Selective monoacylglycerol lipase inhibitors: Antinociceptive vs. cannabimimetic effects in mice

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2-arachidonoylglycerol, 2-AG; anandamide, AEA; arachidonic acid, AA; baseline, BL, chronic constriction injury, CCI; cannabinoid 1, CB1; cannabinoid 2, CB2; fatty acid amide hydrolase, FAAH; monoacylglycerol lipase, MAGL; oleylethanolamide, OEA; palmitoylethanolamide, PEA; Δ⁹- tetrahydrocannabinol, THC

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Abstract

The endogenous cannabinoid 2-arachidonoylglycerol (2-AG) plays an important role in a variety of physiological processes, but its rapid breakdown by monoacylglycerol lipase (MAGL) results in short-lived actions. Initial MAGL inhibitors were limited by poor selectivity and low potency. Here, we tested JZL184 and MJN110, MAGL inhibitor that possesses increased selectivity and potency, in mouse behavioral assays of neuropathic pain (chronic constrictive injury of the sciatic nerve; CCI), interoceptive cannabimimetic effects (drug discrimination paradigm), and locomotor activity in an open field test. MJN110 (1.25 and 2.5 mg/kg) and JZL184 (16 and 40 mg/kg) significantly elevated 2-AG and decreased arachidonic acid, but did not affect anandamide (AEA) in whole brain. Both MAGL inhibitors significantly reduced CCI-induced mechanical allodynia with the following potencies [ED_{50} (95% CL) values in mg/kg: MJN110 (0.43 (0.30-0.63) > JZL184 (17.8 (11.6-27.4))], and also substituted for the potent cannabinoid receptor agonist CP55,940 in the drug discrimination paradigm [ED_{50} (95% CL) values in mg/kg: MJN110 (0.84 (0.69-1.02)) > JZL184 (24.9 (14.6-42.5))]. However, these compounds elicited differential effects on locomotor behavior. Similar to CB₁ receptor agonists, JZL184 produced hypomotility, while MJN110 increased locomotor behavior and did not produce catalepsy or hypothermia. Although these results suggest that MAGL inhibitors hold promise for the treatment of neuropathic pain, their substitution for CP55,940 in the drug discrimination assay may not be desirable for a therapeutic drug. Nonetheless, in contrast to CB₁ receptor agonists, each MAGL inhibitor tested elicited few or no pharmacological effects in other standard assays used to infer cannabimimetic activity.
Introduction

Neuropathic pain is a debilitating chronic pain condition, but available pharmacotherapies lack efficacy and are associated with various adverse side-effects (Nightingale, 2012). Monoacylglycerol lipase (MAGL), the major serine hydrolase responsible for degradation of the most abundant endocannabinoid in the central nervous system, 2-arachidonoylglycerol (2-AG) (Dinh et al., 2002), represents a promising target for the treatment of neuropathic pain and other pain-related therapeutic indications. MAGL inhibitors block 2-AG degradation leading to increased levels of this endocannabinoid and consequently lead to increased signaling at cannabinoid CB1 and CB2 receptors. Initial compounds used to inhibit MAGL in whole animals, such as methylarachidonylfluorophosphonate and N-arachidonyl maleimide have poor selectivity when administered systemically (Makara et al., 2005; Saario et al., 2005; Burston et al., 2008), which makes it difficult to discern whether the observed pharmacological effects were due to MAGL inhibition, off-target actions (e.g., another serine hydrolase), or combination of MAGL inhibition and off-target effects. While an in vitro study showed that URB602 lacks selectivity between MAGL and FAAH (Vandevoorde et al., 2007), in vivo studies found that intracerebral (Hohmann et al., 2005), systemic (Wiskerke et al., 2012), or intraplantar (Guindon et al., 2011) URB602 administration raises 2-AG levels, but not AEA levels.

The recent development of MAGL inhibitors with increased selectivity compared with previous compounds has provided useful tools to investigate consequences of inhibiting this enzyme in the whole animal. Results from several preclinical studies employing the first selective MAGL inhibitor, JZL184 (Long et al., 2009a), suggested that this enzyme represents a unique target to treat pain-related conditions. Acute administration of JZL184 was reported to reduce nociception in acetic acid-induced abdominal stretching, the carrageenan model of inflammatory pain, the chronic constrictive injury of the sciatic nerve (CCI) model, cisplatin-
induced neuropathy model, and capsaicin-induced behavioral sensitization (Kinsey et al., 2009; Long et al., 2009a; Guindon et al., 2010; Spradley et al., 2010; Ghosh et al., 2013; Kinsey et al., 2013). Importantly, JZL184 elicited a decreased spectrum of other cannabimimetic effects associated with $\Delta^9$-tetrahydrocannabinol (THC) and other CB$_1$ receptor agonists. Although JZL184 is approximately 450 fold more selective in inhibiting MAGL than fatty acid amide hydrolase (FAAH) and does not alter AEA brain levels upon acute administration, repeated administration of high dose produces a 2-3 fold increase in this endocannabinoid (Schlosburg et al., 2010). Another limitation of JZL184 is its decreased potency in inhibiting rat MAGL activity ($IC_{50}$ value = 262 nM) compared with mouse MAGL activity ($IC_{50}$ value = 10 nM) (Chang et al., 2012). MJN110, another recently developed selective MAGL inhibitor, showed markedly increased potency in rat and no significant cross-reactivity with FAAH (Niphakis et al., 2013). These compounds provide tools to compare the pharmacological effects of structurally distinct MAGL inhibitors in preclinical animal models of pain and assays indicative of cannabimimetic activity (e.g., drug discrimination, hypothermia, catalepsy, and hypomotility).

The primary purpose of the present study was to compare directly the pharmacological effects of JZL184 and MJN110 in the chronic constriction nerve injury (CCI) model of neuropathic pain as well as in assays indicative of cannabimimetic effects. JZL184 has already been shown to reduce allodynia in the CCI model of neuropathic pain (Kinsey et al., 2009; Kinsey et al., 2013). MJN110 reduces nociception in a rat diabetic neuropathy model (Niphakis et al., 2013), but has yet to be tested in the CCI model. A major challenge in developing direct-acting CB$_1$ receptor agonists as therapeutic agents is their cannabimimetic side effects. Although JZL184 administered in a vehicle consisting of alkamuls-620, ethanol, and saline did not elicit common cannabimimetic effects (i.e., catalepsy and hypothermia), it produced others (i.e., decreased locomotor activity and partial substitution for THC in the drug discrimination paradigm) (Long et al., 2009b; Long et al., 2009c). In contrast, MJN110 remains to be assessed.
Thus, the second objective of this study was to compare the cannabimimetic “side effect” profile of these drugs, with an emphasis on locomotor activity, and interoceptive effects assessed in the drug discrimination paradigm. Additionally, we tested whether MJN110 produces other common cannabimimetic actions, including antinociception in the tail withdrawal assay, catalepsy in the bar test, and hypothermia. Finally, we quantified whole brain levels of 2-AG as well as arachidonic acid. Of note, MAGL serves as a major biosynthetic enzyme of arachidonic acid in brain (Nomura et al., 2011). Because JZL184 also inhibits FAAH, we also quantified brain levels of the prevalent FAAH substrates AEA and noncannabinoid fatty acid amides (i.e., palmitoylethanolamide (PEA) and oleoylethanolamide (OEA)).
Methods

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Body mass of mice ranged from 18 to 35 g. Four to five mice were housed per cage in all experiments, except in the drug discrimination experiments, in which subjects were singly housed. Animals were maintained in a 12/12 h light/dark cycle (0600 h on/1800 h off) in a temperature (20–22 °C) and humidity (55 ± 10 %) controlled AAALAC-approved facility. Animals had ad libitum access to water and food, with the exception of animals used in drug discrimination experiments, which were food restricted to 85-90 % of free feeding body weight. All tests were conducted during the light phase. The sample sizes selected for each treatment group in each experiment were based on previous studies from our laboratory.

All animal protocols were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

Drugs

MJN110 was synthesized in the Cravatt laboratory at the Scripps Research Institute as described previously (Chang et al., 2012; Niphakis et al., 2013). JZL184 (Long et al., 2009a), the CB1 receptor antagonist rimonabant [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide-HCl] (SR141716A), the CB2 receptor antagonist SR144528 [5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,2S,4R)-1,3,3- rimethylbicyclo [2.2.1] hept-2-yl]-1H-pyrazole-3-carboxamide], and CP55,940 [(-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol] were obtained from the National Institute on Drug Abuse (Bethesda, MD). Drugs were dissolved in a vehicle solution consisting of a mixture of ethanol, alkanuls-620 (Sanofi-Aventis, Bridgewater,
NJ), and saline (0.9 % NaCl) in a 1:1:18 ratio. Each drug was given via the intraperitoneal (i.p.) route of administration, with exception of drug discrimination studies, in which injections were given via subcutaneous (s.c.) route of administration. All drugs were administered in a volume of 10 μl/g body mass, with the exception of the drug discrimination experiment assessing 100 mg/kg JZL184, which used an injection volume of 20 μl/g body mass. Drug treatments were randomized in all experiments.

**Behavioral assessment of nociceptive behavior**

Mechanical allodynia and thermal hyperalgesia were used to assess nociceptive behavior following sham or CCI surgery (see below). Prior to surgery, mice were habituated to the testing environment, and then von Frey monofilaments (North Coast Medical, Morgan Hills, CA) were used to establish baseline (BL) responses to light mechanical touch and to assess the development and presence of allodynia after surgery (Murphy et al., 1999). Specifically, the mice were placed on top of a wire mesh screen, with spaces 0.5 mm apart and habituated for approximately 30 min on four consecutive days. Mice were unrestrained, and were singly placed under an inverted Plexiglas basket (8 cm diameter, 15 cm height), with a wire mesh top to allow for unrestricted air flow. The von Frey test utilizes a series of calibrated monofilaments, (2.83 – 4.31 log stimulus intensity) applied randomly to the left and right plantar surface of the hind paw for 3 s. Lifting, licking or shaking the paw was considered a response. After completion of allodynia testing, the mice were placed on a heated (52°C) enclosed Hot Plate Analgesia Meter (Columbus Instruments, Columbus, OH). The latency to jump or lick/shake a hind paw was assessed. A 30 s cut off time was used to avoid potential tissue damage (Garcia-Martinez et al., 2002). For each assay, testing was performed in a blinded fashion and nociceptive behavior was assessed at 0.5, 1, 1.5, 3, 5, and 24 h.
Chronic constriction injury (CCI) surgery

Following baseline (BL) behavioral assessment, the surgical procedure for chronic constriction of the sciatic nerve was completed as previously described (Bennett and Xie, 1988), but modified for mouse (Murphy et al., 1999). In brief, the mice were anesthetized with isoflurane- (induction 5% vol. followed by 2.0% in oxygen), and the mid to lower back and the dorsal left thigh were shaved and cleaned with 75% ethanol. Using aseptic procedures, the sciatic nerve was carefully isolated, and loosely ligated with 3 segments of 5-0 chromic gut sutures (Ethicon, Somerville, NJ). Sham surgery was identical to CCI surgery, but without the loose nerve ligation. The overlying muscle was closed with (1) 4-0 sterile silk suture (Ethicon, Somerville, NJ), and animals recovered from anesthesia within approximately 5 min. Mice were randomly assigned to either CCI or sham surgical group. Mice in both groups were re-assessed for allodynia and thermal hyperalgesia, as described above. Subjects were tested with drug or vehicle between 5 and 18 days after surgery.

Drug discrimination

A separate group of 12 male C57BL/6J mice, trained to discriminate CP55,940 (0.1 mg/kg) from vehicle (30 min pretreatment time), was used to test whether JZL184 or MJN110 would substitute for the training drug. Training and testing were conducted according to general procedures described previously (Long et al., 2009b; Walentiny et al., 2013; Ignatowska-Jankowska et al., 2014), and described in Supplementary Information. Control tests were conducted with the training dose of the drug (0.1 mg/kg CP55,940) or vehicle. For substitution tests, MJN110 (0.25, 0.5, 1.25, 2.5) or JZL 184 (4, 16, 40, or 100 mg) was administered s.c. 120 min before the test session. The JZL184 substitution assessment was conducted in two separate experiments, with the first experiment comparing vehicle vs. 4, 16, and 40 mg/kg JZL184 and the second experiment comparing vehicle vs. 100 mg/kg JZL184. The injections for the second

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JZL184 experiment were double volume (i.e., 20 μl/g body mass). For antagonism studies, mice were injected with rimonabant (1 mg/kg, i.p.) or vehicle 130 min before the test session. Mice were returned to their home cages after each injection and placed in the test chamber immediately before the test period. Full substitution for of training drug was defined as equal to or greater than 80% of responses on the aperture paired with administration of the training drug. Partial substitution was defined as 20-79% of responses on the aperture paired with training drug, and less than 20% of responses on the aperture coupled with training drug was considered as no substitution. The percentage of responses on the “drug” aperture and response rates were recorded for each test session.

**Locomotor activity**

Locomotor activity was assessed in naïve mice placed individually in a clear, dimly lit Plexiglas box (42.7×21.0×20.4 cm) 120 min after injection of JZL184 or MJN110. Activity was monitored for 60 min using Anymaze (Stoelting, Wood Dale, Illinois) software, as described previously (Ignatowska-Jankowska et al., 2014). Distance travelled, running speed, and time spent mobile were measured.

**Triad assay**

Mice were housed individually overnight. Subjects were administered vehicle or MJN110 (5 mg/kg) and 2, 4 and 24 h later were sequentially assessed in the following three procedures: bar test (catalepsy), tail withdrawal test, and rectal temperature. Testing was performed according to previously described procedures (Long et al., 2009b; Schlosburg et al., 2010; Ignatowska-Jankowska, 2014), as detailed in supplementary methods.
Extraction and quantification of endocannabinoids by liquid chromatography-tandem mass spectrometry

2-AG, arachidonic acid, AEA, PEA and OEA levels were quantified from the whole brain of C57BL/6J mice after acute treatment with JZL184, MJN110, or vehicle. Because each MAGL inhibitor significantly attenuated CCI-induced thermal hyperalgesia at 3 h after injection, mice were euthanized via rapid decapitation (at 11:00–17:00 EST) at this time point. The brains were rapidly harvested, snap-frozen in dry ice, and stored at -80°C until the time of processing. Tissues were further processed according to methods described previously (Ramesh et al., 2011; Ignatowska-Jankowska et al., 2014). See supplementary methods for details.

Data analysis

All data are presented as mean ± standard error (SEM). For allodynia testing, psychometric behavioral analysis was performed to compute the log stiffness that would have resulted in the 50% paw withdrawal rate, as previously described (Treutwein and Strasburger, 1999). Briefly, thresholds were estimated by fitting a Gaussian integral psychometric function to the observed withdrawal rates for each of the tested von Frey hairs, using a maximum-likelihood fitting method (Milligan et al., 2001; Wilkerson et al., 2012). Data were analyzed using t-tests, one-way or two-way analysis of variance (ANOVA). Tukey test was used for post hoc analyses of significant one-way ANOVAs. Multiple comparisons following two-way ANOVA were conducted with Bonferroni post hoc comparison. Correlations are reported as Pearson’s r values. Differences were considered significant at the level of p ≤ 0.05. Statistical analysis was performed with IBM SPSS Statistics, version 20.0 (IBM Corp., Armonk, NY).
Results

**MAGL Inhibitors reverse CCI-induced mechanical allodynia and thermal hyperalgesia**

CCI elicited significant allodynia in ipsilateral (p < 0.0001) and contralateral paws (p < 0.0001), as well produced a significant hyperalgesic effect in the hot plate test (p < 0.0001) compared with the sham control mice (Figure 1). JZL184 and MJN110 reversed CCI-induced bilateral mechanical allodynia in dose-related and time-dependent manners (ipsilateral paw: Figure 1A; complete time course of each dose shown in Supplemental Figures 1A and 1B for ipsilateral paw; and contralateral paw: Figure 1B; complete time course for each dose shown in Supplemental Figures 2A and 2B). JZL184 produced maximal anti-allodynic effects at 3 h in ipsilateral [F(3,27) = 12.8, p < 0.0001] and contralateral [F(3,27) = 18.4, p < 0.0001] paws. MJN110 produced maximal anti-allodynic effects at 1 h in ipsilateral [F(5,41) = 16.6, p < 0.001] and contralateral [F(5,41) = 34.3, p < 0.001] paws. The respective ED$_{50}$ (95% CL) values for MJN110 and JZL184 at their optimal time points were 0.43 (0.30-0.63) mg/kg and 17.8 (11.6-27.4) mg/kg. The potency ratio (95% CL) for MJN110 versus JZL184 was 42.7 (24.6-82.9). Neither MAGL inhibitor altered paw withdrawal thresholds in sham mice at any time point (Supplemental Figures 1 and 2).

Each MAGL inhibitor significantly reversed CCI-induced thermal hyperalgesia in dose-related and time-dependent manners (Figure 1C; Supplemental Figures 3A and B). Prior to injection of vehicle or drug, all CCI mice displayed comparable levels of thermal hyperalgesia (p = 0.9). JZL184 [F(3,27) = 8.11, p < 0.05] and MJN110 [F(5,41) = 3.72, p < 0.05] significantly reversed thermal hyperalgesia at 3 h. Neither drug altered hot plate latencies in sham mice (Supplemental Figure 3).

To assess the involvement of CB$_1$ and CB$_2$ receptors in the anti-allodynic and anti-thermal hyperalgesic actions of JZL184 (40 mg/kg; Figure 2A and 2C; Supplemental Figure 4A)
and MJN110 (1.25 mg/kg; Figure 2B and 2D; Supplemental Figure 4B), mice were pretreated with rimonabant (3 mg/kg) or SR144528 (3 mg/kg). JZL184 F(8,48) = 28.1; p < 0.0001 and MJN110 [F(8,48) = 8.26; p < 0.0001] significantly reversed CCI-induced allodynia. Rimonabant blocked the anti-allodynic effects of each MAGL inhibitor (JZL184: p < 0.0001; and MJN110: p < 0.0001). Similarly, SR144528 prevented the anti-allodynic effects of each inhibitor (JZL184: p < 0.0001; and MJN110: p < 0.05). Following allodynia testing, the mice were tested for thermal hyperalgesia in the hot plate test. Again, JZL184 [F(8,48) = 10.9; p < 0.0001] and MJN110 [F(8,48) = 24.6; p < 0.0001] produced significant anti-thermal hyperalgesic effects. Rimonabant significantly reduced the anti-hyperalgesic effects of JZL184 (p < 0.001) and MJN110 (p < 0.001). In contrast, SR144528 did not antagonize the anti-thermal hyperalgesic effects of JZL184 (p = 0.5) or MJN110 (p = 0.6). Rimonabant and SR144528 alone did not alter thermal responses or paw withdrawal thresholds in sham or CCI mice at any time point.

**Evaluation of MAGL inhibitors in the drug discrimination paradigm**

The training dose of CP55,940 fully generalized for itself, while control injections of vehicle did not produce responding on the CP55,940 aperture (Figure 3A). Each MAGL inhibitor increased responding on the aperture associated with CP55,940 (0.1 mg/kg), but with different potencies (Figure 3A). JZL184 [F(4,27) = 9.57, p < 0.001; ED\textsubscript{50} (95% CL) = 24.9 (14.6-42.5) mg/kg] and MJN110 [F(4,32) = 40.3, p < 0.001; ED\textsubscript{50} (95% CL) = 0.84 (0.69-1.02) mg/kg] fully and dose-dependently substituted for CP55,940. MJN110 was 30.4 (18.9-47.6) [potency ratio (95% CL)] more potent than JZL184 in substituting for CP55,940. JZL184 did not alter response rates, but 1.25 and 2.5 mg/kg MJN110 significantly increased rates of responding [F(4,32) = 10.1, p < 0.001]. Rimonabant (1 mg/kg) significantly blocked substitution of MJN110 (2.5 mg/kg) and partial substitution of JZL184 (40 mg/kg) [F(3,13) = 42.9, p < 0.0001].
Rimonabant also significantly decreased response rates regardless of inhibitor treatment \( F(1,13) = 87.3, p < 0.0001 \).

**Locomotor Activity**

As shown in Figure 4, JZL184, and MJN110 differentially affected locomotor activity (see Supplementary Figure 5 for time course data). JZL184 (16 and 40 mg/kg) produced profound decreases in distance travelled \( F(3,30) = 25.0, p < 0.0001 \) and mobility time \( F(3,30) = 52.8, p < 0.0001 \), but did not alter running speed at any dose \( p = 0.08 \). MJN110 significantly increased running speed \( F(3,30) = 9.13, p < 0.001 \) and concomitantly increased the distance travelled \( F(3,30) = 5.6, p < 0.01 \), but did not affect mobility time \( p = 0.5 \). Significant increases in running speed were observed following 1.25 mg/kg and 2.5 mg/kg MJN110 and distance travelled was significantly increased by 1.25 mg/kg MJN110.

**Assessment of acute cannabimimetic effects of MJN110**

MJN110 (5 mg/kg) produced a small, but significant, increase in the tail withdrawal latency \( F(1,11) = 5.43, p < 0.05 \) from 1.25 ± 0.09 s before injection to 3.85 ± 1.12 s 2 h post-injection, when this effect was most pronounced. MJN110 did not elicit cataleptic \( p = 0.4 \) or hypothermic \( p = 0.8 \) effects (Supplementary Figure 6).

**Evaluation of brain endocannabinoid levels following MAGL inhibitor treatment**

The effects of acute administration of each MAGL inhibitor on 2-AG, arachidonic acid, and AEA levels in whole brain 3 h after drug administration are shown in Figure 5. JZL184 \( F(3,25) = 40.1, p < 0.001 \) and MJN110 \( F(3,25) = 41.5, p < 0.001 \) significantly increased 2-AG brain levels. Each of these inhibitors concomitantly decreased whole brain arachidonic acid levels \( JZ184: F(3,25) = 7.9, p < 0.001; MJN110: F(3,25) = 12.4, p < 0.001 \). 2-AG and arachidonic acid brains levels were negatively correlated for both JZL184 \( r = -0.98, p < 0.05 \)
and MJN110 ($r = -0.97$, $p < 0.05$). In contrast, neither of the inhibitors significantly affected brain levels of AEA, OEA, or PEA (Supplementary Figure 7).
Discussion

The primary objective of the present study was to evaluate the impact of MAGL inhibition in reducing neuropathic pain versus eliciting common cannabimimetic side effects. Here, we report that two MAGL inhibitors, JZL184 and MJN110, significantly reversed mechanical allodynia and thermal hyperalgesia in the CCI model of neuropathic pain. These effects were associated with elevated 2-AG and decreased free arachidonic acid in whole brain. However, these inhibitors produced differential effects on locomotor activity at doses that produced full inhibition of MAGL.

Each MAGL inhibitor reversed CCI-induced bilateral allodynia and thermal hyperalgesia. MJN110 was approximately 42 fold more potent than JZL184. The onset of the effects for each drug was 1 h, but MJN110 had a longer duration of action than JZL184. Pharmacokinetic differences between these drugs may account for the quicker onset of peak antinociceptive effects of MJN110 than the maximal effects of JZL184. The anti-allodynic effects of each inhibitor are likely mediated by 2-AG at cannabinoid receptors, as cannabinoid receptor antagonists blocked these anti-allodynic effects. However, given that 2-AG is a major source of arachidonic acid synthesis in brain (Nomura et al., 2011), consequences related to reductions in this lipid may play a contributory role. For example, MAGL inhibition results in decreased levels of prostaglandins (Normura et al., 2011), which are known to play an important role in inflammatory processes (Vane, 1971). In contrast, neither of the MAGL inhibitors increased levels AEA, PEA, or OEA in brain.

In the present study, the anti-allodynic effects of both JZL184 and MJN110 required activation of CB1 and CB2 receptors. Likewise, other reports have demonstrated that both cannabinoid receptors play a necessary role in the antinociceptive effects of JZL184 in neuropathic pain models (Woodhams et al., 2012; Guindon et al., 2013). The current findings demonstrating that CB2 receptors play a necessary role in the mediation of the anti-allodynic
effects of the MAGL inhibitors are divergent with previous mouse CCI studies from our laboratory in which the anti-allodynic effects of JZL184 and KML29 (Kinsey et al., 2009; Kinsey et al., 2013; Ignatowska-Jankowska et al., 2014) were mediated by CB1 receptors, while CB2 receptors did not play a necessary role. A key methodological difference between our previous and present studies is the type of suture used to ligate the sciatic nerve. The previous studies utilized silk sutures that elicited unilateral allodynia of the nerve-injured paw (Kinsey et al., 2009; Kinsey et al., 2010; Kinsey et al., 2011; Kinsey et al., 2013), while in the present study, we utilized chromic gut suture that led to a bilateral alldynia. Reports of CCI-induced allodynia in rodents vary between unilateral and bilateral allodynia depending on the use of silk or chromic gut suture material, respectively (Paulson et al., 2000; Milligan et al., 2006; Jancalek et al., 2010). Accordingly, the bilateral alldynia resulting from chromic gut suture may have elicited an increased inflammatory response that was ameliorated by 2-AG stimulation of CB2 receptors. Indeed, CB2 receptor stimulation has been found to reduce levels of proinflammatory cytokines and secondary signaling molecules associated with neuropathic pain as well as increase levels of the anti-inflammatory cytokine IL-10 (Wilkerson et al., 2012).

In contrast to the necessary role of both cannabinoid receptors in mediating the anti-allodynic effects of JZL184 and MJN110, reversal of CCI-induced thermal hyperalgesia required only the CB1 receptor, while the CB2 receptor was dispensable. Thus, the CB2 receptor plays distinct roles in these two hallmark components of neuropathic pain, which is likely related to different mechanisms subserving the two pain modalities. Thermal hypersensitivity is mediated by lightly myelinated a-δ and myelinated C fibers, while mechanical allodynia is mediated by heavily myelinated a-β fibers (Woolf and Mannion, 1999; Costigan et al., 2009). A disconnect between sensory and thermal alterations in pain modalities has been previously reported in a rat model of peripheral IL-1β induced allodynia and thermal hyperalgesia. In these studies a protein kinase A inhibitor fully blocked alldynia, but did not alter thermal hyperalgesic responses (Kim
et al., 2014). Likewise, in several animal models of pain, TRPV-1 antagonists are sufficient to block or reverse thermal hyperalgesia without producing anti-allodynic effects (Urano et al., 2012; Kim et al., 2014). Therefore, the underpinnings of observed behavioral measurements of allodynia and thermal hyperalgesia are complex and warrant further studies.

Each MAGL inhibitor fully substituted for CP55,940 in the drug discrimination assay, which is in contrast to the effects of MAGL inhibitors in previously reported drug discrimination studies. Specifically, the selective MAGL inhibitor KML29 did not substitute for THC (Ignatowska-Jankowska et al., 2014), which indicates differences between discriminative stimulus effects of those compounds. Moreover, JZL184 only partially substituted for THC (Long et al., 2009b, Wiley et al., 2014); however, the maximum doses of JZL184 tested in these respective prior studies were 40 mg/kg and 30 mg/kg. Higher doses were not assessed in those studies because of solubility constraints. However, in the present study, we found that administration of 100 mg/kg JZL184 in a double volume suspension fully substituted for CP55,940. The finding that MJN110 was more potent than JZL184, is consistent with its lower IC$_{50}$ for MAGL inhibition (Chang et al., 2012; Niphakis et al., 2013). For each inhibitor, substitution/partial substitution occurred at doses that also elicited significant increases of brain 2-AG, but not AEA, and in each case, interoceptive effects were blocked by rimonabant. Although brain endocannabinoid levels were not assessed following 100 mg/kg JZL184, these results are consistent with the notion that the CP55,940-like discriminative stimulus effects of these MAGL inhibitors were mediated by 2-AG activation of the CB$_1$ receptor.

JZL184 has previously been found to produce a subset of effects in the tetrad assay (Long et al., 2009a; Long et al., 2009b; Ignatowska-Jankowska et al., 2014), a paradigm that has been used to infer cannabimimetic activity (Little et al., 1988). It is important to note that cannabinoid receptor agonists are generally more potent in the drug discrimination paradigm than the tetrad assay (Wiley et al., 1995a; Wiley et al., 1995b), which is consistent with the current study.
Specifically, JZL184 produced a small increase in the tail withdrawal latencies (Long et al., 2009) and decreases in spontaneous locomotor activity. Likewise, here we found that MJN110 produced a low magnitude of effect in the warm water tail withdrawal test. Interestingly, MJN110 did not produce hypomotility, but significantly increased running speed during the first 10 min of the test, which led to concomitant increases in distance travelled. Moreover, MJN110 did not produce hypothermic effects. The observations that MJN110 increased running speed are reminiscent with studies showing that low doses of THC and CP55,940 stimulate locomotor behavior (Anderson et al., 1975; Evans et al., 1976; McGregor et al., 1996).

As JZL184 and MJN110 elevated 2-AG in whole brain to a similar degree, the cause of the differential effects of these drugs on locomotor activity remains an open question. One possibility is related to their off-target effects. Specifically, JZL184 inhibits FAAH, albeit with considerable less potency than its inhibition of MAGL (Long et al., 2009b). Nonetheless, acute administration of JZL184 does not lead to an increase in brain AEA levels. MJN110 also inhibits alpha/beta hydrolase domain 6 (ABHD6) (Niphakis et al., 2013), a serine hydrolase that is postsynaptically located and known to metabolize 2-AG (Blankman et al., 2007). Another possibility is that pharmacokinetic differences may have affected absorption and distribution of each drug. Accordingly, these drugs may have led to differential rates of 2-AG elevation in specific neural circuits that mediate locomotor activity.

In conclusion, MAGL inhibitors reliably reverse nociceptive behaviors in the CCI model of neuropathic pain with reduced cannabimimetic side effects. While both JZL184 and MJN110 produced cannabimimetic interoceptive effects in the drug discrimination paradigm, they produced opposing effects on locomotor behavior. In particular, MJN110 reversed nociceptive behavior in the CCI model of neuropathic pain with a two fold greater potency than required to substitute for CP55,940 in the drug discrimination paradigm, and did not produce hypomotility, catalepsy, or hypothermia. More generally, the present study taken together with previous
work (Long et al., 2009b; Kinsey et al., 2009; Schlosburg et al., 2010; Guindon et al., 2011; Guindon et al., 2013; Kinsey et al., 2013; Ignatowska-Jankowska, 2014) suggests that MAGL inhibition represents a promising strategy to alleviate neuropathic pain with decreased incidence of cannabimimetic side effects.
Authors Contributions

Participated in research design: Ignatowska-Jankowska, Wilkerson, Mustafa, Abdullah, Cravatt, Lichtman

Conducted experiments: Ignatowska-Jankowska, Wilkerson, Mustafa, Abdullah

Contributed new reagents or analytic tools: Niphakis, Cravatt

Performed data analysis: Ignatowska-Jankowska, Wilkerson

Wrote or contributed to the writing of the manuscript: Ignatowska-Jankowska, Wilkerson, Wiley, Cravatt, Lichtman
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Footnotes

Authors Bogna Ignatowska-Jankowska and Jenny L. Wilkerson contributed equally to this work.

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Figure Legends

Figure 1. JZL184 and MJN110 reverse CCI-induced allodynia and thermal hyperalgesia in dose-related fashions at their optimal time points, which were 3 h and 1 h, respectively. Von Frey filaments were used to test mechanical allodynia in the ipsilateral paw (A) and contralateral paw (B). Immediately following allodynia assessment, thermal hyperalgesia was assessed in the hot plate assay (C). Filled symbols denote significance from CCI + vehicle. Data reflect mean ± SEM, n = 5-7 mice per group.

Figure 2. The anti-allodynic and anti-thermal hyperalgesic effects of MAGL inhibitors are differentially altered by blockade of CB1 and CB2 receptors. Rimonabant (SR1) and SR144528 (SR2) block anti-allodynic effects of (A) JZL184 (40 mg/kg), and (B) MJN110 (1.25 mg/kg). (C) Rimonabant (3 mg/kg), but not SR144528 (3 mg/kg), blocks anti-thermal hyperalgesic effects of 40 mg/kg i.p. JZL184. (D) Rimonabant, but not SR144528, blocks anti-thermal hyperalgesic effects of MJN110 (1.25 mg/kg). Ipsilateral paw data only are displayed for A,B. *** p<0.0001, ** p<0.005, * p < 0.05 vs. CCI vehicle+vehicle, ### p<0.0001, ## p<0.001, # p<0.05 vs. CCI vehicle+MAGL inhibitor. Data reflect mean ± SEM, n = 5-7 mice per group. Horizontal lines spanning across the ordinates reflect sham values (top lines) and CCI values (bottom lines).

Figure 3. MAGL inhibition with JZL184 (4, 16, 40, 100 mg/kg, n = 7 mice per dose), and MJN110 (0.25, 0.5, 1.25, 2.5 mg/kg, n = 9 mice per dose) produced dose-related substitution for the potent, synthetic cannabinoid receptor agonist CP55,940 in mice trained to discriminate CP55,940 from vehicle (A). Rimonabant (SR1, 1 mg/kg) blocked substitution produced by JZL184 (40 mg/kg), and MJN110 (2.5 mg/kg) (A). JZL184 did not produce significant changes.
in rates of responding, while MJN110 at high doses increased response rates (B). Rimonabant produced significant decrease in rates of responding. Significant changes as compared to vehicle-treated mice are denoted as filled symbols (p < 0.05). Data shown as mean ± SEM.

Figure 4. The MAGL inhibitors JZL184 and MJN110 elicit bidirectional effects on locomotor activity of mice. JZL184 (16 and 40 mg/kg) produced significant decreases in distance travelled (A) and time spent mobile (B), but did not affect average running speed (C). MJN110 (1.25 and 2.5 mg/kg) increased running speed (C) and distance travelled (A), while time mobile was unaffected (B). Filled symbols denote significance from vehicle (p < 0.05). Data shown as mean ± SEM, n = 7-13.

Figure 5. JZL184 (4, 16, 40 mg/kg) and MJN110 (0.25, 1.25, 2.5, 5 mg/kg) produced significant increases in 2-arachidonoylglycerol (2-AG) concentration (A) and concomitant reductions in arachidonic acid (AA) levels (B) in the whole brain of mice. Significant changes as compared to vehicle-treated mice are marked with filled symbols (p < 0.01). Data shown as mean ± SEM, n = 7-8 mouse brains per group.
Figure 1

(A) Ipsilateral

(B) Contralateral

(C) Response Latency (s)
Figure 2
Figure 3
Figure 4

A. Distance travelled [m]

B. Time mobile [s]

C. Speed [m/s]

Legend:

- △ JZL184
- ○ MJN110

X-axis: dose [mg/kg]
Y-axis: vehicle, dose [mg/kg]
Figure 5
Supplementary methods

Drug discrimination

Mice were housed individually in clear plastic cages in a temperature-controlled (20–22 °C) vivarium. Water was available ad libitum except while the mice were in the operant chambers. Training and test sessions were conducted at similar times during the light phase of a 12 h light/dark cycle. Mice were maintained at 85–90% of free-feeding body mass, which was initially calculated based on free feeding body mass before start of deprivation at 11 weeks, and again following each free feeding period that occurred every six months. Eight standard mice operant conditioning chambers that were sound- and light-attenuated (MED Associates, St. Albans, VT) were used for behavioral training and testing. Each operant conditioning chamber (18 x 18 x 18 cm) was equipped with a house light, two nose poke apertures (left and right), and a recessed well centered between the two apertures. A sweetened pellet served as reinforcement and was delivered to the recessed well according to the reinforcement schedule. Fan motors provided ventilation and masking noise for each chamber. Houselights were illuminated during all operant sessions. A computer with Logic “1” interface and MED-PC software (MED Associates) was used to control schedule contingencies and to record data. Mice were trained to respond in one aperture following administration of 0.1 mg/kg CP55,940 and to respond in the opposite aperture following vehicle administration according to a FR10 schedule of reinforcement. Each incorrect response reset the response requirement. Daily injections were administered on a double alternation sequence of drug or vehicle (e.g., drug, drug, vehicle, vehicle). Daily 15-min training sessions were held Monday–Friday until the mice had met two criteria during nine of 10 consecutive sessions: (i) correct completion of the first FR10 (e.g., first 10 consecutive responses on condition appropriate aperture) and (ii) ≥80 % of the total condition appropriate responding. When these two criteria were met, acquisition of the discrimination was established and substitution testing began. Stimulus substitution tests were conducted on Tuesdays and Fridays during 15-min test sessions and training continued on all other days. Thus, test days allowed at least a 72 h washout period from the previous injection of the MAGL inhibitor. During test sessions, responses in either aperture delivered reinforcement according to an FR10 schedule. To qualify for testing, mice must have completed the first FR10 on the correct aperture and ≥80 % of the total condition-appropriate responding on the preceding drug and vehicle sessions.
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Triad assay

Catalepsy was assessed on a bar 0.7 cm in diameter placed 4.5 cm off of the ground. The mouse was placed with its front paws on the bar and a timer (Timer #1) was started. A second timer (Timer #2) was turned on only when the mouse was immobile on the bar, with the exception of respiratory movements. If the mouse moved off the bar, it was placed back on in the original position. The assay was stopped when either Timer #1 reached 60 s, or after the fourth time the mouse moved off the bar, and the cataleptic time was scored as the amount of time on Timer #2. Nociception was then assessed in the tail immersion assay. The mouse was placed head first into a small bag fabricated from absorbent under pads (VWR Scientific Products; 4 cm diameter, 11 cm length) with the tail out of the bag. Each mouse was hand-held and 1 cm of the tail was submerged into a 52 °C water bath. The latency for the mouse to withdraw its tail within a 10 s cut off time was scored. Rectal temperature was assessed by inserting a thermocouple probe 2 cm into the rectum, and temperature was determined by telethermometer (BAT-10 Multipurpose Thermometer, Clifton, NJ, USA). Locomotor activity was assessed 120 min after treatment, for a 60 min period in a Plexiglas cage (42.7 x 21.0 x 20.4 cm) and Anymaze (Stoelting, Wood Dale, Illinois) software was used to determine the percentage of time spent immobile, mean speed and distance travelled.

Measurement of brain endocannabinoid levels

On the day of processing, tissues were weighed and homogenized with 1.4 ml of chloroform/methanol (2:1 v/v containing 0.0348 g of phenylmethylsulfonyl fluoride/ml) after the addition of internal standards to each sample (4 pmol of AEA-d8, 1 nmol of 2-AG-d8, 3.3 nmol PEA-d4, 3 nmol OEA-d4 and 1 nmol AA-d8, Cayman Chemical). Homogenates were then mixed with 0.3 ml of 0.73% w/v NaCl, vortexed, and then centrifuged for 10 min at 3220 × g (4 °C). The aqueous phase plus debris were collected and extracted two more times with 0.8 ml of chloroform. The organic phases from the three extractions were pooled and the organic solvents were evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml of chloroform and mixed with 1 ml of ice-cold acetone. The mixtures were then centrifuged for 5 min at 1811 × g and 4 °C to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml of methanol and placed in autosample vials for analysis.

Liquid chromatography-tandem mass spectrometry with an electrospray ionization source was used to identify and quantify the 2-AG, arachidonic acid, AEA, OEA, and PEA. The chromatographic
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Separation was performed using a Discovery HS C18, 2.1 × 150 mm, 3 μm (Supelco, Bellefonte, PA). The mobile phase consisted of water/methanol (10:90) with 0.1% ammonium acetate and 0.1% formic acid. The following ions were monitored in a multiple-reaction-monitoring positive mode: (348 > 62) and (348 > 91) for AEA; (356 > 62) for AEA-d8; (379 > 287) and (379 > 269) for 2-AG; (387 > 96) for 2-AG-d8; (300 > 62) and (300 > 283) for PEA; (304 > 62) for PEA-d4; (326 > 62) and (326 > 309) for OEA; and (330 > 66) for OEA-d4 and in negative mode: (303 > 259) and (303 > 59) for arachidonic acid and (311 > 267) for arachidonic acid-d8. A calibration curve was constructed for each assay based on linear regression with use of the peak area ratios of the calibrators. The extracted standard curves ranged from 0.039 to 40 pmol for AEA and from 0.0625 to 64 nmol for 2-AG, from 0.039 nmol to 1.25 for PEA and OEA and from 1 nmol to 32 nmol for arachidonic acid.
Supplementary results

**MAGL Inhibitors reverse CCI-induced mechanical allodynia and thermal hyperalgesia**

JZL184 and MJN110 reversed CCI-induced bilateral mechanical allodynia in dose-related and time-dependent manners (ipsilateral paw: Figure 1A-C; complete time course of each dose shown in Supplemental Figures 1A-C for ipsilateral paw; and Supplemental Figures 2A-C for contralateral paw). The effect of JZL184 persisted for 5 h in the ipsilateral \[F(3,27) = 3.50, p < 0.05\] and contralateral \[F(3,27) = 6.90, p < 0.001\] paws. MJN110 had a longer overall duration of action than JZL184. Anti-allodynic effects were detected at 1 h post administration in ipsilateral \[F(5,41) = 16.6, p < 0.001\] and contralateral \[F(5,41) = 34.3, p < 0.001\] paws, and significant differences were still observed by 24 h in either ipsilateral paws. By 24 h post-injection, MJN110 still produced anti-allodynic effects \[F(5,41) = 8.76, p < 0.001\] and \[F(5,41) = 6.955, p < 0.001\]. Also, neither MAGL inhibitor altered paw withdrawal thresholds in sham mice at any time point (Supplemental Figures 1 and 2).

Each MAGL inhibitor also significantly reversed CCI-induced thermal hyperalgesia in dose-related and time-dependent manners (Figure1D-F; Supplemental Figures 3A-C). Prior to injection of vehicle or drug, all CCI mice displayed comparable levels of thermal hyperalgesia \[p = 0.9\]. JZL184 significantly reversed thermal hyperalgesia to varying degrees at 1 h \[F(3,27) = 3.75, p < 0.05\] and 5 h \[F(3,27) = 13.6, p < 0.0001\]. MJN110 produced anti-thermal hyperalgesic effects at 1 h \[F(5,41) = 3.72, p < 0.05\], 5 h \[F(5,41) = 6.96, p < 0.05\], and 24 h \[F(5,41) = 5.74, p < 0.05\]. Neither drug altered hot plate latencies in sham mice (Supplemental Figure 3).

**Locomotor Activity**

JZL184 and MJN110 produced differential effects on locomotor activity (Supplementary Figure 5). JZL184 (16 and 40 mg/kg) produced profound decreases in locomotor activity, as reflected by reductions in distance travelled \[dose x time interaction: F(15,150) = 2.8; p < 0.001\] and time spent mobile \[dose x time interaction: F(15,150) = 5.6; p < 0.0001\]. The mean running speed of animals was not affected by JZL184 at any dose \(p = 0.8\). MJN110 (1.25 and 2.5 mg/kg), produced significant increases in running speed \[main effect of dose: F(5,150) = 6.7; p < 0.01, dose x time interaction: p = 0.08\] and distance travelled \[dose x time interaction: F(5,150) = 2.19; p < 0.01\], but did not affect mobility time. No other changes in locomotor behavior were found following MJN110 administration (Supplementary Figure 5 G, H, I).
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Supplemental Figure 1: MAGL inhibitors reverse CCI-induced mechanical allodynia in the ipsilateral paw in a dose- and time-related manner. (A) Dose response and time course of JZL184 (4, 16, 40 mg/kg i.p.) reversal of CCI-induced allodynia. (B) Dose response and time course of MJN110 (0.125, 0.25, 0.5, 1.25, 5 mg/kg i.p) reversal of CCI-induced allodynia. *** p < 0.0001; ** p < 0.001; * p < 0.05 vs. CCI vehicle. Data reflect mean ± SEM, n = 5-7 mice per group. Horizontal lines spanning across the ordinates reflect sham von Frey values (top lines).
Supplemental Figure 2: MAGL inhibitors reverse CCI-induced mechanical allodynia in the contralateral paw in a dose- and time-related manner. (A) Dose response and time course of JZL184 (4, 16, 40 mg/kg i.p.) reversal of CCI-induced allodynia in contralateral paws. (B) Dose response and time course of MJN110 (0.125, 0.25, 0.5, 1.25, 5 mg/kg i.p) reversal of CCI-induced allodynia in contralateral hindpaws. *** p < 0.0001; ** p < 0.001; * p < 0.05 vs. CCI vehicle. Data reflect mean ± SEM, n=5-7 mice per group. Horizontal lines spanning across the ordinates reflect sham von Frey values (top lines).
Supplemental Figure 3: MAGL inhibitors reverse CCI-induced thermal hyperalgesia in a dose- and time-related manner. (A) Dose response and time course of JZL184 (4, 16, 40 mg/kg i.p.) in reversal of CCI-induced thermal hyperalgesia. (B) Dose response and time course of MJN110 (0.125, 0.25, 0.5, 1.25, 5 mg/kg i.p) in reversal of CCI-induced thermal hyperalgesia. *** p < 0.0001; ** p < 0.001; * p < 0.05 vs. CCI vehicle. Data reflect mean ± SEM, n = 5-7 mice per group. Horizontal lines spanning across the ordinates reflect sham values (top lines).
Supplemental Figure 4: Evaluation of CB1 and CB2 receptors in the anti-allodynic effects of MAGL inhibitors on the contralateral paw. Rimonabant (SR1) and SR144528 (SR2) block anti-allodynic effects of (A) JZL184 (40 mg/kg), (B) MJN110 (1.25 mg/kg). *** p < 0.0001 vs. CCI vehicle+vehicle; # p<0.05; ## p < 0.001, ### p < 0.001 vs. CCI vehicle+MAGL inhibitor. Data reflect mean ± SEM, n = 5-7 mice per group. Horizontal lines spanning across the ordinates reflect sham von Frey values (top lines) and CCI von Frey values (bottom lines).
**Selective monoacylglycerol lipase inhibitors: Antinociceptive vs. cannabimimetic effects in mice**

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Supplementary Figure 5. Time course of locomotor effects of JZL184 and MJN110. JZL184 (16 and 40 mg/kg) produced profound decrease in distance travelled (A) and time spent mobile (C), but did not affect average running speed MJN110 at 1.25 and 2.5 mg/kg also increased speed (E) and distance travelled (D) within first 10 minutes of test, while time mobile was unaffected (F). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 vehicle vs high dose; #p < 0.05, ##p < 0.01; ###p < 0.001; ####p < 0.0001 vehicle vs medium dose of inhibitor. Data shown as mean ± SEM, n = 13 in vehicle-treated group and n = 7 mice in each drug-treated group.
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Supplementary figure 6. MJN110 (5 mg/kg) produced significant antinociceptive effect in the tail withdrawal test at 2 h following treatment (A) without occurrence of hypothermia (B) or catalepsy (C). Data shown as mean ± SEM, n = 6-7 per group,*p<0.05.
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Supplementary Figure 7. Neither JZL184 (4, 16, 40 mg/kg) nor MJN110 (0.25, 1.25, 2.5 mg/kg) affected (A) anandamide (AEA) (p = 0.6, p = 0.7, p = 0.1), (B) oleylethanolamide (OEA) (p = 0.7, p = 0.7, p = 0.2), or (C) palmitoyloethanolamide (PEA) (p = 0.7, p = 0.7, p = 0.3) concentrations in the whole brain of mice. Brains were harvested 3 h after i.p. injections. Data shown as mean ± SEM, n = 7-8 per group.