Effects of Repeated Treatment with the Monoacylglycerol Lipase Inhibitor MJN110 on Pain-Related Depression of Nesting and Cannabinoid 1 Receptor Function in Male and Female Mice

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Repeated MJN110 effects on pain-depressed nesting

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ABSTRACT
MJN110 inhibits the enzyme monoacylglycerol lipase (MAGL) to increase levels of the endocannabinoid (eCB) 2-arachidonoylglycerol (2-AG), an endogenous high-efficacy agonist of cannabinoid 1 and 2 receptors (CB1/2R). MAGL inhibitors are under consideration as candidate analgesics, and we reported previously that acute MJN110 produced partial antinociception in an assay of pain-related behavioral depression in mice. Given the need for repeated analgesic administration in many pain patients and the potential for analgesic tolerance during repeated treatment, this study examined antinociceptive effects of repeated MJN110 on pain-related behavioral depression and CB1R-mediated G-protein function. Male and female ICR mice were treated daily for 7 days in a 2x2 design with (a) 1.0 mg/kg/day MJN110 or its vehicle followed by (b) intraperitoneal injection of dilute lactic acid (IP acid) or its vehicle as a visceral noxious stimulus to depress nesting behavior. After behavioral testing, G-protein activity was assessed in lumbar spinal cord and five brain regions using an assay of CP55,940-stimulated [35S]GTPγS activation. As reported previously, acute MJN110 produced partial but significant relief of IP acid-induced nesting depression on Day 1. After 7 days, MJN110 continued to produce significant but partial antinociception in males, while antinociceptive tolerance developed in females. Repeated MJN110 also produced modest decreases in maximum levels of CP55,940-induced [35S]GTPγS binding in spinal cord and most brain regions. These results indicate that repeated treatment with a relatively low antinociceptive MJN110 dose produces only partial and sex-dependent transient antinociception associated with the emergence of CB1R desensitization in this model of IP acid-induced nesting depression.

Significance Statement
The drug MJN110 inhibits monoacylglycerol lipase (MAGL) to increase levels of the endogenous cannabinoid 2-arachidonoylglycerol and produce potentially useful therapeutic effects including analgesia. This study used an assay of pain-related behavioral depression in mice to show that repeated MJN110 treatment produced (1) weak but sustained antinociception in male mice, (2) antinociceptive tolerance in females, and (3) modest cannabinoid-receptor desensitization that varied by region and sex. Antinociceptive tolerance may limit the utility of MJN110 for treatment of pain.
INTRODUCTION

Although ~20% of adults in the United States report suffering from chronic pain, very few analgesics provide adequate analgesia without deleterious side effects, particularly following repeated administration (Nahin et al., 2023). A goal in analgesic development has been to develop safer candidate analgesics that can alleviate persistent pain, with one class of recently developed candidate analgesics targeting the endocannabinoid (eCB) system (Volkow and Collins, 2017; Donvito et al., 2018). The eCB 2-arachidonoyl glycerol (2-AG) functions as a high-affinity and high-efficacy agonist at cannabinoid 1 and 2 receptors (CB$_{1/2}$Rs) (Stella et al., 1997). In neurons, 2-AG is synthesized post-synaptically in an activity-dependent manner, produces retrograde activation of presynaptic $G_i$-protein-coupled CB$_{1/2}$Rs, and is rapidly metabolized by the enzyme monoacylglycerol lipase (MAGL) (Lu and Mackie, 2016; Donvito et al., 2018). MAGL inhibitors increase 2-AG levels and produce a subset of CB$_{1/2}$R-mediated physiological and behavioral effects similar to those of exogenous CB$_{1/2}$R agonists like $\Delta$9-tetrahydrocannabinol (THC) and CP55,940 (Niphakis et al., 2013; Ignatowska-Jankowska et al., 2015). While direct global activation of CB$_{1/2}$Rs via agonists like THC have shown poor clinical efficacy (Raft et al., 1977; Greenwald and Stitzer, 2000; Wallace et al., 2007; Mun et al., 2020), the potential of harnessing the on-demand feedback-inhibition produced by enhanced eCB-system signaling has led to development of a wide range of MAGL inhibitors. Among these, 2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate (MJN110) has notably high potency and selectivity and produces a dose-dependent and selective increase in brain 2-AG levels along with a subset of cannabimimetic effects (Long et al., 2009; Niphakis et al., 2013; Ignatowska-Jankowska et al., 2015; Owens et al., 2017). However, in contrast to exogenous CB$_{1/2}$R agonists like THC, MJN110 did not produce hypoactivity, hypothermia, or catalepsy (Ignatowska-Jankowska et al., 2015). These results suggest that MJN110 and other MAGL inhibitors may produce a subset of therapeutically useful CB$_{1/2}$R-mediated behavioral effects with minimal cannabimimetic side effects.

Several preclinical studies in rodents have evaluated effectiveness of MAGL inhibitors as candidate analgesics (Donvito et al., 2018). Selective MAGL inhibitors produced dose-dependent and rimonabant-reversible antinociception against a wide range of preclinical pain manipulations that include noxious heat, intraperitoneal acid injection, intraplantar formalin injection, and rodent models of osteoarthritis, cancer-induced bone pain, and neuropathic pain (Long et al., 2009; Schlosburg et al., 2010; Guindon et al., 2011; Niphakis et al., 2013; Ignatowska-Jankowska et al., 2015; Burston et al., 2016; Wilkerson et al., 2016; Curry et al., 2018; Thompson et al., 2020; Diester et al., 2021a). Analgesic tolerance is a potential
complication in pharmacological pain management, particularly in cases of episodic or chronic pain where the analgesic is administered repeatedly. We reported that repeated treatment with high doses of JZL184 in mice produced sustained increases in 2-AG levels, complete tolerance to JZL184 antinociception, and cross-tolerance to antinociceptive effects of exogenous agonists in assays of acute thermal nociception and a chronic-constriction-injury (CCI) model of neuropathy (Schlosburg et al., 2010). However, this tolerance could be avoided by using a lower JZL184 dose that produced either sustained antinociception in the CCI model (Kinsey et al., 2013) or increasing levels of antinociception in a model of chemotherapy-induced neuropathy (Curry et al., 2018). Similarly, repeated treatment in rats with a high dose of 5 mg/kg/day MJN110 produced antinociceptive tolerance in an osteoarthritis model, but antinociception was sustained for seven days when a lower dose of 1.0 mg/kg/day was used (Burston et al., 2016).

Interestingly, in a bone-cancer model, both low and high MJN110 doses produced sustained antinociception (Thompson et al., 2020).

Most studies above used preclinical assays of “pain-stimulated behavior,” in which a behavioral endpoint such as paw or tail withdrawal increased in rate, frequency, or intensity after presentation of a noxious stimulus; however, clinically relevant pain is commonly associated with behavioral depression and functional impairment. We and others have developed preclinical procedures to assess the expression and pharmacological treatment of pain-related behavioral depression (Martin et al., 2004; Negus, 2013; Wilkerson et al., 2018; Negus, 2019; Zhang et al., 2021). For example, we recently reported that intraperitoneal administration of dilute lactic acid (IP acid) served as an acute visceral noxious stimulus to depress nesting behavior in mice, and this pain-related nesting depression could be significantly but only partially alleviated by MJN110 doses of 0.32-3.2 mg/kg (Diester et al., 2021a; Diester et al., 2021b). Daily IP acid injection produces repeated episodes of behavioral depression and permits assessment of effects produced by repeated drug treatments, and we have shown in rats that morphine-induced alleviation of pain-depressed behavior is resistant to tolerance while effects of some antidepressants increase with repeated administration (Miller et al., 2015; Legakis et al., 2020). The present study used this approach to evaluate antinociception produced by repeated 1.0 mg/kg MJN110 treatment in mice treated with repeated IP acid.

To complement behavioral studies, spinal cord and brain tissues were collected at the end of the study and assessed for CB1R-mediated [35S]GTPγS binding (Schlosburg et al., 2010). Regions examined included the lumbar spinal cord, which receives nociceptor input from the peritoneal cavity targeted by the acid noxious stimulus, and the periaqueductal gray, which is a canonical region involved in ascending and descending nociception (Heinricher and Fields,
Pain-depressed behavior has been associated with dysregulation of the mesolimbic dopamine system (Leitl et al., 2014; Serafini et al., 2020). Accordingly, CB₁R-mediated G-protein activity was also examined in the ventral tegmental area, nucleus accumbens, and amygdala (Herkenham et al., 1991; Lazenka et al., 2014a). Lastly, hippocampus was included because it was highly sensitive to downregulation of CB₁R activity by repeated administration of THC or MAGL inhibitors in male rodents (Breivogel et al., 1999; Sim-Selley et al., 2006; McKinney et al., 2008; Schlosburg et al., 2010; Heinricher and Fields, 2013; Todd and Koerber, 2013). Overall, we hypothesized that repeated 1.0 mg/kg/day MJN110 would produce sustained or increasing antinociception with minimal effects on CB₁R function.

MATERIALS AND METHODS

Subjects

Male and female ICR mice (Envigo Laboratories, Indianapolis, IN) were 6-8 weeks old upon arrival, individually housed, and acclimated for at least one week before beginning studies. Mice had ad libitum access to food (Teklad LM-485 Mouse/Rat Diet, Harlan Laboratories) and water in cages (31.75 x 23.50 cm² floor x 15.25 cm high) mounted in a RAIR HD Ventilated Rack (Lab Products, Seaford, DE) with corncob bedding (Harlan Laboratories) and a “nestlet” composed of pressed cotton (Ancare, Bellmore, NY). The temperature-controlled housing room was located in an AAALAC-approved facility and maintained on a 12-hour light/dark cycle (lights on from 7:00 AM to 7:00 PM). All testing occurred during the light phase. 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.

Behavioral Procedures

Studies were conducted in two cohorts of 48 mice tested five months apart (24 female and 24 male mice in each cohort; 96 mice total). Behavioral and pharmacological procedures were identical between the two cohorts as described below, and all mice met an inclusion criterion of building a nest within 24 hr of arrival to the laboratory. After 1 week of acclimation, testing occurred over a 7-day period as shown in Figure 1, and mice were randomly allocated into the following four treatment groups (N=6/sex/treatment/cohort) to receive daily treatment with subcutaneous (SC) injection of 1.0 mg/kg MJN110 or its vehicle (Veh) followed 2 hr later by
IP injection of 0.32% lactic acid or its vehicle (H₂O). The routes of administration, doses, and pretreatment times for MJN110 and IP acid were based on previous studies (Niphakis et al., 2013; Ignatowska-Jankowska et al., 2015; Diester et al., 2021a). In particular, the 1.0 mg/kg/day MJN110 dose was selected based on our previous finding that the antinociception dose-effect curve against 0.32% IP acid-induced nesting depression plateaued at approximated 50% of the maximal possible effect across a dose range of 0.32-3.2 mg/kg, with 1.0 mg/kg/day falling in the middle of the plateau range (Diester et al, 2021a). IP acid injections alternated between the subject’s left and right sides across the —day study. Immediately after the IP injection, old nesting material was removed from the subject’s home cage, two 1-in² nestlet squares were placed 11 inches apart in the center of the opposing short walls of the cage, and the mouse was returned to its home cage for a 90-min nesting session as described previously (Diester et al., 2021b). At the conclusion of the session, 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. Studies were conducted at the same time each day in each mouse (starting at 10:00 AM) to minimize potential environmental or light-cycle confounds. Additionally, mice were weighed prior to injections on each test day. At 24-28h after the last nesting session, mice were euthanized by rapid decapitation, and tissue was collected for studies of cannabinoid agonist-stimulated GTPyS binding.

[^S]GTPyS binding assay.

The following brain regions were collected from each subject as previously described (Lazenka et al., 2014b; Lazenka et al., 2015; Selley et al., 2020): nucleus accumbens (NAc), amygdala, ventral tegmental area (VTA), hippocampus, and periaqueductual gray (PAG). The spinal cord was collected by hydraulic extrusion. First, anterior and posterior borders were marked by Decapitation and hip bone cuts, respectively. A ddH₂O-filled 40 ml syringe fitted with a 200 μl pipette tip was then inserted into the posterior spinal column to gently expel the spinal cord tissue, and the lumbar enlargement was collected (lumbar spinal cord; LSC). All tissue was stored at -80°C. For membrane preparation, samples were homogenized in membrane buffer (Tris-HCl, pH 7.4, 3 mM MgCl₂, 1 mM EGTA) and centrifuged at 40,000 x g for 10 min. The pellet was then homogenized in assay buffer (Tris-HCl, pH 7.4, 3 mM MgCl₂, 0.1 mM EGTA, 100 mM NaCl), and protein was determined using the Bradford method. Membranes (4-6 μg protein) were pretreated with adenosine deaminase for 15 min at 30°C prior to assay, followed by incubation with varying concentrations of CP55,940, 30 μM GDP, and 0.1 nM[^S]GTPyS in assay buffer containing 0.1% BSA for 2 hr at 30°C in a 0.5 ml total volume.
CP55,940 was chosen as the agonist because it has high efficacy and does not stimulate \[^{35}\text{S}]\text{GTPyS}\) binding in any brain region of \(\text{CB}_1\text{R}\) knockout mice (Nguyen et al., 2010), indicating that it produces a selective \(\text{CB}_1\text{R}\) -mediated response in this assay.

Basal binding was determined in the absence of agonist, and nonspecific binding was measured using 20 \(\mu\text{M}\) unlabeled GTPyS. The assay was terminated by filtration through GF/B glass fiber filters, followed by 3 washes with ice-cold Tris buffer. Bound radioactivity was determined by liquid scintillation spectrophotometry. The binding assay and behavioral experiments were conducted in non-blinded conditions.

**Data Analysis**

_Nesting Data._ The primary dependent variable was \% Maximal Nestlet Consolidation on each test day in each mouse, 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 observation period, respectively. This nesting measure was treated as a ratio variable and analyzed by parametric statistics. Because results from the two cohorts were similar (see Supplemental Figure 1 and Supplemental Table 4), all data from the two cohorts were combined to yield 24 mice (12 female, 12 male) for each of the four treatment groups. Data analysis proceeded in a series of steps similar to our previously described method for assessment of sex as a biological variable in studies that include both sexes but are not intended _a priori_ to be powered for detection of sex differences (Diester et al., 2019), with expansion to include the factor of time (days). This approach complies with the National Institutes of Health mandate to include both sexes in preclinical research and to include analyses that segregate results by sex (Miller et al., 2017; Tannenbaum et al., 2019; Galea et al., 2020). All analyses described below were conducted using GraphPad Prism (LaJolla, CA) with a criterion of significance set to \(p<0.05\), inclusion of a Geisser-Greenhouse correction for repeated measures to correct for unequal variance, and _post hoc_ analysis for significant ANOVAs using the Holm-Sidak test.

Our primary analysis focused on data across the 7-day study. Pooled data from both sexes and segregated data for each sex were first analyzed by a repeated-measures three-way ANOVA, with MJN110 and IP acid treatments as between-subject variables and days as a within-subject variable. If the three-way interaction was significant, follow-up two-way ANOVAs were conducted to evaluate treatment effects (MJN110 x Acid) on each day (Kirk, 1995). If the three-way interaction and main effect of days were not significant but there was a significant MJN110 x Acid interaction, data were collapsed across days and analyzed by a two-way ANOVA (MJN110 x Acid). Lastly, to directly compare data from males and females, results were
collapsed across days and analyzed by three-way ANOVA, with MJN110 treatment, IP acid treatment, and Sex as the three between-subject variables.

**Weight Data.** Repeated IP acid may decrease body weight as a surrogate measure for pain-related and analgesic-reversible depression of feeding behavior (Stevenson et al., 2006; Legakis et al., 2020). Accordingly, weights for each subject were collected daily immediately prior to MJN110 or vehicle administration. As with nesting data, our primary analysis focused on pooled data across both sexes and data segregated by sex. Specifically, Day-7 body weights for pooled data, females, and males were expressed as a percentage of Day-1 weights and analyzed by two-way ANOVA (MJN110 x Acid). As a secondary analysis to directly compare data from males and females, raw Day-1 weights and transformed Day-7 weights were also analyzed by three-way ANOVA with treatment groups (MJN110 x Acid) and Sex as the three variables.

**[^35]S\text{GTPyS binding data.** Specific[^35]S\text{GTPyS binding was determined as total non-specific binding. Net-stimulated binding was determined as agonist-stimulated basal binding (determined in the absence of agonist). Concentration-effect data are presented as % stimulation, defined as net-stimulated/basal[^35]S\text{GTPyS binding} x 100%. Concentration-effect curves were fit by non-linear regression using a 4-parameter model with the minimum constrained to zero to obtain $E_{\text{max}}$, log $EC_{50}$ and Hill coefficient values. $E_{\text{max}}$ and log $EC_{50}$ values were analyzed by two-way ANOVA (MJN110/vehicle x acid/H$_2$O) with post-hoc analysis using Tukey’s test. All curve-fitting and statistical analyses were conducted using GraphPad/Prism 9.

**Drugs**
Lactic acid (Fischer Scientific, NH) was diluted in sterile water and administered intraperitoneally. The MAGL- selective inhibitor MJN110 was kindly provided by Dr. Micah Niphakis (Lundbeck La Jolla Research Center, La Jolla, CA) and diluted in a vehicle (Veh) of 1:1:18 ethanol, emulphor (Alkamuls-620; Sanofi-Aventis, Bridgewater, NJ) and saline, and administered subcutaneously. All injections were administered in a volume of 10 ml/kg.
RESULTS

Effects of repeated MJN110 ± IP Acid on nesting

Figure 1 shows the effectiveness of repeated 1.0 mg/kg SC MJN110 to alleviate daily IP acid-induced nesting depression in pooled data from both female and male mice. Briefly, as shown in Figure 1B, vehicle- control mice (Veh/H₂O) nested at high and stable rates across the 7-day study, and MJN110-alone treatment did not alter nesting behavior during repeated administration (MJN110/H₂O). Repeated treatment with IP acid alone (Veh/Acid) produced a significant and sustained depression of nesting, and repeated MJN110 produced partial but significant alleviation of IP acid-depressed nesting (MJN110/Acid). Three-way ANOVA analysis indicated a significant MJN110 x Acid interaction with no main effect of Day or Day x MJN110 x Acid interaction (Figure 1B, see Supplemental Table 1 for statistical results). Accordingly, data were collapsed across days and analyzed by two-way ANOVA (Figure 1C, see Supplemental Table 2 for statistical results), which indicated that IP acid significantly depressed nesting and MJN110 produced a significant but partial alleviation of IP acid effects.

Evaluation of sex as a determinant of treatment effects in pooled data showed no main effect of Sex and no Sex x Treatment interactions (Figure 1C, Supplemental Figure 2, Supplemental Table 1). Data were then segregated by sex for further analysis as recommended by National Institutes of Health guidelines for analysis of sex as a biological variable in preclinical research (Miller et al., 2017; Tannenbaum et al., 2019; Galea et al., 2020), and results are shown in Figure 2. In males, each treatment produced a relatively consistent effect over time, with no significant main effect of Day or Day x Treatment interaction in the three-way ANOVA (Figure 2A, Supplemental Table 1). Male data were collapsed across days, and two-way ANOVA showed both acid-induced depression of nesting and partial but significant antinociception by repeated 1.0 mg/kg MJN110 (Figure 2B, Supplemental Table 2). Conversely, the three-way ANOVA in females showed a significant three-way interaction between Day, MJN110 dose, and IP Acid concentration (Figure 2C, Supplemental Table 1). Follow-up two-way ANOVAs of MJN110 x Acid treatment effects for each day indicated significant acid-induced depression of nesting for all 7 days but significant MJN110 partial antinociception only on Days 1, 3, and 5 (Supplemental Table 3). Figure 2D highlights the results for Days 1 and 7. On Day 1, in agreement with our previously published acute study (Diester et al., 2021a), MJN110 significantly alleviated IP acid-induced depression of nesting in females; however, by Day 7, there was only a main effect of acid treatment and no antinociceptive effect of MJN110.

The apparent antinociceptive tolerance to MJN110 in females may have reflected in part a trend toward a declining nociceptive effect of IP acid. Specifically, as suggested in Figure 2D
and described in more detail in Supplemental Figure 3, there was a trend for decreased IP acid-induced nesting depression over time in females, and this declining nociceptive effect may have reduced sensitivity to MJN110-induced antinociception. Nonetheless, IP acid produced significant nesting depression throughout the 7-day treatment period in females, and by Day 7, MJN110 no longer alleviated this IP acid effect.

**Effects of repeated MJN110 ± IP Acid on body weights**

Figure 3 shows treatment effects on body weights at the end of the study. Two-way ANOVA of data pooled across sexes found main effects of IP acid (to decrease body weights) and MJN110 (to increase body weights), but the interaction was not significant (Figure 3A, Supplemental Table 2). Thus, although MJN110 alleviated IP acid-induced depression of body weight, this effect could not confidently be attributed to antinociception because MJN110 increased body weight regardless of IP acid treatment. Segregating the data by sex showed significant main effects of MJN110 and IP acid treatments in females while males only showed a significant main effect of IP acid treatment, indicating that the main effect of MJN110 treatment was driven by the females (Figure 3B and 3C, Supplemental Table 2).

Supplemental Figure 4 shows body weight data with sex included as a variable to directly compare effects in females and males. On Day 1, males weighed more than females, but within a sex, there were no significant differences in basal weights across groups before the initiation of treatment (Supplemental Figure 4A, Supplemental Table 1). Day 7 data expressed as a percentage of Day 1 weights showed no main effect of Sex and no Sex x Treatment interactions (Supplemental Figure 4B, Supplemental Table 1).

**Effects of repeated MJN110 ± IP acid on CB₁R-mediated G-protein function**

To determine the effect of repeated MJN110 ± IP acid on CB₁R-mediated G-protein activation, CP55,940-stimulated [³⁵S]GTPyS binding was examined in membranes prepared from six dissected central nervous system (CNS) regions: lumbar spinal cord (LSC), periaqueductal gray (PAG), ventral tegmental area (VTA), nucleus accumbens (NAc), amygdala, and hippocampus. Basal [³⁵S]GTPyS binding in pooled sex cohorts ranged from 126 ± 15 pmol/mg in VTA of MJN110/acid-treated mice to 312 ± 32 pmol/mg in amygdala of MJN110/H₂O-treated mice and did not differ between experimental groups in any region (data not shown) except LSC. In LSC, there was a significant main effect of IP acid to slightly decrease basal [³⁵S]GTPyS binding (Supplemental Figure 5). Segregating the data by sex showed no significant differences between groups.
Illustrative concentration-effect curves for CP55,940-stimulated [$^{35}$S]GTPyS binding in LSC and NAc are shown in Figure 4, and data for PAG, VTA, amygdala, and hippocampus are shown in Supplemental Figures 6 and 7. Two-way ANOVA showed no differences in CP55,940 log EC$_{50}$ values between groups in any region examined. Consequently, subsequent analyses focused on E$_{\text{max}}$ values as described below. Significant main effects of MJN110 to decrease CP55,940 E$_{\text{max}}$ values were found in multiple regions and both sexes. By contrast, the IP acid main effect was significant in only two regions (PAG in females and hippocampus in pooled-sex data; see below), and the MJN110 x IP acid interaction was not significant in any region for either sex.

Figure 5 shows E$_{\text{max}}$ values in LSC and PAG. In LSC, there was a main effect of MJN110 to reduce E$_{\text{max}}$ values in both pooled-sex and female mice, but there was no effect in male mice (Figure 5 A-C). In general, the effects of MJN110 were modest in LSC: ~10-15% decreases in E$_{\text{max}}$ values with the largest decrease in H$_2$O-treated female mice (Table 1). In PAG, there was a main effect of MJN110 to reduce E$_{\text{max}}$ values in pooled-sex, female, and male mice (Figure 5 D-F). The magnitude of MJN110-induced decreases in PAG was 13-22%, with the largest decrease seen in H$_2$O-treated male mice (Table 1). There was also a main effect of IP acid to slightly increase the E$_{\text{max}}$ value in PAG of female mice only (Figure 5 E).

Figure 6 shows E$_{\text{max}}$ values in VTA and NAc. In both regions, there was a main effect of MJN110 to decrease CP55,940 E$_{\text{max}}$ values in pooled-sex and male mice, but not in females. The magnitudes of decrease were generally greater in NAc (~10-20%) than VTA (~7-14%), with the largest decrease in NAc of H$_2$O-treated male mice (Table 2).

Supplemental Figure 8 shows E$_{\text{max}}$ values in hippocampus and amygdala. In contrast to the other regions, MJN110 treatment did not significantly alter CP55,940 E$_{\text{max}}$ values in either region, although there was a trend toward a decrease (p = 0.0752) in the pooled-sex cohort. However, in hippocampus, there was a main effect of IP acid to decrease CP55,940 E$_{\text{max}}$ values in pooled-sex mice with a trend in males (p=0.0574), but no significant effect in female mice. The magnitudes of decrease ranged from ~9-14%, with the largest decrease in male H$_2$O-treated mice (Supplemental Table 5).

Across all six regions, there was a general trend toward lesser effects of MJN110 in IP acid- than H$_2$O- treated mice. Thus, these results indicate small to moderate desensitization of CB$_1$R-mediated G-protein activation by repeated administration of 1.0 mg/kg MJN110, and this desensitization generally occurred in: (1) female mice in LSC, (2) H$_2$O-treated mice of both sexes in PAG, and (3) H$_2$O-treated male mice in VTA and NAc.
DISCUSSION

This study evaluated behavioral antinociception and ex vivo changes in CB₁R function produced by chronic treatment with the MAGL inhibitor MJN110 in male and female mice. There were four main findings. First, repeated daily treatment with IP acid produced a significant, sustained, and pain-related depression of nesting behavior, with a potential trend for decreased effectiveness in females. Second, MJN110 produced a weak but sustained partial attenuation of IP acid-induced nesting depression in males, but antinociceptive tolerance developed in females. Third, repeated IP acid decreased body weights, and MJN110 alleviated IP acid-induced weight loss; however, this effect could not confidently be attributed to antinociception because MJN110 increased body weight regardless of the presence or absence of the noxious stimulus. Lastly, evaluation of CB₁R mediated G-protein activity after repeated MJN110 ± IP acid generally showed modest decreases in maximal activation by CP55,940 in MJN110-treated mice, the magnitude of which varied by CNS region, IP acid versus H₂O treatment, and sex.

Antinociceptive effects of repeated MJN110

The present results agree with our previous finding in rats that repeated daily administration of IP acid can produce both (a) consistent daily episodes of pain-related behavioral depression and (b) weight loss as a potential indicator of depressed feeding (Miller et al., 2015; Legakis et al., 2020). In those previous studies, repeated IP acid decreased a positively reinforced operant-conditioned behavior in rats. The present study extended this evidence for repeated IP acid-induced behavioral depression to expression of an unconditioned behavior (nesting) in mice. Additionally, this baseline expression of repeated pain-depressed behavior provided an opportunity to evaluate effects of repeated treatment with 1.0 mg/kg/day MJN110 as a candidate analgesic. The significant antinociception observed on the first day of MJN110 treatment replicated our previous finding that acute treatment with 1.0 mg/kg MJN110 had no effect on nesting behavior when administered alone but produced a significant and partial antinociceptive attenuation of IP acid-induced nesting depression (Diester et al., 2021a). This finding is in agreement with previous studies demonstrating that MJN110, along with other selective MAGL inhibitors like JZL184, can produce acute antinociception in numerous rodent pain models (Long et al., 2009; Schlosburg et al., 2010; Guindon et al., 2011; Niphakis et al., 2013; Ignatowska-Jankowska et al., 2015; Burston et al., 2016; Wilkerson et al., 2016; Curry et al., 2018; Thompson et al., 2020; Diester et al., 2021a).

In contrast to the relatively consistent antinociception observed in studies with acute MAGL-inhibitor treatment, the effects of repeated treatment have been more variable and
depend in part on the chronically administered dose. In general, repeated daily treatment for up to 1 week with high MAGL-inhibitor doses produced initial antinociception that decreased over time as tolerance developed (Schlosburg et al., 2010; Kinsey et al., 2013; Burston et al., 2016; Curry et al., 2018). Conversely, repeated treatment with lower MAGL-inhibitor doses produced either sustained or increasing antinociception over days of treatment without evidence of tolerance. Given these previous results, we expected that repeated treatment here with a relatively low 1.0 mg/kg/day MJN110 dose would produce sustained or increasing antinociception over time in the model of IP acid-induced nesting depression; however, we found no evidence for increasing MJN110 antinociception over days. Moreover, although partial but significant antinociception was sustained both in the analysis of pooled data across sexes and in analysis of the males alone, MJN110 lost its antinociceptive effectiveness in females. This emergence of antinociceptive tolerance in females but not males agrees with previous research that female rats are more vulnerable than males to antinociceptive tolerance with the CB1 agonist THC (Wakley et al., 2015). Previous studies have shown that 1.0 mg/kg MJN110 acutely elevates brain 2-AG levels (Niphakis et al., 2013); however, further studies evaluating changes in endocannabinoids and eicosanoids following repeated administration are needed to better understand possible mechanisms for the antinociceptive tolerance seen in this study. Additionally, studies showing sustained MJN110 antinociception used models of more sustained chronic pain and demonstrated antinociception to be CB2-dependent (Burston et al., 2016; Thompson et al., 2020). We previously demonstrated that CB2 is not required for MJN110 antinociception in this assay of IP acid-induced nesting depression (Diester et al., 2021a). The differential role of CB1Rs vs CB2Rs across pain models is not yet fully understood. Given that CB2Rs are predominantly expressed peripherally on immune cells, one possibility is that effectiveness is that CB2Rs may be especially important in models of inflammatory pain. Future studies evaluating the role of CB2R in MJN110 antinociception are needed to clarify pain models most sensitive to CB2R-mediated MJN110-induced antinociception.

It should be noted that there was a trend for declining IP acid-induced nesting depression over time in females, possibly impacting the loss of MJN110 antinociception by Day 7. Although this study did not track estrous cycle, other studies have shown that visceral pain-related behaviors can fluctuate with mouse and rat estrous cycle (Ji et al., 2008; Escudero-Lara et al., 2021; Tramullas et al., 2021). Additionally, the present study delivered a noxious stimulus in the peritoneal cavity, which is open through the genital canal in females but not males (Solass et al., 2016). Thus, additional work evaluating possible hormonal and anatomical determinants of repeated IP acid effects may be of interest. Nonetheless, a trend for decreased IP acid-
induced nociception might predict increased sensitivity to MJN110 antinociception. This was not observed, supporting the conclusion that antinociceptive tolerance developed in females.

In addition to these effects on nesting, MJN110 treatment also alleviated IP acid-induced weight loss. This MJN110 effect in mice resembles the blockade of IP acid-induced weight loss by the nonsteroidal anti-inflammatory drug ketorolac in rats (Legakis et al., 2020); however, two findings challenge the conclusion that MJN110 effects on body weight reflect antinociception. First, two-way ANOVA results indicated a main effect of MJN110 on body weight, but no interaction between MJN110 and IP acid treatment. A conservative interpretation of this result is that MJN110 increased body weight regardless of IP acid treatment. This would be consistent with a role for endocannabinoids in promoting appetite (Berry and Mechoulam, 2002), and suggests that weight gain should be considered as a possible side effect should MAGL inhibitors advance to clinical testing. Second, when data were segregated by sex, the main effect of MJN110 on body weight was significant only in females, but females developed tolerance to the effects of MJN110 on IP acid-induced nesting depression.

**Effects of IP acid ± MJN110 on CB1R signaling**

Repeated MJN110 treatment decreased maximal stimulation of CB1R-mediated G-protein activation in most CNS regions examined. The effect of MAGL inhibition on CB1R activity has, to our knowledge, previously been assessed using only JZL184 administration. Comparison of our MJN110 results to findings with JZL184 show 1.0 mg/kg/day MJN110 produces a modest reduction compared to high JZL184 doses (<16 mg/kg) (Schlosburg et al., 2010; Kinsey et al., 2013), but greater than lower JZL184 doses (<16 mg/kg) (Kinsey et al., 2013; Ghosh et al., 2015) relative to the antinociceptive potency of each inhibitor. Decreased CB1R activity likely reflected CB1R desensitization (uncoupling from G-protein activation) and/or downregulation. CB1R agonists can produce desensitization at lower doses that do not produce significant downregulation, depending on CNS region (McKinney et al., 2008; Nguyen et al., 2012), suggesting that desensitization contributed to CB1R activity reductions seen here. Regional differences in CB1R adaptation in response to repeated MJN110 were seen as expected; however, the regional pattern depended on multiple factors, including both sex and IP acid co-treatment. In general, co-treatment with IP acid tended to decrease the MJN110-induced CB1R desensitization effect. To our knowledge, noxious stimulus effects on CB1R adaptation to repeated dosing has not previously been reported with either THC or MAGL inhibitors. Males showed greater MJN110-induced decreases in brain CB1R activity, especially in mesolimbic regions. In contrast, females showed greater decreases in LSC CB1R activity.
While this latter effect most closely parallels the antinociceptive tolerance to MJN110 in females, reduced CB₁R activation in PAG might have also contributed. The development of greater antinociceptive tolerance in females is consistent with previous studies that measured THC-mediated antinociception (Wakley et al., 2014). An interesting possibility is that mechanisms underlying tolerance might differ between males and females (Henderson-Redmond et al., 2022). However, reduced CB₁R signaling in NAc, VTA, and/or PAG by repeated MJN110 in IP acid-co-treated males might have contributed to the overall lower behavioral response of male mice to MJN110 over time, especially if these adaptations occurred early in the repeated treatment. The regional profile of CB₁R adaptation in male mice further suggests that tolerance might occur in a different panel of behaviors.

Interestingly, the hippocampus showed no significant adaptation to repeated MJN110, with only a significant main effect of IP acid in the mixed-sex cohort. This is somewhat surprising given that hippocampus generally shows the most robust CB₁R desensitization and downregulation among CNS regions in response to repeated THC [e.g., (Breivogel et al., 1999; Sim-Selley et al., 2006)], including greater THC dose-sensitivity than other regions (McKinney et al., 2008). Hippocampus also showed the greatest decrease in G-protein activation among multiple regions after repeated administration of 40 mg/kg JZL184 (Schlosburg et al., 2010). In contrast, greater CB₁R adaptation was observed in PAG and NAc than hippocampus in the present study. One difference between this study and previous work is that earlier studies used males exclusively. However, no significant MJN110 effect was seen in male hippocampus here, and our previous work found no sex differences in CB₁R desensitization in multiple brain regions including hippocampus, PAG and striatum, in adult rats (Burston et al., 2010). Therefore, it seems that regional differences in the magnitude of CB₁R adaptation with repeated MJN110 treatment may differ from those of THC or JZL184, although comparison using multiple doses of each drug in the same species, strain and sex would be needed to confirm that conclusion.

**Conclusion**

Overall, these findings suggest that it may be challenging to administer MJN110 at doses sufficient to produce even partial alleviation of episodic visceral pain without triggering compensatory antinociceptive tolerance and CB₁R desensitization.
DATA AVAILABILITY STATEMENT
The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Diester, Lichtman, Sim-Selley, Selley, Negus

Conducted experiments: Diester, Balint, Gillespie

Performed data analysis: Diester, Selley, Negus

Wrote or contributed to the manuscript: Diester, Balint, Gillespie, Lichtman, Sim-Selley, Selley, Negus
REFERENCES


Henderson-Redmond AN, Sepulveda DE, Ferguson EL, Kline AM, Piscura MK and Morgan DJ (2022) Sex- specific mechanisms of tolerance for the cannabinoid agonists CP55,940 and delta-9- tetrahydrocannabinol (Delta(9)-THC). *Psychopharmacology (Berl)* **239**:1289-1309.


FOOTNOTES

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FIGURE LEGENDS

Figure 1. MJN110 produced a sustained but partial alleviation of IP acid-induced nesting depression in mice. (A) Schematic illustration of experimental design for the study. (B) Treatment effects on nesting behavior across the seven days of treatment. Abscissa: experimental days. Ordinate: nesting behavior expressed as percent maximal nestlet consolidation. Each point shows mean±SEM for 24 mice (12 per sex). (C) Data from Panel B collapsed across days. Abscissa: treatment with IP H$_2$O or IP Acid. Ordinate: nesting behavior expressed as percent maximal nestlet consolidation. Bars show mean±SEM for 24 mice (12 per sex) in each treatment group, and circles (Veh pretreatment) and triangles (MJN110 pretreatment) show individual data. Asterisks (*) indicates a significant difference as determined by two-way ANOVA and Holm-Sidak post hoc test, p<0.05. Three-way ANOVA results for (B) are shown in Supplemental Table 1, and two-way ANOVA results for (C) are shown in Supplemental Table 2.

Figure 2. Differential effects of repeated MJN110 ± IP acid treatment in female and male mice. Treatment effects on nesting behavior across seven days of treatment in males (Panel A&B) and females (Panel C&D). Abscissae for panels A&C: experimental days. Ordinates for panels A&C: nesting behavior expressed as percent maximal nestlet consolidation, with each point representing mean±SEM for 12 mice per treatment. Abscissae for panels B&D: treatment with IP H$_2$O or IP Acid. Ordinates for panels B&D: nesting behavior expressed as percent maximal nestlet consolidation, with bars representing mean±SEM for 12 mice in each treatment group, and circles (Veh pretreatment) and triangles (MJN110 pretreatment) showing individual data. For males in Panel A, the three-way interaction was not significant, and there was no significant effect of Day or Day x Treatment interaction. Accordingly, data were collapsed across days, evaluated by two-way ANOVA, and results are shown in Panel B. For females in Panel C, the three-way interaction was significant, and data on each day were analyzed by two-way ANOVA, with data for Days 1 and 7 highlighted in Panel D. Asterisks (*) indicate significant effects determined by two-way ANOVA and Holm-Sidak post hoc tests (p<0.05). Three-way ANOVA results for (A) and (C) are shown in Supplemental Table 1. Two-way ANOVA results for (B) are shown in Supplemental Table 2, and two-way ANOVA results for (D) are shown in Supplemental Table 3.
Figure 3. Opposing effects of MJN110 and IP acid treatment on body weight. Abscissae: treatment with IP H$_2$O or IP Acid. Ordinates: bodyweight expressed as percent of baseline Day 1 weights. Bars show mean±SEM for pooled data across both sexes in panel A (24 mice per bar) and for data in females only or males only in panels B and C, respectively (12 mice per sex for each bar). Circles (Veh pretreatment) and triangles (MJN110 pretreatment) show individual data. Significant main effects determined by two-way ANOVA and Holm-Sidak post hoc test (p<0.05) are displayed above each panel. Detailed two-way ANOVA results for all panels are shown in Supplemental Table 2.

Figure 4. Concentration-effect curves of CP55,940-stimulated [$^{35}$S]GTP$_{yS}$ binding in lumbar spinal cord (LSC) and nucleus accumbens (NAc) of repeated MJN110 ± IP acid-treated mice. Abscissae: CP55,940 concentration (log M). Ordinates: % stimulation of [$^{35}$S]GTP$_{yS}$ binding over basal. Panels A-C show data from LSC and panels D-F show data from NAc in pooled sex (A, D), female (B,E) or male (C, F) mice. Data are mean values ± SEM (n = 9-10 or 8-9 per sex per group in LSC and NAc, respectively).

Figure 5. Effects of repeated MJN110 ± IP acid on CP55,940 $E_{max}$ values in pain-modulatory regions: lumbar spinal cord (LSC) and periaqueductal gray (PAG). Abscissae: treatment with IP H$_2$O or IP acid. Ordinates: $E_{max}$ values as % stimulation of [$^{35}$S]GTP$_{yS}$ binding over basal. Panels A-C show data from LSC and panels D-F shows data from PAG in pooled sex (A, D), female (B,E) or male (C, F) mice. Data are mean values ± SEM (n = 9-10 or 6-8 per sex per group in LSC and PAG, respectively).

Figure 6. Effects of repeated MJN110 ± IP acid on CP55,940 $E_{max}$ values in mesolimbic regions: ventral tegmental area (VTA) and nucleus accumbens (NAc). Abscissae: treatment with IP H$_2$O or IP acid. Ordinates: $E_{max}$ values as % stimulation of [$^{35}$S]GTP$_{yS}$ binding over basal. Panels A-C show data from VTA and panels D-F shows data from NAc in pooled sex (A, D), female (B,E) or male (C, F) mice. Data are mean values ± SEM (n = 6-8 or 8-9 per sex per group in VTA and NAc, respectively). * P < 0.05 different from Vehicle/H$_2$O group by post hoc analysis with Tukey’s test.
Table 1. $E_{\text{max}}$ and $EC_{50}$ values of agonist-stimulated $[^3S]GTP_{yS}$ binding in pain-modulatory regions (lumbar spinal cord, LSC; periaqueductal gray, PAG) from repeated MJN110- and vehicle-pretreated mice challenge with IP $H_2O$ or lactic acid

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<th>Challenge</th>
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<th>$E_{\text{max}}$ (% Veh.)</th>
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Concentration-effect curves of CP55,940-stimulated $[^3S]GTP_{yS}$ binding in lumbar spinal cord (LSC) and periaqueductal gray (PAG) were fit by non-linear regression analysis to obtain $E_{\text{max}}$ and $EC_{50}$ values. Values are presented as mean ± SEM ($n = 9-10$ in LSC and 6-8 in PAG per sex per group). % Stim: % stimulation; % Veh.: $E_{\text{max}}$ values normalized to the maximal stimulation obtained in vehicle-treated mice in either the $H_2O$ or acid-treated groups, respectively.
Table 2. E\textsubscript{max} and EC\textsubscript{50} values of agonist-stimulated [\textsuperscript{35}S]GTPyS binding in two mesolimbic regions (ventral tegmental area, VTA; nucleus accumbens, NAc) from repeated MJN110- and vehicle-pretreated mice challenge with IP H\textsubscript{2}O or lactic acid.

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<tr>
<td>Pooled</td>
<td>Vehicle</td>
<td>H\textsubscript{2}O</td>
<td>76.07 ± 2.96</td>
<td>100.00 ± 3.84</td>
<td>-8.05 ± 0.07</td>
</tr>
<tr>
<td>Pooled</td>
<td>MJN110</td>
<td>H\textsubscript{2}O</td>
<td>64.38 ± 2.93</td>
<td>84.74 ± 3.95</td>
<td>-8.06 ± 0.06</td>
</tr>
<tr>
<td>Pooled</td>
<td>Vehicle</td>
<td>Acid</td>
<td>77.45 ± 2.85</td>
<td>100.00 ± 3.56</td>
<td>-8.02 ± 0.04</td>
</tr>
<tr>
<td>Pooled</td>
<td>MJN110</td>
<td>Acid</td>
<td>69.51 ± 3.13</td>
<td>90.18 ± 4.40</td>
<td>-8.14 ± 0.06</td>
</tr>
</tbody>
</table>

Concentration-effect curves of CP55,940-stimulated [\textsuperscript{35}S]GTPyS binding in ventral tegmental area (VTA) and nucleus accumbens (NAc) were fit by non-linear regression analysis to obtain E\textsubscript{max} and EC\textsubscript{50} values. Values are presented as mean ± SEM (n = 6-9 in VTA and 8-9 in NAc per sex per group). % Stim: % stimulation; % Veh.: E\textsubscript{max} values normalized to the maximal stimulation obtained in vehicle-treated mice in either the H\textsubscript{2}O or acid-treated groups, respectively.
Figure 1

A

2 x 2 Experimental Design

<table>
<thead>
<tr>
<th>Veh + H20</th>
<th>Veh + Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJN + H20</td>
<td>MJN + Acid</td>
</tr>
</tbody>
</table>

Week 1 acclimation

1 2 3 4 5 6 7 Day 8 Tissue Collection

Daily Nesting Studies

a) MJN110/Vehicle Pretreatment (SC, 2hrs)

b) IP acid/Vehicle Treatment (IP)

c) 90 minute nesting assay

B

- Veh/H20
- Veh/Acid
- MJN110/H20
- MJN110/Acid

% Maximal Nestlet Consolidation

Days

1 2 3 4 5 6 7

C

- Veh
- MJN110

% Maximal Nestlet Consolidation

H2O Acid

*
Figure 2

A

- ○ Veh/H2O
- ● Veh/Acid
- ▲ MJN110/H2O
- ▼ MJN110/Acid

% Maximal Nestlet Consolidation

Males

Days 1 2 3 4 5 6 7

B

- ○ Veh
- ▲ MJN110

% Maximal Nestlet Consolidation

H2O Acid

C

% Maximal Nestlet Consolidation

Females

Days 1 2 3 4 5 6 7

D

% Maximal Nestlet Consolidation

H2O Acid

Day 1

Day 7
Figure 4

Lumbar Spinal Cord

A. Pooled

- Veh/H2O
- MJN/H2O
- Veh/Acid
- MJN/Acid

B. Female

C. Male

Nucleus Accumbens

D. Pooled

- Veh/H2O
- MJN/H2O
- Veh/Acid
- MJN/Acid

E. Female

F. Male
Figure 5

**Lumbar Spinal Cord**

**A.** Pooled

Main Effects: MJN110 p = 0.0127

**B.** Female

Main Effects: MJN110 p = 0.0050

**C.** Male

**Periaqueductal Gray**

**D.** Pooled

Main Effects: MJN110 p = 0.002

**E.** Female

Main Effects: MJN110 p = 0.0426  
Acid p = 0.0471

**F.** Male

Main Effects: MJN110 p = 0.0303
Figure 6

Ventral Tegmental Area

A. Pooled

Main Effects: MJN110 p = 0.0131

B. Female

C. Male

Main Effects: MJN110 p = 0.0049

Nucleus Accumbens

D. Pooled

Main Effects: MJN110 p = 0.0015

E. Female

F. Male

Main Effects: MJN110 p = 0.0016