, $P < 0.001$). Compared with saline (0), the mean change in the licks:face ratio was 6%, 67%, 162%, and 98% for 0.005, 0.01, 0.03, and 0.05 mg/kg buprenorphine, respectively.

Discussion

Over the past decade, the relief of postsurgical pain in rats and mice has been a major topic of discussion in the laboratory animal medicine field. However, few advances have occurred in terms of the number of analgesics and analgesic combinations with proven efficacy in rodents. Here we present a novel approach for the preclinical evaluation of analgesics in rodents that could help to optimize analgesic administration in rodents by 1) determining dosages that produce optimal analgesic effects and 2) potentially evaluating the effectiveness of readily available but underused analgesics (for example, tramadol) and routes of administration (for example, oral administration). The automated nature of our assay helps to establish a more objective, less invasive approach compared with other behavioral methods for testing analgesic efficacy in rats and mice. The minimally invasive nature of our assay offers similar advantages as those of traditional thermal nociceptive assays by producing no physical trauma (when performed correctly), using a short-lived pain stimulus (when compared with surgical pain models), and allowing animals to be reused.

Our method offers several additional advantages to traditional thermal assays. In our assay, animals are unrestrained and control the amount of nociceptive stimulus received, thus essentially eliminating the need to establish a predetermined cut-off threshold to avoid physical trauma. Instead, the animal subject decides the cut-off threshold to avoid a potentially unpleasant and distressing sensation, a characteristic that allowed us to determine optimal doses. In addition, using capsaicin allows us to lower the thermode testing temperature.

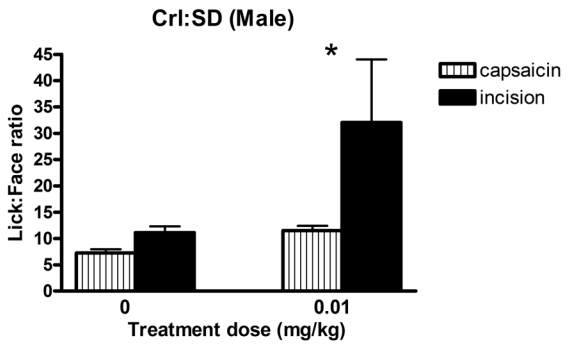

Figure 8. Comparison between capsaicin-induced heat and incision-induced heat hyperalgesia in male rats. The lick:face ratio did not differ between the capsaicin- and incision-induced hyperalgesia models in rats that received saline. However, the lick:face ratio differed significantly (*, $P < 0.05$) in when the nontraumatic (capsaicin) insult was compared with the traumatic (incision) insult after treatment with buprenorphine at 0.01 mg/kg.

The lower temperature minimizes extinction of the task during nonanalgesic dose testing and minimizes the potential for physical trauma due to excessive thermal contact during the testing of analgesic doses. Finally, our assay effectively assesses higher-level cognitive processing, because each animal decides, according to its perceived pain level, whether it will complete the task of maintaining contact with the thermode while obtaining a reward.

The orofacial pain assay uses a minimally invasive, transient pain model that can determine clinically relevant analgesic dosages with minimal physical trauma to the animals being tested. Capsaicin was used to sensitize the transient receptor vanilloid receptor potential 1 (TRVP1) to heat, the potentially painful stimulus used in this assay. TRVP1 is activated by local inflammatory molecules released during inflammation (for example, surgery), heat (>43 °C), and various chemicals.[19] TRVP1 is therefore a key channel for signaling and modulating heat and inflammatory pain.[3,4] Previous studies in mice have documented the importance of TRVP1 in development of incisional pain.[12,17,18] Capsaicin produces the perception of pain by mimicking the physiologic stimulus produced during tissue inflammation.[4] In addition, the perception of pain induced by capsaicin is concentration-dependent, meaning that capsaicin can sensitize TRPV1 to heat at concentrations that do not produce current activation.[19] In our study, capsaicin exposure was controlled by using a low concentration (1% cream) and limiting contact time to 5 min, thus potentially minimizing the perception of pain without noxious thermal contact.

When compared with incisional pain, the perceived hyperalgesic effects of capsaicin application and heat were similar in intensity to those produced by the inflammatory response caused by a traumatic skin incision. Interestingly, rats receiving the different pain stimuli and treated with a buprenorphine dose of 0.01 mg/kg SC showed a significant difference in lick:face ratio; animals that received the incision responded better to the analgesic effects of the low dose of buprenorphine than did those that received capsaicin. This difference can be attributed to the complex central and peripheral inhibitory pain mechanisms that are activated during physical trauma. During physical trauma, peripheral nociceptors are exposed to a variety of chemical agents released from injured cells. The impulses travel the spinal cord and reaches different areas of the brain, where it is perceived as pain. Physical trauma also activates an intrinsic pain control mechanism that modulates the ascending noxious signal through the release of various neurotransmitters, including endogenous opioids.[10] In addition, peripheral effects subsequent to a traumatic event stimulate the upregulation of peripheral opioid receptors and recruitment of endogenous opioid-producing antiinflammatory cells, ultimately producing an antinociceptive peripheral effect.[25] Systemic administration of an opioid agonist is known to produce a more pronounced analgesic effect on injured tissue when compared with noninjured tissue.[24] The endogenous regulatory pain mechanism, in conjunction with the low dose of buprenorphine, could account for the improved analgesic effect observed in the incision model. These findings may indicate a potentially valuable discriminatory sensitivity of our assay that might allow us to isolate and assess the true analgesic potency of a drug. Additional studies are needed to confirm this concept.

Another advantage of our pain assay is its ability to define a dose that would provide a maximal analgesic effect. In male rats, the optimal dose range for buprenorphine at 50 min after administration was 0.03 to 0.05 mg/kg. However, the optimal buprenorphine dose at 50 min after administration was limited to 0.03 mg/kg for female rats. The optimal buprenorphine doses for both male and female rats was within the currently accepted range (0.01 to 0.05 mg/kg).[8] In our model, a dose of 0.05 mg/kg did not provide observable increased analgesic efficacy in female rats. For male mice, both the lower and upper dose of the currently accepted dose range of 0.05 to 0.1 mg/kg[8] provided an analgesic effect at 80 min after administration. However, a dose of 0.1 mg/kg achieved the highest analgesic effect. In female mice, a dose of 0.05 mg/kg appeared to provide the maximal analgesic effect at 80 min after administration. Previous studies have shown that circulating ovarian hormone concentration can alter nociceptive sensitivity depending on the stage of estrous cycle.[13] However, these differences are not observed between male and female mice and rats.[13] Even though we did not evaluate estrous cycle in our female rodents, it is an unlikely source of the difference in our studies. The most likely explanation for the difference in analgesic response observed at 0.1 mg/kg in mice and 0.05 mg/kg in rats might be a difference in the intrinsic efficacy of the drug, resulting in basal shift in nociceptive reactivity. Sex-associated differences in μ-opioid antinociception are well documented.[5] These effects are related to drug potency, with high-efficacy opioids showing minimal to no sex-associated difference in analgesic effect (morphine has a 3-fold difference or less) and low-efficacy opioids showing a more dramatic difference (buprenorphine reportedly is 8 times more potent in male than female rodents and NHP).[2] In light of these results, we conclude that the maximal analgesic efficacy of buprenorphine was achieved at 0.05 mg/kg in female mice and at 0.03 mg/kg in female rats.The higher end of the reported analgesic dose range in the females of the stocks we tested did not provide an increased analgesic advantage and therefore may not provide an additional benefit.

All doses considered to be outside the reported analgesic range for both species (0.005 mg/kg in rats and 0.01 and 0.2 mg/kg in mice) proved ineffective at providing a statistically significant analgesic effect. The results observed at 0.2 mg/kg in mice were the most surprising, because the lick:face ratio obtained when this dose was delivered was no different than that observed when mice were treated with saline. The perceived lack of analgesic efficacy at this dose could be attributed to either overt sedation altering task performance or to a true lack of analgesic efficacy. At a dose of 0.2 mg/kg, male mice exhibited a 50% and 70% reduction in the total number of licks and the total number of face contacts, respectively, when compared

with the saline group. In female mice, there was a 40% reduction in both the total numbers of licks and facial contacts (data not shown). Buprenorphine has been described as an opioid agonist–antagonist with partial binding to μ receptor, triggering agonistic effects, and binding to κ and δ receptors, producing antagonistic effects.[11,23] An inverted bell-shaped antinociceptive dose–response curve has been reported in some pain models, with a dose-dependent decrease in antinociceptive activity at doses higher than those evaluated in the current study.[6,7] Although no conclusion can be made from these findings at this time, they have led us to believe that the antinociceptive effects of buprenorphine are greatly diminished, if not absent, at doses exceeding 0.1 mg/kg in mice. Additional studies are needed to further evaluate the observed decrease in the lick:face ratio of mice. Regardless of the underlying cause, doses higher than 0.1 and 0.05 mg/kg in male and female mice, respectively, appear to provide no additional clinical analgesic benefit at the tested time point and potentially be detrimental (by either providing no analgesia or causing overt sedation in the immediate postoperative period). Additional studies are needed to evaluate whether the results observed continue over time, to assess whether the same observed detrimental effect occurs in rats, and to elucidate the causes of this effect.

From a clinical perspective, we consider that this model might be used to predict effective analgesic dose ranges. For example, for each group of animals tested, the nontreated (temperature at 37 °C plus no capsaicin) lick:faceratio was obtained. In the case of male rats, the mean lick:face ratio was 13.1 ± 3.3 and might be defined as normal nociceptive threshold. Any condition (application of capsaicin or incision and thermal stimulation) that produces a lick:face ratio below the normal nociceptive threshold could therefore be defined as a hyperalgesic stimulus. Likewise, any drug dose that increases the lick:face ratio to normal levels or above during a hyperalgesic stimulus could be considered to provide an analgesic effect. For example, the rat buprenorphine dose of 0.01 mg/kg produced a nonstatistically significant analgesic effect. However, this dose increased the mean lick:face ratio from 7.2 ± 0.8 in the no-analgesic condition to 11.5 ± 0.9, a value very close to the group's mean normal nociceptive threshold (13.1 ± 3.3). Doses of 0.03 and 0.05 mg/kg produced lick:face ratios of 32.6 ± 7.6 and 39.9 ± 4.2, respectively. These values are well above the mean normal nociceptive threshold of 13.1 ± 3.3, therefore apparently providing a definitive analgesic effect. Unfortunately, lick:face ratio from training sessions for both mice and rats could not be used for follow-up statistical analyses since conditions were not standardized (different thermode temperature used, no anesthesia prior to training session).

Because the current study did not evaluate dose efficacy over time, whether analgesic efficacy would improve for the doses considered suboptimal at the time point tested is unknown. Our decision for evaluating the analgesic efficacy at the time points specified in this study (30 and 60 min after pain induction for rats and mice, respectively) were based primarily on the fact that animals needed to be fully awake before being placed in the testing box. To our knowledge, the pharmacokinetics and pharmacodynamics of buprenorphine have not been analyzed previously in the stocks we used. However, such analysis of buprenorphine in male Wistar rats at doses similar to those in the current study indicated that the maximal analgesic effect of buprenorphine occurs at 50 min after injection;[26] our rat testing occurred at 50 min after injection. Only one published report evaluates the pharmacokinetics of buprenorphine in mice, at a dose of 2.4 mg/kg IV;[27] no pharmacodynamics analysis of buprenorphine in mice has been performed previously. Whether our testing at 80 min after injection in mice occurred at the peak analgesic effect is unknown.

Our assay has some limitations. Removing hair from hirsute mice (we attempted to perform the same study in the C57BL/6NCrl strain) proved to be a difficult task without causing skin irritation, and hair clipping alone did not provide sufficient skin contact with the thermodes to provide consistent results. In addition, the evaluation of carprofen and meloxicam in rats at doses of 5 to 10 mg/kg and 1 to 10 mg/kg, respectively, was inconclusive (data not shown), suggesting that the current assay may need to be modified to test the effectiveness of the peripheral analgesic effects of NSAID. Finally, the data shown account only for optimal doses at a specific time point and do not provide information regarding the optimal frequency of administration, a very important component of maintaining sufficient pain relief over time. Additional studies are needed to evaluate the effectiveness of this assay for the evaluation of analgesic efficacy over time.

To summarize, our assay provides a new avenue for the evaluation of centrally acting analgesics yet minimizes both the number of animals needed and the overall pain intensity necessary to obtain valid results. With relatively few animals, a large amount of valuable information can be obtained. For example, differences in the analgesic responses to narrow dose ranges can be evaluated and acceptable dose ranges can be identified. In addition, optimal analgesic doses according to the animal's sex can be obtained. Finally, differences in analgesic efficacy between traumatic and nontraumatic pain induction can be studied. The overall perceived pain is minimized in our method by using a low concentration of capsaicin and limiting contact time. This practice allows us to sensitize the skin but minimize the potential for a painful sensation when heat is not applied. Our assay can be used to assess higher-level cognitive processing because it involves the entire neuraxis (because the animal subjects decide when they feel the sensation of pain), consequently increasing the likelihood of obtaining clinically relevant results. The automated, investigator-independent nature of our assay in conjunction with its high sensitivity make this technique an improvement over traditional noninvasive methods, providing better preliminary data for developing clinically relevant, sex-specific, optimal analgesic recommendations for rats and mice.

Acknowledgments

The project described was supported by Grants for Laboratory Animal Science (GLAS) from the American Association for Laboratory Animal Science. Stipend for veterinary students who worked on the project was provided by the University of Florida Merial Veterinary Scholars Program and Animal Care Services.

JKN is an employee of Velocity Laboratories, a company that provides fee-for-service behavioral testing using operant pain assays.

References

1. **Anderson EM, Mills R, Nolan TA, Jenkins AC, Mustafa G, Lloyd C, Caudle RM, Neubert JK.** 2013. Use of the operant orofacial pain assessment device (OPAD) to measure changes in nociceptive behavior. J Vis Exp **76:**e50336.
2. **Barrett AC.** 2006. Low-efficacy opioids: implications for sex differences in opioid antinociception. Exp Clin Psychopharmacol **14:**1–11.
3. **Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D.** 2000. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science **288:**306–313.

4. **Caterina MJ, Schumacher M, Tominaga M, Rosen TA, Levine JD, Julius D.** 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature **389:**816–824.
5. **Cook CD, Barrett AC, Roach EL, Bowman JR, Picker MJ.** 2000. Sex-related differences in the antinociceptive effects of opioids: importance of rat genotype, nociceptive stimulus intensity, and efficacy at the mu opioid receptor. Psychopharmacology (Berl) **150:**430–442.
6. **Cowan A, Lewis JW, Macfarlane IR.** 1977. Agonist and antagonist properties of buprenorphine, a new antinociceptive agent. Br J Pharmacol **60:**537–545.
7. **Dum JE, Herz A.** 1981. In vivo receptor binding of the opiate partial agonist, buprenorphine, correlated with its agonistic and antagonistic actions. Br J Pharmacol **74:**627–633.
8. **Flecknell PA.** 2009. Laboratory animal anaesthesia. Boston (MA): Elsevier Academic Press.
9. **Flecknell PA, Roughan JV.** 2004. Assessing pain in animals—putting research into practice. Anim Welf **13:**S71–S75.
10. **Flecknell PA, Waterman-Pearson A.** 2000. Pain management in animals. New York (NY): WB Saunders.
11. **Huang P, Kehner GB, Cowan A, Liu-Chen LY.** 2001. Comparison of pharmacological activities of buprenorphine and norbuprenorphine: norbuprenorphine is a potent opioid agonist. J Pharmacol Exp Ther **297:**688–695.
12. **Kissin I, Davison N, Bradley EL.** 2005. Perineural resiniferatoxin prevents hyperalgesia in a rat model of postoperative pain. Anesth Analg **100:**774–780.
13. **Mogil JS, Chesler EJ, Wilson SG, Juraska JM, Sternberg WF.** 2000. Sex differences in thermal nociception and morphine antinociception in rodents depend on genotype. Neurosci Biobehav Rev **24:**375–389.
14. **Mogil JS.** 2009. Animal models of pain: progress and challenges. Nat Rev Neurosci **10:**283–294.
15. **Neubert JK, Rossi HL, Malphurs W, Vierck CJ Jr, Caudle RM.** 2006. Differentiation between capsaicin-induced allodynia and hyperalgesia using a thermal operant assay. Behav Brain Res **170:**308–315.
16. **Neubert JK, Widmer CG, Malphurs W, Rossi HL, Vierck CJ Jr, Caudle RM.** 2005. Use of a novel thermal operant behavioral assay for characterization of orofacial pain sensitivity. Pain **116:**386–395.
17. **Pogatzki-Zahn EM, Shimizu I, Caterina M, Raja SN.** 2005. Heat hyperalgesia after incision requires TRPV1 and is distinct from pure inflammatory pain. Pain **115:**296–307.
18. **Pospisilova E, Palecek J.** 2006. Postoperative pain behavior in rats is reduced after single high-concentration capsaicin application. Pain **125:**233–243.
19. **Rosenbaum T, Simon SA.** 2007. TRVP1 receptors and signal transduction. In: Liedtke W B, Heller S, ed. TRP ion channel function in sensory transduction and cellular signaling cascades. Boca Raton (FL): CRC Press.
20. **Roughan JV, Flecknell PA.** 2003. Evaluation of a short-duration behaviour-based postoperative pain scoring system in rats. Eur J Pain **7:**397–406.
21. **Roughan JV, Flecknell PA.** 2004. Behaviour-based assessment of the duration of laparotomy-induced abdominal pain and the analgesic effects of carprofen and buprenorphine in rats. Behav Pharmacol **15:**461–472.
22. **Roughan JV, Flecknell PA.** 2006. Training in behaviour-based postoperative pain scoring in rats—an evaluation based on improved recognition of analgesic requirements. Appl Anim Behav Sci **96:**327–342.
23. **Sadée W, Rosenbaum JS, Herz A.** 1982. Buprenorphine: differential interaction with opiate receptor subtypes in vivo. J Pharmacol Exp Ther **223:**157–162.
24. **Stein C.** 1995. The control of pain in peripheral tissue by opioids. N Engl J Med **332:**1685–1690.
25. **Stein C, Clark JD, Oh U, Vasko MR, Wilcox GL, Overland AC, Vanderah TW, Spencer RH.** 2009. Peripheral mechanisms of pain and analgesia. Brain Res Rev **60:**90–113.
26. **Yassen A, Olofsen E, Dahan A, Danhof M.** 2005. Pharmacokinetic–pharmacodynamic modeling of the antinociceptive effect of buprenorphine and fentanyl in rats: role of receptor equilibration kinetics. J Pharmacol Exp Ther **313:**1136–1149.
27. **Yu S, Zhang X, Sun Y, Peng Y, Johnson J, Mandrell T, Shukla A, Laizure S.** 2006. Pharmacokinetics of buprenorphine after intravenous administration in the mouse. J Am Assoc Lab Anim Sci **45:**12–16.