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Blockade of Endocannabinoid-Degrading Enzymes Attenuates Neuropathic Pain

S. G. Kinsey, J. Z. Long, S. T. O'Neal, R. A. Abdullah, J. L. Poklis, D. L. Boger, B. F. Cravatt, and A. H. Lichtman

Department of Pharmacology and Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, Virginia (S.G.K., S.T.O., R.A.A., J.L.P., A.H.L.); Skaggs Institute for Chemical Biology and Department of Chemical Physiology, the Scripps Research Institute, La Jolla, California (J.Z.L., B.F.C.); and Skaggs Institute for Chemical Biology and Department of Chemistry, the Scripps Research Institute, La Jolla, California (D.L.B.)

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ABSTRACT

Direct-acting cannabinoid receptor agonists are well known to reduce hyperalgesic responses and allodynia after nerve injury, although their psychoactive side effects have damped enthusiasm for their therapeutic development. Alternatively, inhibiting fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), the principal enzymes responsible for the degradation of the respective endogenous cannabinoids, anandamide (AEA) and 2-arachydonylglycerol (2-AG), reduce nociception in a variety of nociceptive assays, with no or minimal behavioral effects. In the present study we tested whether inhibition of these enzymes attenuates mechanical allodynia, and acetone-induced cold allodynia in mice subjected to chronic constriction injury of the sciatic nerve. Acute administration of the irreversible FAAH inhibitor, cyclohexylcarbamic acid 3′-carbamoylbiphenyl-3-yl ester (URB597), or the reversible FAAH inhibitor, 1-oxo-1-[5-(2-pyridyl)-2-yl]-7-phenylheptane (OL-135), decreased allodynia in both tests. This attenuation was completely blocked by pretreatment with either CB_1 or CB_2 receptor antagonists, but not by the TRPV1 receptor antagonist, capsazepine, or the opioid receptor antagonist, naltrexone. The novel MAGL inhibitor, 4-nitrophenyl 4-(dibenzo[*d*][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate (JZL184) also attenuated mechanical and cold allodynia via a CB_1, but not a CB_2, receptor mechanism of action. Whereas URB597 did not elicit antiallodynic effects in FAAH(−/−) mice, the effects of JZL184 were FAAH-independent. Finally, URB597 increased brain and spinal cord AEA levels, whereas JZL184 increased 2-AG levels in these tissues, but no differences in either endocannabinoid were found between nerve-injured and control mice. These data indicate that inhibition of FAAH and MAGL reduces neuropathic pain through distinct receptor mechanisms of action and present viable targets for the development of analgesic therapeutics.

Although cannabis has been used for thousands of years to treat pain and other ailments, its undesirable psychomimetic effects have dampened enthusiasm for further drug development. Instead, recent research has focused on targeting the endogenous cannabinoid system for the development of new analgesics (Schlosburg et al., 2009). The endogenous cannabinoid system consists of two cloned cannabinoid receptors (CB_1 and CB_2), various proposed endocannabinoid ligands, including anandamide (AEA; Devane et al., 1992) and 2-arachidonylglycerol (2-AG; Mechoulam et al., 1995), and the enzymes that regulate the biosynthesis and catabolism of the endocannabinoids. In particular, fatty acid amide hydrolase (FAAH; Cravatt et al., 1996) and monoacylglycerol lipase (MAGL; Blankman et al., 2007) are the primary catabolic enzymes of AEA and 2-AG, respectively.

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ABBREVIATIONS: CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; 2-AG, 2-arachidonoylglycerol; AEA, anandamide or *N*-arachidonoylethanolamine; CCI, chronic constriction injury; CPZ, capsazepine, *N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2*H*-2-benzazepine-2-carbothioamide; FAAH, Fatty acid amide hydrolase; JZL184, 4-nitrophenyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate; MAGL, monoacylglycerol lipase; naltrexone, 17-(cyclopropylmethyl)-4,5α-epoxy-3,14-dihydroxymorphinan-6-one; OL-135, 1-oxo-1-[5-(2-pyridyl)-2-yl]-7-phenylheptane; SR1, rimonabant, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl; SR2, SR144528, *N*-[(1*S*)-*endo*-1,3,3,-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; TRPV1, transient receptor potential cation channel, subfamily V, member 1; URB597, cyclohexylcarbamic acid 3′-carbamoylbiphenyl-3-yl ester; WIN 55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanonemesylate; SR141716A, rimonabant.

Administration of the irreversible FAAH inhibitor, URB597, or the reversible FAAH inhibitor, OL-135, leads to increased levels of AEA in the brain (Lichtman et al., 2004a; Fegley et al., 2005). In mice, OL-135 produces analgesic effects in the hot-plate, tail-immersion, and formalin tests, via a CB_1 mechanism of action (Lichtman et al., 2004a). Likewise, FAAH(−/−) mice have approximately 10-fold higher levels of anandamide in the brain than wild-type mice (Cravatt et al., 2001). Overall, FAAH(−/−) mice exhibit a CB_1 receptor-mediated analgesic phenotype in a variety of acute and inflammatory pain models (Lichtman et al., 2004b).

Neuropathy often stems from different types of nerve injury, with common symptoms ranging from numbness or mild tingling to moderate to severe pain (Zimmermann, 2001). This pain can present spontaneously, or as increased sensitivity to noxious stimuli (i.e., hyperalgesia), or perceptions of pain in response to non-noxious stimuli (i.e., allodynia). Ligation of the sciatic nerve, known as chronic constriction injury (CCI), is a common model of neuropathic pain in mice that results in thermal hyperalgesia (Lichtman et al., 2004b), mechanical allodynia (Russo et al., 2007), and cold allodynia (Walczak and Beaulieu, 2006). CCI-induced mechanical allodynia and thermal hyperalgesia were attenuated by the mixed CB_1/CB_2 receptor agonist WIN 55,212-2, via both cannabinoid receptor subtypes (La Rana et al., 2008). Likewise, FAAH inhibition reduces neuropathic pain. Repeated injections of URB597 treatment attenuated CCI-induced thermal hyperalgesia and mechanical allodynia, and these effects were completely blocked by either CB_1 or CB_2 receptor antagonists (Russo et al., 2007). In rats, partial nerve ligation also resulted in mechanical allodynia, which was attenuated by OL-135 (Chang et al., 2006). However, in this model, the CB_2 receptor antagonist, SR144528, blocked the antiallodynic effects of OL-135, whereas the CB_1 receptor antagonist, rimonabant, was ineffective. The observation that the μ-opioid antagonist, naloxone, also blocked the antihyperalgesic effects of OL-135 led the authors to conclude that multiple neurochemical mechanisms were involved. The disparate findings between the Chang et al. (2006) and Russo et al. (2007) studies may result from methodological or species differences and warrant further investigation.

Until recently, no selective MAGL inhibitor was available. Although two compounds, URB602 and *N*-arachidonyl maleimide, inhibited MAGL in the brain, both lack specificity and inhibit other serine hydrolases, including FAAH (Hohmann et al., 2005; Vandevoorde et al., 2007; Burston et al., 2008). The recently developed JZL184 is a highly selective and long-lasting MAGL inhibitor that on systemic administration leads to increased 2-AG levels in the brain and CB_1 receptor-mediated hypoalgesic, hypothermic, and locomotor-suppressant effects in mice (Long et al., 2009), but has yet to be evaluated in a neuropathic pain model.

The present study has four objectives. First, we attempted to reconcile the different effects of URB597 and OL-135 by assessing each compound in the mouse CCI pain model. In addition, we examined whether CB_1, CB_2, TRPV1, and opioid receptor antagonists would block the antiallodynic effects caused by FAAH blockade. Second, because FAAH(−/−) mice subjected to CCI showed no phenotypic antihyperalgesia when subjected to noxious heat stimuli (Lichtman et al., 2004b), we investigated the effects of genetic deletion of FAAH on CCI-induced increases in sensitivity to mechanical and cold stimuli. Third, we sought to determine whether the MAGL inhibitor, JZL184, reduces mechanical and cold allodynia in the CCI model. In addition, we assessed the relative involvement of the CB_1 and CB_2 receptors. Fourth, we sought to quantify the impact of CCI and enzyme inhibition on levels of AEA and 2-AG in the brain and spinal cord.

Materials and Methods

Animals. Subjects consisted of male C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) that were approximately 8 weeks of age at the beginning of the study. In addition, male FAAH(−/−) and age-matched FAAH(+/+) mice from the Center Transgenic Colony at Virginia Commonwealth University, backcrossed onto a C57BL/6J (13 generations), were used. Mice were housed in a temperature-(20–22°C) and humidity-controlled, Association for Assessment and Accreditation of Laboratory Animal Care-approved facility, with ad libitum access to food and water. Subjects weighed approximately 25 g, and were housed four to six mice per cage and maintained on a 12:12 light cycle. Based on previous studies from our laboratory (e.g., Lichtman et al., 2004; Long et al., 2009), the sample size for all behavioral studies was 7 to 10 mice/group. All experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. After testing was complete, all mice were humanely sacrificed via CO_2 asphyxia, followed by rapid cervical dislocation.

Chronic Constriction Injury Surgery. Mice were randomly assigned to sham or CCI treatment groups and were given acetaminophen (2.4 mg/ml in drinking water) from 24 h before surgery to 48 h after surgery. Constant anesthesia was maintained throughout surgery by use of 1.5% isoflurane via inhalation. The right hind leg was shaved and the area swabbed with Betadine solution, then ethanol for antisepsis. An incision was made in the skin posterior to the femur, and the sciatic nerve was isolated after separation of the muscle. The nerve was visualized and ligated twice with use of 4-0 chromic gut suture. The surrounding muscle and skin were then sutured with 6-0 nylon. Mice were allowed to recover on paper towels in a heated cage and observed for approximately 2 h before being returned to the colony.

Drugs. URB597 and gabapentin were purchased from Cayman Chemical (Ann Arbor, MI), rimonabant (SR141716A) and SR144528 were obtained from the National Institute on Drug Abuse (Bethesda, MD), naltrexone hydrochloride and capsazepine were purchased from Sigma-Aldrich (St. Louis, MO), and OL-135 was synthesized as described previously (Boger et al., 2005). Drugs were dissolved in a vehicle consisting of ethanol, Alkamuls-620 (sanofi-aventis, Bridgewater, NJ), and saline in a ratio of 1:1:18, and injected intraperitoneally in a volume of 10 μl/g body mass. JZL184 was synthesized as described previously (Long et al., 2009) and was dissolved in a vehicle of 4:1 polyethylene glycol (PEG 200) and Tween 80 (Sigma-Aldrich) and injected intraperitoneally at a volume of 4 μl/g body mass (Long et al., 2009). All solutions were warmed to room temperature before injection.

Nociceptive Testing. Nociceptive testing began two weeks after surgery. Mice were brought into the testing room, weighed, and allowed to acclimate for at least 1 h before injections. Mice were randomly assigned to a drug treatment regimen by a random number generator. After receiving drug, the mice were placed inside ventilated polycarbonate chambers on an aluminum mesh table and allowed to acclimate to the apparatus for 60 min before testing. Thus, for experiments using URB597 and OL-135, total absorption time was 60 min. In the experiments using JZL184, mice were injected, returned to their home cage, and then placed on the test

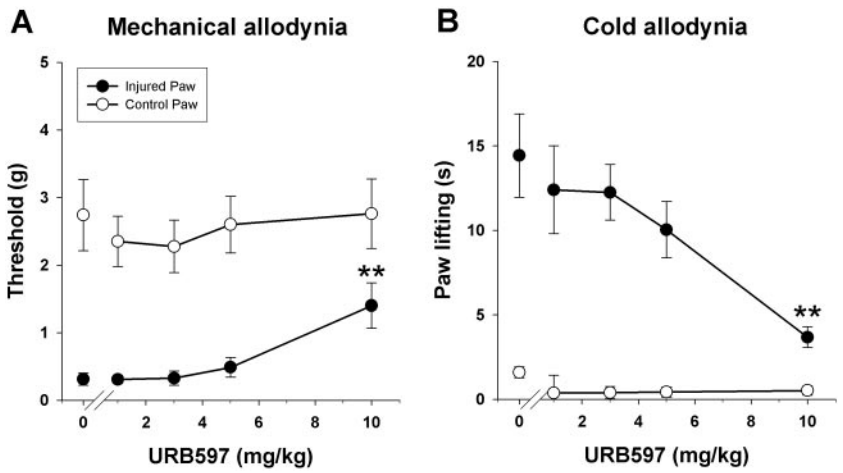

Fig. 1. The irreversible FAAH inhibitor URB597 reduced allodynia in mice subjected to CCI. Mice were injected intraperitoneally with URB597. A, mechanical allodynia was tested 60 min after drug treatment via von Frey test. B, cold allodynia was tested 90 min after drug treatment via acetone-induced cold allodynia test. ○, control paw; ●, CCI paw. Data expressed as mean ± S.E.M. (n = 8–10). ** $p < 0.01$ versus vehicle-treated paw.

table 60 min before testing (total absorption time was 120 min; Long et al., 2009). Mice were pretreated with antagonists 10 min before receiving the FAAH or MAGL inhibitor.

Mechanical allodynia was assessed with von Frey filaments (North Coast Medical, Morgan Hill, CA), using the "up-down" method (Chaplan et al., 1994). The plantar surface of each hind paw was stimulated five times with each filament (0.16–6.0 g), at a frequency of approximately 2 Hz, starting with the 0.6-g filament and increasing until the mouse responded by clutching and/or lifting the paw off the surface of the test apparatus. Paw lifting in response to three or more stimulations was coded as a positive response. Once a positive response was detected, sequentially lower weight filaments were used to assess the sensory threshold for each paw.

Approximately 30 min after the von Frey test, 10 μl of acetone (99% high-performance liquid chromatography grade; Thermo Fisher Scientific, Waltham, MA) was projected via a 100-μl pipette (Rainin Instruments, Woburn, MA) onto the plantar surface of each hind paw (Choi et al., 1994; Decosterd and Woolf, 2000). Acetone was propelled from below via air burst by expressing the pipette, thereby avoiding mechanical stimulation of the paw with the pipette. Total time lifting/clutching each hind paw was recorded with an arbitrary maximum cutoff time of 20 s (Decosterd and Woolf, 2000).

To minimize bias, the experimenter was blinded to surgery condition, genotype, and drug condition, although it should be noted that JZL184 has a distinct yellow tinge in vehicle.

Extraction and Quantification of Endocannabinoids by Liquid Chromatography-Tandem Mass Spectrometry. Fourteen days after CCI/sham surgery, a separate group of mice was used to quantify 2-AG and AEA levels in whole brain and spinal cord after acute treatment with URB597, JZL184, or their respective vehicles. Mice were randomly assigned to surgery and drug treatment groups with use of a random number generator. Pretreatment times were 1 h for URB597, and 2 h for JZL184, along with their respective vehicle controls. Immediately after individual mice were euthanized via CO_2 asphyxiation (at 9:00–11:00 AM EST), the mice were decapitated and their brains and spinal cords were dissected, snap-frozen in dry ice, and stored at −80°C until the time of processing. On the day of processing, tissues were weighed and homogenized with 1.4 ml of chloroform/methanol (2:1 v/v containing 0.0348 g of phenylmethylsulfonyl fluoride/ml) after the addition of internal standards to each sample (2 pmol of AEA-d8 and 1 nmol of 2-AG-d8; Cayman Chemical). Homogenates were then mixed with 0.3 ml of 0.73% w/v NaCl, vortexed, and then centrifuged for 10 min at 4000 rpm (4°C). The aqueous phase plus debris were collected and extracted two more times with 0.8 ml of chloroform. The organic phases from the three extractions were pooled and the organic solvents were evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml of chloroform and mixed with 1 ml of ice-cold acetone. The mixtures were then centrifuged for 5 min at 3000 rpm and 4°C to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml of methanol and placed in autosample vials for analysis.

Liquid chromatography-tandem mass spectrometry was used to quantify AEA and 2-AG. The mobile phase consisted of water/methanol (10:90) with 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery HS C18, 4.6 × 15 cm, 3 μm (Supelco, Bellefonte, PA). The mass spectrometer was run in electrospray ionization in positive mode. Ions were analyzed in a multiple-reaction-monitoring mode and the following transitions were monitored: (348 > 62) and (348 > 91) for AEA; (356 > 62) for AEA-d8; (379 > 287) and (279 > 269) for 2-AG; and (387 > 96) for 2AG-d8. A calibration curve was constructed for each assay based on linear regression with use of the peak area ratios of the calibrators. The extracted standard curves ranged from 0.03 to 40 pmol for AEA and from 0.05 to 64 nmol for 2-AG.

Data Analysis. All data are reported as mean ± S.E.M. and were analyzed by use of one- or two-way analysis of variance. Follow-up comparisons of one-way analysis of variance data used Dunnett's test, and comparisons for two-way comparisons used t tests with Bonferroni correction. A p value of less than 0.05 was considered a statistically significant difference.

Results

CB_1 and CB_2 Receptors Mediate the Antiallodynic Effects of URB597 in von Frey and Acetone-Induced Flinching Assays. CCI caused mechanical allodynia [$F(1, 70) = 83$; $p < 0.0001$; Fig. 1A] and cold allodynia [$F(1, 70) = 109$; $p < 0.0001$; Fig. 1B]. URB597 dose-dependently attenuated allodynia in mice subjected to CCI. In the CCI paw, there was a main effect of drug treatment on mechanical allodynia [$F(4, 39) = 6.5$; $p < 0.001$] and cold allodynia [$F(4, 39) = 5.2$; $p < 0.01$], and follow-up comparisons revealed significantly reduced allodynia at the 10 mg/kg dose in both tests ($p < 0.01$). In both tests, drug treatment had no effect on allodynia in the contralateral, control paw (von Frey, $p = 0.92$; acetone, $p = 0.89$). Because the contralateral paws were not significantly affected by treatments in this or any of the experiments described below, these data are not shown in any subsequent figures.

To assess the receptor mechanism of action underlying the antiallodynic effects of URB597 (10 mg/kg), mice were pretreated with rimonabant (3 mg/kg), SR144528 (3 mg/kg), capsazepine (5 mg/kg), or vehicle 10 min before receiving URB597. There was a significant interaction between

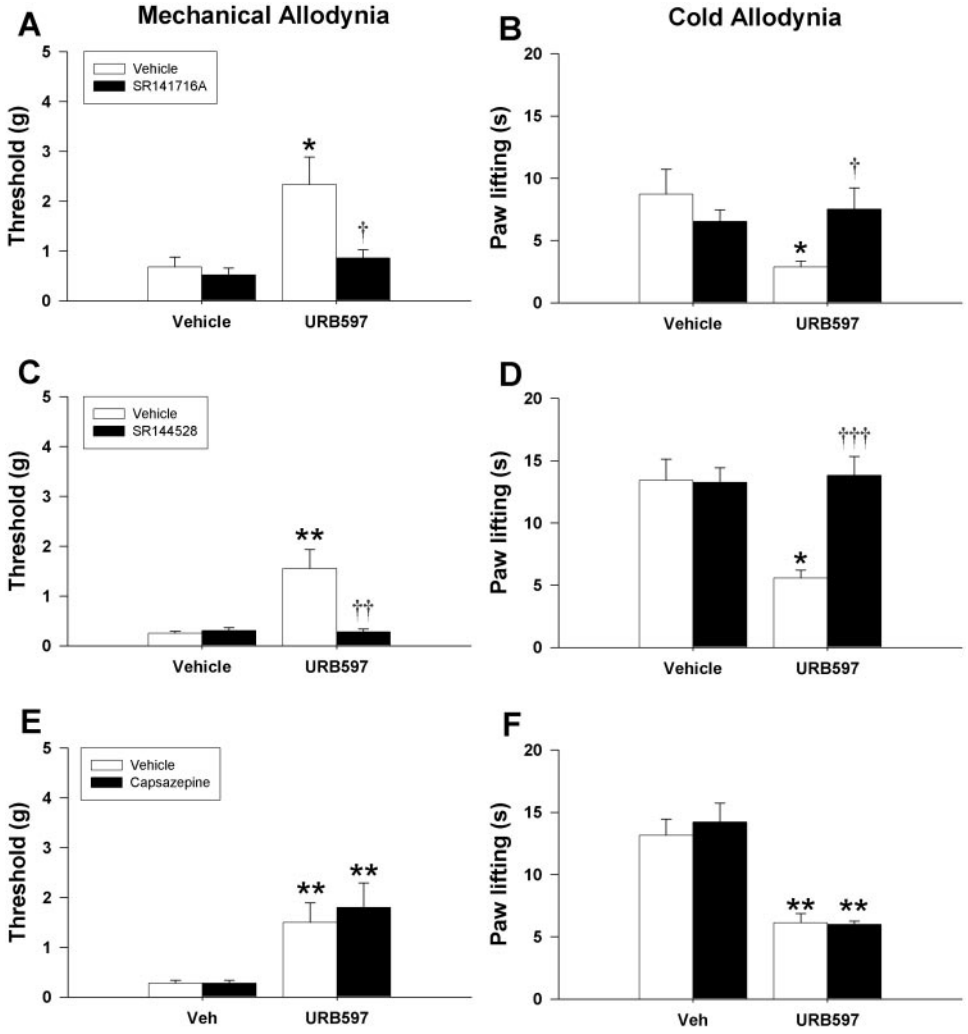

Fig. 2. The irreversible FAAH inhibitor URB597 (10 mg/kg) attenuated allodynia in mice subjected to CCI via CB_1 and CB_2, but not a TRPV1 receptor mediated mechanism of action. Rimonabant (3 mg/kg) completely blocked the antiallodynic effects of URB597 in the von Frey (A) and acetone-induced flinching (B) models. Likewise, the CB_2 receptor antagonist, SR144528 (3 mg/kg), completely blocked the antiallodynic effects of URB597 in the von Frey (C) and acetone-induced flinching (D) models. However, the TPRV1 receptor antagonist, capsazepine (5 mg/kg), did not affect the antiallodynic effects of URB597 in either the von Frey (E) or acetone-induced flinching (F) models. Mechanical allodynia was tested 60 min after URB597 via von Frey test. Cold allodynia was tested 90 min after URB597 via acetone-induced cold allodynia test. Control paws did not differ between treatments. □, vehicle pretreatment; ■, antagonist pretreatment. Data expressed as mean ± S.E.M. (n = 8–9). * $p < 0.05$; ** $p < 0.01$ versus vehicle; † $p < 0.05$; †† $p < 0.01$; ††† $p < 0.001$ versus URB597.

URB597 and pretreatment with rimonabant on mechanical allodynia [$F(1, 32) = 4.329$; $p < 0.05$; Fig. 2A] and cold allodynia [$F(1, 32) = 5.944$; $p < 0.05$; Fig. 2B], indicating CB_1 receptor involvement. URB597 significantly attenuated allodynia in both tests ($p < 0.05$), and this effect was completely blocked by pretreatment with rimonabant ($p < 0.05$). Rimonabant had no effect on allodynia by itself (von Frey, $p = 0.52$; acetone, $p = 0.33$).

There was a significant interaction between URB597 and pretreatment with the selective CB_2 antagonist SR145528 on mechanical allodynia [$F(1, 28) = 11.560$; $p < 0.01$; Fig. 2C] and cold allodynia [$F(1, 28) = 10.146$; $p < 0.01$; Fig. 2D]. URB597 significantly attenuated allodynia in both tests ($p <$ 0.01 and 0.001, respectively), and this effect was completely blocked by pretreatment with SR144528 ($p < 0.01$ and 0.001, respectively). SR144528 had no effect on allodynia by itself (von Frey, $p = 0.47$; acetone, $p = 0.94$).

The selective TRPV1 receptor antagonist, capsazepine, had no effect on the mechanical antiallodynic (URB597 by capsazepine interaction, $p = 0.64$; capsazepine main effect, $p =$ 0.64; Fig. 2E) or cold allodynic (URB597 by capsazepine interaction, $p = 0.58$; capsazepine main effect, $p = 0.67$; Fig. 2F) effects of URB597. URB597 significantly attenuated mechanical [$F(1,28) = 18.9$, $p < 0.001$] and cold allodynia [$F(1,28) = 50$, $p < 0.0001$].

CB_1 and CB_2 Receptors Mediate the Antiallodynic Effects of OL-135 in von Frey and Acetone-Induced Flinching Assays. In this experiment, we assessed whether the reversible FAAH inhibitor, OL-135, would yield a similar pattern of effects as the irreversible FAAH inhibitor, URB597, in the CCI model. As shown in Fig. 3, OL-135 (10 mg/kg) significantly attenuated both mechanical and cold allodynia in mice subjected to CCI. These antiallodynic effects involved both CB_1 and CB_2 receptors, but not TRPV1 or opioid receptors. There was a significant interaction between OL-135 and antagonist pretreatment on mechanical allodynia [$F(4,40) = 9.3$; $p < 0.0001$; Fig. 3A] and cold allodynia [$F(4, 40) = 13.4$; $p < 0.0001$; Fig. 3B]. OL-135 significantly attenuated allodynia in both tests ($p < 0.05$, both tests), and this effect was completely blocked by pretreatment with either rimonabant (3 mg/kg; $p < 0.01$, both tests) or SR144528 (3 mg/kg; $p < 0.05$ and 0.01, respectively). However, capsazepine did not significantly affect the antiallodynic effects of OL-135 (von Frey, $p = 0.22$; acetone, $p = 0.99$).

Because Chang et al. (2006) reported that naloxone significantly attenuated the antiallodynic effects of OL-135 in the rat spinal nerve ligation model, we evaluated whether an opioid receptor mechanism was also involved in the antiallodynic effects of this FAAH inhibitor in the mouse CCI model. As shown in Fig. 3, E and F, naltrexone (1 mg/kg) did not

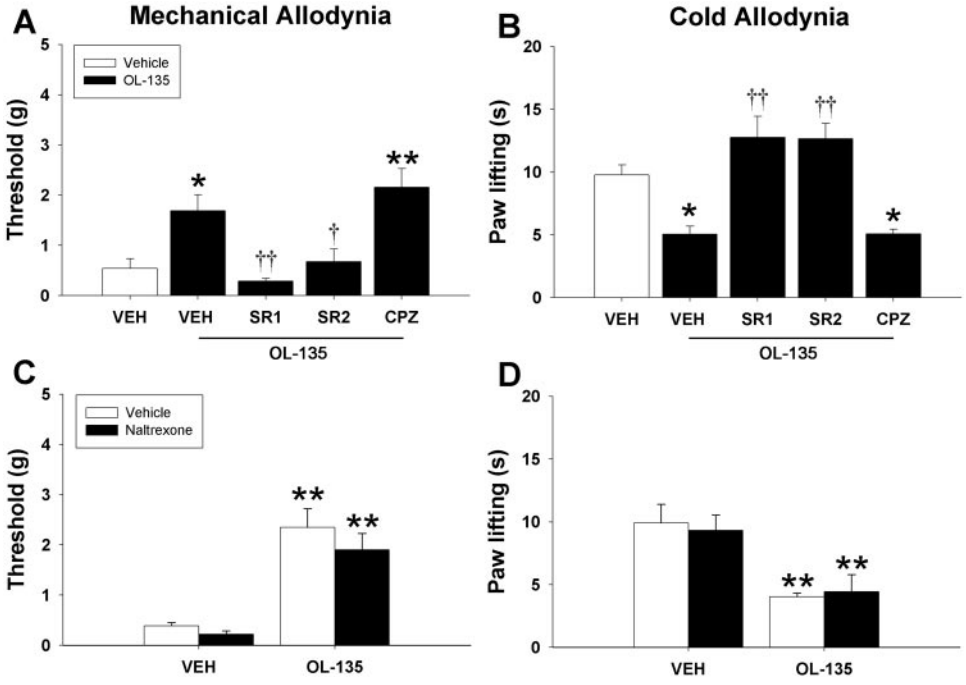

Fig. 3. The reversible FAAH inhibitor OL-135 (10 mg/kg) attenuated allodynia in mice subjected to CCI via CB_1 or CB_2, but not TRPV1 or opioid receptor mediated mechanism of action. Rimonabant (SR1; 3 mg/kg) and SR144528 (SR2; 3 mg/kg) completely blocked the antiallodynic effects of OL-135 in the von Frey (A) and acetone-induced flinching (B) models. However, the TPRV1 receptor antagonist, capsazepine (CPZ; 5 mg/kg) did not affect the antiallodynic effects of OL-135 in either the von Frey (C) or acetone-induced flinching (D) models. Mechanical allodynia was tested 60 min after OL-135 via the von Frey test. Cold allodynia was tested 90 min after OL-135 via the acetone-induced cold allodynia test. Control paws did not differ between treatments. □, vehicle pretreatment; ■, antagonist pretreatment. Data expressed as mean ± S.E.M. (n = 7–9). * $p < 0.05$; ** $p < 0.01$ versus vehicle; † $p < 0.05$; †† $p < 0.01$ versus URB597.

block the antiallodynic effects of OL-135. There was no significant interaction between OL-135 and naltrexone pretreatment on mechanical allodynia ($p = 0.60$; Fig. 3E) or cold allodynia ($p = 0.66$; Fig. 3F). However, OL-135 significantly attenuated allodynia in both mechanical [$F(1, 26) = 45.6$; $p < 0.0001$] and cold tests [$F(1, 26) = 21.3$; $p < 0.0001$], even though this effect was not reversed by pretreatment with naltrexone (von Frey, $p = 0.26$; acetone, $p = 0.94$). In addition, naltrexone given alone had no effect on allodynia (von Frey, $p = 0.10$; acetone, $p = 0.76$).

The Antiallodynic Effects of URB597 and OL-135 Are Mediated through the Suppression of FAAH. In contrast to the antiallodynic effects of URB597 (10 mg/kg) and OL-135 (10 mg/kg) in the von Frey and acetone-induced flinching assays, we reported previously that FAAH(−/−) mice do not display a reduction in thermal hyperalgesia, as assessed in Hargreaves's plantar stimulator test (Lichtman et al., 2004b). Accordingly, in the present experiment, we assessed whether this lack of a thermal hyperalgesic phenotype was modality specific or extended to other types of noxious stimuli. FAAH(−/−) mice displayed identical nociceptive behavior as FAAH(+/+) mice in both the von Frey (Fig. 4, A and C) and acetone (Fig. 4, B and D) assays. Thus, genetic deletion and pharmacological inhibition of FAAH lead to different phenotypes in the mouse CCI model of neuropathic pain. To confirm whether the antiallodynic properties of URB597 and

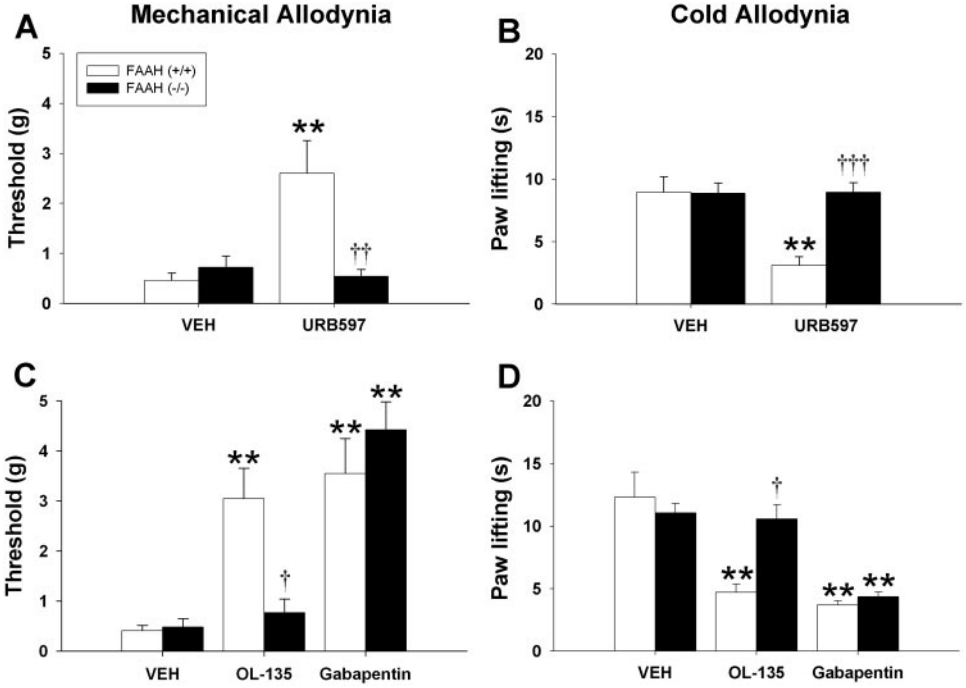

Fig. 4. FAAH (−/−) mice do not display antiallodynic phenotypes in either the von Frey or acetone-induced flinching models. URB597 (10 mg/kg) attenuated CCI-induced allodynia in FAAH(+/+), but not FAAH(−/−) mice in the von Frey (A) and acetone-induced flinching (B) models. Likewise, OL-135 (10 mg/kg) attenuated CCI-induced allodynia in FAAH(+/+), but not FAAH(−/−) mice in the von Frey (C) and acetone-induced flinching (D) models. Gabapentin (50 mg/kg) attenuated allodynia in both strains. Mechanical allodynia was tested 60 min after drug via the von Frey test. Cold allodynia was tested 90 min after drug via the acetone-induced cold allodynia test. Control paws did not differ between treatments. □, FAAH (+/+); ■, FAAH (−/−). Data expressed as mean ± S.E.M. (n = 8). * $p < 0.05$; ** $p < 0.01$ versus vehicle; † $p < 0.05$; †† $p < 0.01$; ††† $p < 0.001$ versus FAAH(+/+).

OL-135 were mediated through FAAH, each drug was tested in FAAH(−/−) and FAAH(+/+) mice. There was no effect of genotype on allodynia in vehicle-treated mice (von Frey, $p = 0.35$; acetone, $p = 0.96$). In contrast, there were significant interactions between URB597 and genotype for mechanical allodynia [$F(1, 26) = 10.4$; $p < 0.05$; Fig. 4A] and cold allodynia [$F(1, 26) = 11.7$; $p < 0.01$; Fig. 4B]. However, URB597 had no effect in FAAH-deficient mice in either mechanical ($p = 0.51$) or cold ($p = 0.96$) allodynia tests.

Next, OL-135 was compared in FAAH(−/−) and (+/+) mice. In addition, the GABA analog gabapentin (50 mg/kg) was assessed in both genotypes, as a positive control. There was a significant interaction between OL-135/gabapentin and strain on mechanical allodynia [$F(2, 42) = 6.3$; $p < 0.01$; Fig. 4C] and cold allodynia [$F(2, 42) = 6.7$; $p < 0.01$; Fig. 4D]. OL-135 elicited antiallodynic effects in FAAH(+/+) mice, but had no effect in FAAH(−/−) mice in either mechanical ($p = 0.39$) or cold allodynia ($p = 0.73$) tests. Unlike the FAAH inhibitors, gabapentin significantly reduced mechanical and cold allodynia in both genotypes.

MAGL Inhibitor JZL184 Attenuated Allodynia via CB_1 Mechanism of Action. In the next set of experiments, we evaluated whether the selective MAGL inhibitor, JZL184, would reduce mechanical and cold allodynia in nerve-injured mice. As shown in Fig. 5, JZL184 (16 mg/kg) was efficacious in reducing mechanical and cold allodynia. In addition, these antiallodynic effects were blocked by rimonabant (3 mg/kg), but not by SR144528 (3 mg/kg). Specifically, a significant interaction was found between JZL184 and antagonist pretreatment on mechanical allodynia [$F(3, 26) = 5.0$; $p < 0.01$; Fig. 5A] and cold allodynia [$F(3, 26) = 15.5$; $p < 0.0001$; Fig. 5B]. JZL184 significantly attenuated allodynia in both tests ($p < 0.05$), and these effects were blocked by rimonabant (von Frey, $p < 0.05$; acetone, $p < 0.01$), but not by SR144528 (von Frey, $p = 0.65$; acetone, $p = 0.46$).

Next, to examine whether JZL184 worked via a FAAH-dependent mechanism of action, we evaluated its effects in FAAH(−/−) and (+/+) mice. JZL184 (16 mg/kg) significantly reduced both mechanical [$F(1, 28) = 42.2$; $p < 0.0001$; Fig. 6A] and cold allodynia [$F(1, 28) = 34.6$; $p < 0.0001$; Fig. 6B] in both genotypes. There was no interaction between drug and genotype in either test of mechanical ($p = 0.82$) or cold allodynia ($p = 0.56$).

Quantification of Endocannabinoid Levels in Brain and Spinal Cord. The levels of AEA and 2-AG were quantified in whole brain and spinal cord of mice 14 days after CCI or sham surgery. Pretreatment times were 1 h for URB597 (10 mg/kg) and 2 h for JZL184 (16 mg/kg), along with their respective vehicles. Because there were no significant differences between the ethoxylated castor oil (Alkamuls-620)/ethanol/saline and polyethylene glycol/Tween 80 vehicles, for AEA or 2-AG in the brain (unpaired t tests, respective p values = 0.17 and 0.53) or spinal cord (unpaired t tests, respective p values = 0.52 and 0.46), these control groups were collapsed. The FAAH inhibitor, URB597, significantly increased AEA levels in brain [$F(2, 29) = 18.8$; $p < 0.0001$; Table 1] and spinal cord [$F(2, 25) = 67.2$; $p < 0.01$] of both CCI and sham-operated mice. URB597 had no effect on 2-AG in either tissue. The MAGL inhibitor, JZL184, significantly increased 2-AG levels in brain [$F(2, 29) = 20.7$; $p < 0.0001$] and spinal cord [$F(2, 25) = 14.9$; $p < 0.0001$] of both CCI and sham-operated mice, but had no effect on AEA in either matrix. Overall, CCI did not affect AEA or 2-AG levels in whole brain (respective p values = 0.59 and 0.40) or whole spinal cord (respective p values = 0.55 and 0.35).

Discussion

Inhibition of FAAH by either irreversible (URB597) or reversible (OL-135) FAAH inhibitors attenuated mechanical and cold allodynia in the CCI model. These antiallodynic effects were blocked by pretreatment with either the CB_1 receptor antagonist/inverse agonist, rimonabant, or the CB_2 receptor antagonist, SR144528. Genetic FAAH-deficient mice showed no difference in CCI-induced allodynia, compared with wild-type mice. URB597 and OL-135 were ineffective in FAAH(−/−) mice, indicating that the observed antiallodynic effects were due to FAAH modulation. The novel MAGL inhibitor, JZL184, also attenuated both mechanical and cold allodynia, although these effects were blocked by rimonabant, but not SR144528 pretreatment. Finally, URB597 increased AEA, but not 2-AG, levels in whole brain and spinal cord, whereas JZL184 elicited the opposite pattern of effects in both matrices.

URB597 has been shown previously to attenuate CCI-induced mechanical allodynia and thermal hyperalgesia

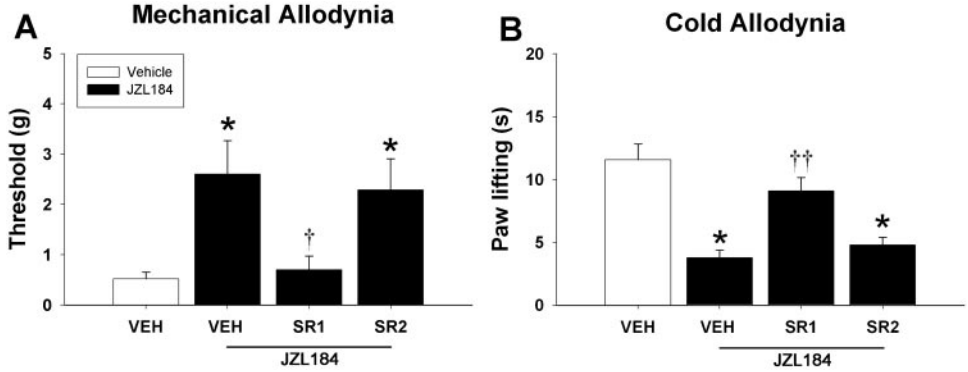

Fig. 5. Pharmacological blockade of MAGL attenuates CCI-induced mechanical and cold allodynia via a CB_1 receptor mediated mechanism of action. The novel MAGL inhibitor JZL184 (16 mg/kg) in the von Frey (A) and acetone-induced flinching (B) models. Rimonabant (SR1; 3 mg/kg) completely blocked the antiallodynic effects of JZL184, whereas SR144528 (SR2; 3 mg/kg) had no effect in the von Frey or acetone-induced flinching models. Mechanical allodynia was tested 120 min after drug via the von Frey test. Cold allodynia was tested 150 min after drug via the acetone-induced cold allodynia test. Control paws did not differ between treatments. □, vehicle; ■, JZL184. Data expressed as mean ± S.E.M. ($n = 7–8$). * $p < 0.05$ versus vehicle; † $p < 0.05$; †† $p < 0.01$ versus JZL184.

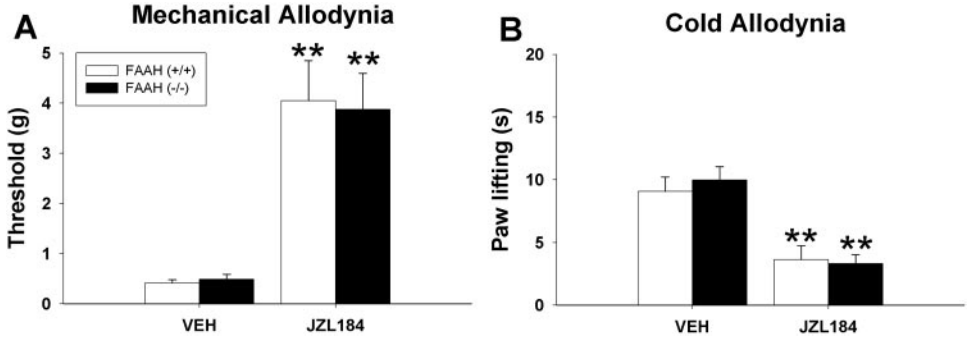

Fig. 6. Pharmacological blockade of MAGL attenuates CCI-induced mechanical and cold allodynia via a FAAH-independent mechanism of action. JZL184 (16 mg/kg) attenuated CCI-induced allodynia in FAAH(+/+) and FAAH(−/−) mice in the von Frey (A) and acetone-induced flinching (B) models. Mechanical allodynia was tested 120 min after drug via the von Frey test. Cold allodynia was tested 150 min after drug via the acetone-induced cold allodynia test. Control paws did not differ between treatments. □, FAAH(+/+); ■, FAAH(−/−). Data expressed as mean ± S.E.M. (n = 8). ** $p <$ 0.01 versus vehicle.

TABLE 1

The enzyme inhibitors, URB597 and JZL184, selectively increased brain and spinal cord levels of AEA and 2-AG, respectively

Fourteen days after CCI/sham surgery, mice were injected with URB597 (10 mg/kg i.p.), JZL184 (16 mg/kg i.p.), or vehicle. Pretreatment times were 1 h for URB597 and 2 h for JZL184, along with each vehicle. LC/MS/MS was used to quantify AEA and 2-AG for each sample. Data are expressed as mean (± S.E.M.). n = 4 to 6.

		Brain		Spine	
		AEA	2-AG	AEA	2-AG
		pmol/g	*nmol/g*	*pmol/g*	*nmol/g*
VEH	CCI	3.76 (0.51)	14.55 (1.95)	7.19 (3.71)	14.77 (3.46)
	Sham	3.30 (0.36)	14.95 (1.91)	5.21 (2.18)	15.82 (3.82)
URB597	CCI	36.30 (9.80)**	13.18 (2.41)	180.44 (89.51)	12.68 (2.90)
	Sham	30.21 (9.37)**	12.05 (1.91)	115.15 (62.42)	10.79 (1.48)
JZL184	CCI	5.90 (1.23)	85.83 (17.75)***	14.07 (2.50)	76.32 (15.58)**
	Sham	5.35 (1.68)	132.16 (36.64)**	12.79 (5.41)	44.64 (15.53)

LC/MS/MS, liquid chromatography-tandem mass spectrometry; VEH, vehicle.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus vehicle.

(Russo et al., 2007). However, approximately one third of patients suffering from neuropathic pain reported a high incidence of sensitivity to cold stimuli, in addition to those with increased sensitivity to heat and/or touch (Attal et al., 2008). Thus, testing for cold sensitivity increases the possible clinical relevance of this model.

OL-135 was previously shown to have analgesic effects in the hot-plate and tail-immersion tests, and these effects were prevented by rimonabant (Lichtman et al., 2004a). In rats, the antihyperalgesic effects of OL-135 were reversed by naloxone, suggesting the involvement of μ-opioid receptors (Chang et al., 2006). In the present study, pretreatment with the opioid antagonist naltrexone had no effect, although either CB_1 or CB_2 receptor antagonists completely blocked the antiallodynic effects of OL-135. Disparities between our findings and those of Chang et al. (2006) may be due to differences in the pain model (spinal nerve ligation versus CCI), species (rats versus mice), or other methodological differences that may have affected stress levels. Of particular interest, the antiallodynic effects of the FAAH inhibitors were fully reversed by pretreatment with either CB_1 or CB_2 antagonists, but were unaffected by the TRPV1 receptor antagonist, capsazepine. It is noteworthy that none of the receptor antagonists administered alone affected either mechanical or cold allodynia, indicating that CB_1, CB_2, opioid, or TRPV1 receptors do not tonically inhibit pain in the mouse CCI model, as measured 2 weeks after injury.

The observation that FAAH(−/−) mice did not show a phenotypic difference in CCI-induced allodynia is in line with a previous study in which CCI-induced thermal hyperalgesia was virtually identical in both genotypes (Lichtman et al., 2004b). Thus, the stimulus modality (i.e., radiant heat versus touch or cold) does seem to be responsible for the lack of a phenotype difference. FAAH(−/−) mice may undergo adaptive changes during development that alter the neuroimmune response to neuropathy. Alternatively, adaptive changes in FAAH knockout mice may differentially affect the nociceptive pathways activated by nerve injury. For example, nerve injury may affect neural cannabinoid receptor function differently in FAAH knockout mice, compared with wild-type mice. In support of this hypothesis, FAAH(−/−) mice no longer showed phenotypic hypoalgesia in the tail immersion or hot-plate tests after CCI surgery (Lichtman et al., 2004b). Genetic FAAH knockout mice were unresponsive to both URB597 and OL-135, but gabapentin or the MAGL inhibitor, JZL184, elicited full antiallodynic effects in these animals. These data confirm that the antiallodynic effects of URB597 and OL-135 are mediated through a FAAH-dependent mechanism. Conversely, these findings indicate that the antiallodynic effects of JZL184 are independent of FAAH. The observations that gabapentin and JZL184 continued to elicit antiallodynic effects in FAAH(−/−) mice demonstrate that these mice are still responsive to antiallodynic treatments.

In the present study and a previous report (Russo et al., 2007), the antiallodynic effects of FAAH inhibition were reversed by CB_1 and CB_2 receptor antagonists. Although CB_2 receptor agonists have analgesic effects, the functional role of the CB_2 receptor in pain circuits is not well understood, even though these receptors are up-regulated in immune cells after injury. For example, microglia show increased CB_2 re-

ceptor expression in mice subjected to CCI compared with noninjured mice (Zhang et al., 2003). Both CB_1 and CB_2 receptors have been implicated in modulating inflammatory pain models (Clayton et al., 2002). After injury, neuropathy is modulated by glia in the dorsal spinal cord, including activated microglia and astrocytes (Ledeboer et al., 2005; Watkins et al., 2007). These cells produce chemokines, which recruit other immune cells to infiltrate the injured tissue, and proinflammatory cytokines including interleukin-1, interleukin-6, and tumor necrosis factor-α. Further nerve injury results, coupled with noxious neural stimulation, resulting in pain perception by the host (Samad et al., 2001; McMahon et al., 2005; Watkins et al., 2007). Blocking this proinflammatory cytokine cascade results in decreased pain after CCI (Milligan et al., 2006). Given the neuroinflammatory nature of the nerve injury in the CCI model, it is not surprising that both CB_1 and CB_2 receptors also play a role in modulating neuropathic pain (Russo et al., 2007; Jhaveri et al., 2008; La Rana et al., 2008). The findings of Russo et al. (2007), taken together with the present data, indicate that both CB_1 and CB_2 receptors are involved in the antiallodynic effects of FAAH inhibition. One explanation for the observation that each cannabinoid receptor plays a necessary role in the antiallodynic effects of FAAH inhibitors is that CB_1 and CB_2 receptors act at different levels of serial nociceptive and/or inflammatory pathways. The CB_2 receptor is expressed predominantly on cells of the immune system, at approximately 10 to 100 times that of CB_1 (Galiègue et al., 1995). However, with the development of improved antibodies, this receptor has now been identified in brain stem neurons (Van Sickle et al., 2005). Although not typically expressed at high levels in healthy tissues, CB_2 receptors are up-regulated in diseased nervous tissue (Wotherspoon et al., 2005). Thus, CB_2 receptor stimulation may decrease allodynia at the level of peripheral nociceptors, spinal nerves, and afferents, or supraspinally. However, CB_1 receptors are most probably involved in neural tissue. Peripheral deletion of CB_1 on nociceptors (with CB_1 preserved in the central nervous system) blocked the analgesic effects of locally and systemically administered cannabinoids (Agarwal et al., 2007).

We were surprised to find that 2-AG seems to attenuate allodynia via a CB_1 receptor mechanism, with no apparent contribution from the CB_2 receptor. Whether these antiallodynic effects are due to direct CB_1 binding on neural tissues or to indirect alterations in neuroinflammation is unknown. One explanation for the difference underlying cannabinoid receptor mechanisms for the antiallodynic effects between MAGL and FAAH inhibitors is that the endogenous cannabinoid may affect different levels of the nociceptive and inflammatory pathways involved in neuropathic pain. The mechanisms for this disconnect are unclear and warrant future investigation.

As shown previously, FAAH inhibition, increased levels of AEA in whole brain and spinal cord, with no effect on 2-AG (Lichtman et al., 2002; Kathuria et al., 2003). Conversely, the novel MAGL inhibitor, JZL184, increased 2-AG in whole brain and spinal cord, but did not affect AEA. In addition to MAGL, two other enzymes have also been identified that regulate a fraction of neuronal 2-AG, ABHD6 and ABHD12 (Blankman et al., 2007). However, MAGL is the predominant enzyme responsible for 2-AG regulation in vivo. We did not observe any differences in endocannabinoid levels between CCI and sham-operated mice. It is possible that differences in AEA levels occurred locally at the site of nerve injury or at the dorsal root ganglia or dorsal horn at the level of injury. Thus, quantifying endocannabinoid levels from the entire cord might have obscured detection of these regional changes. Peripheral nerve damage increased both AEA and 2-AG in the ipsilateral half of the spinal cord (Mitrirattanakul et al., 2006) and the periaqueductal gray and rostral ventral medulla (Petrosino et al., 2007). In addition, FAAH immunoreactivity increased in large myelinated neurons in dorsal root ganglia after nerve injury (Lever et al., 2009). Thus, future studies will quantify the time course of changes in endocannabinoid levels in discrete anatomical structures after nerve injury.

In conclusion, the present findings indicate that pharmacological inhibition of either FAAH or MAGL effectively reduces mechanical and cold allodynia in mice subjected to chronic constriction injury of the sciatic nerve. This reduction of neuropathic pain is mediated via distinct cannabinoid receptor mechanisms of action, which warrant further investigation. These data provide the first evidence that MAGL inhibition attenuates pain in a neuroinflammatory disease model and indicate that inhibition of both FAAH and MAGL is a potential target for the development of analgesic therapeutics.

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Address correspondence to: Dr. Aron Lichtman, PO Box 980613, Richmond, VA 23298. E-mail: alichtma@vcu.edu