Behavioral Battery for Testing Candidate Analgesics in Mice.

I. Validation with Positive and Negative Controls

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Abbreviations: cyclooxygenase$_{1/2}$ (COX), dopamine transporter (DAT), gamma-aminobutyric acid type A (GABA$_A$), mu-opioid receptor (MOR), non-steroidal anti-inflammatory (NSAID), norepinephrine transporter (NET), percent maximal nestlet consolidation (% MNC)

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ABSTRACT

This study evaluated a battery of pain-stimulated, pain-depressed, and pain-independent behaviors for preclinical pharmacological assessment of candidate analgesics in mice. Intraperitoneal injection of dilute lactic acid (IP acid) served as an acute visceral noxious stimulus to produce four pain-related behaviors in male and female ICR mice: stimulation of (1) stretching and (2) facial grimace, and depression of (3) rearing and (4) nesting. Additionally, nesting and locomotion in the absence of the noxious stimulus were used to assess pain-independent drug effects. These six behaviors were used to compare effects of two mechanistically distinct but clinically-effective positive controls (ketoprofen and oxycodone), and two negative controls that are not clinically approved as analgesics but produce either general motor depression (diazepam) or motor stimulation (amphetamine). We predicted that analgesics would alleviate all IP acid effects at doses that did not alter pain-independent behaviors, whereas negative controls would not. Consistent with this prediction, ketoprofen (0.1-32 mg/kg) produced the expected analgesic profile, whereas oxycodone (0.32-3.2 mg/kg) alleviated all IP acid effects except depression of rearing at doses lower than those that altered pain-independent behaviors. For the negative controls, diazepam (1-10 mg/kg) failed to block IP acid-induced depression of either rearing or nesting, and only decreased IP acid-stimulated behaviors at doses that also decreased pain-independent behaviors. Amphetamine (0.32-3.2 mg/kg) alleviated all IP acid effects, but only at doses that also stimulated locomotion. These results support utility of this model as a framework to evaluate candidate-analgesic effects in a battery of complementary pain-stimulated, pain-depressed, and pain-independent behavioral endpoints.
SIGNIFICANCE STATEMENT

Preclinical assays of pain and analgesia often yield false-positive effects with candidate analgesics. This study used two positive-control analgesics (ketoprofen, oxycodone) and two active negative controls (diazepam, amphetamine) to validate a strategy for distinguishing analgesics from non-analgesics by profiling drug effects in a battery of complementary pain-stimulated, pain-depressed, and pain-independent behaviors in male and female mice.
INTRODUCTION

Pain is a major reason for health care utilization (St. Sauver et al., 2013). More than 100 million Americans suffer from chronic pain, and many more are affected by acute pain, costing the US over $600 billion annually (Institute of Medicine, 2011). Despite decades of research, the most commonly used analgesics continue to be nonsteroidal anti-inflammatory drugs (NSAIDSs) and mu opioid receptor (MOR) agonists. Use of these compounds is constrained by limited clinical efficacy for some pain indications (Finnerup et al., 2015) and by side effects that include gastric ulceration for NSAIDS and abuse liability and potentially lethal respiratory depression for MOR agonists that have contributed to the current opioid public health crisis. The opioid crisis in particular has invigorated efforts to discover new, effective, and safe medications for pain treatment. Preclinical testing in laboratory animals will likely play an important role in this drug-discovery effort, but preclinical-to-clinical translational has been poor in analgesic drug development (Mogil, 2009; Yezierski and Hansson, 2018; Negus, 2019; Tappe-Theodor et al., 2019; Gonzalez-Cano et al., 2020; Kandasamy and Morgan, 2020). One of the largest discrepancies between preclinical and clinical pain assessment is the type of behavioral endpoint used to indicate the presence of a pain state and the impact of a drug treatment. Clinical pain in human medicine is primarily measured via verbal reporting, whereas preclinical pain research has focused almost exclusively on reflexive withdrawal behaviors stimulated by noxious stimuli. This creates a major discrepancy for translational research because verbal behavior cannot be measured in animals, and suppression of nocifensive withdrawal reflexes is not a priority for analgesic administration in humans. Moreover, pain manifests as a constellation of behavioral changes in both humans and animals, and adequate assessment requires evaluation of multiple behaviors.

To address this issue, we have argued that preclinical assessment of candidate analgesics would benefit from drug testing in a battery of different pain-related behaviors that include not only conventional pain-stimulated behaviors such as withdrawal reflexes, but also pain-related behavioral depression that models pain-associated functional impairment and behavioral depression in humans.
Complementary assessment of both pain-stimulated and pain-depressed behaviors not only broadens the scope and translational relevance of preclinical behavioral testing but also protects against false-positive effects. For example, drugs that produce sedation or motor impairment can produce false-positive effects in assays of pain-stimulated behavior but not in assays of pain-depressed behavior (Negus, 2019).

Accordingly, the goal of the present study was to validate a battery of pain-stimulated and pain-depressed behaviors for use in mice to test candidate analgesics. Intraperitoneal injection of dilute lactic acid (IP acid) served as a visceral noxious stimulus that models inflammation-associated tissue acidosis as one contributor to inflammatory pain (Koster et al., 1959; Holzer, 2009; Cobos et al., 2012; McMahon et al., 2013; Spahn et al., 2017). IP acid also has the experimental advantage of producing replicable effects with repeated administration, which allows within-subject experimental designs (Le Bars et al., 2001; Stevenson et al., 2006). Drug effects were examined on four different IP acid-induced nociceptive behaviors: (1) stimulation of a stretching (or writhing) response as a conventional pain-stimulated behavior somatotopically directed to the site of noxious stimulus delivery and potentially relying on spinal circuits (Collier et al., 1968; Le Bars et al., 2001), (2) stimulation of facial grimace as pain-stimulated behavior directed to a remote site away from the noxious stimulus and hence requiring supraspinal circuits and potentially reflecting affective pain behaviors (Langford et al., 2010; Matsumiya et al., 2012), (3) depression of rearing as an unconditioned and high-frequency locomotor behavior (Cho et al., 2013; Cobos and Portillo-Salido, 2013), and (4) depression of nesting as a robust and adaptive ethological behavior (Jirkof, 2014; Negus et al., 2015). Drug effects were also evaluated on two additional pain-independent behaviors in the absence of the IP acid noxious stimulus: (1) nesting to assess drug-induced disruption of an adaptive ethological behavior, and (2) horizontal locomotor activity that could be either increased or decreased by test drugs.

To validate this behavioral battery with both clinically effective and ineffective drugs as recommended for translational research (S. Ferreira et al., 2019), effects were compared for two
mechanistically distinct but clinically effective positive-control analgesics (ketoprofen and oxycodone) and two negative-control drugs that are not indicated for use as analgesics but do produce robust behavioral effects in mice (diazepam and amphetamine). Ketoprofen is a cyclooxygenase\textsubscript{1/2} (COX) inhibitor representative of NSAID analgesics, whereas oxycodone is a MOR agonist representative of opioid analgesics (Kantor, 1986; Kalso, 2005; McMahon et al., 2013; Brunton et al., 2018). Diazepam is a gamma aminobutyric acid receptor A (GABA\textsubscript{A})-receptor positive allosteric modulator that produces general motor suppression, and amphetamine is a substrate at dopamine and norepinephrine transporters (DAT and NET, respectively) that produces dopamine and norepinephrine release and subsequent psychomotor-stimulant behavioral effects (Calcaterra and Barrow, 2014; Hutson et al., 2014; Brunton et al., 2018). We hypothesized that ketoprofen and morphine would alleviate both IP acid-stimulated and IP acid-depressed behaviors at doses that did not alter pain-independent behaviors, whereas diazepam and amphetamine would not. Results of this validation study were then used as an empirical framework to interpret effects in the same behavioral battery produced by a series of endocannabinoid catabolic enzyme inhibitors that were evaluated in a companion study (Diester et al., 2021).

\textbf{MATERIALS AND METHODS}

\textbf{Subjects}

Subjects were male and female ICR mice (Harlan Laboratories, Frederick, MD) that were 6-8 weeks old upon arrival to the laboratory. Males weighed 25-45g and females weighed 20-35g throughout the study. In an AAALAC approved facility, mice were housed in cages (31.75cm long x 23.50 cm wide x 15.25cm deep) with corncob bedding (Harlan Laboratories) and a “nestlet ” composed of pressed cotton (Ancare, Bellmore, NY). All mice had ad libitum access to food (Teklad LM-485 Mouse/Rat Diet, Harlan Laboratories) and water, and cages were mounted in a RAIR HD Ventilated Rack (Lab Products, Seaford, DE) in a temperature-controlled room with a 12-hour light/dark cycle (lights on from 6:00 AM to 6:00 PM). Mice used in studies of stretching, grimace, and
rearing were littermates group housed 3/cage, and mice used in nesting studies were individually housed. For group-housed mice, a cardboard tube was added to the cage environment to provide enrichment and minimize fighting. All experiments were performed during the light phase of the daily light/dark cycle beginning at least one week after arrival at the laboratory. Additionally, for singly housed mice in nesting studies, experiments were performed in their home cages at least two days after a cage change. Animal-use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and complied with the National Research Council Guide for the Care and Use of Laboratory Animals.

**Overview of Experimental Design**

The goal of the study was to compare effects of two clinically effective positive-control analgesics (ketoprofen, oxycodone) and two behaviorally active negative controls (diazepam, amphetamine) on a panel of pain-stimulated, pain-depressed, and pain-independent behaviors in mice. Pain-related behaviors were elicited by intraperitoneal (IP) injection of dilute lactic acid as the noxious stimulus, and an initial study determined the potency of IP acid (0-0.32%) to produce these behaviors in two groups of mice. These two groups and all other groups described below consisted of 12 mice (6 males and 6 females) to permit exploratory analysis of sex differences as described previously (Diester et al., 2019). One group of mice was used to evaluate IP acid-induced stimulation of abdominal stretching and facial grimace behaviors as well as IP acid-induced depression of rearing. A second group was used to evaluate IP acid-induced depression of nesting behavior. Thus, behavioral assessment focused on two pain-stimulated behaviors (stretching, grimace) and two pain-depressed behaviors (rearing, nesting). In each group, all mice received all IP acid concentrations in a within-subject repeated-measures design. The order of presentation for different acid concentrations was randomized across mice using a Latin-square design, and tests were conducted once per week in each mouse. On the basis of this initial study, a concentration of 0.32% IP acid was used as the noxious stimulus for all subsequent studies with test drugs. Antinociception dose-effect
curves for each test drug were determined in two groups of mice, one to assess drug effects on IP acid-induced changes in stretching, facial grimace, and rearing, and a second to assess drug effects on IP acid-induced depression of nesting. Effects of each test drug on pain-independent behaviors were determined in two additional groups of mice, one to assess drug effects on control nesting in the absence of the IP acid noxious stimulus, and a second to assess drug effects on locomotion in the absence of the noxious stimulus. In each group, all mice received all drug doses, dose order was randomized across mice using a Latin-square design, and tests were conducted once per week in each mouse. Doses for each drug were varied in 0.5 or 1.0 log-unit increments across a ≥10-fold dose range with the intent of progressing from low doses that produced little or no effect to high doses that produced either significant antinociception on one or more endpoints of pain-related behavior or significant changes in nesting or locomotor activity as pain-independent behaviors. Data were analyzed to evaluate the degree to which each test drug alleviated pain-related behaviors at doses below those that altered pain-independent behaviors.

Test drug doses, pretreatment times, and supporting references were as follows: the cyclooxygenase_{1/2} inhibitor ketoprofen, 0.1-32 mg/kg, 30 min (Negus et al., 2015); the mu-opioid receptor agonist oxycodone, 0.32-10 mg/kg, 30 min (Beardsley et al., 2004); the gamma aminobutyric acid receptor A (GABA_A) receptor positive allosteric modulator diazepam, 1-10 mg/kg, 30 min (Rosland et al., 1987; Schwienteck et al., 2017); and the dopamine and norepinephrine transporter substrate amphetamine, 0.32-10 mg/kg, 30 min (Tyler and Tessel, 1979; Nevins et al., 1993).

**Behavioral Procedures**

**Stretching/Grimace/Rearing Procedure.** Studies of stretching, grimace, and rearing were conducted in a procedure room separate from the housing room. Mice were acclimated for at least 1 hr to the procedure room at least one day before the first test day, and on all subsequent test days, mice were again acclimated to the procedure room for at least 1 hr before testing. Testing was initiated by subcutaneous (SC) administration of the specified test drug dose followed by return of the
mouse to its home cage for the pretreatment time specified above. Subsequently, mice received IP acid immediately before being placed into individual plexiglass cylinders (4” diameter) and filmed for 20.5 min without any researchers in the room. Videos were later scored for stretching, facial grimace, and rearing by two trained and blinded observers, and scores across the two observers were averaged. The number of stretches and rears was counted for the first 20-min of the observation period. A stretch was defined as a horizontal extension of the abdomen followed by extension of at least one hind limb. A rear was defined as vertical extension of the mouse with both front paws off the ground followed by return of at least one front paw to the ground. Importantly, a rear was not counted when a mouse was resting on its hind legs without vertical extension (e.g. during grooming). Facial grimace was scored during the last 0.5 min of the observation period by evaluating ptosis and ear position with criteria similar to those described previously (Langford et al., 2010). Specifically, ptosis was scored on a graded scale with 0 = eyes fully open, 0.5 = eyes half to a quarter closed, and 1 = eyes fully closed. Ear position was also scored on a graded scale by reference to a line drawn following the top of the whisker line along the snout. If the center tip of the mouse’s ear was above the line, it was scored as a 0, through the center of the ear was a 0.5, and below was a 1. Scores for ptosis and ear position were assigned based on observer impressions for the entire 0.5 min observation period and summed to yield a total score for each mouse, with a minimum score of 0 and maximum score of 2.

**Nesting Procedure.** A nesting procedure described previously (Negus et al., 2015) was modified to accommodate testing in the housing room and provide a continuous quantitative dependent variable. Additionally, mice were excluded from nesting studies if they failed to nest during the initial acclimation week in the housing room (3 mice over the course of the study). On test days, mice received a SC injection with the specified test drug before being returned to their home cages on the housing rack. After the specified pre-treatment time, mice were removed from their cage and received either IP acid or vehicle. Old nesting material was removed, two 1-in² nestlet squares were placed 11 in apart in the center of the opposing short walls of the cage (see Supp. Figure 1), and the
mouse was again returned to its home cage. After a 90-min nesting period with no researcher in the
room, the cage top was removed, the position of the nestlets was photographed from above, and the
distance between the center of mass for each nestlet was measured to the nearest quarter inch. For
the initial study to examine the potency and time course of IP acid effects, nestlet position was also
evaluated every 30 min during the 90 min nesting period (Supp. Figure 1); however, for all remaining
experiments, nestlet position was evaluated only at 90 min to minimize cage disturbances during the
experiment. The primary dependent variable was % Maximal Nestlet Consolidation (%MNC), defined
as \([(11-\text{End})/11] \times 100\), where 11 and End were the distances in inches between the nestlets at the
start and end of the nesting period, respectively.

**Locomotor Procedure.** Horizontal locomotor activity was assessed during 30-min session in
16.8 cm wide x 12.7 cm deep x 12.7 cm high boxes housed in sound-attenuating chambers (Med
Associates, St. Albans, VT) and located in a procedure room separate from the housing room. Each
box had black plexiglass walls, a clear plexiglass ceiling equipped with a house light, bar floors, and
six photobeams arranged at 3 cm intervals across the long wall and 1 cm above the floor. Beam
breaks were monitored by a microprocessor operating Med Associates software. The primary
dependent variable was the total number of beam breaks, excluding consecutive interruptions of the
same beam, during the 30-min session. Test sessions were conducted twice a week with at least 48
hr between sessions. A different group of 12 mice (6 male, 6 female) was used to test each drug.
Within each group, all mice received all doses, and dose order was counterbalanced across mice
using a Latin square design. On test days, mice were brought to the procedure room at least 2 hr
before session onset. After SC test-drug administration, mice were returned to their home cages for
the 30-min pretreatment interval, then placed into the locomotor activity boxes at session onset.

**Data Analysis**

Stretching, rearing, nesting, and locomotor data were treated as ratio variables and analyzed
by parametric statistics, whereas facial grimace was treated as an ordinal variable and analyzed with
non-parametric statistics. Data for each treatment on each endpoint were analyzed in four phases as we have described previously (Diester et al., 2019). As this study was designed based on power to assess treatment effects for pooled data (N=12), data for males and females were first pooled and evaluated using a repeated-measures one-way ANOVA followed by Dunnett’s post hoc test for parametric data or Friedman’s test followed by Dunn’s post hoc for non-parametric data. Second, data were segregated by sex and evaluated by repeated-measures one-way ANOVA to assess drug effects within each sex, and as with pooled data, a significant ANOVA was followed by Dunnett’s post hoc test for parametric data, whereas a significant Friedman’s test was followed by Dunn’s post hoc for non-parametric data. Third, male and female parametric data for a given endpoint were analyzed by two-way ANOVA to directly compare data from males and females, with sex as a between-subjects factor and IP acid concentration or drug dose as a within-subjects factor. A significant sex x treatment interaction was followed by a Holm-Šídák post hoc test. For non-parametric data, multiple t-tests with correction for multiple comparisons were used. These first three steps of data analysis were performed using GraphPad Prism (LaJolla, CA) with a criterion for significance of p<0.05. Lastly, results were submitted to power analyses to calculate the Cohen’s f effect size, achieved power (1-\(\beta\)), and the total number of animals predicted as necessary to detect a significant effect given the effect size, \(\alpha = 0.05\) and power (1-\(\beta\)) = 0.8 using the free statistical analysis program G*Power (Faul et al., 2007). There is currently no consensus method for power analysis of non-parametric data so grimace data was not submitted for further power analyses in this study (Lehmann, 2006; Motulsky, 2020).

**Drugs**

Lactic acid (Fisher Scientific, Hampton, NH) was diluted in sterile water and administered IP. Ketoprofen was obtained as a commercially available solution (100 mg/mL; Ford Dodge, IA) and diluted in sterile saline. Oxycodone and amphetamine were provided by the National Institute on Drug Abuse Supply Program (Bethesda, MD), and were prepared in sterile saline. Diazepam was obtained
as a commercially available solution (5 mg/ml, Hospira, Lake Forest, IL) and diluted in 1:4:5 ethanol, propylene glycol, and saline. All test drugs were administered SC in volumes of 0.1 to 0.9 ml.

RESULTS

Effects of IP Lactic Acid Alone

Figure 1 shows the effects of IP injection with vehicle or increasing concentrations of lactic acid on stretching, facial grimace, rearing, and nesting behaviors. After IP vehicle, stretching and facial grimace scores were low, whereas rearing and nesting scores were high. IP acid produced concentration-dependent increases in both stretching and grimace, with significant increases for both pain-stimulated behavioral endpoints at 0.18% and 0.32% (see Table 1 for all statistical results with pooled data from both sexes). Conversely, IP acid produced concentration-dependent decreases in rearing and nestlet consolidation, with significant decreases for rears at 0.18% and 0.32% and significant decreases in nesting for all three acid concentrations. The time course of nesting behavior in 30-min intervals after vehicle or acid treatment is shown in Supplemental Figure 1. Results of statistical analysis to examine sex as a determinant of IP acid effects are shown in Supplemental Table 1. With these sample sizes, only stretching showed a significant sex x dose interaction; however, further post hoc analysis showed no difference between males and females for any concentration compared to vehicle (Supp. Fig. 2). Based on these results, a concentration of 0.32% IP acid was used for all subsequent studies with test drugs.

Overview of Data Presentation

Each test drug was evaluated for its potency and effectiveness to block each of the four IP acid-induced behaviors, and results are shown in Figures 2-3. Additionally, each test drug was administered alone in the nesting and locomotor procedures to evaluate general behavioral effects in the absence of the IP acid noxious stimulus, and results are shown in Figure 4. Figure 5 compares the potencies of each test drug to significantly attenuate IP acid effects and to alter nesting and
locomotor behaviors when the test drug was administered alone. An optimal test-drug profile would be significant attenuation of all IP acid-induced behaviors at doses that did not affect nesting or locomotion when the test drug was administered alone. Table 1 shows the results of ANOVA and power analyses for pooled data across sexes. Supplemental Tables 1-2 show results of ANOVA and power analyses for data segregated by sex. Supplemental Tables 3-7 report results of statistical analysis to examine sex as a determinant of effects for each drug on each endpoint, and figures are included for selected effects when there was either a significant main effect of sex or a significant dose x sex interaction.

Effects of Ketoprofen and Oxycodone

Figure 2 shows the effectiveness of the COX inhibitor ketoprofen and the MOR agonist oxycodone to block IP acid-induced pain-related behaviors. Ketoprofen (0.1-10 mg/kg) significantly blocked IP acid-stimulated stretching and facial grimace, and also blocked IP acid-induced depression of both rearing and nesting. Ketoprofen alone at doses up to 32 mg/kg had no effect on nesting or locomotion (Figure 4). Thus, ketoprofen blocked all IP acid-induced changes in behaviors at doses that had no effect on nesting or locomotion when ketoprofen was administered alone (Figure 5). Table 1 summarizes the ANOVA and power analysis results for these pooled data. Results of statistical analysis to examine sex as a determinant of ketoprofen effects are shown in Supplemental Table 4. No endpoint showed a significant sex x dose interaction, and only nesting in the presence of IP acid had a significant main effect of sex (greater nesting in females; Supp. Figure 3).

Oxycodone (0.32-3.2 mg/kg) significantly blocked IP acid-stimulated stretching at all three doses and facial grimace at 1.0 and 3.2 mg/kg. No oxycodone dose tested blocked IP acid-depressed rearing, and only 1.0 mg/kg significantly attenuated IP acid-depressed nesting to a mean %MNC of 42.2±10.96. Oxycodone alone significantly decreased nesting at 10 mg/kg, and significantly stimulated locomotion at 3.2 and 10 mg/kg (Figure 4). Thus, oxycodone decreased IP acid-stimulation of both stretching and facial grimace and attenuated acid-induced depression of nesting at doses
lower than those that produced significant effects on nesting and locomotion when administered alone (Figure 5). The ANOVA and power analysis results for these pooled data are summarized in Table 1. Supplemental Table 5 shows the results of the statistical analyses to examine sex as a determinant for oxycodone effects. No endpoint showed a significant main effect of sex for the given sample and effect sizes, and only nesting in the absence of the noxious stimulus produced a significant sex x dose interaction; however, further post hoc analysis did not reveal an individual dose being significantly different between males and females (Supp. Figure 4).

**Effects of Diazepam and Amphetamine**

Figure 3 shows the effects of the GABA<sub>α</sub> positive allosteric modulator diazepam and the DAT/NET substrate amphetamine on IP acid-induced pain-related behaviors. The highest dose of diazepam (10 mg/kg) significantly attenuated IP acid-stimulated stretching and grimace, but diazepam did not attenuate IP acid-induced depression of either rearing or nesting; rather, 10 mg/kg diazepam exacerbated IP acid-induced depression of rearing. Diazepam administered alone significantly decreased nesting at 10 mg/kg and significantly decreased locomotion at 3.2 and 10 mg/kg (Figure 4). Thus, diazepam reduced IP acid-stimulated stretching and grimace only at a dose that exacerbated IP acid-induced depression of rearing and significantly decreased nesting and locomotion when administered alone (Figure 5). ANOVA and power analysis data summarizing these results is shown in Table 1. Results of statistical analysis to examine sex as a determinant of diazepam are shown in Supplemental Table 6. No endpoints in the presence of IP acid resulted in a significant sex x dose interaction or main effect of sex for the given sample and effect sizes. Diazepam alone did produce a significant main effect of sex in locomotion, but further post hoc analysis showed no individual dose to be significantly different between males and females (Supplemental Figure 4).

Amphetamine (0.32-3.2 mg/kg) significantly reduced IP acid-induced stimulation of both stretching and facial grimace at the highest dose tested. Additionally, 3.2 mg/kg amphetamine
attenuated IP acid-induced depression of rearing and nesting. Amphetamine delivered alone (0.32-10 mg/kg) significantly decreased nesting at 10 mg/kg and significantly increased locomotion at both 3.2 and 10 mg/kg (Figure 4). Thus, amphetamine blocked all IP acid-induced behaviors, but only at a dose that significantly increased locomotion when administered in the absence of the noxious stimulus (Figure 5). Table 1 summarizes the ANOVA and power analysis results for these pooled data. Supplemental Table 7 shows the results of the statistical analyses to examine sex as a determinant for amphetamine effects. No endpoints showed a significant sex x dose interaction or main effect of sex for the given sample and effect sizes.

**DISCUSSION**

This study compared the effects of two clinically effective analgesics (ketoprofen, oxycodone) and two active negative controls (diazepam and amphetamine) on a panel of pain-stimulated, pain-depressed, and pain-independent behaviors in male and female mice. There were three main findings. First, IP acid served as an effective chemical noxious stimulus to produce a concentration-dependent stimulation of stretching and facial grimace and depression of rearing and nesting as putative pain-related behaviors. Test drugs could then be evaluated for their profiles of antinociceptive effectiveness to alleviate these IP acid effects. Second, the positive-control analgesics ketoprofen and oxycodone produced antinociception in assays of both pain-stimulated and pain-depressed outcome measures (with ketoprofen being the most effective) at doses below those that altered nesting and/or locomotion in the absence of the IP acid noxious stimulus, whereas the negative controls diazepam and amphetamine did not. These results suggest that analgesic potential of test drugs can be predicted by higher potency to alleviate pain-related stimulation and depression of behavior than to produce pain-independent motor disruption in mice. Lastly, sex differences in drug effects were not a primary focus of the present study and few sex differences were identified; however, the inclusion of equal numbers of male and female mice permitted exploratory power analysis of sex differences that could guide future studies designed to focus on sex as a primary...
variable of interest. Overall, this study outlines an experimental design and a framework of results with positive and negative controls that can be used to study and interpret effects of candidate analgesic drugs.

Effects of IP lactic acid as a noxious stimulus

The present results confirm and extend previous studies in finding that IP injection of dilute acid serves as an effective noxious stimulus to stimulate stretching (Koster et al., 1959; Collier et al., 1968; Stevenson et al., 2006; Booker et al., 2009; Do Carmo et al., 2009; Miller et al., 2012; Bagdas et al., 2016; Alexander et al., 2019) and facial grimace (Langford et al., 2010) and to depress rearing (Cho et al., 2013; Cobos and Portillo-Salido, 2013) and nesting (Negus et al., 2015; Lewter et al., 2017; Alexander et al., 2019). The present study builds on these previous results by showing that potency of IP lactic acid was similar across all four endpoints. Additionally, our approach assessed stretching, facial grimace, and rearing simultaneously during the same video sessions to provide for efficient data collection on both pain-stimulated and pain-depressed behaviors in the same subject. Nesting was assessed in different subjects tested in their home cages, which provided an opportunity to assess generality of results across different subjects and testing environments.

Effects of ketoprofen and oxycodone

The present results agree with previous evidence that ketoprofen and other COX inhibitors are effective to produce antinociception against a range of chemical and inflammatory pain stimuli producing an array of pain-related behaviors including stretching (Seguin et al., 1995; Bagdas et al., 2016; Alexander et al., 2019), facial grimace (Leach et al., 2012; Matsumiya et al., 2012; Tuttle et al., 2018; Cho et al., 2019; de Almeida et al., 2019), depression of rearing (Matson et al., 2007; Nagase et al., 2012) and depression of nesting (Negus et al., 2015; Oliver et al., 2018; Alexander et al., 2019). In the present study, ketoprofen potency and efficacy were similar to alleviate IP acid-induced stimulation of stretching and facial grimace and depression of nesting, and although ketoprofen was
less potent to alleviate IP acid-induced depression of rearing, it did significantly restore rearing at 10 mg/kg. The antinociceptive effectiveness of ketoprofen on endpoints of both pain-stimulated and pain-depressed behaviors provides one source of evidence to suggest that ketoprofen effects reflected sensory blockade of the acid noxious stimulus rather than production of nonspecific motor effects that might impede expression of nociceptive behaviors. This conclusion is further supported by the finding that antinociceptive ketoprofen doses did not alter nesting or locomotion in the absence of the IP acid noxious stimulus. Previous studies have also reported ketoprofen antinociception at doses without evidence of motor disruption (Niemegeers et al., 1975; Julou et al., 1976; Negus et al., 2015; Alexander et al., 2019). Taken together, these findings are consistent with the clinical analgesic efficacy of ketoprofen and other COX inhibitors for treatment of inflammatory pain (Moore and McQuay, 2013), and they also demonstrate sensitivity of this panel of behavioral endpoints to a clinically effective analgesic as a positive control.

The MOR agonist oxycodone is also a clinically effective analgesic to treat inflammatory and other types of pain, but it produced a profile of effects distinct from that of ketoprofen. Like ketoprofen, oxycodone attenuated acid-induced stimulation of stretching and facial grimace and depression of nesting at doses that did not alter locomotion or nesting in the absence of the noxious stimulus. This agrees with other evidence to suggest that MOR agonists can alleviate some inflammation-related pain-stimulated and pain-depressed behaviors at doses that do not alter other pain-independent behaviors (Matson et al., 2007; Cobos et al., 2012; Cobos and Portillo-Salido, 2013; Bagdas et al., 2016; Lewter et al., 2017; Negus, 2019). However, unlike ketoprofen, oxycodone produced only a partial alleviation of acid-induced depression of nesting, and it failed to alleviate acid-induced depression of rearing. Previous studies have also reported limited effectiveness of MOR agonists to alleviate some types of pain-related behavioral depression, and this limited efficacy appears to reflect MOR agonist-induced motor effects that prevent antinociceptive restoration of function in assays of pain-depressed behavior (Matson et al., 2007; Cobos et al., 2012; Elhabazi et al., 2012; Cho et al., 2013; Kendall et al., 2016). In the present study, for example, oxycodone significantly increased
locomotion and produced a small though nonsignificant decrease in control nesting at 3.2 mg/kg. These motor effects could have contributed to the descending limb of the inverted-U shaped dose-effect curve for oxycodone effects on IP acid-induced nesting depression and prevented expression of oxycodone antinociception on acid-induced rearing depression (which was also less responsive to ketoprofen, see above). Taken together, these results suggest that this panel of behaviors is also sensitive to MOR agonist analgesics and provides data that can be used to interpret interactions between analgesic and motoric drug effects.

**Effects of diazepam and amphetamine**

The GABA_A receptor positive allosteric modulator diazepam produced significant antinociception against both IP acid-stimulated stretching and grimace, a finding that agrees with other evidence for antinociception by diazepam and related benzodiazepines in preclinical assays of pain-stimulated behaviors elicited by chemical noxious stimuli (Rosland et al., 1987; Fidecka and Pirogowicz, 2002; Munro et al., 2008; Chiba et al., 2009). However, as in these previous studies, diazepam antinociception was observed only at a high dose that also produced evidence of motor impairment in the absence of the noxious stimulus, suggesting that apparent antinociception reflected motor impairment rather than analgesia. This conclusion was further supported by the failure of diazepam to alleviate IP acid-induced depression of either rearing or nesting, and indeed, diazepam only exacerbated IP acid-induced depression of rearing. Moreover, this conclusion is also consistent with the lack of diazepam analgesic effectiveness in humans (Raft et al., 1977) and for the absence of a clinical indication for diazepam as a stand-alone treatment for pain (Physician's Desk Reference, 2020). Overall, these results illustrate the vulnerability of traditional assays of pain-stimulated behavior to false-positive effects with drugs that produce sedation or motor impairment. A traditional strategy to address this vulnerability has been to compare drug potency to produce antinociception with potency to impair motor performance in the absence of the noxious stimulus, but this approach is not always reliable (Seguin et al., 1995). Combined drug evaluation in complementary assays of both
pain-stimulated and pain-depressed behavior as shown here can further reduce false positives and enhance selectivity for clinically effective analgesics while also probing drug effects on clinically relevant outcome measures related to behavioral depression and functional impairment (Cobos et al., 2012; Negus et al., 2015; Bagdas et al., 2016; Negus, 2019).

The psychostimulant and dopamine/norepinephrine releaser amphetamine produced antinociception on all four behavioral endpoints only at a dose (3.2 mg/kg) that also significantly stimulated pain-independent locomotor activity, although this dose did not alter pain-independent nesting behavior. These data are consistent with previous evidence that amphetamine and other related psychostimulants (e.g. dopamine transporter inhibitors like cocaine) can produce antinociception in assays of both pain-stimulated behavior (Tocco and Maickel, 1984; Gatch et al., 1999; Connor et al., 2000; Alexander et al., 2019) and pain-depressed behavior (Matson et al., 2007; Negus et al., 2012; Rosenberg et al., 2013). However, as in the present study, amphetamine and related psychostimulants generally produce antinociception only at doses that also stimulate behavior in the absence of a noxious stimulus (suggesting a lack of behavioral selectivity), and they do not always restore pain-related behavioral depression in preclinical studies (Matson et al., 2007; Alexander et al., 2019) (suggesting limited effectiveness). Although amphetamine is not approved for use as an analgesic, it has been found to produce weak analgesic effects in humans and can augment the analgesic effects of opioids (Dalal and Melzack, 1998; Westfall and Westfall, 2011). These analgesic effects may be related to effectiveness of amphetamine and related psychostimulants to alleviate pain-related depression of mesolimbic dopamine signaling (Wood, 2008; Leitl et al., 2014; Martikainen et al., 2018; Watanabe and Narita, 2018). As increased dopamine also can cause increases in general motor behavior, this proves difficult to parse true analgesia from psychostimulant effects. With regard to its effects on pain-independent behaviors in the present study, the inverted-U shaped dose-effect curve for amphetamine in the assay of locomotor activity is consistent with previous studies (Rethy et al., 1970; Tyler and Tessel, 1979). In particular, the decrease in locomotion at 10 mg/kg likely reflected recruitment of stereotypies that competed with
and reduced horizontal locomotion (Tyler and Tessel, 1979; Matson et al., 2007). High baseline nesting near the ceiling of the assay’s dynamic range may have prevented detection of any stimulation of nesting by lower amphetamine doses; however, depression of nesting by 10 mg/kg amphetamine may also have reflected stereotypies that competed with and reduced nesting behavior.

**Authorship Contributions:**

*Participated in research design:* Diester, Negus

*Conducted experiments:* Diester, Santos, Moerke

*Performed data analysis:* Diester

*Wrote or contributed to the writing of the manuscript:* Diester, Santos, Negus
REFERENCES


**Footnotes**

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Figure Legends

Figure 1. Effects of IP lactic acid on stretching, facial grimace, rearing and nesting behaviors in male and female mice. Abscissae: concentration of lactic acid diluted in sterile water and administered IP in a volume of 10 ml/kg (log scale). Ordinates: number of stretches (A), grimace score (B), number of rears (C), and nesting expressed as percent maximum nestlet consolidation (D). Each point shows mean ± SEM for 12 mice (6 male, 6 female). Filled symbols indicate a significant difference from vehicle (Veh) as determined by RM one-way ANOVA and Dunnett’s post hoc test for parametric data (A, C & D) or by Friedman’s and Dunn’s post hoc test for nonparametric data (B), p<0.05. Results of ANOVA and power analysis for each panel are shown in Table 1.

Figure 2. Effects of the clinically-effective positive controls ketoprofen and oxycodone on IP acid-induced pain behaviors. Abscissae: doses of ketoprofen or oxycodone in mg/kg (log scale). Ordinates: number of stretches (A), grimace score (B), number of rears (C), and nesting expressed as percent maximum nestlet consolidation (D). Each point shows mean ± SEM for 12 mice (6 male, 6 female). Filled symbols indicate a significant difference from vehicle (Veh) as determined by RM one-way ANOVA and Dunnett’s post hoc test for parametric data (A, C & D) or by Friedman’s and Dunn’s post hoc test for nonparametric data (B), p<0.05. Results of ANOVA and power analysis data for each panel are shown in Table 1.

Figure 3. Effects of the active negative controls diazepam and amphetamine on IP acid-induced pain behaviors. Abscissae: doses of diazepam or amphetamine delivered SC in mg/kg (log scale). Ordinates: number of stretches (A), grimace score (B), number of rears (C), and nesting expressed as percent maximum nestlet consolidation (D). Each point shows mean ± SEM for 12 mice (6 male, 6 female). Filled symbols indicate a significant difference from vehicle (Veh) as determined by RM one-way ANOVA and Dunnett’s post hoc test for parametric data (A, C & D) or by Friedman’s
and Dunn’s post hoc test for nonparametric data (B), p<0.05. Results of ANOVA and power analysis data for each panel are shown in Table 1.

Figure. 4. Effects of all drugs on nesting and locomotion in the absence of the IP acid noxious stimulus. Abscissae: Doses of ketoprofen, diazepam, oxycodone and amphetamine delivered in mg/kg (log scale). Ordinates: nesting expressed as percent maximum nestlet consolidation (A) and locomotor counts (B). Each point shows mean ± SEM for 12 mice (6 male, 6 female). Filled symbols indicate a significant difference from vehicle (Veh) as determined by RM one-way ANOVA and Dunnett’s post hoc test. Results of ANOVA and power analysis data for each panel are shown in Table 1.

Figure. 5. Drug profiles for comparing potency to produce antinociceptive effects versus general behavioral disruption. Abscissae: Drug dose for either ketoprofen (A), oxycodone (B), diazepam (C) or amphetamine (D). Open bars span the dose range over which each drug significantly attenuated IP acid-induced stimulation of stretching (S), facial grimace (G) or IP acid-induced depression of rearing (R) or nesting (N). In each panel, bars for pain-stimulated behaviors (PSB) and pain-depressed behaviors (PDB) are shown above and below the dose line, respectively. The gray zone in each panel spans doses over which each drug disrupted nesting and/or locomotion when administered alone in the absence of the IP acid noxious stimulus. Ketoprofen (A) did not alter nesting or locomotion at doses up to 32 mg/kg, so no gray zone is indicated.
**Table 1. Summary of power analysis results from pooled one-way ANOVA data from Figures 1-4**

<table>
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<tr>
<th></th>
<th>IP Acid</th>
<th>Ketoprofen</th>
<th>Oxycodone</th>
<th>Diazepam</th>
<th>Amphetamine</th>
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<td>Stretch + Acid</td>
<td>F(2.188, 24.07)=15.29; p&lt;0.0001*</td>
<td>-</td>
<td>F(1.578, 17.36)=8.075; p=0.0052*</td>
<td>F(2.307, 25.38)=6.478; p=0.0039*</td>
<td>F(2.201, 26.41)=6.822; p=0.0033*</td>
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<td>Rear + Acid</td>
<td>F(1.938, 21.32)=7.251; p=0.0042*</td>
<td>0.58</td>
<td>F(2.403, 26.43)=6.641; p=0.003*</td>
<td>F(2.066, 22.72)=3.224; p=0.0571</td>
<td>F(2.44, 29.28)=3.131; p=0.0496*</td>
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<td>Grinace</td>
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<td>-</td>
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<td>F(2.188, 24.07)=29.17; p&lt;0.0001*</td>
<td>F(1.634, 17.98)=4.157; p=0.0396*</td>
<td>F(2.28, 25.08)=2.984; p=0.0628</td>
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<tr>
<td>F=25.37; p&lt;0.0001*</td>
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*Current Power: Power≥0.8*
Figure 2

A. Stretching

- △ Ketoprofen
- ○ Oxycodone

B. Grimace

C. Rearing

D. Nesting

- % Maximal Nestlet Consolidation
Figure 3

A. Stretching

- Diazepam
- Amphetamine

B. Grimace

C. Rearing

D. Nesting

% Maximal Nestlet Consolidation

Dose (mg/kg)