1521-0103/377/2/242–253$35.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

https://doi.org/10.1124/jpet.121.000497
J Pharmacol Exp Ther 377:242–253, May 2021

Behavioral Battery for Testing Candidate Analgesics in Mice. II. Effects of Endocannabinoid Catabolic Enzyme Inhibitors and Δ9-Tetrahydrocannabinol[S]

C. M. Diester, A. H. Lichtman, and S. S. Negus

Department of Pharmacology and Toxicology (C.M.D., A.H.L., S.S.N.), School of Pharmacy (A.H.L.), Virginia Commonwealth University, Richmond, Virginia

Received January 5, 2021; accepted February 22, 2021

ABSTRACT

Enhanced signaling of the endocannabinoid (eCB) system through inhibition of the catabolic enzymes monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) has received increasing interest for development of candidate analgesics. This study compared effects of MAGL and FAAH inhibitors with effects of Δ9-tetrahydrocannabinol (THC) using a battery of pain-stimulated, pain-depressed, and pain-independent behaviors in male and female mice. Intraperitoneal injection of dilute lactic acid (IP acid) served as an acute visceral noxious stimulus to stimulate two behaviors (stretching, facial grimace) and depress two behaviors (rearing, nesting). Nesting and locomotion were also assessed in the absence of IP acid as pain-independent behaviors. THC and a spectrum of six eCB catabolic enzyme inhibitors ranging from MAGL- to FAAH-selective were assessed for effectiveness to alleviate pain-related behaviors at doses that did not alter pain-independent behaviors. The MAGL-selective inhibitor MJN110 produced the most effective antinociceptive profile, with 1.0 mg/kg alleviating IP acid effects on stretching, grimace, and nesting without altering pain-independent behaviors. MJN110 effects on IP acid–depressed nesting had a slow onset and long duration (40 minutes to 6 hours), were blocked by rimonabant, and tended to be greater in females. As inhibitors increased in FAAH selectivity, antinociceptive effectiveness decreased. PF3845, the most FAAH-selective inhibitor, produced no antinociception up to doses that disrupted locomotion. THC decreased IP acid–stimulated stretching and grimace at doses that did not alter pain-independent behaviors; however, it did not alleviate IP acid–induced depression of rearing or nesting. These results support further consideration of MAGL-selective inhibitors as candidate analgesics for acute inflammatory pain.

SIGNIFICANCE STATEMENT

This study characterized a spectrum of endocannabinoid catabolic enzyme inhibitors ranging in selectivity from monoacylglycerol lipase–selective to fatty acid amide hydrolase–selective in a battery of pain-stimulated, pain-depressed, and pain-independent behaviors previously pharmacologically characterized in a companion paper. This battery provides a method for prioritizing candidate analgesics by effectiveness to alleviate pain-related behaviors at doses that do not alter pain-independent behaviors, with inclusion of pain-depressed behaviors increasing translational validity and decreasing susceptibility to motor-depressant false positives.

Introduction

Every year, millions of people suffer from acute and chronic pain, placing a heavy burden on the United States healthcare system (Institute of Medicine (US) Committee on Advancing Pain Research, Care, and Education, 2011). Nonsteroidal anti-inflammatory drugs and μ-opioid receptor agonists represent the most commonly used classes of analgesics; however, limited clinical efficacy, chronic dosing constraints, and the ongoing opioid public health crisis have further invigorated a decades-long effort to develop new analgesics. One emerging class of candidate analgesics consists of drugs targeting the endocannabinoid (eCB) system (Donvito et al., 2018). The main cannabinoid 1 and 2 receptors ($CB_{1/2}R$) are $G_{i/o}$ G protein-coupled receptors widely expressed throughout the central nervous system and periphery. These receptors can be activated by exogenous orthosteric agonists [e.g., Δ9-tetrahydrocannabinol (THC)] or by endogenous lipids that include 2-arachidonyl glycerol (2-AG) and anandamide, which provide synaptic-level feedback inhibition through retrograde signaling (Lu and Mackie, 2016).

Although multiple studies have shown poor clinical efficacy alongside unwanted psychomimetic and motor-impairing effects with $CB_{1/2}R$ agonists as analgesics (Raft et al., 1977; Greenwald and Stitzer, 2000; Wallace et al., 2007; Kraft et al., 2008; Mun et al., 2020), increasing evidence suggests analgesic potential for inhibitors of the main eCB catabolic degradative enzymes, monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH), to enhance endogenous levels of 2-AG and

Supported by National Institutes of Health National Institute on Drug Abuse [Grant R01DA030404] and [Grant F31DA051163].
The authors have no conflicts of interest to report.
https://doi.org/10.1124/jpet.121.000497.
[S] This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: 2-AG, 2-arachidonyl glycerol; CB_1R, cannabinoid 1 receptor; CB_2R, cannabinoid 2 receptor; $CB_{1/2}R$, cannabinoid 1 and 2 receptor; eCB, endocannabinoid; FAAH, fatty acid amide hydrolase; IP acid, intraperitoneal injection of dilute lactic acid; MAGL, monoacylglycerol lipase; THC, Δ9-tetrahydrocannabinol.

anandamide, respectively. For example, pharmacological inhibition of MAGL and FAAH elicits antinociceptive effects in assays with neuropathic, inflammatory, and thermal noxious stimuli (Long et al., 2009a; Schlosburg et al., 2009; Nomura et al., 2011; Booker et al., 2012; Fowler, 2012; Dalmann et al., 2015; Burston et al., 2016; Habib et al., 2019; Thompson et al., 2020). However, an FAAH inhibitor failed as an analgesic in a clinical trial for pain due to osteoarthritis of the knee (Huggins et al., 2012; von Schaper, 2016), and clinical results have not yet been published with MAGL-selective or dual MAGL/FAAH inhibitors. A major issue in the preclinical development of analgesics is translational validity of behavioral endpoints used to measure pain (Mogil, 2009; Cobos and Portillo-Salido, 2013; Negus, 2019). The vast majority of assays in preclinical research measure pain-stimulated behaviors, in which putative pain manipulations increase expression of the target behavior, and antinociception is exhibited as a decrease in that behavior. Importantly, general behavioral suppression can produce false-positive evidence for antinociception in preclinical assays of pain-stimulated behaviors (Yezierski and Hansson, 2018; Negus, 2019). Moreover, in terms of face validity, suppression of reflexive pain-stimulated behaviors is not a primary endpoint for clinical treatment of pain. In contrast, pain-related functional impairment and behavioral depression are major drivers for healthcare utilization (St. Sauver et al., 2013), with restoration of these behaviors as a primary outcome measure for treatment (Dworkin et al., 2005; U.S. Department of Health and Human Services, 2019). Additionally, pain-depressed behaviors are routinely used in veterinary medicine to diagnose and treat pain (National Research Council (US) Institute for Laboratory Animal Research, 1996), and because antinociception is indicated by an increase in the target behavior, assays of pain-depressed behavior are not susceptible to false-positive effects from general motor depressants (Negus, 2019). Accordingly, we have argued that preclinical assays for development of candidate analgesics should include measures of pain-related behavioral depression to promote preclinical-to-clinical translation and protect against false-positive effects with drugs that produce motor impairment (Negus et al., 2015; Diester et al., 2021).

eCB catabolic enzyme inhibitors have not been extensively evaluated in preclinical studies that included assays of pain-depressed behavior, but existing data suggest that MAGL inhibitors may be more effective than FAAH inhibitors (Kwilasz et al., 2014; Wilkerson et al., 2018). The main purpose of this study was built on these previous results by testing a spectrum of eCB catabolic enzyme inhibitors in a battery of pain-stimulated, pain-depressed, and pain-independent mouse behaviors described and validated in a companion study (Diester et al., 2021). Intraperitoneal injection of dilute lactic acid (IP acid) served as an acute visceral noxious stimulus to model tissue acidosis as a contributor to inflammatory pain (Koster et al., 1959; Holzer, 2009), and the behavioral battery was used to compare antinociceptive and motor-disruptive effects of the direct $CB_{1/2}R$ agonist Δ9-tetrahydrocannabinol (THC) and the following spectrum of eCB catabolic enzyme inhibitors: the MAGL-selective inhibitors MJN110 and JZL184, the dual MAGL/FAAH inhibitors JZL195 and SA57, and the FAAH-selective inhibitors URB597 and PF3845 (Fig. 1). We predicted a more favorable profile of antinociceptive effects without motor impairment for MAGL-selective inhibitors than for dual or FAAH-selective inhibitors or THC. Additionally, previous work has shown the antinociceptive effects of MAGL and FAAH to be $CB_{1/2}R$-mediated in neuropathic and inflammatory pain models, with some inconsistencies for the involvement of CB_2R in MAGL effects (Kinsey et al., 2009; Naidu et al., 2010; Booker et al., 2012; Ignatowska-Jankowska et al., 2015; Burston et al., 2016). Accordingly, the time course and $CB_{1/2}R$ antagonism were assessed for the most effective eCB catabolic enzyme inhibitor. Lastly, there is some evidence for sex differences in cannabinoid antinociception (Cooper and Craft, 2018; Blanton et al., 2021), and a recent mandate from the National Institutes of Health encourages inclusion of both males and females in preclinical studies to assess any sex differences in effects. We addressed this issue by including equal numbers of male and female mice and analyzing results for sex differences using a stepwise strategy described previously (Diester et al., 2019).

Materials and Methods

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 25–45 g and females weighed 20–35 g throughout the study. In an American Association for Accreditation of Laboratory Animal Care-approved facility, mice were housed in cages (31.75 cm long × 23.50 cm wide × 15.25 cm 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 (Laboratory 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 and in studies of locomotor activity were littermates, group housed 3 per 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 1 week after arrival at the laboratory. Additionally, for singly housed mice in

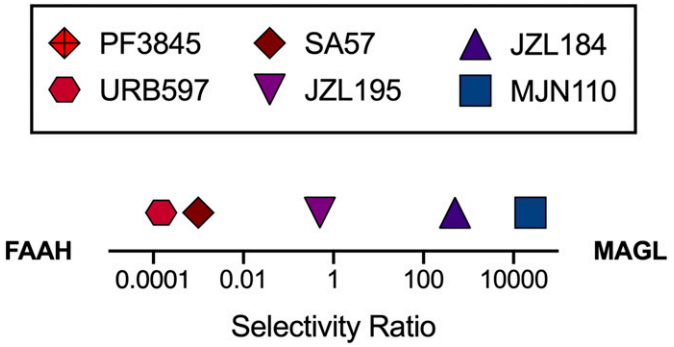

Fig. 1. Selectivity of test compounds for the main endocannabinoid catabolic enzymes MAGL and FAAH based on competitive substrate binding and activity-based protein profiling assays. Data were obtained from the literature as cited below, with competitive substrate binding used for calculating selectivity if available and activity-based protein profiling used if no substrate binding data were available. All data are from assays using mouse brain tissue. No data for MAGL binding could be found for either endpoint for PF3845, which is considered to be a highly selective FAAH inhibitor. As a result, no selectivity ratio could be calculated for this inhibitor. PF3845: Ahn, 2009 (main paper denoting FAAH selectivity); URB597: Kathuria et al., 2003; SA57: Niphakis et al., 2012; JZL195: Long et al., 2009c; JZL184: Long et al., 2009a; MJN110: Niphakis et al., 2013.

nesting studies, experiments were performed in their home cages at least 2 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 this study was to compare effects of the direct cannabinoid receptor agonist THC and a series of eCB catabolic enzyme inhibitors covering a broad spectrum of selectivity for MAGL versus FAAH on a battery of pain-stimulated, pain-depressed, and pain-independent behaviors in mice as described in the companion paper by Diester et al. (2021). Pain-related behaviors were elicited by intraperitoneal injection of dilute lactic acid, and antinociception dose-effect curves for each test drug were determined in two groups of mice: 1) one to assess drug effects on IP acid–induced changes in stretching, facial grimace, and rearing, and 2) a second to assess drug effects on IP acid–induced depression of nesting. Two additional groups were used to test effects of each test drug on two pain-independent behaviors: 1) one group to assess control nesting in the absence of the IP acid noxious stimulus, and 2) a second group to assess locomotion in the absence of the noxious stimulus. To further probe the effects observed with the MAGL-selective inhibitor MJN110, two additional nesting groups were used to assess time course and sensitivity to cannabinoid receptor antagonists. Each group consisted of 12 mice (six males, six females) to provide adequate power for detection of drug effects and permit exploratory analysis of sex differences as described previously (Diester et al., 2019). In general, all mice in a given group received all doses of a single drug, 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. The only exception was for eCB catabolic enzyme inhibitors tested in locomotor studies, in which each group of mice was used to test vehicle and two eCB catabolic enzyme inhibitors at the highest dose tested in antinociception studies. 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 cannabinoid receptor 1/2 direct agonist Δ9-tetrahydrocannibinol, 1.0–30 mg/kg, 30 minutes (Grim et al., 2017); the selective MAGL >> FAAH inhibitors (Fig. 1) MJN110, 0.1–10 mg/kg, 2 hours (Niphakis et al., 2013; Ignatowska-Jankowska et al., 2015) and JZL184, 3.2–32 mg/kg, 2 hours (Long et al., 2009a; Ignatowska-Jankowska et al., 2015; Wiebelhaus et al., 2015); the dual MAGL = FAAH inhibitor (Fig. 1) JZL195, 3.2–32 mg/kg, 2 hours (Long et al., 2009c; Anderson et al., 2014; Hruba et al., 2015); the dual MAGL < FAAH inhibitor (Fig. 1) SA57, 1.0–10 mg/kg, 2 hours (Niphakis et al., 2012; Owens et al., 2016); the selective MAGL << FAAH inhibitors (Fig. 1) URB597, 1.0–10 mg/kg, 60 minutes (Kinsey et al., 2009; Naidu et al., 2009; Naidu et al., 2010) and PF3845, 1.0–32 mg/kg, 2 hours (Booker et al., 2012; Wiebelhaus et al., 2015).

Behavioral Procedures

Stretching/Grimace/Rearing Procedure. Studies of stretching, grimace, and rearing were conducted in a procedure room separate from the housing room, and each drug was tested in a different group of mice. Mice were acclimated for at least 1 hour to the procedure room at least 1 day before the first test day, and on all subsequent test days, mice were again acclimated to the procedure room for at least 1 hour before testing. Testing was initiated by subcutaneous administration of the specified test drug dose followed by return of the mouse to its home cage for the specified pretreatment time. Subsequently, mice received IP acid immediately before being placed into individual Plexiglas cylinders (4″ diameter, 10″ high) and filmed for 20.5 minutes 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 minutes 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 minutes 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-minute 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. Nesting was evaluated in each mouse's home cage in the vivarium beginning at least 1 week after arrival, and each drug was tested in a different group of mice. Additionally, all mice nested during the initial acclimation week; thus, all were included in the study. On test days, mice received a subcutaneous injection with the specified test drug before being returned to their home cages on the housing rack. After the specified pretreatment time, mice were removed from their cage and received either IP acid (for studies of drug effects on IP acid–depressed nesting) or vehicle (for studies of drug effects on control nesting in the absence of the noxious stimulus). Old nesting material was removed, two 1-square-inch nestlet squares were placed 11 inches apart in the center of the opposing short walls of the cage [see (Diester et al., 2021), Supplemental Fig. 1], and the mouse was again returned to its home cage. After a 90-minute 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. Nestlet position was evaluated only at 90 minutes to minimize cage disturbances during the experiment. The primary dependent variable was percent maximal nestlet consolidation, 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.

Time course and antagonism studies were conducted in two additional groups of mice. For time-course studies, 1.0 mg/kg MJN110 was administered at different pretreatment times ranging from 10 minutes to 24 hours, and the sequence of pretreatment times was presented in a Latin square order across mice. For antagonism studies, mice received a subcutaneous pretreatment of vehicle, the CB_1R antagonist rimonabant (3 mg/kg) or the CB_2R antagonist SR144528 (3 mg/kg), 10 minutes before 1.0 mg/kg MJN110 or vehicle, and the nesting session commenced 2 hours later. Again, these treatments were administered in a Latin square design. Antagonist doses and pretreatment times were based on previous studies (Booker et al., 2012; Ignatowska-Jankowska et al., 2015).

Locomotor Procedure. Locomotor activity studies were conducted in a procedure room separate from the housing room. One group of mice was used to test a range of THC doses. Three additional groups were used to test the eCB enzyme inhibitors, with vehicle and a single high dose of each of two eCB enzyme inhibitors tested in each group. Mice were acclimated for at least 1 hour to the procedure room at least 1 day before the first test day, and on all subsequent test days, mice were again acclimated to the procedure room for at least 1 hour

before testing. After acclimation on a given test day, the test drug was administered subcutaneously, and mice were returned to their home cages for the designated pretreatment interval before being placed into locomotor activity boxes for 30 minutes. Horizontal locomotor activity was assessed in 16.8 cm wide × 12.7 cm deep × 12.7 cm high boxes housed in sound-attenuating chambers (Med Associates, St. Albans, VT). Each box had black Plexiglas walls, a clear Plexiglas 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-minute session.

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 nonparametric 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 nonparametric 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 nonparametric 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 drug dose as a within-subjects factor. A significant sex × treatment interaction was followed by a Holm-Šídák post hoc test. For nonparametric 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 nonparametric data, so grimace data were not submitted for further power analyses in this study (Lehmann, 2006).

Drugs

Lactic acid (Fisher Scientific, Hampton, NH) was diluted in sterile water and administered intraperitoneally. THC (20 mg/m in ethanol), JZL184, URB597, and PF3845 were provided by the National Institute on Drug Abuse Supply Program (Bethesda, MD). MJN110 and SA57 were kindly provided by Dr. Michah Niphakis, currently at Lundbeck La Jolla Research Center. JZL195 was purchased from Tocris/Bio-Techne (Minneapolis, MN). The CB_1R antagonist rimonabant (SR141716A) and CB_2R antagonist SR144528 were provided by the National Institute on Drug Abuse Supply Program (Bethesda, MD). All drugs were prepared in a vehicle of 1:1:18 ethanol, emulphor (Alkamuls-620; Sanofi-Aventis, Bridgewater, NJ), and saline. In general, final concentrations were prepared as clear solutions; however, final concentrations of JZL184 (≥1.0 mg/ml) and JZL195 (≥3.2 mg/ml) were suspensions thoroughly shaken immediately before preparation of syringes and injection. All test drugs and antagonists were administered subcutaneously in volumes of 0.1–0.9 ml.

Results

Overview of Data Presentation. Figures. 2–5 show antinociception dose-effect curves for each drug on each endpoint of IP acid–induced behaviors (stimulation of stretching and facial grimace in left panels, depression of rearing and nesting in right panels). Figure 6 shows effects of each compound on nesting and locomotion in the absence of the noxious stimulus to determine IP acid–independent effects of each test drug. Figure 7 compares the potencies of each drug to produce antinociceptive effects versus IP acid–independent effects, in which an ideal drug would produce antinociception at doses below those that produce IP acid–independent effects. Figure 8 shows the time course, $CB_{1/2}R$ antagonism, and sex differences for the MAGL-selective inhibitor MJN110. ANOVA results and power analyses for pooled data across sexes are presented in Table 1. Supplemental Tables 1 and 2 show ANOVA results for data segregated by sex, and Supplemental Tables 3–9 show results from two-way ANOVA examining sex as a determinant of effects for each test drug on each endpoint. For any significant main effects of sex or sex × dose interactions, additional supplemental figures are added and denoted in the results below.

Effects of Δ9-THC. Figure 2 shows that the direct $CB_{1/2}R$ agonist THC significantly decreased IP acid–stimulated stretching (3.2–10 mg/kg) and facial grimace (10 mg/kg) but failed to alter IP acid–induced depression of either rearing or nesting. Figure 6 shows that THC significantly decreased both nesting and locomotion in the absence of the noxious stimulus at 30 mg/kg. Thus, THC displayed a 0.5–1.0 log higher potency to produce antinociception compared with general motor effects in the absence of the noxious stimulus, but only for IP acid–stimulated behaviors, as it had no significant effect for either IP acid–depressed behavior (Fig. 7). Additionally, there were no main effects of sex or dose × sex interactions for any endpoint (Supplemental Table 3).

Effects of MAGL >> FAAH–Selective Inhibitors. Figure 3 shows antinociceptive effects of the MAGL >> FAAH–selective inhibitors MJN110 and JZL184. MJN110 significantly decreased both IP acid–stimulated stretching and grimace at 1.0 mg/kg, but only stretching was significantly decreased at 3.2 mg/kg. MJN110 did not alter IP acid–induced depression of rearing, but doses of 0.32–3.2 mg/kg attenuated IP acid–induced depression of nesting. No dose of MJN110 up to 10 mg/kg altered nesting in the absence of the noxious stimulus, and 3.2 mg/kg did not alter locomotion (Fig. 6). Thus, MJN110 produced significant antinociception for three of the four IP acid–induced behaviors at doses that did not alter IP acid–independent behaviors (Fig. 7). IP acid–depressed nesting was the only endpoint that showed a significant effect of sex as a determinant of MJN110 effects, where one-way ANOVAs segregated by sex showed a significant effect for females and not males, and two-way ANOVA showed a main effect of sex (females > males) (Supplemental Fig. 1; Supplemental Tables 1, 2, and 4). Additional evaluation of sex as a determinant of MJN110 effects on IP acid–induced depression of nesting is described below.

JZL184 did not significantly alter IP acid–stimulated stretching at any dose, but 32 mg/kg JZL184 significantly decreased facial grimace. No dose significantly altered IP acid–induced depression of rearing, but 3.2 and 32 mg/kg attenuated depression of nesting. No dose up to 32 mg/kg of

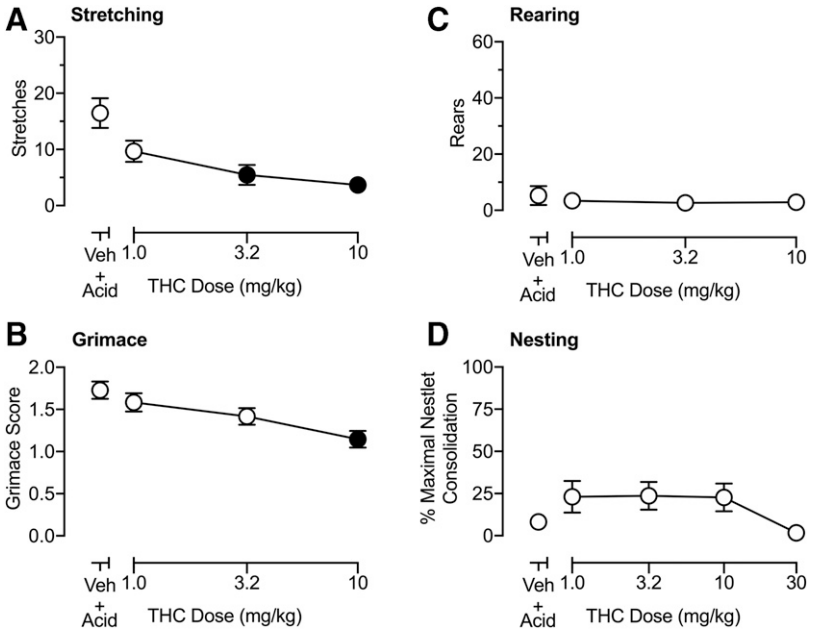

Fig. 2. Effects of THC on IP acid–induced stretching, facial grimace, rearing, and nesting behaviors in male and female mice. Abscissae: dose of THC delivered subcutaneously in milligrams per kilogram (log scale). Ordinates: number of stretches (A), grimace score (B), number of rears (C), and nesting expressed as percent maximum nestlet consolidation (D). Each point shows ±S.E.M. for 12 mice (six male, six female). Filled symbols indicate a significant difference from vehicle (Veh) as determined by repeated-measures one-way ANOVA and Dunnett's post hoc test for parametric data (A, C, and 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.

JZL184 altered nesting in the absence of IP acid, and 32 mg/kg did not significantly affect locomotion (Fig. 6). Thus, JZL184 produced antinociception for one IP acid–stimulated behavior (facial grimace) and one IP acid–depressed behavior (nesting) at doses that did not significantly alter IP acid–independent behaviors (Fig. 7). Two-way ANOVA of sex differences showed main effects of sex for JZL184 effects on IP acid–stimulated stretching (male > females), IP acid–induced depression of rearing (males > females), and nesting in the absence of the noxious stimulus (males > females), but there were no sex × dose interactions on any endpoint (Supplemental Fig. 1; Supplemental Table 5).

Effects of Dual MAGL and FAAH Inhibitors. Figure 4 shows antinociceptive effects of the dual MAGL ≅ FAAH inhibitor JZL195 and the dual MAGL < FAAH inhibitor SA57. JZL195 significantly blocked IP acid–stimulated stretching at 32 mg/kg but did not block acid-stimulated facial grimace. Although IP acid–depressed rearing was not affected, IP acid–depressed nesting was attenuated at 32 mg/kg. No dose of JZL195 up to 32 mg/kg affected nesting in the absence of IP acid, and 32 mg/kg did not alter locomotor behavior (Fig. 6). Thus, JZL195 had antinociceptive effectiveness on one IP acid–stimulated behavior (stretching) and one IP acid–depressed behavior (nesting) at doses that did not

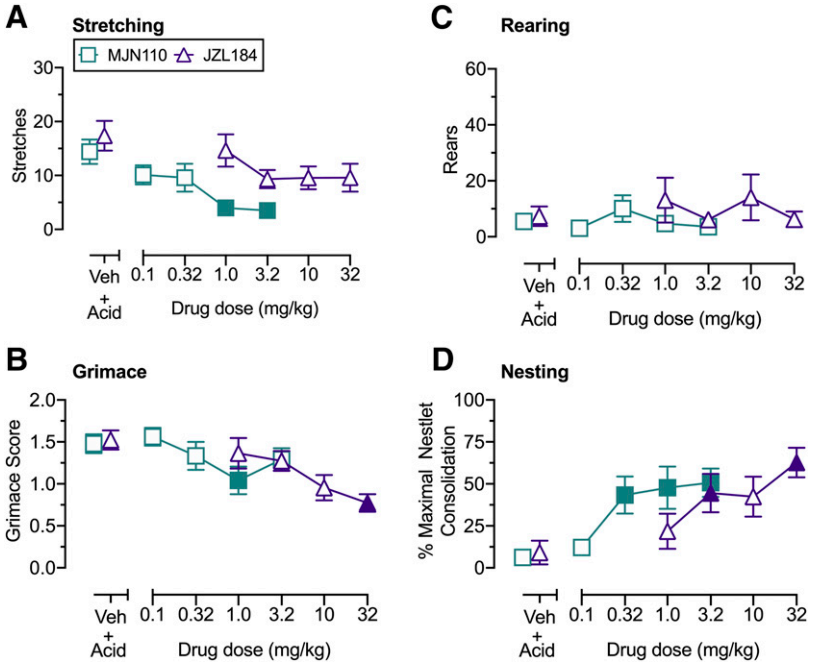

Fig. 3. Effects of MAGL-selective inhibitors MJN110 and JZL184 on IP acid–induced stretching, facial grimace, rearing, and nesting behaviors in male and female mice. Abscissae: doses of MJN110 or JZL184 delivered subcutaneously in milligrams per kilogram (log scale). Ordinates: number of stretches (A), grimace score (B), number of rears (C), and nesting expressed as percent maximum nestlet consolidation (D). Other details as in Fig. 2.

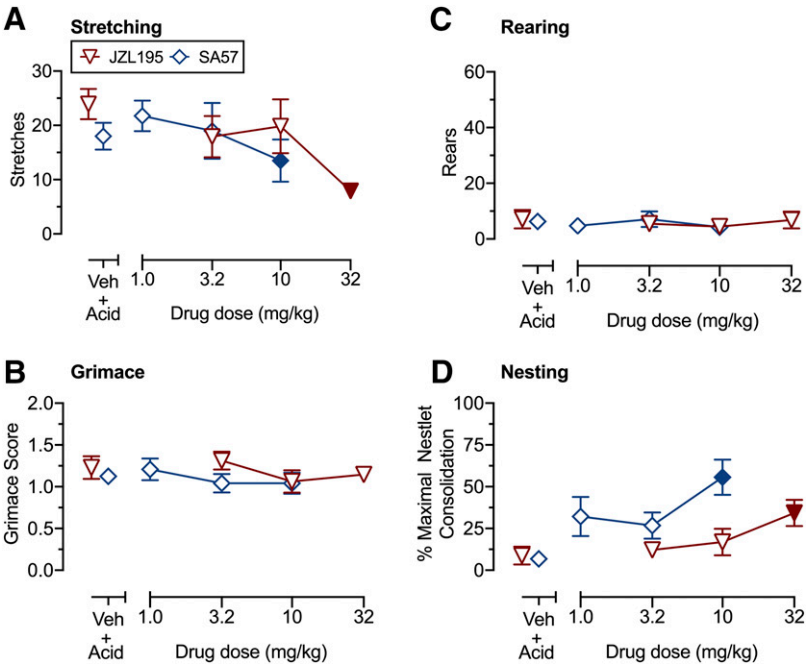

Fig. 4. Effects of dual MAGL and FAAH inhibitors JZL195 and SA57 on IP acid–induced stretching, facial grimace, rearing, and nesting behaviors in male and female mice. Abscissae: doses of JZL195 or SA57 delivered subcutaneously in milligrams per kilogram (log scale). Ordinates: number of stretches (A), grimace score (B), number of rears (C), and nesting expressed as percent maximum nestlet consolidation (D). Other details as in Fig. 2.

produce effects on acid-independent behaviors (Fig. 7). In analysis of sex as a determinant of JZL195 effects, there was a main effect of sex in locomotion (females > males), but there were no sex × dose interactions on any endpoint (Supplemental Fig. 2; Supplemental Table 6).

SA57 also significantly blocked IP acid–stimulated stretching (10 mg/kg) but not facial grimace. SA57 did not alter IP acid–depressed rearing, but it significantly attenuated IP acid–depressed nesting. No dose of SA57 significantly affected nesting or locomotion in the absence of the noxious stimulus (Fig. 6). Thus, SA57 blocked IP acid–stimulated stretching and attenuated IP acid–induced depression of nesting at doses that did not significantly alter nesting or locomotion when administered alone (Fig. 7). For SA57, there was a significant main effect of sex for stretching (females < males) and locomotion (females > males), but there were no sex × dose interactions on any endpoint (Supplemental Fig. 2; Supplemental Table 7).

Effects of MAGL << FAAH–Selective Inhibitors. Figure 5 shows the antinociceptive effectiveness of the MAGL << FAAH–selective inhibitors URB597 and PF3845. URB597 did not significantly block either IP acid–stimulated behavior at any dose tested, and it also did not alter IP acid–induced depression of rearing. It attenuated IP acid–depressed nesting at 10 mg/kg,

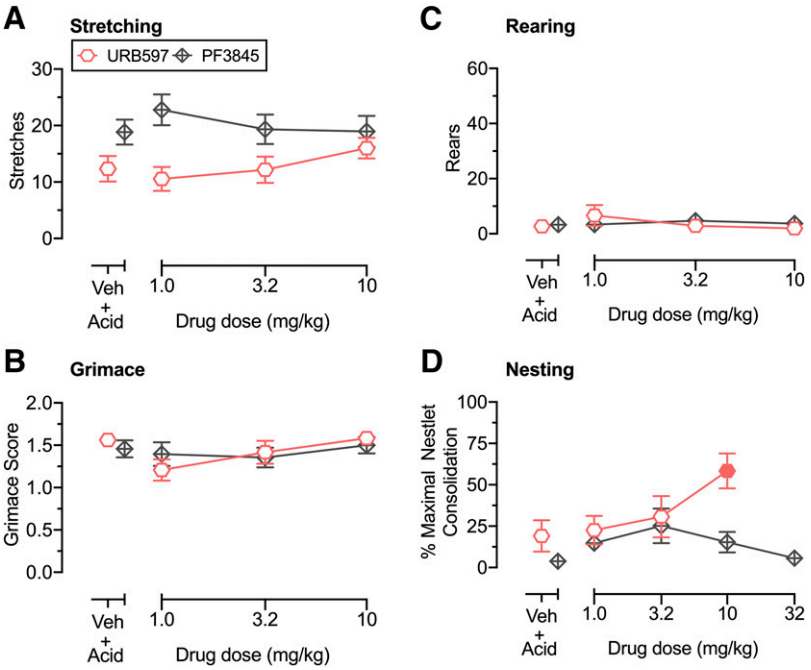

Fig. 5. Effects of FAAH-selective inhibitors URB597 and PF3845 on IP acid–induced stretching, facial grimace, rearing and nesting behaviors in male and female mice. Abscissae: doses of URB597 and PF3845 delivered subcutaneously in milligrams per kilogram (log scale). Ordinates: number of stretches (A), grimace score (B), number of rears (C), and nesting expressed as percent maximum nestlet consolidation (D). Other details as in Fig. 2.

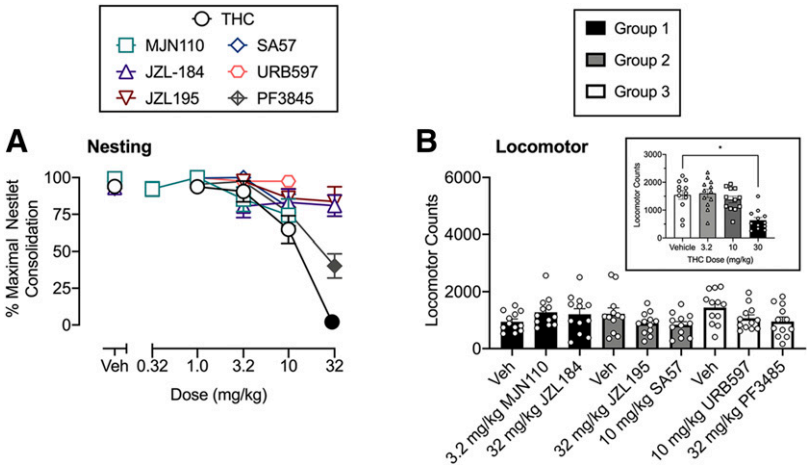

Fig. 6. Effects of endocannabinoid catabolic enzyme inhibitors and THC on nesting and locomotor behaviors in male and female mice in the absence of the IP acid noxious stimulus. Abscissae: doses of endocannabinoid catabolic inhibitors or THC in milligrams per kilogram. Doses of endocannabinoid catabolic enzyme inhibitors for locomotor studies were the highest tested in antinociception assays, whereas THC [inset in (B)] was tested across a range of doses. Ordinates: nesting expressed as percent maximum nestlet consolidation (A), and locomotor counts (B). Each point shows ±S.E.M. for 12 mice (six male, six female). Filled symbols in (A) and the asterisk in (B) inset indicate a significant difference from vehicle (Veh) as determined by repeated-measures one-way ANOVA and Dunnett's post hoc test, $P < 0.05$. Results of ANOVA and power analysis data for each panel are shown in Table 1.

and no dose of URB597 significantly affected nesting or locomotion when administered in the absence of IP acid (Fig. 6). Thus, URB597 showed antinociceptive effectiveness for only one IP acid–depressed behavior (nesting) at a dose that did not alter nesting or locomotion in the absence of the noxious stimulus (Fig. 7). Analysis of sex as a determinant of URB597 effects showed no significant effects of sex (Supplemental Table 8).

PF3845 did not produce antinociception on any endpoint. The highest dose of 32 mg/kg significantly decreased nesting in the absence of the noxious stimulus and produced a non-significant trend toward reduced locomotion (Fig. 6). Thus, PF3845 did not produce antinociceptive effects up to doses that decreased behavior in the absence of the noxious stimulus (Fig. 7). There were no sex differences for any endpoints (Supplemental Table 9).

Time Course, $CB_{1/2}R$ Antagonism, and Sex Differences for MJN110 Effects. Follow-up studies in the assay of IP acid–depressed nesting were conducted with 1.0 mg/kg MJN110 because it produced antinociception on the greatest number of endpoints. Results are shown in Fig. 8, and statistical results are reported in the figure legend. In time-course studies, MJN110 significantly attenuated IP

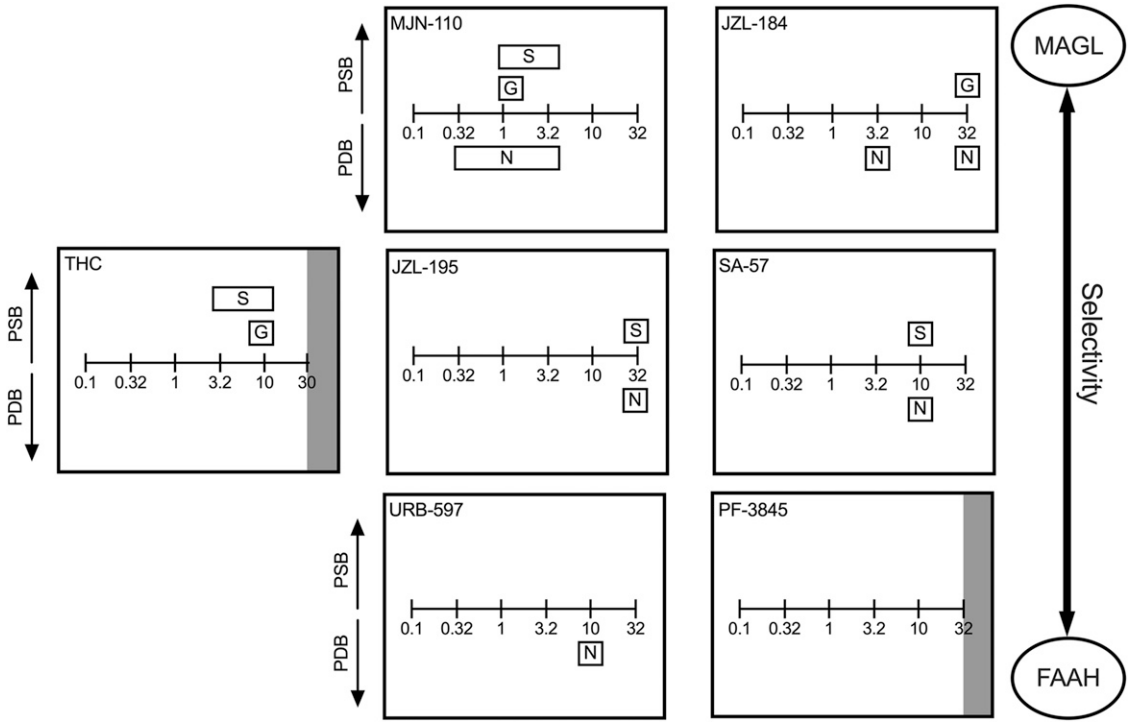

Fig. 7. Drug profiles for comparison of potency to produce antinociceptive effects on IP acid–stimulated and IP acid–depressed behaviors vs. general behavioral disruption. Abscissae: drug dose in milligrams per kilogram (log scale). Boxed letters (S, G, R, N) denote the dose range over which each drug significantly attenuated IP acid–induced stimulation of stretching (S) or facial grimace (G) or IP acid–induced depression of rearing (R) or nesting (N). In each panel, boxes for pain-stimulated behaviors (PSB) and pain-depressed behaviors (PDB) are shown above and below the dose axis, respectively. The gray zone at the right edge of panels for THC and PF3845 shows doses that produced motor disruption in assays of nesting and/or locomotion in the absence of the IP acid noxious stimulus. For the other drugs, no tested dose altered nesting or locomotion in the absence of the noxious stimulus, and no gray zone is indicated. The test compounds for inhibition of the two main endocannabinoid degradative enzymes, MAGL and FAAH, are organized by their selectivity, going from MAGL-selective to FAAH-selective, as indicated by the selectivity arrows on the right.

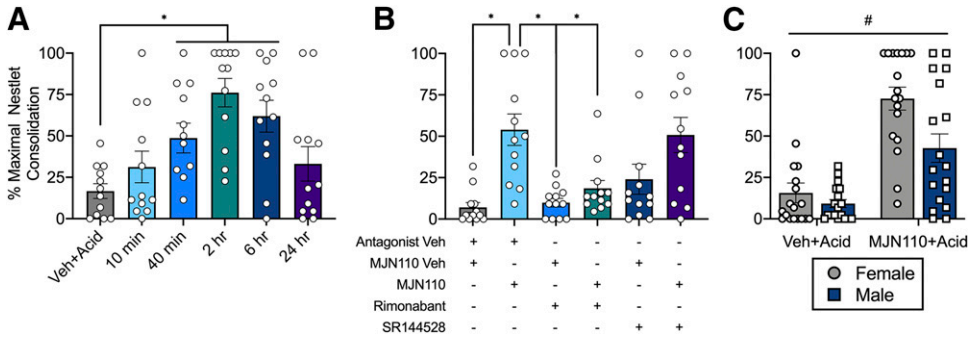

Fig. 8. Effects of time, cannabinoid receptor antagonism, and sex on MJN110 attenuation of IP acid–induced depression of nesting in male and female mice. Abscissae: (A) pretreatment time of 1.0 mg/kg MJN110 before administration of 0.32% IP acid, (B) treatments for assessment of the CB_1R-selective antagonist rimonabant (3 mg/kg) and the CB_2R-selective antagonist SR144528 (3 mg/kg), and (C) effect of sex on 1.0 mg/kg MJN110 antinociception, all delivered subcutaneously in a volume of 10 ml/kg. Ordinates: nestlet consolidation expressed as percent maximal nestlet consolidation. An asterisk (*) indicates a significant difference from vehicle (Veh) + Acid (A and C) or from Antagonist Veh:MJN110 (B), as determined by repeated-measures one-way ANOVA and Dunnett's post hoc test (A and B) or two-way ANOVA and Holm-Šídák post hoc test (C), $P < 0.05$. Statistical results are as follows: (A) significant main effect of treatment [$F(2.738, 30.12) = 7.367$; $P = 0.001$]; (B) significant main effect of treatment [$F(3.931, 43.24) = 7.84$; $P < 0.0001$]. (C) The # indicates that there was a significant main effect of treatment [$F(1, 34) = 53.41$; $P < 0.0001$] and sex [$F(1, 34) = 7.661$; $P = 0.0091$] but no treatment × sex interaction [$F(1, 34) = 3.605$; $P = 0.0661$].

acid–induced depression of nesting with pretreatment times from 40 minutes to 6 hours. In antagonism studies, MJN110 antinociception was significantly blocked by the CB_1R antagonist but not by the CB_2R antagonist SR144528. The effects of 2 hours of pretreatment with vehicle and 1.0 mg/kg MJN110 were determined in a total of 18 male and 18 female mice from the original study (Fig. 3D) and from the two follow-up studies (2 hours of pretreatment for time-course study, vehicle pretreatment for antagonism study). Figure 8C compares effects of vehicle and 1.0 mg/kg MJN110 for all these male and female mice. Two-way ANOVA indicated only significant main effects of dose (1.0 mg/kg MJN110 > vehicle) and sex (females > males); the dose × sex interaction approached, but did not reach, the criterion for statistical significance.

Discussion

Effects of Δ9-THC. This study used a battery of pain-stimulated, pain-depressed, and pain-independent behaviors in male and female mice to compare effects of THC and six eCB catabolic enzyme inhibitors ranging from MAGL-selective to FAAH-selective. Our results agree with previous work that showed subcutaneous THC to be more potent to decrease IP acid–stimulated stretching than to produce locomotor impairment (Booker et al., 2009). In contrast, THC is less potent in assays with acute thermal and mechanical noxious stimuli, resulting in relatively similar potencies to produce antinociception and locomotor impairment (Sofia et al., 1975; Cravatt et al., 2001; Varvel, 2005; Grim et al., 2016; Britch et al., 2017; Grim et al., 2017). The present study extended this literature by including facial grimace as a second pain-stimulated behavior and assessment of the two pain-depressed endpoints, nesting and rearing. THC was less potent and effective to decrease IP acid–stimulated facial grimace than stretching. Moreover, in agreement with our previous work with pain-depressed behaviors in rats (Kwilasz and Negus, 2012; Leitl and Negus, 2016), THC failed to attenuate IP acid–induced depression of either nesting or rearing. The poor efficacy of THC to alleviate signs of pain-related behavioral depression aligns with clinical studies that show weak or no analgesic efficacy of THC on measures of acute pain in humans (Raft et al., 1977; Greenwald and Stitzer, 2000; Wallace et al., 2007; Lötsch et al., 2018; Mun et al., 2020). As such, these results support the translational validity of preclinical assays of pain-depressed behaviors.

Effects of MAGL >> FAAH–Selective Inhibitors. Previous studies have demonstrated that selective MAGL inhibition produces antinociception in assays of pain-stimulated behavior through increasing central 2-AG levels and enhancing endogenous eCB tone (Niphakis et al., 2013; Ignatowska-Jankowska et al., 2015; Donvito et al., 2018). Although other studies have shown antinociceptive effectiveness of MAGL-selective inhibitors in models of acute thermal pain and chronic inflammatory or neuropathic pain (Niphakis et al., 2013; Ignatowska-Jankowska et al., 2015; Burston et al., 2016; Wilkerson et al., 2018; Thompson et al., 2020), our study extended this literature by assessing their effectiveness using an acute visceral inflammatory stimulus. MJN110 potency to decrease IP acid–stimulated stretching was similar to its potency in inflammatory/neuropathic models and higher than its potency for acute thermal nociception. We further extended the literature by assessing IP acid–stimulated facial grimace and IP acid–depressed nesting and rearing. The potency of MJN110 was similar in assays of IP acid–stimulated grimace and stretching, but the effect on grimace was small and not dose-dependent. Although MJN110 did not alleviate IP acid–induced depression of rearing, it did alleviate IP acid–depressed nesting with a stable level of antinociceptive effectiveness across a 10-fold dose range. Doses that produced antinociception did not alter pain-independent behaviors in our study; however, MJN110 doses of 1.25 and 2.5 mg/kg increased locomotor speed and distance traveled in another study (Ignatowska-Jankowska et al., 2015). Accounting for possible differences in sensitivity between these two measures of

TABLE 1
Summary of power analysis results from pooled one-way ANOVA data from Figs. 1–5

Male and Female	F Statistic, *P* Value	Current Effect Size (Cohen's f)	Current Power	Sample Size: Power ≥ 0.8	Friedman Statistic; *P* Value
		△9-Tetrahydrocannabinol			
Stretch + acid	F(2.290, 25.19) = 11.83; $P = 0.0001$*	1.040	0.994	7	—[a]
Rear + acid	F(1.433, 15.77) = 0.362; $P = 0.6325$	0.753	0.94	9	—
Grimace	—	—	—	—	F = 13.09; $P = 0.0044$*
Nesting + acid	F(2.469, 27.16) = 2.083; $P = 0.1352$	0.435	0.431	25	—
Nesting	F(2.351, 25.86) = 45.27; $P < 0.0001$*	0.44	0.427	25	—
Locomotor	F(2.352, 25.87) = 11.64; $P = 0.0001$	1.028	0.994	7	—
		MJN110			
Stretch + acid	F(2.955, 32.51) = 6.231; $P = 0.0019$*	0.753	0.94	9	—
Rear + acid	F(2.045, 22.49) = 0.9844; $P = 0.3908$	0.299	0.201	56	—
Grimace	—	—	—	—	F = 10.65; $P = 0.0308$*
Nesting + acid	F(2.528, 27.80) = 6.5; $P = 0.0028$*	0.768	1	5	—
nesting	F(1.927, 21.19) = 2.789; $P = 0.0857$	0.503	0.407	27	—
Locomotor	F(1.453, 15.98) = 2.166; $P = 0.1556$	0.445	0.33	34	—
		JZL184			
Stretch + acid	F(3.580, 35.80) = 2.726; $P = 0.0496$	0.522	0.662	14	—
Rear + acid	F(1.992, 19.92) = 0.4745; $P = 0.6283$	0.217	0.116	>100	—
Grimace	—	—	—	—	F = 16.66; $P = 0.0023$*
Nesting + acid	F(2.777, 30.55) = 5.316; $P = 0.0054$*	0.695	0.88	11	—
Nesting	F(2.072, 22.79) = 0.6424; $P = 0.5406$	0.241	0.145	84	—
Locomotor	F(1.453, 15.98) = 2.166; $P = 0.1556$	0.445	0.33	34	—
		JZL195			
Stretch + acid	F(2.440, 26.84) = 5.007; $P = 0.0102$*	0.675	0.821	12	—
Rear + acid	F(2.413, 26.55) = 0.2673; $P = 0.8066$	0.157	0.092	>100	—
Grimace	—	—	—	—	F = 1.664; $P = 0.645$
Nesting + acid	F(2.252, 24.78) = 3.782; $P = 0.0325$*	0.587	0.668	16	—
Nesting	F(1.696, 18.65) = 1.157; $P = 0.3279$	0.299	0.184	64	—
Locomotor	F(1.516, 16.68) = 2.768; $P = 0.1024$	0.502	0.416	26	—
		SA57			
Stretch + acid	F(2.256, 24.82) = 4.219; $P = 0.0228$*	0.619	0.718	14	—
Rear + acid	F(2.278, 25.06) = 0.3463; $P = 0.7377$	0.179	0.103	>100	—
Grimace	—	—	—	—	F = 3.058; $P = 0.3828$
Nesting + acid	F(1.972, 21.7) = 4.391; $P = 0.0255$*	0.631	0.691	15	—
Nesting	F(1.109, 12.20) = 5.032; $P = 0.0413$*	0.677	0.565	19	—
Locomotor	F(1.516, 16.68) = 2.768; $P = 0.1024$	0.502	0.416	26	—
		URB597			
Stretch + acid	F(2.643, 29.07) = 1.256; $P = 0.306$	0.337	0.282	38	—
Rear + acid	F(1.346, 14.81) = 1.129; $P = 0.3258$	0.32	0.185	65	—
Grimace	—	—	—	—	F = 6.200; $P = 0.1023$
Nesting + acid	F(2.321, 23.21) = 4.102; $P = 0.0251$*	0.641	0.71	13	—
Nesting	F(1.631, 17.94) = 0.5785; $P = 0.5373$	0.229	0.125	>100	—
Locomotor	F(1.938, 21.31) = 3.418; $P = 0.0529$	0.557	0.57	19	—
		PF3845			
Stretch + acid	F(2.541, 27.95) = 0.5712; $P = 0.6111$	0.227	0.144	82	—
Rear + acid	F(2.051, 22.56) = 0.252; $P = 0.7847$	0.15	0.084	>100	—
Grimace	—	—	—	—	F = 0.0826; $P = 0.9938$
Nesting + acid	F(1.769, 19.46) = 2.045; $P = 0.160$	0.432	0.35	31	—
Nesting	F(2.350, 25.85) = 17.43; $P < 0.0001$*	1.259	0.999	5	—
Locomotor	F(1.938, 21.31) = 3.418; $P = 0.0529$	0.557	0.57	19	—

[a]Dashes indicate statistical analyses that were not conducted in accordance with parametric or nonparametric data.

locomotion, this hyperlocomotive effect is particularly intriguing, as it is diametrically opposed to typical $CB_{1/2}R$ agonist-induced hypolocomotion (Fig. 6) (Stark and Dews, 1980; Varvel, 2005; Wiebelhaus et al., 2015; Grim et al., 2017).

Further assessment for the antinociceptive time course of 1.0 mg/kg MJN110 in the assay of IP acid–depressed nesting agreed with previous studies that showed behavioral effects and increased 2-AG levels starting after ~1 hour and lasting several hours (Niphakis et al., 2013; Ignatowska-Jankowska et al., 2015). Although all reports agree with our results showing MJN110 antinociception is blocked by rimonabant to implicate CB_1R, the role of CB_2R is less clear (Ignatowska-Jankowska et al., 2015; Burston et al., 2016; Thompson et al., 2020). This may reflect differential recruitment of CB_2R across the noxious stimuli assessed in these studies, with neuropathic and chronic inflammatory manipulations being more likely to enhance CB_2R expression and signaling (Burston et al., 2013; Donvito et al., 2018). Our studies agree with previous evidence that CB_2R is not necessary for MJN110 antinociception in acute-pain models (Ignatowska-Jankowska et al., 2015). The role of sex as a determinant of MNJ110 effects is discussed below.

The other MAGL-selective inhibitor, JZL184, did not significantly alleviate IP acid–stimulated stretching (Long et al., 2009a; Sakin et al., 2015). Similar to MJN110, it weakly reduced facial grimace and alleviated IP acid–induced depression of nesting, but not rearing, at doses that did not alter pain-independent behaviors. The poor effectiveness of JZL184 on the present measures of pain-stimulated behavior contrasts with previous evidence for antinociception across multiple acute pain–stimulated endpoints (Long et al., 2009b,c; Schlosburg et al., 2010; Kamimura et al., 2018). Additionally, the failure of JZL184 to alter pain-independent behaviors contrasts with previous evidence that JZL184 doses of 20–40 mg/kg increase (Aliczki et al., 2013; Bedse et al., 2018) or decrease (Long et al., 2009b; Kinsey et al., 2011; Ignatowska-Jankowska et al., 2015; Wiebelhaus et al., 2015) other behaviors. These discrepancies may reflect two issues. First, these previous studies only included male subjects, and the role of sex as a determinant of drug effects is discussed below. Second, JZL184 was delivered subcutaneously as a suspension in this study. Methods for JZL184 preparation in previous reports are unclear, but communication with authors and published results show varying preparation techniques aimed at enhancing solubility, including preparation immediately prior to injection, heating JZL184 solutions, and use of different vehicles (Kinsey et al., 2009; Long et al., 2009b,c). These alterations alongside intraperitoneal administration may produce pharmacokinetic differences that result in higher effective doses and stronger antinociception than observed here. However, effectiveness of JZL184 to alleviate IP acid–induced nesting depression suggests that behaviorally active doses were tested.

Effects of Dual MAGL and FAAH Inhibitors. Previous evidence supports enhanced antinociceptive effects for dual inhibition of MAGL and FAAH in comparison with selective inhibition for either enzyme alone to alleviate acute thermal noxious stimuli (Long et al., 2009c). Our results did not support this for an acute visceral noxious stimulus because the dual MAGL/FAAH inhibitors JZL195 and SA57 were not more effective than the selective MAGL inhibitors to produce antinociception. Specifically, both JZL195 and SA57 alleviated IP acid–induced stimulation of stretching and depression of nesting at doses that did not alter pain-independent behaviors, but neither drug alleviated IP acid–induced grimace or depression of rearing. The potency of JZL195 to reduce IP acid effects on stretching and nesting in the present study (32 mg/kg s.c.) was similar to its potency to produce antinociception in an assay of warm-water tail withdrawal in mice (20 mg/kg i.p.) (Long et al., 2009c). Long et al. (2009c) also found that 20 mg/kg i.p. JZL195 decreased locomotor activity and produced catalepsy, and other groups have also shown decreased locomotor activity or Rotarod performance at similar or lower doses (Anderson et al., 2014; Adamson Barnes et al., 2016). Similarly, SA57 decreased IP acid–stimulated stretching in the present study at a similar potency (10 mg/kg s.c.) shown to produce acute thermal antinociception (12.5 mg/kg i.p.) (Wilkerson et al., 2017), but previous studies also found that intraperitoneal administration of similar SA57 doses produced many undesirable cannabimimetic effects, including increased immobility, catalepsy, and decreased rectal temperature (Wiebelhaus et al., 2015; Wilkerson et al., 2017). The more selective effects of JZL195 and SA57 on pain-related versus pain-independent behaviors in the present study may again reflect pharmacokinetic differences due to use of subcutaneous versus intraperitoneal administration.

Effects of MAGL << FAAH–Selective Enzyme Inhibitors. Our results do not support FAAH-selective inhibitors as candidate analgesics for acute visceral pain. URB597 produced antinociception on only one endpoint (IP acid–induced nesting depression), and PF3845 failed to produce antinociception on any endpoint up to a dose that depressed locomotion. The effectiveness of URB597 to alleviate IP acid–induced nesting suppression agrees with previous evidence for URB597 antinociception in other assays of pain-depressed behavior (Miller et al., 2012; Kwilasz et al., 2014); however, URB597 did not alleviate depression of rearing, and in contrast to previous studies (Naidu et al., 2009; Miller et al., 2012; Kwilasz et al., 2014), URB597 was not effective to alleviate IP acid–stimulated behaviors. There is a similar discrepancy in the literature on URB597 antinociception in assays of acute thermal pain–stimulated behaviors (Kathuria et al., 2003; Miller et al., 2012). PF3845 was the most FAAH-selective inhibitor in this study, and it produced the least favorable profile of effects. Previous studies have reported small but significant antinociceptive effectiveness of PF3845 on some acute pain–stimulated behaviors (Schlosburg et al., 2009; Grim et al., 2014; Ghosh et al., 2015), but such effects were not detected here. Moreover, the failure of PF3845 to alleviate behavioral depression produced by IP acid in the present study agrees with failure of PF3845 to alleviate depression of marble burying in mice by a chronic constriction injury to the sciatic nerve (Wilkerson et al., 2018). The decrease in IP acid–independent nesting supports previous studies that have shown a similar potency to decrease marble burying and intracranial self-stimulation in mice (Kinsey et al., 2011; Wiebelhaus et al., 2015); however, these same doses were shown to have no effect on immobility time, operant food responding, or distance traveled (Kinsey et al., 2011; Ghosh et al., 2015; Wiebelhaus et al., 2015).

Sex as a Determinant of Drug Effects. The present study was not powered to detect sex differences, but both females and males were included to conduct a secondary analysis using a strategy to provide preliminary insights on the role of sex as a determinant of drug effects (Diester et al., 2019). In general, sex-dependent antinociception was rare,

and there were no sex × dose interactions for any drug on any endpoint. However, MJN110 consistently produced higher antinociceptive effects in females than males in the assay of IP acid–induced nesting depression, whereas JZL184 produced a larger effect in males across three of the six endpoints. This discrepancy may be due in part to tissue-dependent differential activity of MJN110 and JZL184 with serine hydrolases other than MAGL, such as ABHD6 and C16:0 and C18:1 monoaclyglycerolss (Long et al., 2009b; Niphakis et al., 2013). Further studies will be required to examine expression and mechanisms of sex differences in effects of eCB catabolic enzyme inhibitors.

Conclusions

There were three main findings in this study. First, THC significantly attenuated IP acid–stimulated stretching and facial grimace at doses that did not produce general motor disruption, but THC did not alleviate IP acid–induced depression of either rearing or nesting. Second, for the eCB catabolic enzyme inhibitors, the MAGL-selective inhibitor MJN110 produced antinociception without motor disruption on three of four endpoints, including significant alleviation of IP acid–induced depression of nesting, whereas the FAAH-selective inhibitor PF3845 failed to produce antinociception on any endpoint up to a dose that did produce motor disruption. Other eCB catabolic enzyme inhibitors produced effects between these extremes. Lastly, time course and antagonism studies for MJN110 in the assay of IP acid–induced depression of nesting indicated a long duration of antinociceptive action (40 minutes to 6 hours) and mediation by CB_1R but not CB_2R. Overall, these results support further consideration of MAGL-selective inhibitors, especially MJN110, as candidate analgesics.

Authorship Contributions

Participated in research design: Diester, Lichtman, Negus.

Conducted experiments: Diester.

Performed data analysis: Diester.

Wrote or contributed to the writing of the manuscript: Diester, Lichtman, Negus.

References

Adamson Barnes NS, Mitchell VA, Kazantzis NP, and Vaughan CW (2016) Actions of the dual FAAH/MAGL inhibitor JZL195 in a murine neuropathic pain model. *Br J Pharmacol* **173**:77–87.

Ahn K, et al. (2009) Discovery and characterization of a highly selective FAAH inhibitor that reduces inflammatory pain. *Chem Biol* **16** (4):411–420 PMC2692831.

Aliczki M, Zelena D, Mikics E, Varga ZK, Pinter O, Bakos NV, Varga J, and Haller J (2013) Monoacylglycerol lipase inhibition-induced changes in plasma corticosterone levels, anxiety and locomotor activity in male CD1 mice. *Horm Behav* **63**: 752–758.

Anderson WB, Gould MJ, Torres RD, Mitchell VA, and Vaughan CW (2014) Actions of the dual FAAH/MAGL inhibitor JZL195 in a murine inflammatory pain model. *Neuropharmacology* **81**:224–230.

Bedse G, Bluett RJ, Patrick TA, Romness NK, Gaulden AD, Kingsley PJ, Plath N, Marnett LJ, and Patel S (2018) Therapeutic endocannabinoid augmentation for mood and anxiety disorders: comparative profiling of FAAH, MAGL and dual inhibitors. *Transl Psychiatry* **8**:92.

Blanton HL, Barnes RC, McHann MC, Bilbrey JA, Wilkerson JL, and Guindon J (2021) Sex differences and the endocannabinoid system in pain. *Pharmacol Biochem Behav* **202**:173107.

Booker L, Kinsey SG, Abdullah RA, Blankman JL, Long JZ, Ezzili C, Boger DL, Cravatt BF, and Lichtman AH (2012) The fatty acid amide hydrolase (FAAH) inhibitor PF-3845 acts in the nervous system to reverse LPS-induced tactile allodynia in mice. *Br J Pharmacol* **165**:2485–2496.

Booker L, Naidu PS, Razdan RK, Mahadevan A, and Lichtman AH (2009) Evaluation of prevalent phytocannabinoids in the acetic acid model of visceral nociception. *Drug Alcohol Depend* **105**:42–47.

Britch SC, Wiley JL, Yu Z, Clowers BH, and Craft RM (2017) Cannabidiol-Δ^9-tetrahydrocannabinol interactions on acute pain and locomotor activity. *Drug Alcohol Depend* **175**:187–197.

Burston JJ, Mapp PI, Sarmad S, Barrett DA, Niphakis MJ, Cravatt BF, Walsh DA, and Chapman V (2016) Robust anti-nociceptive effects of monoacylglycerol lipase inhibition in a model of osteoarthritis pain. *Br J Pharmacol* **173**:3134–3144.

Burston JJ, Sagar DR, Shao P, Bai M, King E, Brailsford L, Turner JM, Hathway GJ, Bennett AJ, Walsh DA, et al. (2013) Cannabinoid CB2 receptors regulate central sensitization and pain responses associated with osteoarthritis of the knee joint. *PLoS One* **8**:e80440.

Cobos EJ and Portillo-Salido E (2013) "Bedside-to-Bench" behavioral outcomes in animal models of pain: beyond the evaluation of reflexes. *Curr Neuropharmacol* **11**: 560–591.

Cooper ZD and Craft RM (2018) Sex-dependent effects of cannabis and cannabinoids: a translational perspective. *Neuropsychopharmacology* **43**:34–51.

Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, and Lichtman AH (2001) Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci USA* **98**:9371–9376.

Dalmann R, Daulhac L, Antri M, Eschalier A, and Mallet C (2015) Supra-spinal FAAH is required for the analgesic action of paracetamol in an inflammatory context. *Neuropharmacology* **91**:63–70.

Diester CM, Banks ML, Neigh GN, and Negus SS (2019) Experimental design and analysis for consideration of sex as a biological variable. *Neuropsychopharmacology* **44**:2159–2162.

Diester CM, Santos EJ, Moerke MJ, and Negus SS (2021) Behavioral battery for testing candidate analgesics in mice. I. Validation with positive and negative controls. *J Pharmacol Exp Ther* **377**(2):232–241.

Donvito G, Nass SR, Wilkerson JL, Curry ZA, Schurman LD, Kinsey SG, and Lichtman AH (2018) The endogenous cannabinoid system: a budding source of targets for treating inflammatory and neuropathic pain. *Neuropsychopharmacology* **43**:52–79.

Dworkin RH, Turk DC, Farrar JT, Haythornthwaite JA, Jensen MP, Katz NP, Kerns RD, Stucki G, Allen RR, Bellamy N, et al.; IMMPACT (2005) Core outcome measures for chronic pain clinical trials: IMMPACT recommendations. *Pain* **113**: 9–19.

Faul F, Erdfelder E, Lang A-G, and Buchner A (2007) G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods* **39**:175–191.

Fowler CJ (2012) Monoacylglycerol lipase - a target for drug development? *Br J Pharmacol* **166**:1568–1585.

Ghosh S, Kinsey SG, Liu Q-S, Hruba L, McMahon LR, Grim TW, Merritt CR, Wise LE, Abdullah RA, Selley DE, et al. (2015) Full fatty acid amide hydrolase inhibition combined with partial monoacylglycerol lipase inhibition: augmented and sustained antinociceptive effects with reduced cannabimimetic side effects in mice. *J Pharmacol Exp Ther* **354**:111–120.

Greenwald MK and Stitzer ML (2000) Antinociceptive, subjective and behavioral effects of smoked marijuana in humans. *Drug Alcohol Depend* **59**:261–275.

Grim TW, Ghosh S, Hsu KL, Cravatt BF, Kinsey SG, and Lichtman AH (2014) Combined inhibition of FAAH and COX produces enhanced anti-allodynic effects in mouse neuropathic and inflammatory pain models. *Pharmacol Biochem Behav* **124**:405–411.

Grim TW, Morales AJ, Gonek MM, Wiley JL, Thomas BF, Endres GW, Sim-Selley LJ, Selley DE, Negus SS, and Lichtman AH (2016) Stratification of cannabinoid 1 receptor (CB1R) agonist efficacy: manipulation of CB1R density through use of transgenic mice reveals congruence between in vivo and in vitro assays. *J Pharmacol Exp Ther* **359**:329–339.

Grim TW, Morales AJ, Thomas BF, Wiley JL, Endres GW, Negus SS, and Lichtman AH (2017) Apparent CB_1 receptor rimonabant affinity estimates: combination with THC and synthetic cannabinoids in the mouse in vivo triad model. *J Pharmacol Exp Ther* **362**:210–218.

Habib AM, Okorokov AL, Hill MN, Bras JT, Lee M-C, Li S, Gossage SJ, van Drimmelen M, Morena M, Houlden H, et al. (2019) Microdeletion in a FAAH pseudogene identified in a patient with high anandamide concentrations and pain insensitivity. *Br J Anaesth* **123**:e249–e253.

Holzer P (2009) Acid-Sensitive Ion Channels and Receptors, in *Sensory Nerves. Handbook of Experimental Pharmacology* (Canning B and Spina D eds) **194**, Springer, Berlin, Heidelberg.

Hruba L, Seillier A, Zaki A, Cravatt BF, Lichtman AH, Giuffrida A, and McMahon LR (2015) Simultaneous inhibition of fatty acid amide hydrolase and monoacylglycerol lipase shares discriminative stimulus effects with Δ9-tetrahydrocannabinol in mice. *J Pharmacol Exp Ther* **353**:261–268.

Huggins JP, Smart TS, Langman S, Taylor L, and Young T (2012) An efficient randomised, placebo-controlled clinical trial with the irreversible fatty acid amide hydrolase-1 inhibitor PF-04457845, which modulates endocannabinoids but fails to induce effective analgesia in patients with pain due to osteoarthritis of the knee. *Pain* **153**:1837–1846.

Ignatowska-Jankowska B, Wilkerson JL, Mustafa M, Abdullah R, Niphakis M, Wiley JL, Cravatt BF, and Lichtman AH (2015) Selective monoacylglycerol lipase inhibitors: antinociceptive versus cannabimimetic effects in mice. *J Pharmacol Exp Ther* **353**:424–432.

Institute of Medicine (US) Committee on Advancing Pain Research, Care, and Education (2011) *Relieving Pain in America: A Blueprint for Transforming Preveition, Care, Education, and Research*, The National Academics Press, Washington, D.C.

Kamimura R, Hossain MZ, Unno S, Ando H, Masuda Y, Takahashi K, Otake M, Saito I, and Kitagawa J (2018) Inhibition of 2-arachydonoylgycerol degradation attenuates orofacial neuropathic pain in trigeminal nerve-injured mice. *J Oral Sci* **60**: 37–44.

Kathuria S, Gaetani S, Fegley D, Valiño F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, et al. (2003) Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med* **9**:76–81.

Kinsey SG, Long JZ, O'Neal ST, Abdullah RA, Poklis JL, Boger DL, Cravatt BF, and Lichtman AH (2009) Blockade of endocannabinoid-degrading enzymes attenuates neuropathic pain. *J Pharmacol Exp Ther* **330**:902–910.
Kinsey SG, O'Neal ST, Long JZ, Cravatt BF, and Lichtman AH (2011) Inhibition of endocannabinoid catabolic enzymes elicits anxiolytic-like effects in the marble burying assay. *Pharmacol Biochem Behav* **98**:21–27.
Koster R, Anderson M, and De Beer EJ (1959) Acetic Acid for Analgesic Screening. *Federation Proceedings* **18**:412–417.
Kraft B, Frickey NA, Kaufmann RM, Reif M, Frey R, Gustorff B, and Kress HG (2008) Lack of analgesia by oral standardized cannabis extract on acute inflammatory pain and hyperalgesia in volunteers. *Anesthesiology* **109**:101–110.
Kwilasz AJ, Abdullah RA, Poklis JL, Lichtman AH, and Negus SS (2014) Effects of the fatty acid amide hydrolase inhibitor URB597 on pain-stimulated and pain-depressed behavior in rats. *Behav Pharmacol* **25**:119–129.
Kwilasz AJ and Negus SS (2012) Dissociable effects of the cannabinoid receptor agonists 9-tetrahydrocannabinol and CP55940 on pain-stimulated versus pain-depressed behavior in rats. *J Pharmacol Exp Ther* **343**:389–400.
Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, Lacroix-Fralish ML, et al. (2010) Coding of facial expressions of pain in the laboratory mouse. *Nat Methods* **7**:447–449.
Lehmann EL (2006) *Nonparametrics: Statistical Methods Based on Ranks*, Springer, New York.
Leitl MD and Negus SS (2016) Pharmacological modulation of neuropathic pain-related depression of behavior: effects of morphine, ketoprofen, bupropion and [INCREMENT]9-tetrahydrocannabinol on formalin-induced depression of intracranial self-stimulation in rats. *Behav Pharmacol* **27**:364–376.
Long JZ, Li W, Booker L, Burston JJ, Kinsey SG, Schlosburg JE, Pavón FJ, Serrano AM, Selley DE, Parsons LH, et al. (2009a) Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. *Nat Chem Biol* **5**:37–44.
Long JZ, Nomura DK, and Cravatt BF (2009b) Characterization of monoacylglycerol lipase inhibition reveals differences in central and peripheral endocannabinoid metabolism. *Chem Biol* **16**:744–753.
Long JZ, Nomura DK, Vann RE, Walentiny DM, Booker L, Jin X, Burston JJ, Sim-Selley LJ, Lichtman AH, Wiley JL, et al. (2009c) Dual blockade of FAAH and MAGL identifies behavioral processes regulated by endocannabinoid crosstalk in vivo. *Proc Natl Acad Sci USA* **106**:20270–20275.
Lötsch J, Weyer-Menkhoff I, and Tegeder I (2018) Current evidence of cannabinoid-based analgesia obtained in preclinical and human experimental settings. *Eur J Pain* **22**:471–484.
Lu H-C and Mackie K (2016) An introduction to the endogenous cannabinoid system. *Biol Psychiatry* **79**:516–525.
Miller LL, Picker MJ, Umberger MD, Schmidt KT, and Dykstra LA (2012) Effects of alterations in cannabinoid signaling, alone and in combination with morphine, on pain-elicited and pain-suppressed behavior in mice. *J Pharmacol Exp Ther* **342**: 177–187.
Mogil JS (2009) Animal models of pain: progress and challenges. *Nat Rev Neurosci* **10**:283–294.
Mun CJ, Letzen JE, Peters EN, Campbell CM, Vandrey R, Gajewski-Nemes J, DiRenzo D, Caufield-Noll C, and Finan PH (2020) Cannabinoid effects on responses to quantitative sensory testing among individuals with and without clinical pain: a systematic review. *Pain* **161**:244–260.
Naidu PS, Booker L, Cravatt BF, and Lichtman AH (2009) Synergy between enzyme inhibitors of fatty acid amide hydrolase and cyclooxygenase in visceral nociception. *J Pharmacol Exp Ther* **329**:48–56.
Naidu PS, Kinsey SG, Guo TL, Cravatt BF, and Lichtman AH (2010) Regulation of inflammatory pain by inhibition of fatty acid amide hydrolase. *J Pharmacol Exp Ther* **334**:182–190.
Negus SS (2019) Core outcome measures in preclinical assessment of candidate analgesics. *Pharmacol Rev* **71**:225–266.
National Research Council (US) Institute for Laboratory Animal Research (1996) *Guide for the Care and Use of Laboratory Animals*, National Academy of Sciences, Washington, D.C.
Negus SS, Neddenriep B, Altarifi AA, Carroll FI, Leitl MD, and Miller LL (2015) Effects of ketoprofen, morphine, and kappa opioids on pain-related depression of nesting in mice. *Pain* **156**:1153–1160.
Niphakis MJ, Cognetta AB III, Chang JW, Buczynski MW, Parsons LH, Byrne F, Burston JJ, Chapman V, and Cravatt BF (2013) Evaluation of NHS carbamates as a potent and selective class of endocannabinoid hydrolase inhibitors. *ACS Chem Neurosci* **4**:1322–1332.
Niphakis MJ, Johnson DS, Ballard TE, Stiff C, and Cravatt BF (2012) O-hydroxyacetamide carbamates as a highly potent and selective class of endocannabinoid hydrolase inhibitors. *ACS Chem Neurosci* **3**:418–426.
Nomura DK, Morrison BE, Blankman JL, Long JZ, Kinsey SG, Marcondes MCG, Ward AM, Hahn YK, Lichtman AH, Conti B, et al. (2011) Endocannabinoid hydrolysis generates brain prostaglandins that promote neuroinflammation. *Science* **334**:809–813.
Owens RA, Ignatowska-Jankowska B, Mustafa M, Beardsley PM, Wiley JL, Jali A, Selley DE, Niphakis MJ, Cravatt BF, and Lichtman AH (2016) Discriminative stimulus properties of the endocannabinoid catabolic enzyme inhibitor SA-57 in mice. *J Pharmacol Exp Ther* **358**:306–314.
Raft D, Gregg J, Ghia J, and Harris L (1977) Effects of intravenous tetrahydrocannabinol on experimental and surgical pain. Psychological correlates of the analgesic response. *Clin Pharmacol Ther* **21**:26–33.
Sakin YS, Dogrul A, Ilkaya F, Seyrek M, Ulas UH, Gulsen M, and Bagci S (2015) The effect of FAAH, MAGL, and Dual FAAH/MAGL inhibition on inflammatory and colorectal distension-induced visceral pain models in Rodents. *Neurogastroenterol Motil* **27**:936–944.
Schlosburg JE, Blankman JL, Long JZ, Nomura DK, Pan B, Kinsey SG, Nguyen PT, Ramesh D, Booker L, Burston JJ, et al. (2010) Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nat Neurosci* **13**:1113–1119.
Schlosburg JE, Kinsey SG, and Lichtman AH (2009) Targeting fatty acid amide hydrolase (FAAH) to treat pain and inflammation. *AAPS J* **11**:39–44.
Sofia RD, Vassar HB, and Knobloch LC (1975) Comparative analgesic activity of various naturally occurring cannabinoids in mice and rats. *Psychopharmacology (Berl)* **40**:285–295.
St Sauver JL, Warner DO, Yawn BP, Jacobson DJ, McGree ME, Pankratz JJ, Melton LJ III, Roger VL, Ebbert JO, and Rocca WA (2013) Why patients visit their doctors: assessing the most prevalent conditions in a defined American population. *Mayo Clin Proc* **88**:56–67.
Stark P and Dews PB (1980) Cannabinoids. I. Behavioral effects. *J Pharmacol Exp Ther* **214**:124–130.
Thompson AL, Grenald SA, Ciccone HA, BassiriRad N, Niphakis MJ, Cravatt BF, Largent-Milnes TM, and Vanderah TW (2020) The endocannabinoid system Alleviates pain in a murine model of cancer-induced bone pain. *J Pharmacol Exp Ther* **373**:230–238.
U.S. Department of Health and Human Services (2019) *Pain Management Best Practices Inter-Agency Task Force Report: Updates, Gaps, Inconsistencies, and Recommendations*, U.S. Department of Health and Human Services, Washington, D. C.
Varvel SA, Bridgen DT, Tao Q, Thomas BF, Martin BR, and Lichtman AH (2005) Δ9-tetrahydrocannbinol accounts for the antinociceptive, hypothermic, and cataleptic effects of marijuana in mice. *J Pharmacol Exp Ther* **314**:329–337.
von Schaper E (2016) Bial incident raises FAAH suspicions. *Nat Biotechnol* **34**:223.
Wallace M, Schulteis G, Atkinson JH, Wolfson T, Lazzaretto D, Bentley H, Gouaux B, and Abramson I (2007) Dose-dependent effects of smoked cannabis on capsaicin-induced pain and hyperalgesia in healthy volunteers. *Anesthesiology* **107**:785–796.
Wiebelhaus JM, Grim TW, Owens RA, Lazenka MF, Sim-Selley LJ, Abdullah RA, Niphakis MJ, Vann RE, Cravatt BF, Wiley JL, et al. (2015) Δ9-tetrahydrocannabinol and endocannabinoid degradative enzyme inhibitors attenuate intracranial self-stimulation in mice. *J Pharmacol Exp Ther* **352**:195–207.
Wilkerson JL, Curry ZA, Kinlow PD, Mason BL, Hsu K-L, van der Stelt M, Cravatt BF, and Lichtman AH (2018) Evaluation of different drug classes on transient sciatic nerve injury-depressed marble burying in mice. *Pain* **159**: 1155–1165.
Wilkerson JL, Ghosh S, Mustafa M, Abdullah RA, Niphakis MJ, Cabrera R, Maldonado RL, Cravatt BF, and Lichtman AH (2017) The endocannabinoid hydrolysis inhibitor SA-57: intrinsic antinociceptive effects, augmented morphine-induced antinociception, and attenuated heroin seeking behavior in mice. *Neuropharmacology* **114**:156–167.
Yezierski RP and Hansson P (2018) Inflammatory and neuropathic pain from bench to bedside: what went wrong? *J Pain* **19**:571–588.

Address correspondence to: S. S. Negus, Department of Pharmacology and Toxicology, Virginia Commonwealth University, 410 N. 12th St., Richmond, VA. E-mail: sidney.negus@vcuhealth.org