The Selective Monoacylglycerol Lipase Inhibitor MJN110 Produces Opioid-Sparing Effects in a Mouse Neuropathic Pain Model


Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, Virginia (J.L.W., T.W.G., M.A.M., R.A.A., J.L.P., W.L.D., H.A., M.L.B., L.E.W., A.H.L.); and The Skaggs Institute for Chemical Biology and Department of Chemical Physiology, The Scripps Research Institute, La Jolla, California (M.J.N., B.F.C.)

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ABSTRACT

Serious clinical liabilities associated with the prescription of opiates for pain control include constipation, respiratory depression, pruritus, tolerance, abuse, and addiction. A recognized strategy to circumvent these side effects is to combine opioids with other antinociceptive agents. The combination of opiates with the primary active constituent of cannabis (Δ9-tetrahydrocannabinol) produces enhanced antinociceptive actions, suggesting that cannabinoid receptor agonists can be opioid sparing. Here, we tested whether elevating the endogenous cannabinoid 2-arachidonoylglycerol through the inhibition of its primary hydrolytic enzyme monoacylglycerol lipase (MAGL), will produce opioid-sparing effects in the mouse chronic constriction injury (CCI) of the sciatic nerve model of neuropathic pain. The dose-response relationships of i.p. administration of morphine and the selective MAGL inhibitor 2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate (MJN110) were tested alone and in combination at equieffective doses for reversal of CCI-induced mechanical allodynia and thermal hyperalgesia. The respective ED50 doses (95% confidence interval) of morphine and MJN110 were 2.4 (1.9–3.0) mg/kg and 0.43 (0.23–0.79) mg/kg. Isobolographic analysis of these drugs in combination revealed synergistic antiallodynic effects. Acute antinociceptive effects of the combination of morphine and MJN110 required μ-opioid, CB1, and CB2 receptors. This combination did not reduce gastric motility or produce subjective cannabimimetic effects in the drug discrimination assay. Importantly, combinations of MJN110 and morphine given repeatedly (i.e., twice a day for 6 days) continued to produce antiallodynic effects with no evidence of tolerance. Taken together, these findings suggest that MAGL inhibition produces opiate-sparing events with diminished tolerance, constipation, and cannabimimetic side effects.

Introduction

Opiates are widely accepted for the treatment of chronic pathologic pain (Ballantyne and Mao, 2003); however, their untoward side effects including constipation, pruritus, and respiratory depression (Campbell et al., 2015) diminish their clinical utility. Additionally, the long-term use of opiates for the management of chronic pain leads to tolerance, dependence, and carries a high abuse potential (Thomas et al., 2015). The combination of opiates with other classes of analgesics represents a promising strategy to minimize these deleterious side effects. In particular, accounts of combining opiates and cannabinoids for the treatment of pain-related injuries dates back to ancient Greece, where archaeological evidence describes the use of a cannabis and opioid salve for athletic injury (Bartels et al., 2006). In current times, preclinical studies have established that the combination of opiates and cannabinoids produce enhanced antinociceptive effects. Coadministration of the primary psychoactive component of marijuana, Δ9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam, 1964), and morphine produces synergistic antinociceptive effects in acute pain tests (Cichewicz and Welch, 2003) and in the rat Freund's complete adjuvant-induced arthritic model (Cox et al., 2007). Additionally, the potent synthetic cannabinoid receptor agonist CP55,940 produces a leftward shift of the morphine dose response curve in the acetic acid abdominal stretching pain assay (Miller et al., 2012). Human studies have also shown that treatment with cannabinoids enhances the analgesic effects of opioids in...
patients suffering from advanced cancer pain (Johnson et al., 2010, 2013).

THC has been well characterized to exert its actions through activation of CB1 (Devane et al., 1988; Matsuda et al., 1990; Pertwee, 1997) and CB2 (Munro et al., 1993; Pertwee et al., 2007) receptors. The CB1 receptor is responsible for the multitude of the behavioral actions of THC and is predominately expressed in presynaptic neurons (Huang et al., 2001; Szabo and Schlicker, 2005), especially GABAergic interneurons (Kantha et al., 1999) in the brain, while the CB2 receptor is highly expressed in immune cells (Galiègue et al., 1995; Pettit et al., 1996; Schatz et al., 1997; Carayon et al., 1998). The endocannabinoid ligands 2-arachidonoylglycerol (2-AG) (Sugiura et al., 1995; Mechoulam and Deutsch, 2005) and N-arachidonylethanolamine (AEA) (Felder et al., 1996; Martin et al., 1999) are enzymatically regulated, bind both CB1 and CB2 receptors, and play important roles in many physiologic functions. The actions of these endocannabinoids are short-lived because of rapid degradation by their respective primary hydrolytic enzymes, monoacylglycerol lipase (MAGL) (Dinh et al., 2002; Long et al., 2009) and fatty acid amide hydrolase (Crawatt et al., 1996, 2001; Kathuria et al., 2003). Elevating these endogenous cannabinoids through the inhibition of their catabolic enzymes has broad implications on a wide range of physiologic processes, specifically with regard to neuronal and immune functioning, both of which are critical mediators of pathologic pain (Calignano et al., 1998; Russo et al., 2007; Kinsey et al., 2009, 2010, 2011; Guindon et al., 2011, 2013). Notably, the combination of the fatty acid amide hydrolase inhibitor URB597 and morphine produced additive antinociceptive effects in the acetic acid abdominal stretching assay (Miller et al., 2012). However, the antinociceptive effects of a MAGL inhibitor in combination with morphine have yet to be reported.

The present study tested whether increasing endogenous 2-AG, using the selective MAGL inhibitor 2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate (MJNI110), which possesses increased potency and selectivity over other MAGL inhibitors (Nipahki et al., 2013; Ignatowska-Jankowska et al., 2015), would enhance the antinociceptive effects of morphine in the chronic constriction injury (CCI) of the sciatic nerve model of neuropathic pain. We also used the selective receptor antagonists rimonabant, SR144528, and naloxone to examine the respective contributions of CB1, CB2, and μ-opioid receptors in mediating the observed antinociceptive effects. Since significant untoward side effects associated with opioids and cannabinoids could limit therapeutic utility when given in combination, we examined the consequences of dual administration of morphine and MJN110 on gastric motility, to infer opioid-induced constipation (Ross et al., 2008) and cannabimimetic interoceptive effects in mice trained to discriminate the potent cannabinoid receptor agonist CP55,940 from vehicle in the drug discrimination paradigm (Long et al., 2009; Walentyi et al., 2013; Ignatowska-Jankowska et al., 2014). Additionally, because the antinociceptive effects of morphine (Cichewicz and Welch, 2003; Smith et al., 2007) and MAGL inhibitors (Schlosburg et al., 2010) undergo tolerance after repeated administration, we examined the consequences of repeated injections of MJN110 and morphine given in combination in the CCI model of neuropathic pain. Finally, we quantified levels of endocannabinoids in brain and spinal cord following acute or repeated administration of the combination of morphine and MJN110 in mice subjected to CCI.

Materials and 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-hour light/12-hour dark cycle (6:00 AM on/6:00 PM off) in a temperature-controlled (20–22°C) and humidity-controlled (55% ± 10%) Association for Assessment and Accreditation of Laboratory Animal Care International (Frederick, MD) 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. 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.

Drugs. MJN110 was synthesized in the Cravatt Laboratory at the Scripps Research Institute as described previously (Chang et al., 2012, 2013). The CB1 receptor antagonist rimonabant [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-1-methyl-1H-pyrazole-3-carboxamide-HCl; the CB2 receptor antagonist SR144528 [5-(4-chloro-3-methylphenyl)-1-(4-methylphenyl)ethyl]-N-[1S,2S,4R)-cis-3-[2-hydroxy-4,1-dimethylthiophenyl]phenyl-trans-4-(3-hydroxypropyl)cyclohexanol]; and morphine sulfate were generously supplied from the National Institute on Drug Abuse (Bethesda, MD). Naloxone was purchased from Sigma-Aldrich (St. Louis, MO). All drugs, except morphine, were dissolved in a vehicle solution consisting of a mixture of ethanol, alkamuls-620 (Sanofi-Aventis, Bridgewater, NJ), and saline (0.9% NaCl) in a 1:1:8 ratio. Morphine sulfate was dissolved in sterile saline (Hospira Inc., Lake Forest, IL). Each drug was given via the i.p. route of administration. All drugs were administered in a volume of 10 μl/kg body mass. All experiments employed a 1-hour absorption period for MJN110, based on previous studies (Ignatowska-Jankowska et al., 2015), and a 30-minute drug absorption period for all other drugs. In studies in which MJN110 and morphine were coadministered, MJN110 was given at 0 minutes, morphine was administered at 30 minutes, and behavioral testing commenced at 60 minutes.

Chronic Constriction Injury Surgery. Following baseline behavioral assessment, the surgical procedure for chronic constriction of the sciatic nerve was completed as previously described (Bennett and Xie, 1988), but modified for the mouse (Ignatowska-Jankowska et al., 2015). In brief, the mice were anesthetized with isoflurane (induction 5% by volume, 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 three segments of 5-0 chronic gut sutures (Ethicon, Somerville, NJ). Sham surgery was identical to CCI surgery, but without the loose nerve ligation. The overlying muscle was closed with 4-0 sterile silk suture (Ethicon), and animals recovered from anesthesia within approximately 5 minutes. Mice were randomly assigned to either the CCI or sham surgical group. Mice in both groups were reassessed for allodynia and thermal hyperalgesia, as described subsequently. Subjects were tested with drug or vehicle between 5 and 18 days after surgery.

Behavioral Assessment of Nociceptive Behavior. Mechanical allodynia and thermal hyperalgesia were used to assess nociceptive behavior following sham or CCI surgery (described previously). Prior to surgery, mice were habituated to the testing environment, and von Frey monofilaments (North Coast Medical, Morgan Hills, CA) were used to establish baseline responses to light mechanical touch and compared with post-surgery thresholds (Ignatowska-Jankowska et al., 2015). During allodynia testing, the mice were placed on top of a wire mesh screen, with spaces 0.5 mm apart and habituated for approximately 30 minutes 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 airflow. 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 seconds. Lifting, licking, or shaking the paw was considered a response. After completion of the 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-second cutoff time was used to avoid potential tissue damage (Garciá-Martínez et al., 2002). For each assay, testing was performed in a blinded fashion.

For repeated administration experiments, mice were given vehicle, 0.0818 mg/kg MJN110, 0.469 or 10 mg/kg morphine, or a combination of 0.0818 mg/kg MJN110 and 0.469 mg/kg morphine twice daily for 5.5 days. Then, mice underwent behavioral testing on day 6. The combination of 0.0818 mg/kg MJN110 and 0.469 mg/kg morphine produced significant antinoceceptive effects; however, when administered alone each reflected threshold doses.

**Extraction and Quantification of Endocannabinoids by Liquid Chromatography–Tandem Mass Spectrometry.** 2-AG, arachidonic acid (AA), AEA, palmitoylethanolamide (PEA), oleoylthanolamine (OEA), prostaglandin E2, and prostaglandin D2 levels of arachidonic acid (AA), AEA, palmitoylethanolamide (PEA), and oleoylthanolamine (OEA) were quantified from the spinal cord and whole brain of C57BL/6J mice after acute administration of 0.143 mg/kg MJN110, 0.821 mg/kg morphine, the combination of MJN110 and morphine, or 1:1:18 vehicle via i.p. injection. The tissues were collected upon completion of behavioral assessment and processed for quantification of 2-AG, AA, AEA, PEA, and OEA. Because equivalent doses of MJN110 and morphine in combination significantly attenuated CCI-induced allodynia and thermal hyperalgesia at 30 minutes after the final morphine injection, mice were euthanized via rapid decapitation (at 11:00 AM) at this time point. Spinal cords and brains were rapidly frozen in liquid nitrogen 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 the Supplemental Material for details.

**Gastric Motility Assay.** Mice were food deprived for 24 hours and were then given i.p. injection of vehicle, 10 mg/kg morphine, 0.821 mg/kg morphine (the calculated suprathereshold dose of the effective 25% dose of morphine to produce antiallodynia), 0.1432 mg/kg MJN110 (the calculated suprathereshold dose of the effective 25% dose of MJN110 to produce antiallodynia), or the combination of 0.821 mg/kg morphine plus 0.1432 mg/kg MJN110. Twenty minutes following the morphine or equivnlent vehicle injection the subjects were administered charcoal by oral gavage. Intestines were harvested 10 minutes postgavage, and the length of charcoal transit through the gut was measured relative to its total length, as previously described (Ross et al., 2008).

**Drug Discrimination.** A separate group of C57BL/6J male mice, trained to discriminate CP55,940 (0.1 mg/kg) from vehicle, was used to test whether vehicle, 5 mg/kg MJN110 (which fully blocks MAGL), 10 mg/kg morphine, 0.821 mg/kg morphine (the calculated suprathereshold dose of the effective 25% dose of morphine to produce antiallodynia), 0.1432 mg/kg MJN110 (the calculated suprathereshold dose of the effective 25% dose of MJN110 to produce antiallodynia), or the combination of 0.821 mg/kg morphine plus 0.1432 mg/kg MJN110. Twenty minutes following the morphine or equivalent vehicle injection the subjects were administered charcoal by oral gavage. Intestines were harvested 10 minutes postgavage, and the length of charcoal transit through the gut was measured relative to its total length, as previously described (Ross et al., 2008).

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Training and testing were conducted according to general procedures described previously (Long et al., 2009; Walenty et al., 2013; Ignatowska-Jankowska et al., 2014). The percentage of responses on the drug aperture and response rates was recorded for each test session. Mice were maintained at 85%–90% of free-feeding body mass by restricting daily ration of standard rodent chow. Eight standard mice operated 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 × 18 × 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 (14 mg sweetened pellets; Bio-Serv, Frenchtown, NJ) 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 a 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 fixed ratio schedule of reinforcement after every 10th response (FR10). Each incorrect response reset the response requirement. Data from five sessions were examined on a double alternation sequence of drug or vehicle (e.g., drug, drug, vehicle, vehicle). Daily 15-minute training sessions were held Monday to Friday until the mice had met two criteria during nine of 10 consecutive sessions: 1) correct completion of the first FR10 (e.g., first 10 consecutive responses on condition appropriate aperture) and 2) >80% of the total condition appropriate responding. When these two criteria were met, acquisition of the discrimination was established and substitution testing began. Fifteen-minute stimulus substitution tests were conducted no more than twice per week with at least 72 hours between test sessions. Training continued on other days. During test sessions, responses in either aperture delivered reinforcement according to the FR10 schedule. Testing required that mice completed the first FR10 on the correct aperture and >80% of the total condition-appropriate responding on the preceding drug and vehicle sessions. Control tests were then conducted with the training dose of the drug (0.1 mg/kg CP55,940) or vehicle. For substitution tests, MJN110, morphine sulfate, or effective doses of both compounds were administered before the test session. Full substitution for the training drug was defined as >80% of responses on the aperture paired with administration of the training drug. The range of 20%–80% of responses on the aperture paired with the training drug was considered as partial substitution, and <20% of responses on the aperture coupled with the training drug was considered as no substitution (Solinas et al., 2006).

**Data Analysis.** All data are presented as mean ± S.E.M. For alldynia 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). 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., 2012a,b). In all studies, data from the ipsilateral paw were statistically similar to the contralateral paw. Thus, only ipsilateral paw values are reported. Data were analyzed using t tests and one- or two-way analysis of variance. The Tukey test was used for post hoc analyses of significant one-way analysis of variance. Multiple comparisons following two-way analysis of variance were conducted with Bonferroni post hoc comparison.

The ED50 dose and lower equally effective dose values as well as 95% confidence limits (Bliss, 1967) were calculated using a standard linear regression analysis of the linear portion of the dose response curve for morphine, MJN110, or the combination of morphine and MJN110 that reversed ipsilateral allodynia. The theoretical additive ED50 value of the combined drugs was calculated from the individual dose-response curves to determine synergistic, additive, or subadditive interactions. The combination was assumed to equal the sum of the effects of each drug. For dose-addition analysis, the ED50 of MJN110 was plotted on the abscissa (x axis) and the isoeffective dose of morphine was plotted on the ordinate (y axis). A line connecting the two points represents the theoretical additive effect of morphine and MJN110 dose combinations. The drug mixture ED50 value was determined by linear regression since the overall mixture dose MJN110 and morphine doses were summed. The experimentally derived ED50 values (Zadd) from the dose response curves of the ratios were compared with the predicted additive ED50 values (Zadd). If the ED50 values of the Zmax are below those of Zadd and the confidence intervals do not overlap, then the interaction is considered synergistic (Tallarida, 2001, 2006). The statistical difference between the theoretical additive and experimental...
Results

The Combination of MJN110 and Morphine Reverses CCI-Induced Allodynia in a Synergistic Fashion. Morphine \( F(2,14) = 33.6; P < 0.0001 \) (Fig. 1A) and MJN110 \( F(4,24) = 11.9; P < 0.0001 \) (Fig. 1B) dose dependently reversed CCI-induced allodynia. The respective ED50 values (95% confidence limits) of morphine and MJN110 were 2.4 (1.87–3.04) mg/kg and 0.430 (0.233–0.793) mg/kg (Ignatowska-Jankowska et al., 2015). In a separate experiment, we assessed the dose-response relationship of eiqueffective doses of each drug in combination, and found a leftward shift in the dose-response relationship compared with either compound given alone (Fig. 1, A and B). Isobolographic analysis revealed a synergistic interaction between these drugs (Fig. 1C). The calculated experimental \( Z_{\text{mix}} \) value [0.286 (0.075–0.497) mg/kg] was significantly less than the calculated theoretical \( Z_{\text{add}} \) value [1.432 (1.387–1.477) mg/kg], and below the line of additivity. These compounds alone and in combination also reversed CCI-induced thermal hyperalgesia for morphine \( F(2,14) = 30.93; P < 0.0001 \) and MJN110 \( F(4,24) = 9.216; P < 0.001 \) (Supplemental Fig. 1).

Antinociceptive Effects of Combined Morphine and MJN110: Assessment of \( \mu \)-opioid, CB1, and CB2 Receptors. Combined administration of the calculated suprathreshold doses of morphine (0.821 mg/kg) and MJN110 (0.1432 mg/kg) produced full reversal of CCI-induced allodynia and thermal hyperalgesia. This combination of drugs was used to examine the involvement of CB1, CB2, and \( \mu \)-opioid receptors. As can be seen in Fig. 2, this combination significantly reversed allodynia as assessed with von Frey filaments \( F(5,49) = 133.8; P < 0.0001 \), as well as thermal hyperalgesia as assessed in the hot plate test \( F(4,24) = 28.11; P < 0.001 \). The \( \mu \)-opioid receptor antagonist naloxone and the CB2 receptor antagonist rimonabant fully blocked both the antiallodynic (Fig. 2A) and antithermal hyperalgesic (Fig. 2B) effects of this combination of drugs \( (P < 0.001) \). The CB2 receptor antagonist SR144528 partially reversed the antiallodynic effects of this combination \( (P < 0.05) \) (Fig. 2A) but did not block the antithermal hyperalgesic effects in the hot plate test \( (P = 0.41) \) (Fig. 2B).

Combination of Suprathreshold Doses of MJN110 and Morphine Alters Spinal Levels of 2-AG and AA. Endocannabinoids were quantified in brain and spinal cord following suprathreshold doses of morphine (0.821 mg/kg) and MJN110 (0.1432 mg/kg) alone and in combination in mice that had been subjected to CCI surgery. Acute MJN110 either alone or in combination with morphine elevated 2-AG [main effect of MJN110: \( F(1,15) = 15.18; P < 0.001 \) (Fig. 3A) in spinal cord, but not in brain \( (P = 0.75) \) (Fig. 3B). Acute 0.821 mg/kg morphine + vehicle in CCI mice did not alter spinal cord levels of 2-AG [interaction effect \( P = 0.353 \); morphine effect \( P = 0.287 \)]. However, this dose of acute morphine significantly increased spinal levels of AA, although this elevation was not observed in mice receiving 0.1432 mg/kg MJN110 and 0.821 morphine [significant interaction between morphine and MJN110: \( F(1,15) = 8.06; P < 0.001 \) (Fig. 3C)]. However, no changes were detected in whole brain \( (P = 0.55) \) (Fig. 3D). This acute combination of MJN110 and morphine did not alter either spinal \( (P = 0.90) \) (Fig. 3E) or brain AEA \( (P = 0.31) \) (Fig. 3F). Additionally, no differences of OEA (spinal cord: \( P = 0.39 \); brain: \( P = 0.39 \)) or PEA (spinal cord: \( P = 0.39 \); brain: \( P = 0.11 \)) were found (Supplemental Fig. 2).
and MJN110 (0.1432 mg/kg), which given in combination produced full reversal of CCI-induced allodynia and thermal hyperalgesia, were further tested for common side effects of morphine and cannabinoids. The charcoal transit assay was used as a functional measure of constipation/gastric transport inhibition, a common untoward side effect of morphine. Subjective cannabinomimetic effects of these drugs given in combination were tested in mice trained to discriminate CP55,940 from vehicle in the drug discrimination paradigm. Morphine (10 mg/kg) produced a significant reduction in gastrointestinal transit compared with vehicle; however, morphine (0.821 mg/kg) alone, MJN110 (0.1432 mg/kg) alone, or the combination of these doses did not significantly alter gastrointestinal transit [F(4,25) = 16.88; P < 0.0001] (Fig. 4A). As shown in Fig. 4B, high dose MJN110 (5 mg/kg) fully substituted for CP55,940, but mice responded on the vehicle aperture following administration of morphine (0.821 mg/kg) alone, MJN110 (0.1432 mg/kg) alone, or the combination of these drugs [F(5,59) = 95.49; P < 0.0001]. Additionally, 5 mg/kg MJN110 increased response rates, while 0.821 mg/kg morphine alone or in combination with 0.1432 mg/kg MJN110 reduced response rates compared with vehicle rates [F(5,59) = 22.71; P < 0.0001] (Fig. 4C).

Combination of MJN110 and Morphine Retains Its Antiallodynic and Antithermal Hyperalgesic Effects Following Repeated Administration. Because neuropathic pain requires chronic administration of analgesics, we conducted two experiments to test whether combined administration of morphine and MJN110 given repeatedly would retain its antiallodynic and antihyperalgesic effects. The first experiment examined the impact of suprathereshold doses of MJN110 (0.1432 mg/kg) and morphine (0.821 mg/kg) administered in combination twice daily for 6 days. Behavior was examined each day before and after the first daily injection. The full time course of the experiment is shown in (Supplemental Fig. 3). Mice displayed tolerance to the antinociceptive effects of 10 mg/kg morphine by day 4 (Supplemental Fig. 3), which persisted for the entire study for allodynia [F(4,26) = 86.62; P < 0.0001], as well as for thermal hyperalgesia [F(4,26) = 59.3; P < 0.0001] (Fig. 5). In contrast, mice receiving morphine (0.821 mg/kg) and MJN110 (0.1432 mg/kg) in combination showed complete reversal of antiallodynia (Fig. 5A) and thermal hyperalgesia (Fig. 5B), both acutely and after repeated administration (P < 0.05).

In the second experiment, we examined the consequences of repeated administration of threshold doses of morphine and MJN110 in combination based on their individual values. Thus, mice received repeated administration of the combination of MJN110 (0.0818 mg/kg) and morphine (0.469 mg/kg) in the CCI model of neuropathic pain. The full time course of the experiment is shown in (Supplemental Fig. 4). Although acute administration of this combination failed to reverse
CCI-induced behavior, repeated administration resulted in full antiallodynic [drug and time interaction: $F(2,14) = 15.72; P < 0.0001$] and antithermal hyperalgesic [drug and time interaction: $F(2,14) = 12.95; P = 0.0005$] effects (Fig. 6, A and B). Endocannabinoid levels and related lipids from the repeated combination of MJN110 and morphine were examined in spinal cord and whole brain (Fig. 6; (Supplemental Fig. 5). Repeated administration of either the threshold dose of MJN110 (0.0818 mg/kg) alone or in combination with morphine (0.469 mg/kg) increased levels of 2-AG in spinal cord [main effect of MJN110: $F(1,17) = 14.95; P = 0.0004$] (Fig. 6C) and brain [main effect of MJN110: $F(1,17) = 20.88; P < 0.0003$] (Fig. 6D). In addition, MJN110 (0.0818 mg/kg) alone or in combination with morphine (0.469 mg/kg) significantly decreased spinal cord AA levels [main effect of MJN110: $F(1,17) = 11.61; P < 0.01$] (Fig. 6E), but did not alter brain AA levels ($P = 0.30$) (Fig. 6F). Morphine (0.469 mg/kg) produced significant increases in AEA in spinal cord [main effect of morphine: $F(1,17) = 8.825; P < 0.01$] (Supplemental Fig. 5A), but no alteration of AEA was observed in brain tissue ($P = 0.25$) (Supplemental Fig. 5B). There were no significant changes in spinal PEA ($P = 0.58$) (Supplemental Fig. 5C), brain PEA ($P = 0.60$) (Supplemental Fig. 5D), spinal OEA ($P = 0.12$) (Supplemental Fig. 5E) or brain OEA ($P = 0.37$) (Supplemental Fig. 5F) levels.

**Discussion**

Previous preclinical and clinical work has demonstrated that THC can be opioid sparing. Specifically, the combination of THC and morphine produced synergistic antinociceptive effects in the rat complete Freund’s adjuvant model of arthritis (Cox et al., 2007). Likewise, compared with opioid treatment only, chronic pain patients treated with both cannabis and opioids reported feeling less pain (Abrams et al., 2011). The present study makes the unique observation that inhibition of MAGL also represents a potential opioid-sparing strategy. Here, we report that combined administration of the MAGL inhibitor MJN110 and morphine produces synergetic...
antiallodynic effects in the mouse CCI model of neuropathic pain that required both μ-opioid and cannabinoid receptors. Repeated administration of suprathreshold or threshold doses of MJN110 and morphine given in combination for 6 days retained antiallodynic and antithermal hyperalgesic effects. Acute administration of these effective doses alone or in combination did not inhibit gastric motility, reflective of constipation, a common side effect of morphine. Moreover, this combination did not substitute for the high-efficacy CB1 receptor agonist CP55,940 in the drug discrimination assay. However, it should be noted that the combination of MJN110 and morphine decreased drug discrimination operant response rates, which

![Figure 4](image-url)

Fig. 4. The calculated suprathreshold doses of morphine (0.821 mg/kg) and MJN110 (0.1432 mg/kg) given in combination do not inhibit gastric motility or substitute for the subjective effects of the cannabinoid receptor agonist CP55,940. (A) Inhibition of gastric motility is not observed either alone or in the combination of equally effective doses of MJN110 or morphine. However, 10 mg/kg morphine significantly inhibited gastric motility. The ordinate depicts the percent of intestinal transit for a charcoal gavage, relative to intestinal total length, and the abscissa depicts the treatment group. (B) Either alone or in the combination, the equally effective doses of MJN110 or morphine does not substitute for the potent, synthetic cannabinoid receptor agonist CP55,940 in the drug discrimination paradigm. In contrast, MJN110 (5 mg/kg) fully substitutes for CP55,940. In this graph the ordinate depicts the percentage of responses in the drug-associated aperture and the abscissa depicts the treatment group. (C) MJN110 (5 mg/kg) increases response rates, while 0.821 mg/kg morphine decreases response rates. The ordinate depicts the response rate as the number of responses per minute and the abscissa depicts the treatment group. *** $P < 0.0001$, ** $P < 0.005$, * $P < 0.05$ versus vehicle control. Data reflect mean ± S.E.M., n = 8–11 mice per group.
may have resulted from potential alterations in motor function or motivation. Nonetheless, these findings provide proof of principle that MAGL inhibitors possess promise as an opioid-sparing strategy.

The combination of threshold doses of MJN110 and morphine did not produce observable side effects in either inhibiting gastric motility or producing cannabimimetic effects in the drug discrimination assay, indicating dissociation between these effects and the antinociceptive effects. However, the present study did not assess the full dose-response relationship of these drugs in combination to infer whether these drugs offer protection against one another’s side effects. Interestingly, other studies demonstrated that THC, as well as fatty acid amide hydrolase inhibitors and MAGL inhibitors, block opioid withdrawal somatic signs (Cichewicz and Welch, 2003; Ramesh et al., 2011, 2013). The present results suggest that a low dose of MJN110 and morphine in combination produced enhanced antinociception, while decreasing the likelihood of at least some untoward side effects.

Other key observations in the present study are that the combination of threshold doses of MJN110 and morphine did not significantly attenuate mechanical allodynia or thermal hyperalgesia following acute administration. However, 6 days of daily injections of this combination fully reversed both of these nociceptive actions in CCI mice. In contrast, repeated injections of fully analgesic doses of either morphine or MAGL inhibitors resulted in tolerance as well as downregulation/desensitization of μ-opioid and CB₁ receptors, respectively (Selley et al., 1997; Stafford et al., 2001; Chanda et al., 2010; Schlosburg et al., 2010). However, low doses of the MAGL inhibitor JZL184 given repeatedly retained its antinociceptive properties and did not reduce CB₁ receptor function (Kinsey et al., 2013). Also, it has been established that low doses of morphine and THC retained their antinociceptive properties upon repeated administration (Smith et al., 2007). The doses of MJN110 given repeatedly in the present study only partially inhibited MAGL (Niphakis et al., 2013; Ignatowska-Jankowska et al., 2015), which likely accounts for the lack of tolerance. The present study also demonstrates that the antiallodynic effects of MJN110 and morphine involved CB₂ receptors, which do not downregulate after repeated administration of CB₂ receptor agonists, and their analgesic effects do not undergo tolerance (Deng et al., 2015). Thus, several distinct underlying cellular mechanisms are likely to account for the enhanced antinociceptive effects resulting from combined administration of morphine and MJN110.

The present study shows that CCI did not elicit any significant changes in spinal or brain levels of 2-AG, AEA, or other quantified lipids compared with sham mice. However, acute administration of a suprathreshold dose of MJN110 (0.1432 mg/kg) produced a significant, albeit small, increase of 2-AG in the spinal cord [i.e., 30.76% ± 8.44% (mean ± S.E.M.)] compared with the CCI mice that received repeated injections of vehicle, although no significant differences were found in the brain. Interestingly, an acute suprathreshold dose of morphine (0.821 mg/kg), which produced partial reversal of allodynia and thermal hyperalgesia, also elevated AA in the spinal cord. These findings expand on previous work showing that MJN110 elevates 2-AG at lower doses than needed to reduce AA (Ignatowska-Jankowska et al., 2015). The present study also examined the consequences of repeated administration of combined threshold doses of MJN110 (0.0818 mg/kg) and morphine (0.469 mg/kg). Compared with the CCI group that received repeated injections of vehicle, repeated MJN110 (with or without morphine) significantly elevated spinal [i.e., 64.94% ± 10.10% (mean ± S.E.M.)] and brain [i.e., 68.27% ± 12.64% (mean ± S.E.M.)] levels of 2-AG, while reducing AA in the spinal cord [i.e., 35.02% ± 6.37% (mean ± S.E.M.)], but not in the brain. A caveat of these results is that the present study used tissue from whole brain and whole spinal cord; therefore, it is possible that CCI surgery or drug manipulations may affect lipid levels in discrete brain or spinal regions. Nonetheless, these results demonstrate that acute administration of a suprathreshold dose of MJN110 increases spinal 2-AG levels, while repeated administration of a threshold dose of MJN110 increases 2-AG in the spinal cord and brain and decreases AA in the spinal cord. The augmented antinociceptive effects of combined administration of morphine and MJN110 may be mediated through interactions between cannabinoid receptors and μ-opioid receptors expressed throughout the central nervous system.
Notably, CB1 receptors are expressed within the periphery on paw pad nociceptive terminals (Richardson et al., 1998), dorsal root ganglia (Hohmann and Herkenham, 1999), primary afferent nerves within the superficial lamina of the dorsal horn of the spinal cord (Hohmann et al., 1999; Morisset and Urban, 2001), spinal cord interneurons (Jennings et al., 2001), trigeminal sensory neurons (Price et al., 2003), and within periaqueductal gray neurons (Mailleux and Vanderhaeghen, 1992). CB2 receptors are well characterized on peripheral macrophage and lymphocytes (Bouaboula et al., 1993; Munro et al., 1993; Gaulégue et al., 1995) and within the CNS on microglia (Romero-Sandoval et al., 2009), with some reports showing CB2 receptors on spinal cord dorsal horn sensory neurons after injury (Wotherspoon et al., 2005). μ-Opioid receptors are expressed on nociceptors within the periphery (Schmidt et al., 2012), the superficial lamina of the dorsal horn (Hohmann et al., 1999; Morisset and Urban, 2001), spinal cord interneurons (Jennings et al., 2001), trigeminal sensory neurons (Price et al., 2003), and within periaqueductal gray neurons (Mailleux and Vanderhaeghen, 1992). CB2 receptors are well characterized on peripheral macrophage and lymphocytes (Bouaboula et al., 1993; Munro et al., 1993; Gaulégue et al., 1995) and within the CNS on microglia (Romero-Sandoval et al., 2009), with some reports showing CB2 receptors on spinal cord dorsal horn sensory neurons after injury (Wotherspoon et al., 2005). μ-Opioid receptors are expressed on nociceptors within the periphery (Schmidt et al., 2012), the superficial lamina of the dorsal horn (Arvidsson et al., 1995; Hohmann et al., 1999), the periaqueductal gray neurons (Kalyuzhny et al., 1996; Commons et al., 2000), peripheral macrophages, and CNS microglia (Makman et al., 1995; Roy et al., 1996). Accordingly, CB2 receptor activation may reduce nociception by increasing the anti-inflammatory cytokine IL-10, decreasing the proinflammatory cytokine IL-1β (Wilkinson...
et al., 2012a,b), decreasing the AKT-Erk1/2 pathway (Merighi et al., 2012), and reducing the mRNA of the critical chemokine MCP1/CCL2 (Deng et al., 2015). Recent evidence suggests a role of MCP-1/CCL2 in opioid tolerance (Zhao et al., 2012). Given that μ-opioid and cannabinoid receptors are widely expressed in both the CNS and periphery, it is important to note that the observed behavioral effects may be due to receptor function at the peripheral nociceptors (Desroches et al., 2014), dorsal root ganglia (Khasabova et al., 2004), dorsal horn of the spinal cord (da Fonseca Pacheco et al., 2008; Desroches et al., 2014), and periadaqueductal gray and other brain regions (Paldy et al., 2008; Wilson-Poe et al., 2015) implicated in the regulation of nociception. CB1 and CB2 receptors appeared to play a differential role in mediating the antinociceptive effects of morphine and MJN110 given in combination. The CB1 and μ-opioid receptors were necessary for both the antiallodynic and antihyperalgesic effects of the combination of these compounds. In contrast, SR144528, a CB2 receptor antagonist, produced partial reduction in antiallodynic effects but did not significantly attenuate the antithermal hyperalgesic effects of combined morphine and MJN110 treatment, which may be due to a lack of functional CB2 receptors on peripheral nociceptor terminals. The CCI model is a neuropathic pain model that has both a peripheral and CNS component, and modulation at either one of these anatomic locations in this model may be sufficient to produce antinoiception. It will be important in future studies to examine the combination of MJN110 and morphine in other antinociception. It will be important in future studies to examine the combination of MJN110 and morphine in other antinociceptive effects shown here were opioid receptor and cannabinoid receptor dependent, although the neural circuits that mediate the enhanced antinociceptive effects remain to be described. Overall, these results indicate that MAGL inhibition represents a novel therapeutic avenue to decrease doses of opioids needed for clinical pain control.

Authorship Contributions

Participated in research design: Wilkerson, Poklis, Banks, Dewey, Akbarali, Wise, Cravatt, Lichtman.

Conducted experiments: Wilkerson, Mustafa, Abdullah.

Contributed new reagents or analytic tools: Niphakis, Grim, Cravatt.

Performed data analysis: Wilkerson, Grim, Mustafa, Poklis, Wise, Lichtman.

Wrote or contributed to the writing of the manuscript: Wilkerson, Banks, Wise, Cravatt, Lichtman.

References


Hollister LE, and Gearheart LF (1987) Evidence that morphine and morphine-like compounds induce constipation or cannabinoid subjective effects. Importantly, these antinoiceptive effects did not undergo tolerance after 6 days of repeated administration. The enhanced antiallodynic and antihyperalgesic effects shown here were opioid receptor and cannabinoid receptor dependent, although the neural circuits that mediate the enhanced antinociceptive effects remain to be described. Overall, these results indicate that MAGL inhibition represents a novel therapeutic avenue to decrease doses of opioids needed for clinical pain control.
Opioid-Sparing Effects of Endocannabinoids


**Address correspondence to:** Dr. Jenny L. Wilkerson, Department of Pharmacology and Toxicology, Virginia Commonwealth University, P.O. Box 980613, Richmond, VA 23298-0613. E-mail: jenny.wilkerson@vcuhealth.org