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Title: The selective monoacylglycerol lipase inhibitor MJN110 produces opioid sparing effects in a mouse neuropathic pain model

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Running Title: Opioid sparing effects of endocannabinoids

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Abbreviations

2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; ABHD6, alpha beta hydrolase domain-containing protein 6; CCI, chronic constriction injury; CNS, central nervous system; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; THC, Δ9-tetrahydrocannabinol; TNF-α, tumor necrosis factor alpha

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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, Δ^9-tetrahydrocannabinol, produces enhanced antinociceptive actions, suggesting that cannabinoid receptor agonists can be opioid sparing. Here, we tested whether elevating the endogenous cannabinoid 2-arachidonylglycerol (2-AG) 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 equi-effective 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 anti-allodynic effects. Acute antinociceptive effects of the combination of morphine and MJN110 required μ-opioid, CB_1, and CB_2 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 six days) continued to produce anti-allodynic effects with no evidence of tolerance. These findings, taken together, suggest that MAGL inhibition produces opiate sparing events with diminished tolerance, constipation, and cannabimimetic side effects.

Introduction

Whereas opiates are widely accepted for the treatment of chronic pathological pain (Ballantyne and Mao, 2003), 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.*, 2014). 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 combination of opiates and cannabinoids produce enhanced antinociceptive effects. Co-administration of the primary psychoactive component of marijuana, delta-9-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 CB_1 (Devane *et al.*, 1988; Matsuda *et al.*, 1990; Pertwee, 1997) and CB_2 (Munro *et al.*, 1993; Pertwee *et al.*, 2007) receptors. Whereas the CB_1 receptor is responsible for the multitude of the behavioral actions of THC and is predominately expressed on presynaptic neurons (Huang *et al.*, 2001; Szabo and Schlicker, 2005), especially GABAergic interneurons (Katona, 1999) in the brain, the CB_2 receptor is highly expressed on immune cells (Carayon *et al.*, 1998; Galiegue, 1995; Pettit *et al.*, 1996; Schatz *et al.*, 1997). The endocannabinoid ligands 2-arachidonyl glycerol (2-AG) (Mechoulam and Deutsch, 2005, Sugiura *et al.*, 1995) and N-arachidonoylethanolamine (anandamide; AEA) (Felder *et al.*, 1996; Martin *et al.*, 1999) are enzymatically regulated, bind both CB_1 and CB_2 receptors, and play important roles in many physiological functions. The actions of these endocannabinoids are short-lived because of rapidly degradation by their

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respective primary hydrolytic enzymes monoacylglycerol lipase (MAGL) (Dinh *et al.*, 2002; Long *et al.*, 2009) and fatty acid amide hydrolase (FAAH) (Cravatt *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 physiological processes, specifically with regard to neuronal and immune functioning, both of which are critical mediators of pathological pain (Calignano *et al.*, 1998; Guindon *et al.*, 2011; 2013; Kinsey *et al.*, 2009; 2010; 2011a,b; Russo *et al.*, 2007). Notably, combination of the FAAH 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 MJN110, which possesses increased potency and selectivity over other MAGL inhibitors (Ignatowska-Jankowska *et al.*, 2015; Niphakis *et al.*, 2013), would enhance the antinociceptive effects of morphine in the chronic constriction injury of the sciatic nerve (CCI) model of neuropathic pain. We also used the selective receptor antagonists rimonabant, SR144528, and naloxone to examine the respective contributions of CB_1, CB_2 and μ-opioid receptors in mediating the observed antinociceptive effects. As 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.*, 2009b; Walentiny *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 combination morphine and MJN110 in mice subjected to CCI.

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.

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

2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate (MJN110) was synthesized in the Cravatt laboratory at the Scripps Research Institute as described previously (Chang *et al.,* 2012; Niphakis *et al.,* 2013). The CB_1 receptor antagonist rimonabant [*N*- (piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide-HCl], the CB_2 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], CP55,940 [(-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)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 (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 1:1:18 ratio. Morphine sulfate was dissolved in sterile saline (Hospira Inc, Lake Forest, IL). Each drug was given via the intraperitoneal (i.p.) route of administration. All drugs were administered in a volume of 10 μl/g body mass. All experiments employed a 1 h absorption period for MJN110, based on previous studies (Ignatowska-Jankowska *et al.*, 2015), and a 30 min drug absorption period for all other drugs. In

studies in which MJN110 and morphine were co-administered, MJN110 was given at 0 min, morphine was administered at 30 min, and behavioral testing commenced at 60 min.

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 the mouse (Ignatowska-Jankowska *et al.,* 2015). 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 three 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 below. 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 (see above). 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 (BL) responses to light mechanical touch and compared with post-surgery thresholds (Murphy *et al.,* 1999). 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 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.

For repeated administration experiments, mice were given vehicle, 0.0818 mg/kg MJN110, 0.469 or 10 mg/kg morphine, or combination of 0.0818 mg/kg MJN110 and 0.469 mg/kg morphine twice daily for 5.5 days and then underwent behavioral testing on day 6. Combination of 0.0818 mg/kg MJN110 and 0.469 mg/kg morphine produced significant antinociceptive effects, but reflected threshold doses when administered alone.

Extraction and quantification of endocannabinoids by liquid chromatography-tandem mass spectrometry

2-AG, arachidonic acid (AA), AEA, palmitoylethanolamide (PEA), oleoylethanolamine (OEA), prostaglandin E2 (PGE2), and prostaglandin D2 (PGD2) levels 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 intraperitoneal 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 min after the final morphine injection, mice were euthanized via rapid decapitation (at 11:00–17:00 EST) at this time point. Spinal cords and 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.

Gastric motility assay

Mice were food deprived for 24 h and were then given i.p. injection of vehicle, 10 mg/kg morphine, 0.821 mg/kg morphine (the calculated supra-threshold dose of the effective 25% dose of morphine to produce anti-allodynia), 0.1432 mg/kg MJN110 (the calculated supra-threshold dose of the effective 25% dose of MJN110 to produce anti-allodynia), or the combination of 0.821 mg/kg morphine + 0.1432 MJN110. Twenty min following the morphine or equivalent

vehicle injection the subjects were administered charcoal by oral gavage. Intestines were harvested 10 min post gavage, 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 supra-threshold dose of the effective 25% dose of morphine to produce anti-allodynia), 0.1432 mg/kg MJN110 (the calculated supra-threshold dose of the effective 25% dose of MJN110 to produce anti-allodynia), or the combination of 0.821 mg/kg morphine + 0.1432 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). 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 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 (14 mg sweetened pellets; Bio-Serv, Frenchtown, NJ, USA) 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

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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. Fifteen min stimulus substitution tests were conducted no more than twice per week with at least 72 h between test sessions. Training continued on other days. During test sessions, responses in either aperture delivered reinforcement according to an FR10 schedule. Testing criteria 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 training drug was considered as partial substitution, and < 20% of responses on the aperture coupled with training drug was considered as no substitution (Solinas et *al*., 2006).

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). 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, 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.

The ED_{50} 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 ED_{50} 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 ED_{50} 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 ED_{50} value was determined by linear regression as the overall mixture dose MJN110 and morphine doses were summed). The experimentally derived ED_{50} values (Z_{mix}) from the dose response curves of the ratios were compared to the predicted additive ED_{50} values (Z_{add}). If the ED_{50} values of the Z_{mix} are below those of Z_{add} and the confidence intervals (CI) do not overlap, then the interaction is considered synergistic (Tallarida, 2001; 2006). The statistical difference between the theoretical additive ED_{50} value and the experimental ED_{50} value was analyzed using a Fisher's exact test (Naidu *et al.*, 2009). Differences were considered significant at the level of $p \leq 0.05$. Statistical analysis was performed with GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, CA).

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Results

The combination of MJN110 and morphine reverses CCI- induced allodynia in a synergistic fashion

Morphine [$F(2,14) = 33.6$; $P < 0.0001$; Figure 1A] and MJN110 [$F(4,24) = 11.9$; $P < 0.0001$; Figure 1B] dose-dependently reversed CCI-induced allodynia. The respective ED50 values (95% CL) 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 equi-effective doses of each drug in combination, and found a leftward shift in the dose-response relationship compared with either compound given alone (Figure 1A, B). Isobolographic analysis revealed a synergistic interaction between these drugs (Figure 1C). The calculated experimental Z_{mix} (0.286 (0.075-0.497) mg/kg was significantly less than the calculated theoretical Z_{add} (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 Figure 1).

Antinociceptive effects of combined morphine and MJN110: Assessment of μ-opioid, CB_1, and CB_2 receptors

Combined administration of the calculated supra-threshold 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 utilized to examine the involvement of CB_1, CB_2, and μ-opioid receptors. As can be seen in Figure 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 μ-opioid receptor antagonist naloxone and the CB_1 receptor antagonist rimonabant fully blocked both the anti-allodynic (Figure 2A) and anti-thermal hyperalgesic (Figure 2B) effects of this combination of drugs ($P < 0.001$). The CB_2 receptor antagonist SR144528 partially reversed the anti-allodynic effects of this combination (Figure 2A; $P < 0.05$), but did not block the anti-hyperalgesic effects in the hot plate test (Figure 2B; $P = 0.41$).

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Combination of supra-threshold doses of MJN110 and morphine alters spinal levels of 2-AG and AA.

Endocannabinoids were quantified in brain and spinal cord following supra-threshold 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: $F(1,15) = 15.18$; $P < 0.001$; Figure 3A] in spinal cord, but not in brain ($P = 0.75$; Figure 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 arachidonic acid, though this elevation was not observed in mice receiving 0.1432 mg/kg MJN110 and 0.821 morphine [significant interaction between morphine and MJN110:$F(1,15) = 8.06$; $P < 0.001$; Figure 3C]. However, no changes were detected in whole brain ($P = 0.55$, Figure 3D). This acute combination of MJN110 and morphine did not alter either spinal ($P = 0.90$; Figure 3E) or brain AEA ($P = 0.31$; Figure 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 Figure 2).

Combined administration of supra-threshold doses of MJN110 and morphine does not elicit common cannabimimetic or opioid actions indicative of side effects.

The supra-threshold doses of morphine (0.821 mg/kg) 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 utilized as a functional measure of constipation/gastric transport inhibition, a common untoward side effect of morphine. Subjective cannabimimetic effects of these drugs given in combination were tested in mice trained to discriminate CP55,940 from vehicle in the drug discrimination paradigm.

Whereas morphine (10 mg/kg) produced a significant reduction in gastrointestinal transit compared to vehicle, morphine (0.821 mg/kg) alone, MJN110 (0.1432 mg/kg) alone, or the combination of these doses did not significantly alter gastrointestinal transit [$F(4,25) = 16.88$; $P < 0.0001$; Figure 4A].

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As shown in Figure 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 to vehicle rates [$F(5,59) = 22.71$; $P < 0.0001$; Figure 4C]

Combination of MJN110 and morphine retains its anti-allodynic and anti-thermal 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 anti-allodynic and anti-hyperalgesic effects. The first experiment examined the impact of supra-threshold doses of MJN110 (0.1432 mg/kg) and morphine (0.821 mg/kg) administered in combination twice daily for six days. Behavior was examined each day before and after the first daily injection. The full time course of the experiment is shown in Supplemental Figure 3. Mice displayed tolerance to the antinociceptive effects of 10 mg/kg morphine by day 4 (Supplemental Figure 3), which persisted for the entire study for allodynia [$F(4,26) = 86.62$; $P < 0.0001$], as well as thermal hyperalgesia [$F(4,26) = 59.3$; $P < 0.0001$] (Figure 5). In contrast, mice receiving morphine (0.821 mg/kg) and MJN110 (0.1432 mg/kg) in combination showed complete reversal of anti-allodynia (Figure 5A) and thermal hyperalgesia (Figure 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 (Table 1). 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 Figure 4. Although acute administration of this combination failed to reverse CCI-induced behavior, repeated administration resulted in full anti-allodynic [drug and time interaction: $F(2,14) = 15.72$; $P < 0.0001$] and anti-thermal hyperalgesic [drug and time interaction: $F(2,14) = 12.95$; $P < 0.05$] effects (Figure 6A,B). Endocannabinoid levels and related lipids from the repeated combination of MJN110 and morphine were examined in spinal cord and whole brains (Figure 6, Supplemental Figure 5). Repeated administration of

either the threshold dose of MJN110 (0.0818 mg/kg) alone or combination with morphine (0.469 mg/kg) increased levels of 2-AG in spinal cord [main effect of MJN110: $F(1,17) = 14.95$; $P = 0.0004$; Figure 6C] and brain [main effect of MJN110:$F(1,17) = 20.88$; $P < 0.0003$, Figure 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:$F(1,17) = 11.61$; $P < 0.01$; Figure 6E], but did not alter brain AA levels ($P = 0.30$; Figure 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 Figure 5A], but no alteration of AEA was observed in brain tissue ($P = 0.25$; Supplemental Figure 5B). There were no significant changes in spinal PEA ($P = 0.58$; Supplemental Figure 5C), brain PEA ($P = 0.60$; Supplemental Figure 5D), spinal OEA ($P = 0.12$; Supplemental Figure 5E) or brain OEA ($P = 0.37$; Supplemental Figure 5F) levels.

Discussion

Previous preclinical and clinical work has demonstrated that THC can be opioid sparing. Specifically, 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 anti-allodynic effects in the mouse CCI model of neuropathic that required both mu opioid and cannabinoid receptors. Repeated administration of supra-threshold or threshold doses of MJN110 and morphine given in combination for six days retained anti-allodynic and anti-thermal 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 CB_1 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 may have resulted from potential alterations in motor function or motivation. Nonetheless, these findings provide proof of principle that MAGL inhibitors possess promise as 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 a 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 FAAH inhibitors and MAGL inhibitors, block opioid withdrawal somatic signs (Cichewicz & 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, six 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 down-regulation/desensitization of μ-opioid and CB_1 receptors, respectively (Selley *et al.,* 1997; Stafford *et al.,* 2001; Schlosburg *et al.,* 2010; Chanda *et al.,* 2010). However, low dose of the MAGL inhibitor JZL184 given repeatedly retained its antinociceptive properties and did not reduce CB_1 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 inhibits 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 anti-allodynic effects of MJN110 and morphine involved CB_2 receptors, which do not downregulate after repeated administration of CB_2 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 supra-threshold dose of MJN110 (0.1432 mg/kg) produced a significant, albeit small, increase of 2-AG (i.e. 30.76 $\pm$ 8.44 % (mean $\pm$ SEM)) compared with the CCI mice that received repeated injections of vehicle) in the spinal cord, though no significant differences were found in brain. Interestingly, an acute supra-threshold 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 MJN110 (0.0818 mg/kg) and morphine (0.469 mg/kg). Compared with CCI group that received repeated injections of vehicle, repeated MJN110 (with or without morphine) significantly elevated spinal (i.e. 64.94 $\pm$ 10.10 % (mean $\pm$ SEM)) and brain (i.e. 68.27 $\pm$ 12.64 % (mean $\pm$ SEM)) levels of 2-AG, while reducing AA in spinal cord (i.e. 35.02 $\pm$ 6.37 % (mean $\pm$ SEM)), but not in brain. A caveat of these results is that the present study used tissue from whole brain

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and whole spinal cord; thus, it is possible CCI surgery or the drug manipulations may affect lipid levels in discrete brain or spinal regions. Nonetheless, these results demonstrate that acute administration of a supra-threshold dose of MJN110 increases spinal 2-AG levels, while repeated administration of a threshold doses of MJN110 increases 2-AG in 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 mu opioid receptors expressed throughout the CNS and periphery. Notably, CB_1 receptors are expressed within the periphery on paw pad nociceptive terminals (Richardson *et al.,* 1998), dorsal root ganglia (DRG) (Hohmann & Herkenham, 1999b), primary afferent nerves within the superficial lamina of the dorsal horn of the spinal cord (Hohmann *et al.,* 1999a; Morsset & Urban, 2001), spinal cord interneurons (Jennings *et al.,* 2001), trigeminal sensory neurons (Price *et al.,* 2003), and within periaqueductal grey neurons (Mailleux & Vanderhaeghen, 1992). CB_2 receptors are well characterized on peripheral macrophage and lymphocytes (Munro *et al.,* 1993; Bouaboula *et al.,* 1993; Galiegue *et al.,* 1995) and within the CNS on microglia (Romero Sandoval *et al.,* 2009), with some reports showing CB_2 receptors on spinal cord dorsal horn sensory neurons after injury (Wotherspoon *et al.,* 2005). Mu 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.,* 1999a), the periaqueductal (Kalyuzhny *et al.,* 1996; Commons *et al.,* 2000), peripheral macrophages, and CNS microglia (Markman *et al.,* 1995; Roy *et al.,* 1996). Accordingly, CB_2 receptor activation may reduce nociception by increasing the anti-inflammatory cytokine IL-10, decreasing the proinflammatory cytokine IL-1β, (Wilkerson et al., 2012 a,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), DRGs (Khasabova *et. al*, 2004), the dorsal horn of the spinal cord (da Fonseca Pacheco *et. al*, 2008; Desroches *et. al*, 2014), the periaqueductal gray and other brain regions (Paldy *et. al*, 2008; Wilson-Poe *et. al*, 2012) implicated in the regulation of nociception. CB_1 and CB_2 receptors appeared to play a differential role in mediating the antinociceptive

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effects of morphine and MJN110 given in combination. The CB_1 and mu opioid receptors were necessary for both the anti-allodynic and anti-hyperalgesic effects of the combination of these compounds. In contrast, SR144528, a CB_2 receptor antagonist, produced a partial reduction in anti-allodynic effects, but did not significantly attenuate the anti-thermal hyperalgesic effects of combined morphine and MJN110 treatment, which may be due to a lack of functional CB_2 receptors on peripheral nociceptor terminals. The CCI model is a neuropathic pain model that has both a peripheral and central nervous system component, and modulation at either one of these anatomical locations in this model may be sufficient to produce antinociception. It will be important in future studies to examine the combination of MJN110 and morphine in other, more centrally mediated neuropathic pain models, such as the Chung model of spinal nerve ligation (Chung *et al.*, 2004). The findings shown here suggest that activation of CB_1 and CB_2 receptors may play a critical role in augmenting μ-opioid receptor antinociceptive effects.

In conclusion, the present study demonstrates that the MAGL inhibitor MJN110, which increased spinal 2-AG levels, interacted in a synergistic manner with morphine to reverse allodynia and thermal hyperalgesia in a mouse model of neuropathic pain, without the side effects of opioid-induced constipation or cannabinoid subjective effects. Importantly, these antinociceptive effects did not undergo tolerance after six days of repeated administration. The enhanced anti-allodynic and anti-hyperalgesic effects shown here were opioid receptor and cannabinoid receptor dependent, though 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

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Footnotes

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Legends for Figures

Figure 1. Systemic morphine and MJN110 reverse CCI-induced allodynia. (A) Intraperitoneal morphine reverses CCI-induced allodynia in a dose-related manner 30 min after administration. Equi-effective doses of morphine and MJN110 given in combination produces a leftward shift of the dose-response curve. For these graphs the left ordinate depicts the psychometric calculated absolute log stimulus intensity as measured in the log of mg pressure for the 50% threshold response. The right ordinate depicts the same data as the left ordinate, but transformed into grams. The abscissa depicts the dose of drug administered. (B) Intraperitoneal MJN110 reverses CCI-induced allodynia in a dose-related manner 1 h after administration, as originally published and reprinted with permission (Ignatowska-Jankowska *et al.*, 2015). An equi-effective combination of morphine and MJN110 produces a leftward shift of dose-response curve. (C) The equi-effective combination of morphine and MJN110 produces a synergistic effect, as it falls below the line of additivity. For this graph, the derived ED_{50} of MJN110 in mg/kg was plotted on the abscissa and the isoeffective dose of morphine was plotted on the ordinate. Filled symbols denote significance from CCI + vehicle ($P < 0.05$). Data reflect mean ± SEM, n = 5-7 mice/group.

Figure 2. The anti-allodynic and anti-thermal hyperalgesic effects of the calculated equi-effective supra-threshold doses of morphine (0.821 mg/kg) and MJN110 (0.1432 mg/kg) given in combination, require mu-opioid and CB_1 receptors, but are differentially altered by a CB_2 receptor antagonist. Rimonabant, SR144528, and naloxone block anti-allodynic effects of combined morphine and MJN110 administration in CCI-induced (**A)** allodynia, (**B**) but there is no effect of SR144528 in the blockade of thermal hyperalgesia. For these graphs the ordinate depicts the paw withdrawal latency in seconds and the abscissa depicts the treatment group. *** $p<0.0001$, ** $p<0.005$, * $p < 0.05$ vs. CCI vehicle+vehicle, ## $p<0.001$ vs. CCI MJN110 + morphine. Data reflect mean ± SEM, n=6 mice per group.

Figure 3. Acute administration of supra-threshold doses of morphine (0.821 mg/kg) and MJN110 (0.1432 mg/kg) given in combination alters spinal 2-AG and AA in neuropathic mice. (A) MJN110 produces an overall increase in spinal 2-AG but (B) no change in brain 2-AG. (C) This dose of morphine produces significant elevations of spinal AA, which is not present in mice

receiving MJN110 alone or in combination with morphine, with (D) no change in brain AA. No changes were detected in (E) spinal AEA or (F) brain AEA. In these graphs the ordinate depicts the concentration of endocannabinoid lipid and the abscissa depicts treatment group. *** $p < 0.001$ vs. CCI conditions not receiving MJN110 (i.e., vehicle+vehicle and morphine+vehicle). Data reflect mean ± SEM, n=5-6 mice per group.

Figure 4. The calculated supra-threshold 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 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 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 treatment group. *** $p<0.0001$, ** $p<0.005$, * $p < 0.05$ vs. Vehicle control, Data reflect mean ± SEM, n=8-11 mice per group.

Figure 5. The anti-allodynic and anti-thermal hyperalgesic effects of supra-threshold doses of morphine (0.821 mg/kg) and MJN110 (0.1432 mg/kg) given in combination, do not undergo tolerance after six days of repeated administration (A) Repeated administration of the combination of MJN110 and morphine does not lead to tolerance. Compared to i.p. vehicle morphine injected mice, the effective dose combination of MJN110 and morphine produces a reversal from (A) allodynia and (B) thermal hyperalgesia in CCI-treated mice for six days. However, mice injected with 10 mg/kg display acute analgesic effects that undergo tolerance upon repeated administration. ** $p < 0.01$, * $p < 0.05$ vs. CCI + vehicle+vehicle. Data reflect mean ± SEM, n = 5-7 mice/group.

Figure 6. The effects of repeated threshold doses of morphine (0.469 mg/kg) and MJN110 (0.0818 mg/kg). (A,B) Repeated administration of the combination of MJN110 and morphine leads to enhanced antinociceptive effects. Compared to i.p. vehicle + vehicle injected mice, the combination of MJN110 and morphine is inactive after the first injection, but six days of daily administration fully reverses (A) CCI-induced allodynia and (B) and CCI-induced thermal hyperalgesia. Tissues were collected 30 min after the final drug injection on day 6. Repeated administration of the combination of MJN110 or MJN110 + morphine significantly increases 2-AG levels within CCI-treated mice in (C) spinal cord and (D) whole brain tissues. (E) Repeated administration of equi-effective threshold combinations of MJN110 or MJN110 + morphine reduces AA in spinal cord, but not in (F) whole brain tissue. *** $p < 0.001$, ** $p < 0.01$ vs. CCI conditions not receiving MJN110 (i.e., vehicle+vehicle and morphine+vehicle). Data reflect mean ± SEM, n = 5-7 mice/group.

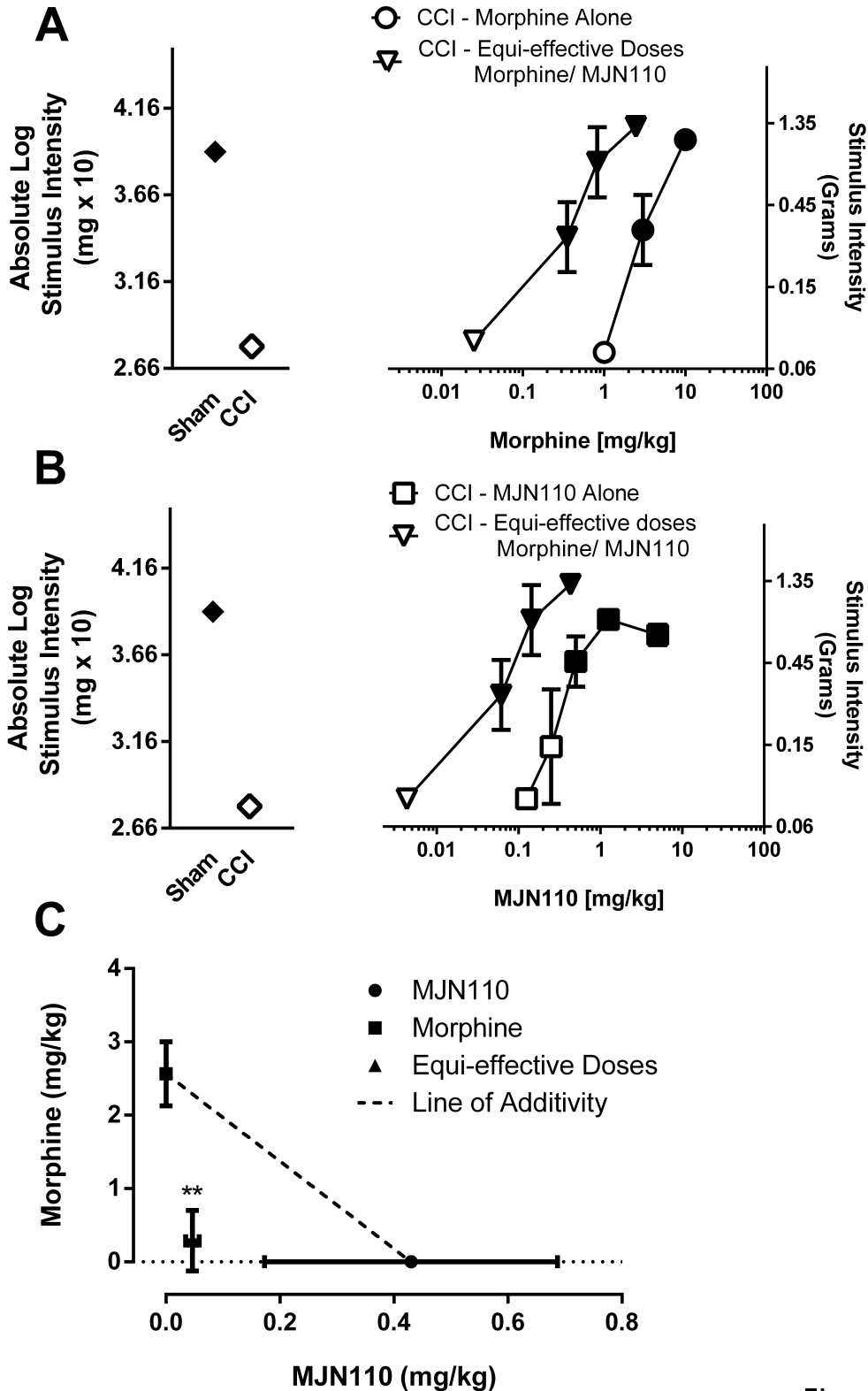
A
CCI - Morphine Alone
CCI - Equi-effective Doses
Morphine/ MJN110
Absolute Log
Stimulus Intensity
(mg x 10)
4.16
3.66
3.16
2.66
Sham
CCI
1.35
0.45
0.15
0.06
Stimulus Intensity
(Grams)
0.01
0.1
1
10
100
Morphine [mg/kg]
B
CCI - MJN110 Alone
CCI - Equi-effective doses
Morphine/ MJN110
Absolute Log
Stimulus Intensity
(mg x 10)
4.16
3.66
3.16
2.66
Sham
CCI
1.35
0.45
0.15
0.06
Stimulus Intensity
(Grams)
0.01
0.1
1
10
100
MJN110 [mg/kg]
C
MJN110
Morphine
Equi-effective Doses
Line of Additivity
Morphine (mg/kg)
4
3
2
1
0
**
0.0
0.2
0.4
0.6
0.8
MJN110 (mg/kg)

Figure 1

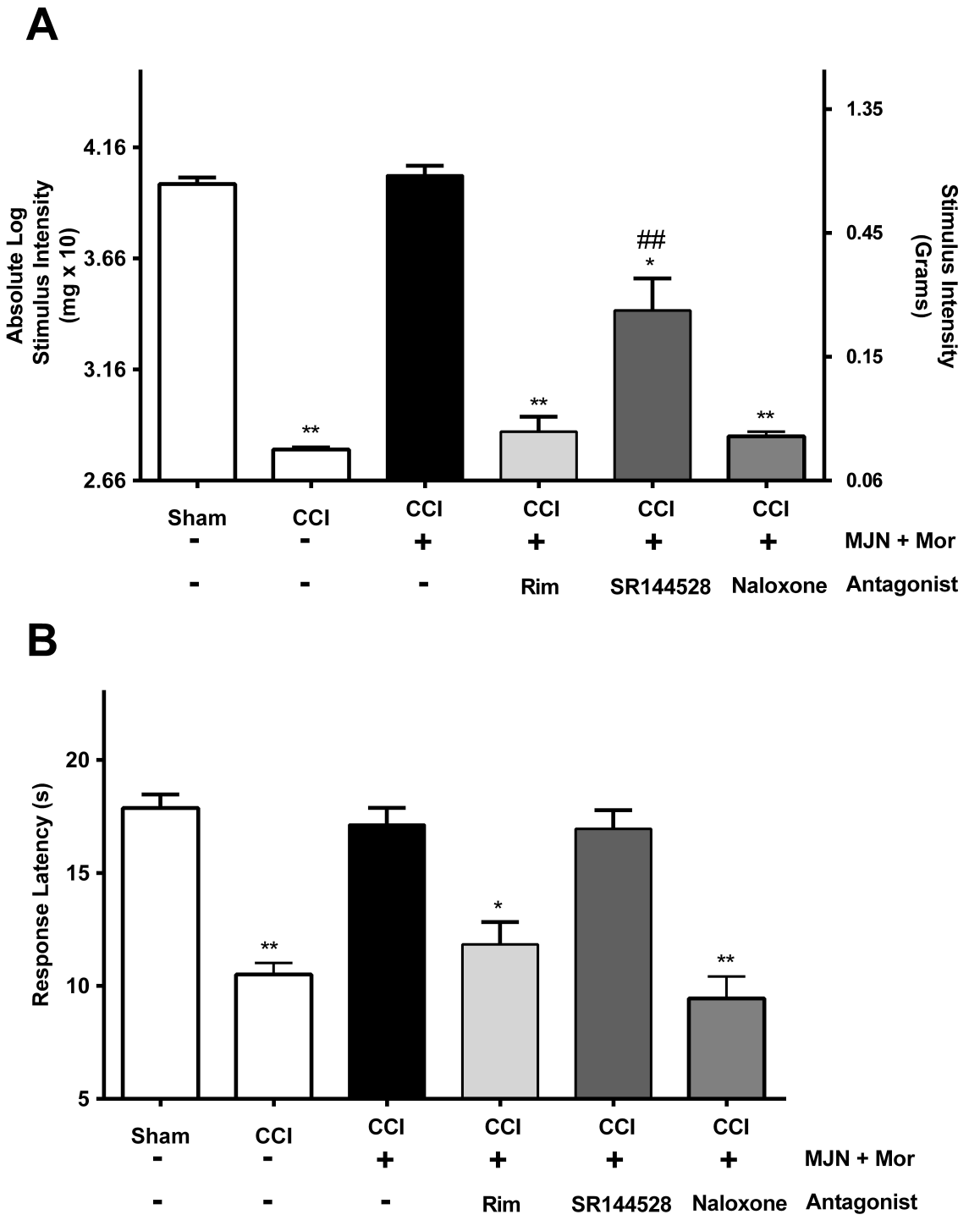
A
Absolute Log Stimulus Intensity (mg x 10)
4.16
3.66
3.16
2.66
1.35
0.45
0.15
0.06
Stimulus Intensity (Grams)
**
**
##
*
**
Sham
CCI
CCI
CCI
CCI
CCI
MJN + Mor
-
-
+
+
+
+
-
-
-
Rim
SR144528
Naloxone
Antagonist
B
Response Latency (s)
20
15
10
5
**
*
**
Sham
CCI
CCI
CCI
CCI
CCI
MJN + Mor
-
-
+
+
+
+
-
-
-
Rim
SR144528
Naloxone
Antagonist

Figure 2

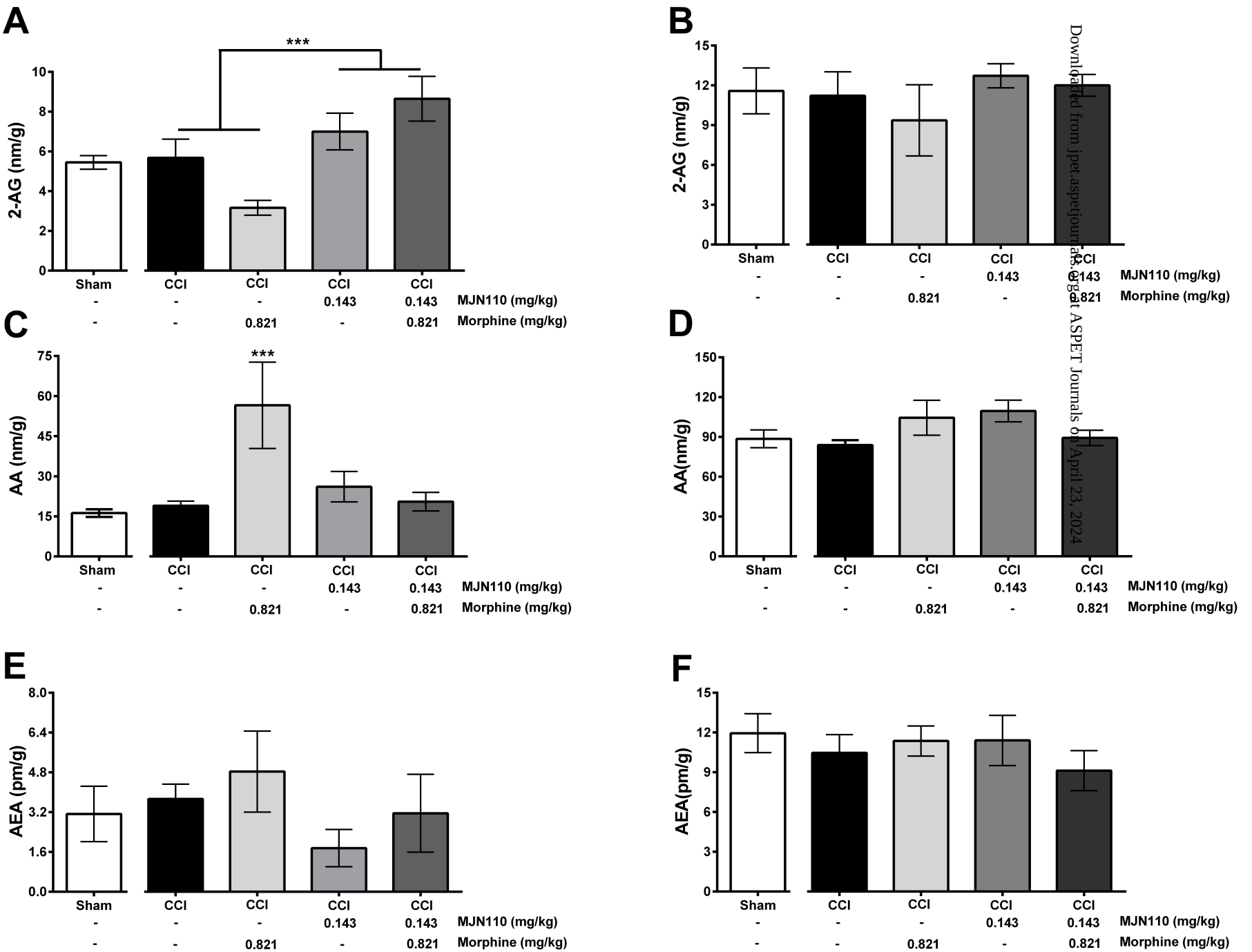

Figure 3

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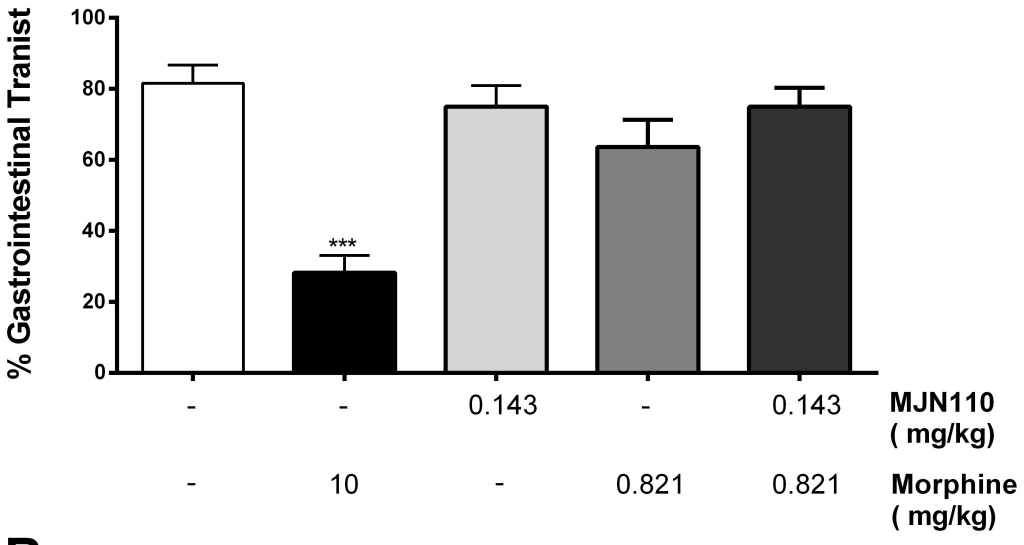

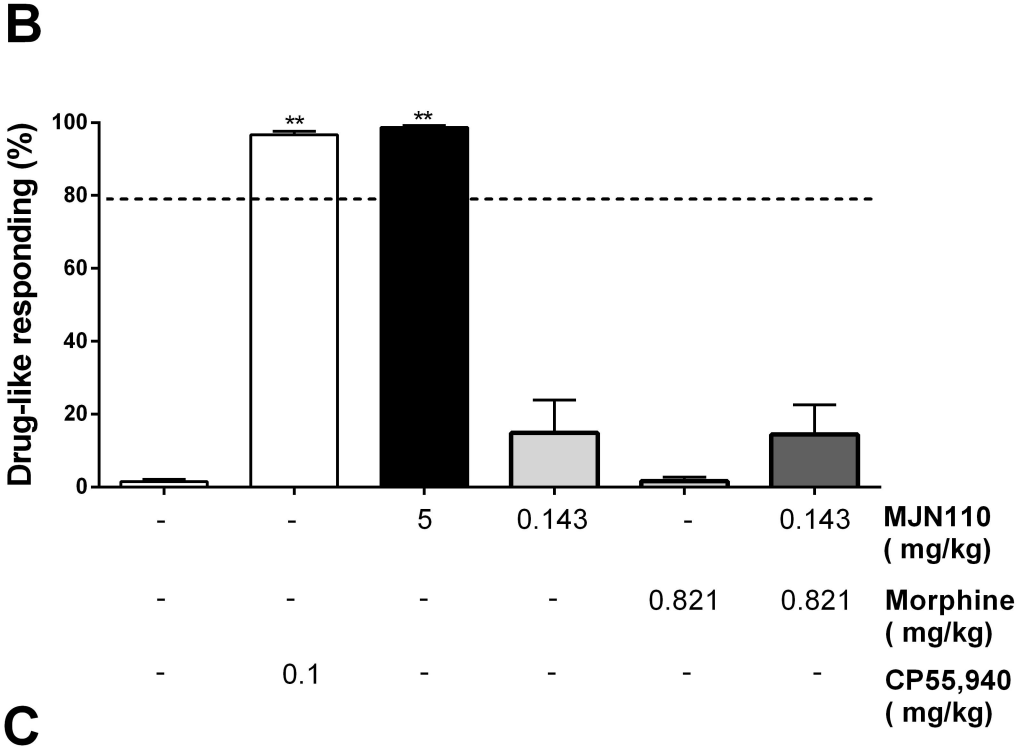

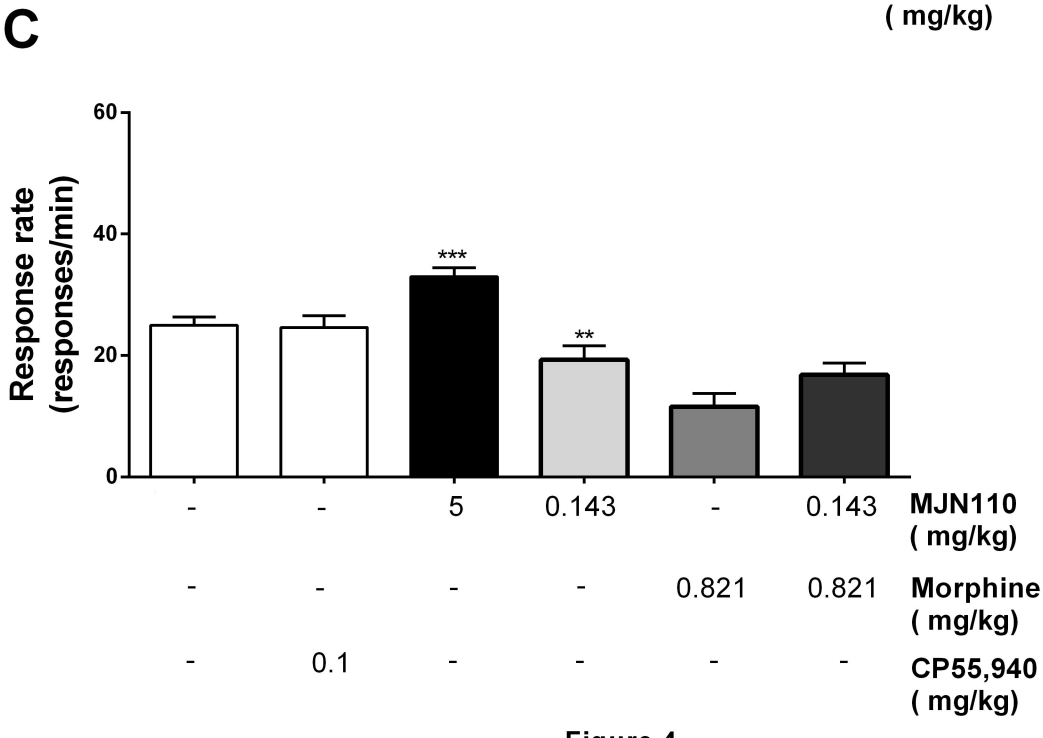

Figure 4

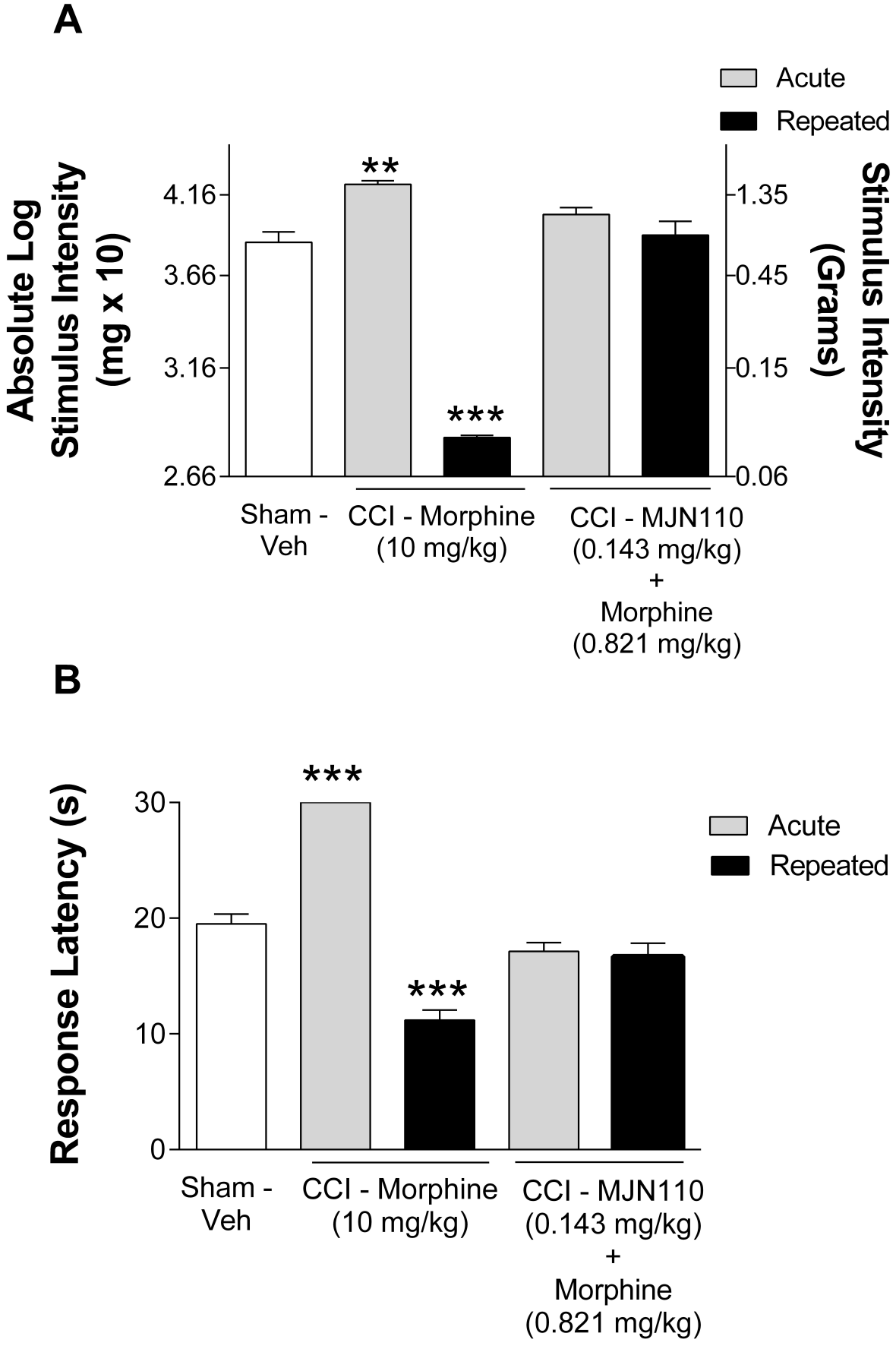
A
Acute
Repeated
Absolute Log Stimulus Intensity (mg x 10)
Stimulus Intensity (Grams)
4.16
3.66
3.16
2.66
1.35
0.45
0.15
0.06
**
***
Sham - Veh
CCI - Morphine (10 mg/kg)
CCI - MJN110 (0.143 mg/kg) + Morphine (0.821 mg/kg)
B
Response Latency (s)
30
20
10
0
***
***
Acute
Repeated
Sham - Veh
CCI - Morphine (10 mg/kg)
CCI - MJN110 (0.143 mg/kg) + Morphine (0.821 mg/kg)

Figure 5

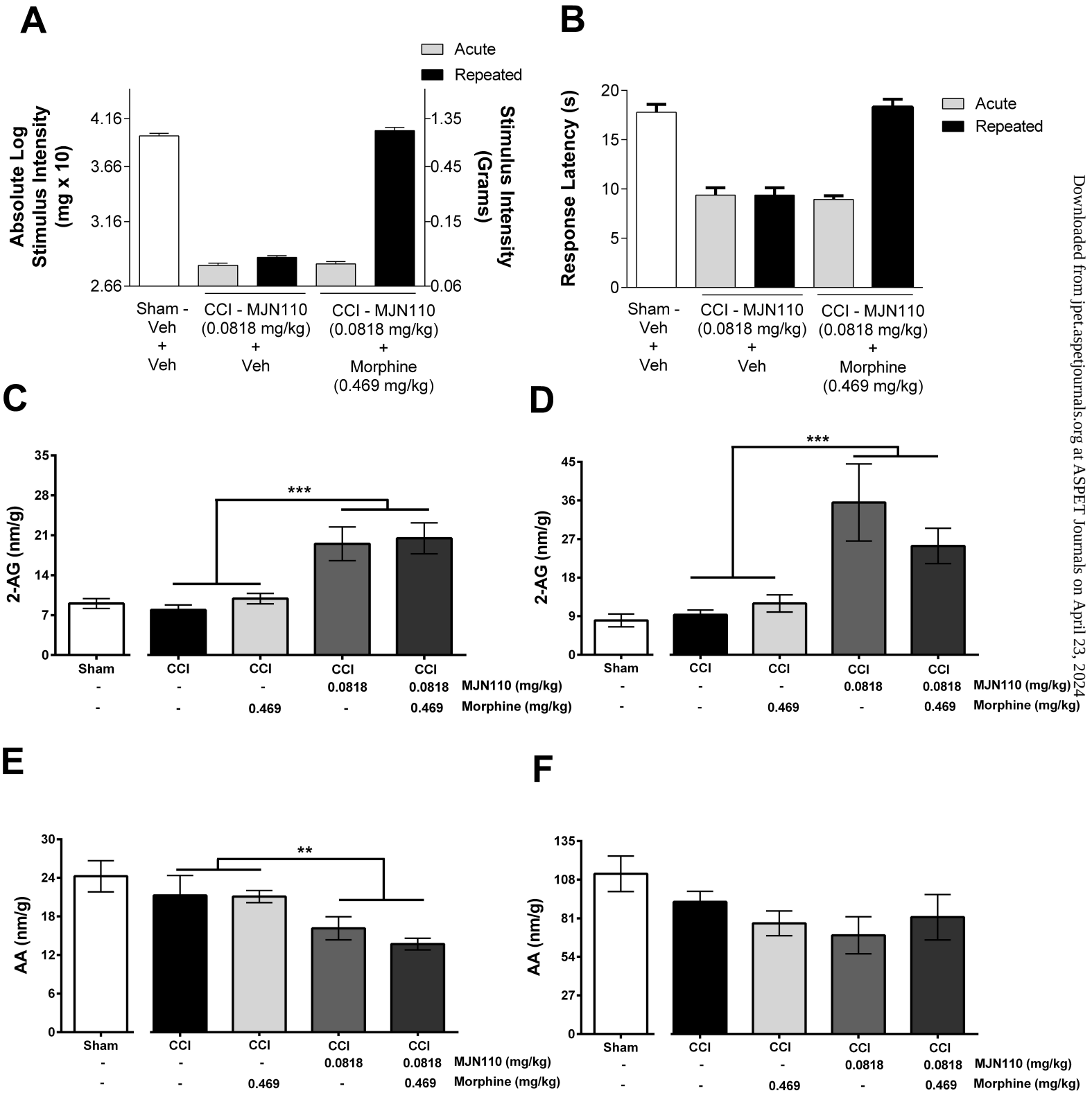

Figure 6