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Full Fatty Acid Amide Hydrolase Inhibition Combined with Partial Monoacylglycerol Lipase Inhibition: Augmented and Sustained Antinociceptive Effects with Reduced Cannabimimetic Side Effects in Mice

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ABSTRACT

Inhibition of fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL), the primary hydrolytic enzymes for the respective endocannabinoids *N*-arachidonoylethanolamine (AEA) and 2-arachidonylglycerol (2-AG), produces antinociception but with minimal cannabimimetic side effects. Although selective inhibitors of either enzyme often show partial efficacy in various nociceptive models, their combined blockade elicits augmented antinociceptive effects, but side effects emerge. Moreover, complete and prolonged MAGL blockade leads to cannabinoid receptor type 1 (CB_1) receptor functional tolerance, which represents another challenge in this potential therapeutic strategy. Therefore, the present study tested whether full FAAH inhibition combined with partial MAGL inhibition would produce sustained antinociceptive effects with minimal cannabimimetic side effects. Accordingly, we tested a high dose of the FAAH inhibitor PF-3845 (*N*-3-pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidinecarboxamide; 10 mg/kg) given in combination with a low dose of the MAGL inhibitor JZL184 [4-nitrophenyl 4-(dibenzo[*d*][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate] (4 mg/kg) in mouse models of inflammatory and neuropathic pain. This combination of inhibitors elicited profound increases in brain AEA levels (>10-fold) but only 2- to 3-fold increases in brain 2-AG levels. This combination produced significantly greater antinociceptive effects than single enzyme inhibition and did not elicit common cannabimimetic effects (e.g., catalepsy, hypomotility, hypothermia, and substitution for Δ^9-tetrahydrocannabinol in the drug-discrimination assay), although these side effects emerged with high-dose JZL184 (i.e., 100 mg/kg). Finally, repeated administration of this combination did not lead to tolerance to its antiallodynic actions in the carrageenan assay or CB_1 receptor functional tolerance. Thus, full FAAH inhibition combined with partial MAGL inhibition reduces neuropathic and inflammatory pain states with minimal cannabimimetic effects.

Introduction

Inhibition of fatty acid amide hydrolase (FAAH; Cravatt et al., 1996, 2001) or monoacylglycerol lipase (MAGL; Dinh et al., 2002), the respective primary hydrolytic enzymes for *N*-arachidonoylethanolamine (AEA; Devane et al., 1992) and

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ABBREVIATIONS: [^{35}S]GTPγS, guanosine 5′-O-(3-[^{35}S]thio)triphosphate; AA, arachidonic acid; AEA, *N*-arachidonoylethanolamine; 2-AG, 2-arachidonoylglycerol; AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; AM630, 1-[2-(morpholin-4-yl)ethyl]-2-methyl-3-(4-methoxybenzoyl)-6-iodoindole; ANOVA, analysis of variance; BSA, bovine serum albumin; CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; CCI, chronic constrictive injury of the sciatic nerve; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CP55,940, 2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol; D-AP-5, D-2-amino-5-phosphonovaleric acid; FAAH, fatty acid amide hydrolase; IPSC, inhibitory postsynaptic current; JZL184, 4-nitrophenyl 4-(dibenzo[*d*][1,3]dioxol-5-yl(hydroxy)methyl) piperidine-1-carboxylate; MAGL, monoacylglycerol lipase; PF-3845, *N*-3-pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidinecarboxamide; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethylbicyclo [2.2.1]heptan2-yl]-5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-1*H*-pyrazole-3-carboxamide; THC, Δ^9-tetrahydrocannabinol; WIN55,212-2, *R*-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-1-naphthalenylmethanone mesylate.

2-arachidonylglycerol (2-AG; Mechoulam et al., 1995; Sugiura et al., 1995), represent potential strategies to alleviate pain but with reduced cannabimimetic side effects. Pharmacologic inhibition or genetic deletion of FAAH (Cravatt et al., 2001; Kathuria et al., 2003) and MAGL (Long et al., 2009a; Chanda et al., 2010; Schlosburg et al., 2010) elevates respective AEA and 2-AG levels in the brain. Blockade of either enzyme produces antinociceptive effects in a variety of acute and chronic preclinical models of pain (Kathuria et al., 2003; Chang et al., 2006; Jhaveri et al., 2007; Russo et al., 2007; Kinsey et al., 2009; Spradley et al., 2010; Guindon et al., 2011, 2013; Ghosh et al., 2013). Inhibitors of these enzymes often lack full efficacy in neuropathic and inflammatory pain models, which reflects a potential limitation for their clinical development. On the other hand, combined inhibition of the major endocannabinoid catabolic enzymes with the dual FAAH-MAGL inhibitor JZL195 produced enhanced antinociceptive effects in several preclinical pain models compared with individual inhibition of these enzymes (Long et al., 2009d; Anderson et al., 2014); however, JZL195 also produced hypomotility, catalepsy, Δ^9-tetrahydrocannabinol (THC)–like subjective effects in the drug-discrimination assay, and impaired spatial memory performance in the Morris water-maze task (Long et al., 2009d; Wise et al., 2012; Anderson et al., 2014). Although these cannabimimetic actions represent drawbacks in the strategy of dual inhibition of FAAH and MAGL for the treatment of pain, JZL195 was approximately 3-fold more potent in reversing complete Freund adjuvant–induced allodynia than in producing catalepsy and hypomotility (Anderson et al., 2014).

Tolerance represents another challenge in the development of new analgesics, as many conditions require chronic drug treatment. The consequences of prolonged inhibition of MAGL markedly contrast with those of sustained FAAH blockade. Whereas repeated administration of high doses of FAAH inhibitors produce sustained analgesia, without loss of cannabinoid receptor type 1 (CB_1) receptor function (Falenski et al., 2010; Schlosburg et al., 2010), and FAAH-deficient mice display a CB_1 receptor–mediated hypoalgesic phenotype (Lichtman et al., 2004), complete blockade of MAGL leads to tolerance, physical dependence, impaired endocannabinoid-dependent synaptic plasticity, and CB_1 receptor downregulation and desensitization in select brain regions, but it does not affect CB_2 receptor expression in the spleen (Chanda et al., 2010; Schlosburg et al., 2010; Ignatowska-Jankowska et al., 2014). Prolonged partial MAGL inhibition with repeated administration of low doses of JZL184 [4-nitrophenyl 4-(dibenzo[*d*][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate] (e.g., $\leq$8 mg/kg) maintains its anxiolytic-like effects (Sciolino et al., 2011), as well as its antinociceptive actions in the chronic constrictive injury of the sciatic nerve (CCI) and carrageenan models of pain (Ghosh et al., 2013; Kinsey et al., 2013). Repeated administration of low-dose JZL184 ($\leq$8 mg/kg) does not lead to CB_1 receptor downregulation or desensitization (Kinsey et al., 2013).

In the present study, we tested whether full inhibition of FAAH in combination with partial MAGL inhibition would elicit enhanced antinociceptive effects compared with single enzyme inhibition. To this end, we examined the impact of coadministration high-dose PF-3845 (10 mg/kg) and low-dose JZL184 (4 mg/kg) in the carrageenan model of inflammatory pain and the CCI model of neuropathic pain. The selected doses of these respective drugs fully inhibit FAAH (Ahn et al., 2009) and partially inhibit (i.e., approximately 40%) MAGL (Long et al., 2009c). Moreover, repeated administration of these inhibitors given individually does not lead to CB_1 receptor downregulation or desensitization (Schlosburg et al., 2010; Kinsey et al., 2013). Potential cannabimimetic side effects of this drug combination were assessed using common in vivo measures that are sensitive to direct-acting CB_1 receptor agonists (i.e., catalepsy, locomotor activity, thermal antinociception, and hypothermia) (Little et al., 1988) and for THC-like effects in the drug-discrimination assay (McMahon et al., 2008). In addition, we tested whether repeated injections of PF-3845 and JZL184, in combination, would produce sustained antiallodynic effects in carrageenan-treated mice. After completion of the behavioral study, the mice were euthanized and CB_1 receptor expression and function was assessed using [^{3}H]SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride] binding and CP55,940 (2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol)-stimulated guanosine 5′-O-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS)–stimulated binding assays. Finally, inhibition of postsynaptic currents (IPSCs) induced by WIN55,212-2 (*R*-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-1-naphthalenylmethanone mesylate), a nonselective cannabinoid receptor agonist, was examined in cingulate cortex, a brain region implicated in pain (Zhuo, 2006) and known to contain a high concentration of CB_1 receptors (Herkenham et al., 1990) that undergo desensitization after 6 days of daily injections of 40 mg/kg JZL184 (Schlosburg et al., 2010).

Materials and Methods

Subjects. Male C57BL/6J mice from The Jackson Laboratory (Bar Harbor, ME) that weighed between 18 and 25 g served as subjects and were housed in a temperature- (20–22°C) and humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care–approved facility, with a 12-hour light/dark cycle. Four to five mice were housed per cage and were given unlimited access to food and water in their home cages. The Virginia Commonwealth University Institutional Animal Care and Use Committee approved all animal protocols, which were in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals, Institute of Laboratory and Animal Resources. At the conclusion of behavioral studies, mice were euthanized via CO_2 asphyxia, followed by rapid cervical dislocation. For the binding and endocannabinoid quantification studies, the animals were euthanized by decapitation without anesthesia.

Drugs. JZL184, PF-3845 (*N*-3-pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidinecarboxamide), rimonabant, SR144528 [*N*-[(1*S*)-endo-1,3,3-trimethylbicyclo [2.2.1]heptan2-yl]-5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-1*H*-pyrazole-3-carboxamide], and THC were obtained from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). Deuterated standards for AEA and 2-AG were purchased from Cayman Chemical (Ann Arbor, MI). CP55,940, GDP, and unlabeled GTPγS were purchased from Sigma-Aldrich (St. Louis, MO), and [^{35}S]GTPγS was purchased from PerkinElmer (Waltham, MA).

Drug Administration. Drugs were dissolved in a vehicle that consisted of a mixture of ethanol, alkamuls-620 (Sanofi, Bridgewater, NJ), and saline in a ratio of 1:1:18 parts, and sonicated as needed. In the drug-discrimination experiment, however, the vehicle consisted of a mixture containing propylene glycol, Tween 80, and saline in a ratio of 1:1:18 parts. Each drug was given via the intraperitoneal route of administration in a volume of 10 μl/g of body mass.

Carrageenan-Induced Inflammatory Pain. Paw edema and inflammatory pain were induced by giving an intraplantar injection of 0.3% carrageenan (Sigma-Aldrich) in a 20-μl volume through a 30-gauge needle into the left hind paw, as previously described (Ghosh et al., 2013). Paw thickness was measured using electronic digital calipers (Traceable Calipers, Friendswood, TX) before and 5 hours after carrageenan administration, which corresponds to peak edema, and mechanical allodynia was assessed 5 hours after carrageenan administration. These time points have been used previously by our laboratory (Ghosh et al., 2013).

Neuropathic Pain Model. Mice were subjected to CCI of the sciatic nerve, as described previously (Kinsey et al., 2009). Anesthesia was induced under isoflurane anesthesia. The right hind leg was shaved, and the area was swabbed with betadine solution and then ethanol. A small incision was made in the skin posterior to the femur, the muscle was separated, and the sciatic nerve was isolated and ligated twice with 5-0 (1.0 metric) black silk-braided suture (Surgical Specialties Corporation, Reading, PA). The surrounding muscle and skin were then sutured with 6-0 nylon. Mice were placed in a heated cage to recover from anesthesia before being returned to the vivarium. Mice were tested for allodynia approximately 1 week after CCI surgery to establish baseline levels of allodynia.

Mechanical Allodynia. Carrageenan-injected mice and CCI mice were assessed for mechanical allodynia using von Frey filaments (North Coast Medical, Morgan Hill, CA), as previously described (Ghosh et al., 2013). The mice were placed inside ventilated polycarbonate chambers on an elevated aluminum mesh table and allowed to acclimate to the apparatus for 60 minutes before testing. 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 licking and/or lifting the paw off the surface of the test apparatus. Three or more responses of five stimulations were coded as a positive response.

Mice were transported to the testing room, weighed, randomly assigned to the different treatment regimens, and allowed to acclimate for at least 1 hour before injections. JZL184 and PF-3845 were administered 2 hours before carrageenan (i.e., 7 hours before testing) to test whether the combination prevents allodynia. In experiments assessing CB_1 and CB_2 receptor involvement, the respective antagonists rimonabant (1 mg/kg) and SR144528 (3 mg/kg) were administered 30 minutes before the enzyme inhibitors (i.e., 7.5 hours before testing). We previously established that these doses of antagonist and time points effectively antagonize the pharmacologic effects of JZL184 alone in the carrageenan assay (Ghosh et al., 2013). In the carrageenan reversal experiment, the compounds were injected 3 hours after carrageenan (i.e., 2 hours before testing). Similarly, in the CCI experiment, the compounds were given 2 hours before allodynia testing. This pretreatment time was selected because both PF-3845 (Ahn et al., 2009) and JZL184 (Long et al., 2009a) elicit maximal elevations of endocannabinoids within 2 hours of administration that persist for several hours. Moreover, both drugs reduce carrageenan-induced (Ghosh et al., 2013) and CCI-induced allodynia (Kinsey et al., 2011, 2013) within this time frame.

To assess the impact of repeated administration of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) on paw edema and mechanical allodynia in the carrageenan study, the following groups of mice were tested: (group 1) vehicle for 6 days, (group 2) vehicle for 5 days, and challenged with JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) on day 6 and (group 3) JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) for 6 days. On test days, mice were administered their respective treatments 2 hours before carrageenan was injected. Edema and mechanical allodynia were assessed 5 hours later.

Cannabimimetic Behavior Assessments. Subjects were divided into six groups (n = 8–10 mice/group) to assess cannabimimetic effects of combined administration of PF-3845 (10 mg/kg i.p.) and the dose-response relationship of JZL184 (0, 4, 16, 40, or 100 mg/kg i.p.), with the sixth group receiving two injections of vehicle. The highest JZL184 dose (100 mg/kg) was achieved by using double-volume injections. Baseline measurements for rectal temperature were taken immediately before injection. After a 2-hour pretreatment, total time spent immobile was analyzed using ANYmaze Video Tracking Software (Stoelting, Wood Dale, IL) during a 10-minute locomotor activity test. At the conclusion of this test, the subjects were removed from activity chambers and assessed for catalepsy and hypothermia. Catalepsy was assessed using the bar test, in which the forelimbs of each mouse were placed on a horizontal bar during a 60-second test in which time immobile was measured (Schlosburg et al., 2010). Rectal temperature was determined by inserting a thermocouple probe 2.0 cm into the rectum and temperature was obtained from a BAT-10 telethermometer (Physitemp Instruments, Clifton, NJ).

Drug Discrimination. Drug-discrimination experiments were conducted in mouse-operant conditioning chambers (MedAssociates, St. Albans, VT) that were housed within ventilated, sound-attenuating enclosures, as previously described (McMahon et al., 2008). The center of one wall of the operant conditioning chamber contained a light (i.e., house light) positioned above a hole 2.2 cm in diameter through which milk could be obtained after the operant "nose-poke" procedure. Condensed milk in a volume of 0.01 ml was available via a dipper that could be raised from a tray positioned outside the hole. On the opposite wall were three recessed holes (2.2-cm diameter) spaced 5.5 cm apart, and each of these holes contained a photo beam and a light. The center of each hole was positioned 1.6 cm from the floor. Mice were trained to discriminate THC (5.6 mg/kg, 30-minute pretreatment period) from vehicle using a FR-10 schedule during 30-minute test sessions. In the substitution experiments, PF-3845 (10 mg/kg) or vehicle and JZ184 (4 mg/kg) or vehicle were administered 2 hours before the 30-minute test session. A semi-Latin square design was used to counterbalance the order of drug testing.

Measurement of Brain Lipids. Mice that had been administered acute injections of PF-3845 (10 mg/kg) or vehicle and JZL184 (4 mg/kg) or vehicle 2 hours before carrageenan were humanely euthanized via rapid decapitation immediately after testing (i.e., approximately 7 hours after drug administration). Their brains were rapidly removed, frozen on dry ice, and stored at −80°C until processing. On the day of processing, the preweighed tissues were 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 AEA-d_8, 1 nmol 2-AG-d_8, and 1 nmol arachidonic acid (AA)-d_8]. Homogenates were mixed with 0.3 ml of 0.73% w/v NaCl and then centrifuged for 10 minutes at 3220g (4°C). The aqueous phase and debris were collected and extracted again twice 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 cold acetone. The mixtures were centrifuged for 5 minutes at 1811g (4°C) to precipitate protein. 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 auto sample vials for analysis. Liquid chromatography-tandem mass spectrometry was used to quantify AEA, 2-AG, and AA. The mobile phase consisted of methanol/water (90:10) with 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery HS C18, 2.1 mm × 15 cm, 3 μm (Supelco, Bellefonte, PA). Ions were analyzed in multiple reaction monitoring mode, and the following transitions were monitored in positive mode: (348 > 62) and (348 > 91) for AEA, (356 > 62) for AEA-d_8, (379 > 287) and (379 > 269) for 2-AG, (387 > 96) for 2-AG-d_8; in negative mode: (303 > 259) and (303 > 59) for AA and (311 > 267) for AA-d_8. A calibration curve was constructed for each assay based on linear regression using the peak area ratios of the calibrators. The extracted standard curves ranged from 0.039 to 40 pmol for AEA, from 0.0625 to 64 nmol for 2-AG, and from 1 to 32 nmol for AA.

[^{3}H]SR141716A Binding. Mice were injected with PF-3845 (10 mg/kg) or vehicle and JZL184 (4 mg/kg) or vehicle for 5 consecutive days and euthanized 24 hours after the final injection. An additional group of mice was given a daily injection of 40 mg/kg JZL184 for 5 days

and euthanized on day 6. The brains were hemisected along the longitudinal fissure, and half of each brain was used for [^{3}H]SR141716A binding; the other half was used for CP55,940-stimulated [^{35}S]GTPγS binding (see below), as previously described (Falenski et al., 2010; Schlosburg et al., 2010). The tissue was placed in 15 ml of cold membrane buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4) and homogenized. Saturation analysis was performed by incubating 30 μg of membrane protein with 0.2–3 nM [^{3}H]SR141716A (rimonabant) in 50 mM Tris-HCl, 3 mM $MGCl_2$, 0.1 mM EGTA, 100 mM NaCl, pH 7.4 (assay buffer) including bovine serum albumin (BSA) (0.5 g/100 ml) for 90 minutes at 30°C. Nonspecific binding was determined using 2 μM unlabeled rimonabant, and total binding was determined in the absence of unlabeled rimonabant. The reactions were terminated using vacuum filtration through a Whatman GF/B glass fiber filter presoaked in Tris buffer containing 5 $g{\cdot}l^{-1}$ BSA (Tris–BSA) and three washes with 4°C Tris-BSA. Bound radioactivity was evaluated by liquid scintillation spectrophotometry at 45% efficiency after extraction in Budget-Solve scintillation fluid (Research Products International Corp., Mount Prospect, IL).

Agonist-Stimulated [^{35}S]GTPγS Binding. The other half of each brain was processed for CP55,940-stimulated [^{35}S]GTPγS binding. Tissue was centrifuged at 50,000*g* for 10 minutes at 5°C. The supernatant was removed, and samples were resuspended in 15 ml of TME membrane buffer. Centrifugation was repeated, the pellet resuspended in assay buffer, and the protein concentration determined. Membranes then were pretreated with adenosine deaminase (10 mU/ml) for 15 minutes at 30°C. Membrane protein (10 μg) was incubated in assay buffer with 100 mM NaCl, 0.1% BSA, 30 μM GDP, 0.1 nM [^{35}S]GTPγS, and varying concentrations of CP55, 940 (0.01–10 μM) for 2 hours at 30°C. Nonspecific binding was determined using 20 μM unlabeled GTPγS. Basal binding was determined in the absence of agonist. The incubation was terminated by rapid filtration through GF/B glass fiber filters and three washes with ice-cold Tris–HCl (pH 7.4). Liquid scintillation spectrophotometry was used to evaluate bound radioactivity after the extraction of filters in Budget-Solve scintillation fluid.

Electrophysiology. C57BL/6J mice were given daily intraperitoneal injections for 6 days of 1) vehicle + vehicle, 2) 4 mg/kg JZL184 + vehicle, 3) 4 mg/kg JZL184 + 10 mg/kg PF-3845, or 4) 40 mg/kg JZL184 + 10 mg/kg PF-3845. Two hours after the final injection, the mice were anesthetized under isoflurane inhalation and decapitated. To determine the involvement of CB_1 and CB_2 receptors in this assay, we tested whether the respective receptor antagonists AM251 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; 2 μM) or AM630 (1-[2-(morpholin-4-yl)ethyl]-2-methyl-3-(4-methoxybenzoyl)-6-iodoindole; 2 μM) would block WIN55,212-2–depressed IPSCs. Cingulate cortex slices (300 μm thick) were cut using a vibratome (Leica, Wetzlar, Germany) and prepared as previously described (Schlosburg et al., 2010; Pan et al., 2011). The slices were prepared at 4–6°C in a solution containing (in mM): 220 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgSO_4$, 26 $NaHCO_3$, 10 glucose, and 1 sodium ascorbate. The slices were transferred into artificial cerebrospinal fluid containing (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose and were allowed to recover for at least 1 hour at room temperature. All solutions were saturated with 95% O_2 and 5% CO_2.

Whole-cell voltage-clamp recordings were made using a patch-clamp amplifier (Multiclamp 700B) under infrared-differential contrast interference microscopy. Data acquisition and analysis were performed using a digitizer (DigiData 1440A) and analysis software pClamp 10 (Molecular Devices, Sunnyvale, CA). Pyramidal neurons in layer V of the anterior cingulate cortex were identified visually based on pyramidal-shaped soma with a prominent apical dendrite. Additionally, the pyramidal neurons exhibit spike-frequency adaptation in response to depolarizing current injections (Satake et al., 2008). To evoke IPSCs in layer V pyramidal neurons, the stimulation electrode was placed in layer V of cingulate cortex. The pipettes were filled with an internal solution containing (in mM): 80 K-gluconate, 60 KCl, 10 HEPES, 0.2 EGTA, 2 $MgCl_2$, 4 MgATP, 0.3 Na_2GTP, and 10 Na_2-phosphocreatine (pH 7.2 with KOH). Glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μM) and D-2-amino-5-phosphonovaleric acid (D-AP-5, 20 μM) were present in the ACSF throughout the experiments. Series resistance (15–30 MΩ) was monitored throughout the recordings, and data were discarded if the resistance changed by more than 20%. All recordings were performed at 32 ± 1°C by using an automatic temperature controller.

Data Analyses. Paw edema data are expressed as the difference in paw thickness between the 5-hour and preinjection measurements. Paw withdrawal thresholds to the von Frey filaments in the carrageenan-injected and contralateral (i.e., control) paws at the 5-hour time point were used to assess mechanical allodynia and at least10 days after CCI surgery. The magnitude of the antiallodynic effects of the enzyme inhibitors alone and in combination were calculated as percent maximum possible effect (%MPE) by the following formula: %MPE = 100 · (enzyme inhibitor von Frey threshold value − vehicle von Frey threshold value)/sham von Frey value − vehicle von Frey threshold value). All data are depicted as mean ± S.E.M. Data were analyzed using *t* tests, one-way analysis of variance (ANOVA), or two-way ANOVA. Tukey-Kramer post hoc analysis was used for all tests comparing different treatment groups. Bonferroni planned comparisons were used to assess the data in receptor binding and activation studies. [^{3}H]SR141716A and [^{35}S]GTPγS binding data were fit by nonlinear regression analysis to obtain B_{max} and K_D or E_{max} values, respectively. The pharmacologic effects of THC were analyzed using a mixed-design ANOVA vehicle + vehicle, and JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) pretreatment was the between-subjects variable; THC dose was the within-subjects variable, followed by Bonferroni post hoc test to compare each pretreatment with vehicle at individual THC doses. Differences of $P < 0.05$ were considered statistically significant. IPSC amplitude was normalized to baseline. The depression (percentage) of IPSCs by WIN55,212-2 was calculated as follows: 100 × [mean amplitude of IPSCs during the last 5 minutes of treatment/mean amplitude of baseline IPSCs]. Data sets were compared with Student's *t* test. All the results were considered significant at $P < 0.05$.

Results

Combination of Full FAAH Inhibition and Partial MAGL Inhibition Produces Augmented Antinociceptive Effects with Reduced Cannabimimetic Side Effects. The first experiment evaluated the individual or combined effects of JZL184 (4 mg/kg) and PF-3845 (10 mg/kg) administered before intraplantar administration of carrageenan (Fig. 1A). As shown in Fig. 1B, combined administration of these enzyme inhibitors fully prevented carrageenan-induced allodynia (79% + 14% MPE), whereas PF-3845 (41% + 4% MPE) or JZL184 (40% + 5% MPE) showed partial efficacy [$F(4,35) = 17.2$, $P < 0.001$]. Neither single nor combined enzyme inhibition affected paw withdrawal thresholds in the control paws. Inhibition of FAAH or MAGL partially reduced carrageenan-induced paw edema, and dual inhibition did not produce further antiedematous effects [$F(3,28) = 51.2$, $P < 0.001$; Fig. 1C]. Immediately after behavioral testing, the mice were euthanized, brains were harvested, and endocannabinoid levels were quantified. PF-3845, given alone or in combination with JZL184, led to a 10-fold increase in AEA levels [$F(4,35) = 389.0$, $P < 0.001$; Fig. 1D]. JZL184 (4 mg/kg), given alone or in combination with PF-3845, produced a 3-fold elevation in 2-AG levels [$F(4,35) = 99$, $P < 0.001$; Fig. 1E]. The elevation in brain 2-AG was not accompanied by significant reductions in the levels of AA ($P = 0.41$; Fig. 1F). On the other hand, full blockade of MAGL produced by JZL184 (40 mg/kg) elevated 2-AG levels 8-fold

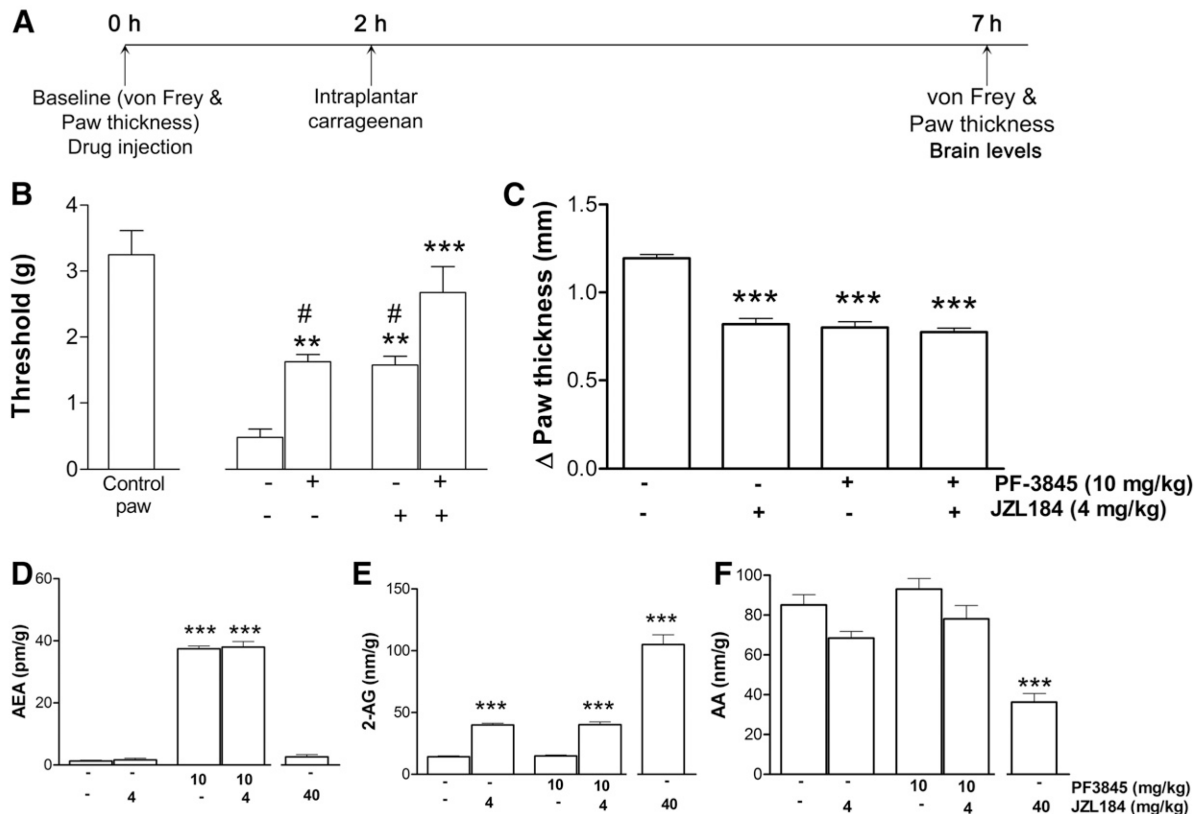

Fig. 1. Combined administration of high-dose PF-3845 (10 mg/kg) and low-dose JZL184 (4 mg/kg) completely prevented carrageenan-induced mechanical allodynia (A) but did not further reduce carrageenan-induced paw edema (B) compared with single enzyme inhibition. (C) High-dose PF-3845 (10 mg/kg) administered alone or in combination with low-dose JZL184 (4 mg/kg) produced a 10-fold increase AEA brain levels, but JZL184 (4 or 40 mg/kg) did not affect brain AEA levels. (D) Acute administration of low-dose JZL184 (4 mg/kg) administered alone or in combination with high-dose PF-3845 (4 mg/kg) produced a 3-fold increase in brain 2-AG levels. High-dose JZL184 (40 mg/kg) produced a 10-fold increase in brain 2-AG levels, but PF-3845 did not influence brain 2-AG levels. (E) High-dose JZL184 (40 mg/kg) significantly decreased AA brain levels, but none of the other treatments had an effect. (F) Experimental time line. Drugs were administered 2 hours before the intraplantar injection of carrageenan. Mechanical allodynia was measured 5 hours after carrageenan, and paw thickness was measured before and 5 hours after carrageenan administration. Mice were euthanized immediately after behavioral testing (i.e., approximately 7 hours after drug administration), and brains were harvested to quantify lipid concentrations. $^{**}P < 0.01$; $^{***}P < 0.001$ versus vehicle; $^{\#}P < 0.05$ versus control paw (Tukey-Kramer post hoc tests). Data are presented as mean ± S.E.M., n = 7 or 8 mice/group.

[Fig. 1E] and reduced brain AA levels 2-fold [$F(4,35) = 18.92$, $P < 0.001$; Fig. 1F].

To determine whether cannabinoid receptors mediate the antiallodynic and antiedematous effects of combined FAAH and MAGL inhibition, we used the respective CB_1 and CB_2 receptor antagonists rimonabant (1 mg/kg) and SR144528 (3 mg/kg). As shown in Fig. 2A, rimonabant [$F(1,21) = 21.9$, $P < 0.001$], as well as SR144528 [$F(1,21) = 20.5$, $P < 0.001$], completely prevented the antiallodynic effects of combined administration PF-3845 and JZL184. In contrast SR145528 [$F(1,21) = 23.0$, $P < 0.001$], but not rimonabant [$P = 0.73$], significantly prevented the antiedematous effects of the

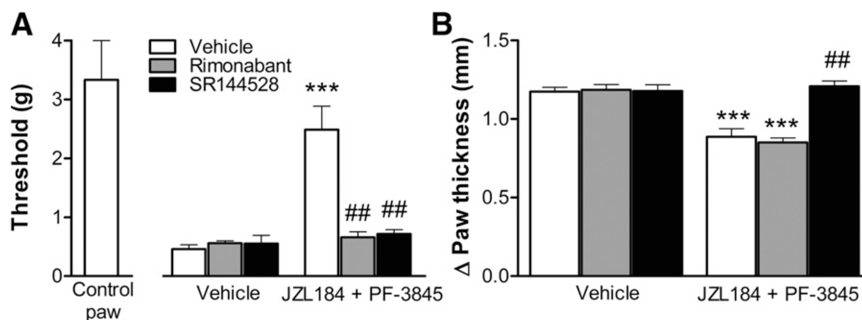

Fig. 2. Assessment of cannabinoid receptors mediating the antiallodynic and antiedematous effects produced by combined administration of JZL184 (4 mg/kg) and PF-3845 (10 mg/kg). (A) Rimonabant (1 mg/kg) or SR144528 (3 mg/kg) blocked the antiallodynic effects of the inhibitors given in combination. (B) SR144528 (3 mg/kg), but not rimonabant (1 mg/kg), blocked the antiedematous effects of the inhibitors given in combination. Mechanical allodynia was measured 5 hours after carrageenan and paw thickness was measured prior to and 5 hours after carrageenan administration. JZL184 + PF-3845 or vehicle was injected 2 hours before carrageenan. Rimonabant or SR144528 was injected 30 minutes before vehicle or JZL184 + PF-3845. $^{***}P < 0.001$ versus vehicle/vehicle; $^{\#\#}P < 0.05$ versus vehicle/JZL184 + PF-3845 (Bonferroni test). Data are presented as mean ± S.E.M., n = 6 mice/group.

combination (Fig. 2B). Finally, in the absence of an endocannabinoid catabolic enzyme inhibitor, neither rimonabant nor SR144528 significantly affected carrageenan-induced edema or allodynia. Thus, these findings indicate that the antiallodynic effects of PF-3845 and JZL184 require both CB_1 and CB_2 receptors, but the edematous effects of these enzyme inhibitors require only CB_2 receptors.

We next evaluated whether dual FAAH and MAGL inhibition would reverse nociceptive behavior in the carrageenan model of inflammation, as well as in the CCI model of neuropathic pain (Fig. 3A). As shown in Fig. 3B, combined administration of high-dose PF-3845 (10 mg/kg) and low-dose JZL184 (4 mg/kg) produced greater antiallodynic effects (85% ± 4% MPE) than mice treated with either PF-3845 (27% ± 7% MPE) or JZL184 (35% ± 6% MPE) [$F(4,25) = 9.21$, $P < 0.001$]. Moreover, mice administered the combination of these inhibitors exhibited nearly identical paw withdrawal thresholds between the carrageenan-injected and contralateral control paws, suggesting full antiallodynic actions. The combination did not affect paw withdrawal thresholds in the control paws. In addition, single or dual enzyme inhibition partially reduced carrageenan-induced paw edema [$F(3,20) = 15.30$, $P < 0.001$], but the combination of PF-3845 and JZL184 did not elicit augmented antiedematous effects compared with single enzyme inhibition (Fig. 3C). Combination of PF-3845 and JZL184 also produced enhanced antiallodynic effects in the CCI model of neuropathic pain (62% ± 8% MPE) compared with either PF-3845 (42% ± 7% MPE) or JZL184 (37% ± 8% MPE) alone [$F(4,29) = 27.5$; $P < 0.001$; Fig. 3D]; however, combination of the enzyme inhibitors only partially reversed the allodynia, as von Frey withdrawal thresholds significantly differed from the paw withdrawal thresholds of the sham mice. Moreover, the magnitude of effect of dual FAAH and MAGL inhibition was less in reducing CCI-induced allodynia than in reducing carrageean-induced allodynia.

In the next experiments, we evaluated the effects of the combination of PF-3845 and JZL184 in common assays used to infer cannabimimetic pharmacologic effects. PF-3845 (10 mg/kg) and JZL184 (4 mg/kg) given alone or in combination did not substitute for THC in the drug-discrimination paradigm and did not affect operant response rates (Fig. 4, A and B). Assessment of JZL184 (0, 4, 16, 40, or 100 mg/kg), in combination with 10 mg/kg PF-3845 on locomotor activity (Fig. 4C), catalepsy (Fig. 4D), and hypothermia (Fig. 4E) revealed that 100 mg/kg JZL184, in combination with PF-3845, significantly differed from the vehicle condition.

Repeated Administration of High-Dose PF-3845 and Low-Dose JZL184: Maintained Antiallodynic and Antiedematous Actions, without CB_1 Receptor Downregulation or Desensitization. As shown in Fig. 5A, combination PF-3845 (10 mg/kg) and JZL184 (4 mg/kg), given acutely or repeatedly, produced a similar reduction of carrageenan-induced allodynia [$F(5,44) = 5.85$, $P < 0.001$]. Similarly, the antiedematous effects of dual FAAH-MAGL inhibition were virtually identical in magnitude after acute and repeated administration [$F(2,22) = 8.47$, $P < 0.01$; Fig. 5B].

As previously reported, repeated treatment of high-dose JZL184 (40 mg/kg) reduced whole-brain CB_1 receptor levels by 33.3% and produced a 56.3% reduction in maximal CP55,940-stimulated G protein activity. Simultaneous repeated administration of high-dose PF-3845 (10 mg/kg) + low dose JZL184 (4 mg/kg), as well as each treatment alone, however, did not

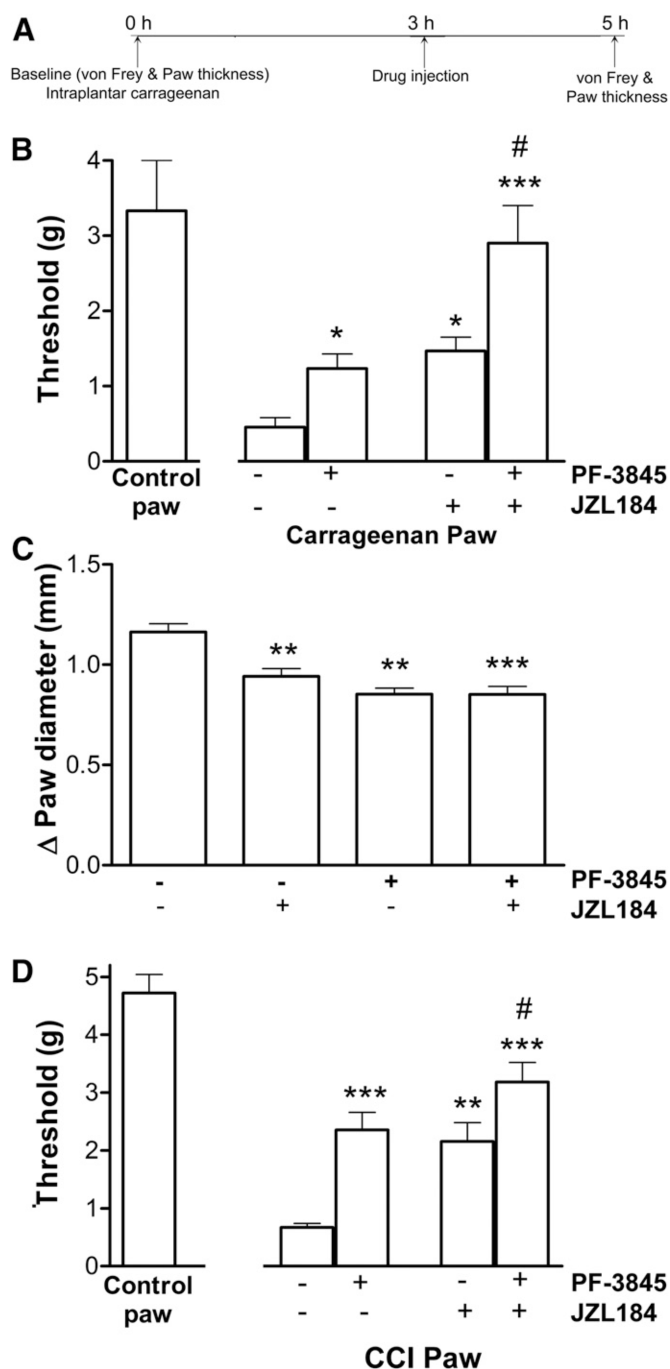

Fig. 3. Simultaneous full FAAH inhibition and partial MAGL inhibition fully reverses inflammatory and neuropathic nociception. (A) Experimental timeline. Drugs were administered 3 hours after carrageenan. Paw thickness was measured before and 5 hours after carrageenan administration and mechanical allodynia was measured 5 hours after carrageenan. Combined administration of high-dose PF-3845 (10 mg/kg) and low-dose JZL184 (4 mg/kg) significantly reversed mechanical allodynia (B), but it did not further reduce paw edema (C). Compared with single enzyme inhibition in the carrageenan model of inflammatory pain. (D) Combined administration of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) produced enhanced antiallodynic effects compared with either JZL184 or PF-3845 alone in the CCI model of neuropathic pain. Mechanical allodynia was assessed 2 hours after drug administration. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus vehicle; #$P < 0.05$ versus single enzyme inhibition (Tukey-Kramer post hoc tests). Data presented as mean ± S.E.M., $n = 6$ mice/group (B and C) and $n = 20$ mice/treatment (D).

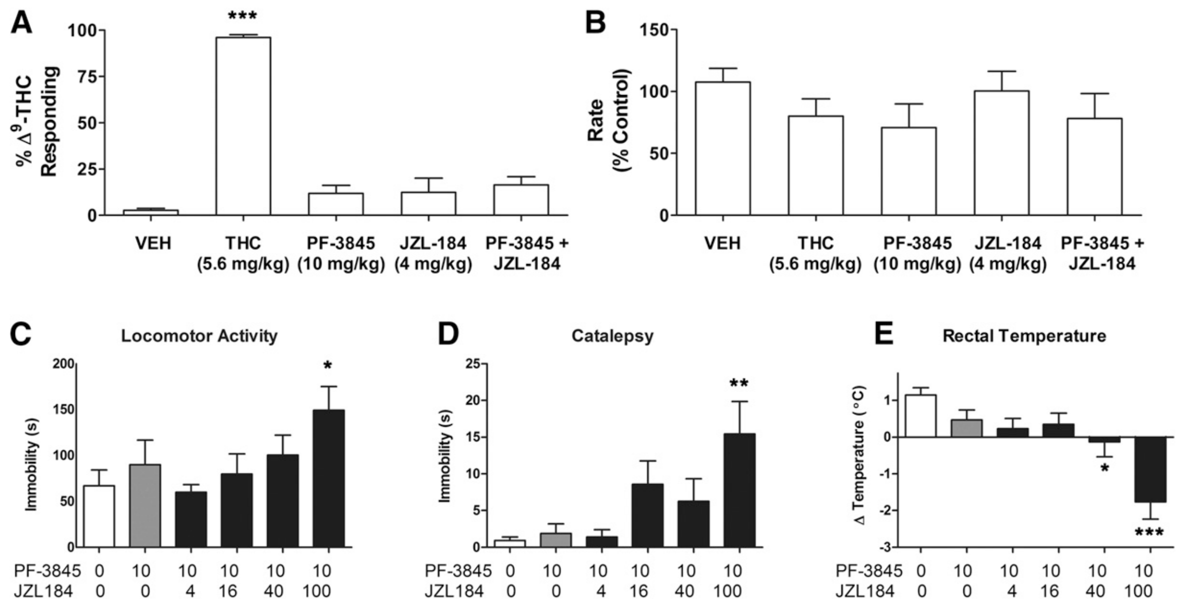

Fig. 4. Evaluation of combined administration of PF-3845 and JZL184 on cannabimimetic measures. Administration of PF-3845 (10 mg/kg) and JZL184 (4 mg/kg) given separately or in combination does not substitute for THC (A) or affect response rates (B) in the drug-discrimination assay. Mice were trained to discriminate THC (5.6 mg/kg i.p.) from vehicle. The combination of PF-3845 (10 mg/kg) and JZL184 (100 mg/kg) produced significant locomotor depressive (C), cataleptic (D), and hypothermic (E) effects. Baseline rectal temperature was 36.3 ± 0.1°C (mean ± S.E.M.) $*P < 0.5$; $**P < 0.01$; $***P < 0.001$ versus vehicle-vehicle. Values reflect mean ± S.E.M., n = 10 mice.

significantly alter CB_1 receptor levels (Fig. 5C) or CP55,940-stimulated G protein activity (Fig. 5D) compared with the vehicle + vehicle control group.

Finally, we examined whether repeated administration of the combination treatment for 6 days attenuates WIN55,212-2 depression of IPSCs in the cingulate cortex, a brain region critically involved in the regulation of pain (Zhuo, 2006) and that desensitizes after repeated administration of 40 mg/kg JZL184 (Schlosburg et al., 2010). To infer which cannabinoid receptors mediate the acute actions of WIN55,212-2 on IPSCs, we used the selective CB_1 receptor antagonist AM251 (2 μM) and the selective CB_2 receptor antagonist AM630 (2 μM). Bath application of WIN55,212-2 (2 μM) induced significant depression of IPSCs (61.9% ± 5.5% of baseline). This depression

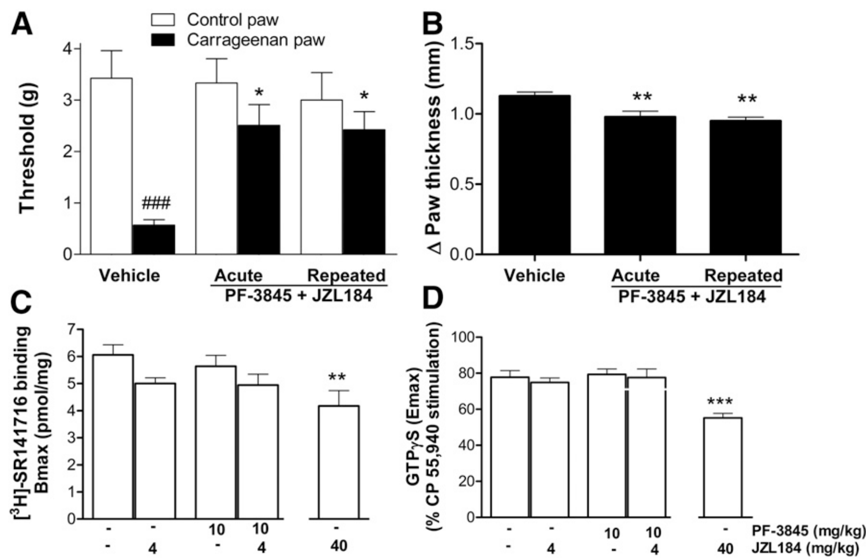

Fig. 5. Repeated administration of combined JZL184 and PF-3845 produces sustained antiallodynic (A) and antiedematous (B) effects in the carrageenan model of inflammatory pain. Acute administration or repeated (6 days daily) administration of low-dose or high-dose PF-3845 (10 mg/kg) plus low dose JZL 184 (4 mg/kg) reduced carrageenan-induced mechanical allodynia and paw edema to a similar magnitude. Intraplantar carrageenan was administered 2 hours after drug administration. Mechanical allodynia was tested 5 hours after carrageenan and paw thickness was measured before and 5 hours after carrageenan administration. CB_1 receptor binding (C) and receptor-mediated G protein activation (D) are maintained following repeated injections of high dose PF-3845 and low-dose JZL184 administered separately or in combination; however, 6 days of daily injections of high-dose JZL184 (40 mg/kg) reduced both the [^{3}H]SR141716A B_{max} and agonist-stimulated G protein activity. Mice were treated for 6 days with vehicle + vehicle, vehicle + JZL184 (4 mg/kg), vehicle + PF-3845 (10 mg/kg), JZL184 + PF-3845 or vehicle + JZL184 (40 mg/kg) $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ versus vehicle; $^{###}P < 0.001$ versus control paw. Data are presented as mean ± S.E.M., n = 8 or 9 mice/group.

was blocked by the CB_1 receptor selective antagonist AM251 (91.3% ± 4.1% of baseline; $P < 0.01$ versus control), but it was not significantly affected by AM630 (67.8% ± 4.1% of baseline; $P = 0.40$ versus control; Fig. 6A). These findings indicate that CB_1 receptors, not CB_2 receptors, mediate WIN55,212-2–induced depression of IPSCs.

We next examined cingulate slices from mice given a daily injection for 6 days of the following: 1) vehicle + vehicle, 2) vehicle + 4 mg/kg JZL184, 3) 4 mg/kg JZL184 + 10 mg/kg PF-3845, or 4) 40 mg/kg JZL184 + 10 mg/kg PF-3845. In the vehicle + vehicle group, WIN55,212-2 induced similar depression of IPSCs between groups 1 and 2 (vehicle + vehicle: 61.9% ± 5.5% of baseline; vehicle + 4 mg/kg JZL184: 67.4% ± 5.2% of baseline; $P = 0.48$; Fig. 6, A and B), suggesting that repeated JZL184 at this low dose did not induce significant CB_1 receptor desensitization. In a previous study, we reported no apparent CB1 receptor desensitization in mice given a daily injection of PF-3845 (10 mg/kg) for 6 days (Schlosburg et al., 2010). Consistent with this result, we found that the magnitude of WIN55,212-2–induced depression of IPSCs in the cingulate cortex did not significantly differ between mice treated with JZL184 (4 mg/kg) plus vehicle and JZL184 (4 mg/kg) plus PF-3845 (10 mg/kg) (66.4% ± 5.4% of baseline, $n = 8$; $P = 0.90$; Fig. 6B); however, WIN55,212-2–induced depression of IPSCs was significantly attenuated in mice treated with JZL184 (40 mg/kg) plus PF-3845 (10 mg/kg) (86.6% ± 5.3% of baseline, $n = 7$) compared with that in mice treated with JZL184 (4 mg/kg) plus PF-3845 (10 mg/kg) ($P < 0.05$; Fig. 6B).

Discussion

Simultaneous inhibition of the primary endocannabinoid catabolic enzymes FAAH and MAGL produces augmented antinociceptive effects compared with either enzyme alone in acute and chronic laboratory animal models of pain (Long et al., 2009d; Niphakis et al., 2013; Anderson et al., 2014). However, dual blockade of these enzymes can also elicit cannabimimetic side effects, including catalepsy, hypomotility, deficits in learning and memory, and THC-like interoceptive cues (Long et al., 2009d; Anderson et al., 2014; Ignatowska-Jankowska et al., 2014; Wiley et al., 2014; Hruba et al., 2015; Walentiny et al., 2015). Moreover, prolonged and complete MAGL inactivation results in tolerance accompanied with CB_1 receptor downregulation and desensitization (Chanda et al., 2010; Schlosburg et al., 2010; Schlosburg et al., 2014). On the other hand, repeated administration of low-dose JZL184 produces sustained antinociceptive effects without evidence of CB_1 receptor functional tolerance (Kinsey et al., 2013). Thus, the present study tested whether full FAAH inhibition combined with partial MAGL inhibition would produce enhanced antiallodynic effects in the carrageenan and CCI models of pain. Here, we show that partial MAGL inhibition with low-dose JZL184 (4 mg/kg) and full FAAH blockade with high-dose PF-3845 (10 mg/kg) completely prevents and reverses carrageenan-induced mechanical allodynia and partially reverses CCI-induced allodynia, but it does not elicit cannabimimetic side effects commonly associated with CB_1 receptor agonists. Finally, repeated daily administration of this combination of inhibitors over 6 days continued to block carrageenan-induced allodynia, with no evidence of CB_1 receptor functional tolerance.

To infer the separation between the antiallodynic effects and side effects of combined FAAH and MAGL inhibition, we examined the dose-response relationship of JZL184 (4–100 mg/kg) in combination with a high dose of PF-3845 (10 mg/kg) in common assays reflective of cannabimimetic activity. Although 4 mg/kg JZL184 plus PF-3845 completely reversed carrageenan-induced allodynia, significant cataleptic, hypothermic, and locomotor depressant effects emerged only after 100 mg/kg JZL184 in combination of PF-3845. Similarly, whereas we found that 4 mg/kg JZL184 plus PF-3845 did not substitute for THC in the drug-discrimination assay, the combination of 10 mg/kg PF-3845 and 40 mg/kg JZL184 partially substituted and 120 mg/kg JZL184 fully substituted for THC (Hruba et al., 2015); however, the different vehicles used in the drug-discrimination experiment (i.e., 1:1:18 mixture of propylene glycol, Tween-80, and saline) compared with the other experiments (i.e., 1:1:18 mixture of ethanol, alkamuls-620, and saline) limit direct comparison. Nonetheless, observations that the dose of JZL184 in combination with PF-3845 that elicited significant antiallodynic effects in CCI and carrageenan assays was 25-fold lower than the dose required to produce significant cataleptic, hypothermic, and motor depressive effects represent a reasonable

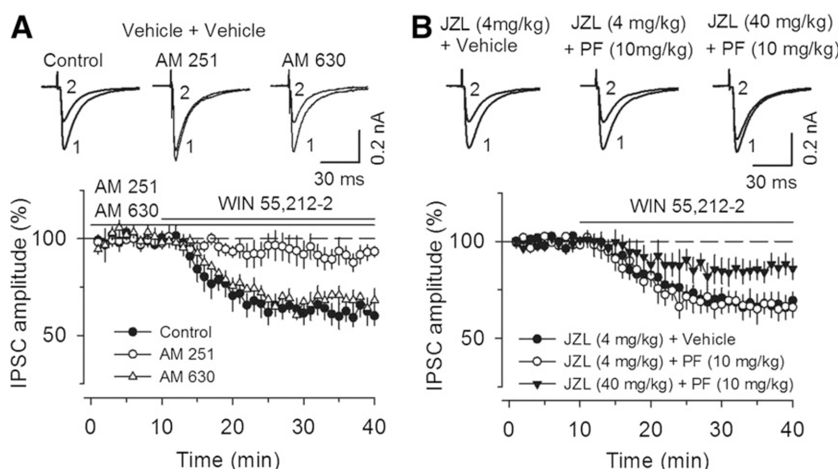

Fig. 6. Effects of repeated in vivo administration of JZL184 alone or in combination with PF-3845 (10 mg/kg) on WIN55,212-2 (2 μM)–induced depression of IPSCs in the cingulate cortex. (A) The CB_1 receptor antagonist AM251 (2 μM) blocked WIN55,212-2–induced depression of IPSCs ($P < 0.01$), whereas the CB_2 receptor antagonist AM630 (2 μM) had no significant effect ($P = 0.4$). (B) Mice treated for 6 days with daily injections of low-dose JZL184 (JZL; 4 mg/kg) alone or in combination with PF-3845 (PF) display similar magnitude of WIN55,212-2–induced depression of IPSCs compared with that in mice treated with repeated vehicle shown in (A) ($P = 0.48$). However, WIN55,212-2 induced significantly less depression of IPSCs in mice treated with high-dose JZL184 (40 mg/kg) plus PF-3845 than in mice in each other group ($P < 0.05$). Data presented as mean ± S.E.M. and were obtained from multiple slices (n = 6–8) of three mice per group.

separation between antiallodynic effects and cannabimimetic side effects.

Although the present study found that repeated administration of combined administration of high-dose PF-3845 and low-dose JZL184 maintained antiedematous and antiallodynic effects in the carrageenan model, we did not examine the consequences of repeated administration in the CCI model of neuropathic pain; however, previous studies have shown that the antiallodynic effects of low-dose JZL184 (i.e., ≤8 mg/kg) alone (Ghosh et al., 2013; Kinsey et al., 2013) or high-dose PF-3845 alone (Schlosburg et al., 2010) did not undergo tolerance following repeated injections in the carrageenan and CCI pain models. Moreover, the present study also found that repeated administration of PF-3845 (10 mg/kg) and JZL184 (4 mg/kg) did not lead to CB_1 receptor downregulation or desensitization in the whole brain. Because repeated injections of this JZL184 dose resulted in brain region–dependent CB_1 receptor desensitization (Schlosburg et al., 2010), the use of whole brain may have obfuscated regional differences. Therefore, we examined the impact of six daily injections of 10 mg/kg PF-3845 combined with either 4 or 40 mg/kg JZL184 on the magnitude of WIN55,212-2–induced depression of IPSCs in the cingulate cortex, which we show is a CB_1 receptor mediated effect (Fig. 6A). Repeated administration of PF-3845 (10 mg/kg) in combination with high-dose JZL184 (40 mg/kg) significantly reduced WIN55,212-2–induced depression of IPSCs in this brain region, similar to the actions of repeated injections of JZL184 (40 mg/kg) alone in this assay (Schlosburg et al., 2010). Repeated administration of PF-3845 (10 mg/kg) in combination with low-dose of JZL184 (4 mg/kg) did not, however, lead to CB_1 receptor functional tolerance in the cingulate cortex.

Rimonabant, as well as SR144528, completely prevented the antiallodynic actions of combined PF-3845 and JZL184 in the carrageenan model of inflammatory pain, indicating that CB_1 and CB_2 receptors play necessary roles in this response. Likewise, the antiallodynic effects JZL195 in the complete Freund adjuvant model of inflammatory pain required both cannabinoid receptors (Jayamanne et al., 2006; Anderson et al., 2014). Selective inhibition of MAGL alone in the carrageenan model produces antiallodynic effects of MAGL inhibitors requiring activation of both cannabinoid receptors, whereas the antiedematous effects of the drugs are mediated via CB_2 receptors (Ghosh et al., 2013; Ignatowska-Jankowska et al., 2014). Similarly, CB_2 receptors, but not CB_1 receptors, mediate the antiedematous effects of the FAAH inhibitor URB597 in the carrageenan model (Holt et al., 2005). The lack of further antiedematous effects by dual inhibition of FAAH and MAGL, compared with inhibition of either enzyme alone, may be due to a “ceiling effect” and further indicates underlying differences between the antiallodynic and antiedematous actions of endogenous cannabinoids in the carrageenan model of inflammatory pain. Finally, the failure of rimonabant or SR144528 given alone to alter the magnitude of carrageenan-induced allodynia and edema is consistent with previous work showing no tonic involvement of CB_1 and CB_2 receptors in this inflammatory model of pain (Holt et al., 2005; Ghosh et al., 2013).

The question of why dual FAAH and MAGL inhibition produces enhanced antiallodynic effects remains unanswered. Several nonmutually exclusive explanations may account for the enhanced effects. First, the enhancement may have resulted from simply an increase in the total quantity of endocannabinoids to stimulate cannabinoid receptors. It is important to note that 4 mg/kg JZL184 produced an approximately 3-fold increase in brain 2-AG levels, irrespective of coadministration of PF-3845, and PF-3845 produced a greater than 10-fold increase of brain AEA levels, regardless of the presence of JZL184. The fact that 2-AG levels are approximately three orders of magnitude higher than AEA levels in whole brain, however, negates this idea of mass action. Alternatively, it is plausible that simultaneous inhibition of MAGL and FAAH produces augmented antiallodynic effects because the respective endocannabinoids activate distinct CB_1 and CB_2 receptor-mediated circuits. A third explanation is that, along with the increased levels of AEA and 2-AG, other lipids regulated by FAAH and MAGL may contribute to the augmented antiallodynic effects. In particular, FAAH metabolizes other bioactive fatty acid amides besides AEA, including *N*-palmitoylethanolamine, *N*-oleoylethanolamine, and oleamide (Cravatt et al., 1996, 2001), as well as *N*-acyl-taurines (Saghatelian et al., 2006), which activate various transient receptor potential channels and peroxisome proliferator-activated receptor-α receptors. In addition, MAGL plays an important role in the biosynthesis of AA in the brain, and its blockade results in reduced AA, and concomitant reductions in prostaglandins (Nomura et al., 2011). The observation that 4 mg/kg JZL184 did not alter AA levels in whole brain tends to argue against the involvement of reduced prostaglandin levels. On the other hand, the use of whole brain extracts may have obfuscated the detection of regional differences in AA, as well as endocannabinoids in regions associated with pain (e.g., cingulate cortex, periaqueductal gray, rostral ventral medulla, spinal cord, dorsal root ganglia). Moreover, measurement of interstitial levels of AEA and 2-AG in relevant regions provides deeper insight regarding the relationship between inhibiting FAAH and MAGL and elevated endocannabinoid levels that are likely to play a role in cannabinoid receptor signaling than levels determined from whole brain lipid extracts or even from discrete regions (Wiskerke et al., 2012). Thus, combined blockade of FAAH and MAGL produces multiple neurochemical alterations that may account for increased antiallodynic effects compared with cannabimimetic effects (e.g., catalepsy, hypothermia, hypomotility, and THC substitution).

Whereas full and simultaneous blockade of FAAH and MAGL elicits enhanced reductions in nociceptive behavior in preclinical assays of pain, the occurrence of motor deficits (e.g., hypomotility, catalepsy) and THC-like subjective effects represent challenges for translational implications. Moreover, development of tolerance and CB_1 receptor functional tolerance produced by prolonged MAGL inhibition are further drawbacks for this type of strategy. Thus, the results presented here suggest that complete blockade of FAAH combined with partial blockade of MAGL not only increases antiallodynic efficacy but also circumvents occurrence of cannabimimetic side effects and CB_1 receptor functional tolerance. Thus, tailoring drugs to possess differential selectivity in the inhibition of the principal endocannabinoid catabolic enzymes may provide a novel strategy to treat clinical pain states. In conclusion, the present study demonstrates that full inhibition of FAAH in combination with partial inhibition of MAGL produces enhanced antiallodynic effects compared with single enzyme inhibition but with reduced untoward side effects and no evidence of CB_1 receptor functional tolerance.

Authorship Contributions

Participated in research design: Ghosh, Kinsey, Wise, Liu, McMahon, Grim, Selley, Sim-Selley, Abdullah, Cravatt, Lichtman.

Conducted experiments: Ghosh, Kinsey, Liu, Hruba, Abdullah, Merritt.

Performed data analysis: Ghosh, Wise, Kinsey, Liu, McMahon, Selley, Sim-Selley, Abdullah, Merritt, Grim.

Wrote or contributed to the writing of the manuscript: Ghosh, Kinsey, Liu, McMahon, Selley, Sim-Selley, Cravatt, Lichtman.

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