

**Full FAAH inhibition combined with partial monoacylglycerol lipase inhibition: Augmented and sustained antinociceptive effects with reduced cannabimimetic side effects in mice**

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Nonstandard Abbreviations

AA: arachidonic acid;

AEA: anandamide, N-arachidonylethanolamine;

2-AG: 2-arachidonoylglycerol;

CB<sub>1</sub>: cannabinoid receptor type 1;

CB<sub>2</sub>: cannabinoid receptor type 2;

CP55,940: 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol;

FAAH: fatty acid amide hydrolase;

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;

THC:  $\Delta^9$ -tetrahydrocannabinol;

WIN55,212-2: R-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate;

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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*-arachidonylethanolamine (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 CB<sub>1</sub> 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 (10 mg/kg) given in combination with a low dose of the MAGL inhibitor JZL184 (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 2-3 fold increases in brain 2-AG levels. Importantly, 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 THC 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 anti-allodynic actions in the carrageenan assay or CB<sub>1</sub> 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; Cravatt et al., 2001)) or monoacylglycerol lipase (MAGL (Dinh et al., 2002)), the respective primary hydrolytic enzymes for *N*-arachidonylethanolamine (AEA (Devane et al., 1992)) and 2-arachidonylglycerol (2-AG; (Mechoulam et al., 1995; Sugiura et al., 1995)) represent potential strategies to alleviate pain, but with reduced cannabimimetic side effects. Pharmacological 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 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; Ghosh et al., 2013; Guindon et al., 2013). However, 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, as compared with individual inhibition of these enzymes (Long et al., 2009d; Anderson et al., 2014). However, JZL195 also produced hypomotility, catalepsy, 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 three-fold more potent in reversing complete Freund adjuvant (CFA)-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 CB<sub>1</sub> receptor function (Falenski et al., 2010; Schlosburg et al., 2010), and FAAH deficient mice display a CB<sub>1</sub> receptor-mediated hypoalgesic phenotype (Lichtman et al., 2004), complete blockade of MAGL leads to tolerance, physical dependence, impaired endocannabinoid-dependent synaptic plasticity, and CB<sub>1</sub> receptor down-regulation and desensitization in select brain regions, but does not affect CB<sub>2</sub> receptor expression in spleen (Chanda et al., 2010; Schlosburg et al., 2010; Ignatowska-Jankowska et al., 2014). However, prolonged partial MAGL inhibition with repeated administration of low doses of JZL184 (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). Importantly, repeated administration of low dose JZL184 ( $\leq 8$  mg/kg) does not lead to CB<sub>1</sub> 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 co-administration high dose PF-3845 (10 mg/kg) and low dose JZL184 (4 mg/kg) in the carrageenan model of inflammatory pain and 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<sub>1</sub> receptor down-regulation/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<sub>1</sub> 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 anti-

allodynic effects in carrageenan-treated mice. Following completion of the behavioral study, the mice were euthanized and CB<sub>1</sub> receptor expression and function assessed using [<sup>3</sup>H]SR141716A binding and CP55,940-stimulated [<sup>35</sup>S]GTPγS-stimulated binding assays. Finally, inhibition of IPSCs induced by WIN55,212-2, 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<sub>1</sub> receptors (Herkenham et al., 1990) that undergo desensitization following six days of daily injections of 40 mg/kg JZL184 (Schlosburg et al., 2010).

## Methods

**Subjects.** Male C57BL/6J mice from 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 AAALAC-approved facility, with a 12/12 h 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 guide for the care and use of Laboratory animals (Institute of Laboratory and Animal Resources, 1996). At the conclusion of behavioral studies, mice were euthanized via CO<sub>2</sub> 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, rimonabant, SR144528, 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 GTP $\gamma$ S were purchased from Sigma (St. Louis, MO) and [<sup>35</sup>S]GTP $\gamma$ S was purchased from PerkinElmer (Waltham, MA).

**Drug administration.** Drugs were dissolved in a vehicle that consisted of a mixture of ethanol, alkamuls-620 (Sanofi-Aventis, Bridgewater, NJ), and saline in a ratio of 1:1:18 parts, and sonicated as needed. However, in the drug discrimination experiment, 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 (i.p.) route of administration in a volume of 10  $\mu$ l/g body mass.

**Carrageenan-induced inflammatory pain.** Paw edema and inflammatory pain were induced by giving an intraplantar injection of 0.3% carrageenan (Sigma, St Louis, MO), in a 20  $\mu$ l volume through a 30 gauge needle into the hind left paw, as previously described (Ghosh et al., 2013). Paw thickness was measured with electronic digital calipers (Traceable Calipers, Friendswood, TX) before and 5 h after

carrageenan administration, which corresponds to peak edema, and mechanical allodynia was assessed 5 h following 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 one 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 min 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 h before injections. JZL184 and PF-3845 were administered 2 h before carrageenan (i.e., 7 h before testing) to test whether the combination prevents allodynia. In experiments assessing CB<sub>1</sub> and CB<sub>2</sub> receptor involvement, the respective antagonists rimonabant (1 mg/kg) and SR144528 (3 mg/kg) were administered 30 min prior to the enzyme inhibitors

(i.e., 7.5 h before testing). We previously established that these doses of antagonist and time points effectively antagonize the pharmacological effects of JZL184 alone in the carrageenan assay (Ghosh et al., 2013). In the carrageenan reversal experiment, the compounds were injected 3 h after carrageenan (i.e., 2 h before testing). Similarly, in the CCI experiment, the compounds were given 2 h 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 h 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; Kinsey et al., 2013) within this time frame.

In order 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 h before carrageenan was injected. Edema and mechanical allodynia were assessed 5 h 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 prior to injection. Following a 2 h pretreatment, total time spent immobile was analyzed using ANYmaze Video Tracking Software (Stoelting, Wood Dale, IL) during a 10 min 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 s 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 following the operant “nose poke” procedure. Condensed milk in a volume of 0.01 cc was available via a dipper that could be raised from a tray positioned outside the hole. On the opposite wall, there were three recessed holes (2.2-cm diameter) spaced 5.5 cm apart, and each of these holes contained a photobeam 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 min pretreatment period) from vehicle using a FR-10 schedule, during 30 min test sessions. In the substitution experiments, PF-3845 (10 mg/kg) or vehicle and JZ184 (4 mg/kg) or vehicle were administered 2 h before the 30 min test session. A semi-Latin square design was used to counter-balance 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 h before carrageenan were humanely euthanized via rapid decapitation immediately after testing (i.e., approximately 7 h 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 pre-weighed tissues were homogenized with 1.4 ml chloroform: methanol (2:1 v/v containing 0.0348g phenylmethylsulfonyl fluoride/ml) after the addition of internal standards to each sample (2 pmol AEA-d8, 1 nmol 2-AG-d8, and 1 nmol arachidonic acid (AA)-d8). Homogenates were mixed with 0.3 ml of 0.73% w/v NaCl and then centrifuged for 10 min at 3220 g (4°C). The aqueous phase and debris were collected and extracted again twice with 0.8 ml 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 chloroform and mixed with 1 ml cold acetone. The mixtures were centrifuged for 5 min at 1811 g (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 methanol and placed in auto sample vials for analysis. LC/MS/MS was used to quantify AEA, 2-AG, and arachidonic acid. 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 X 15 cm, 3 micron (Supelco, USA). 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-d8; (379 > 287) and (379 > 269) for 2-AG ; (387 > 96) for 2-AG-d8 in negative mode: (303 > 259) and (303 > 59) for arachidonic acid and (311 > 267) for arachidonic acid-d8. A calibration curve was constructed for each assay based on linear regression 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 arachidonic acid.

**[<sup>3</sup>H]SR141716A binding.** Mice were injected with PF-3845 (10 mg/kg) or vehicle and JZL184 (4 mg/kg) or vehicle for five consecutive days and were euthanized 24 h after the final injection. An additional group of mice was given a daily injection of 40 mg/kg JZL184 for five days and euthanized on day 6. The brains were hemisected along the longitudinal fissure and half of each brain was used for [<sup>3</sup>H]SR141716A binding and the other half was used for CP55,940-stimulated [<sup>35</sup>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<sub>2</sub>, 1 mM EGTA, pH 7.4) and homogenized. Saturation analysis was performed by incubating 30 μg membrane protein with 0.2–3 nM [<sup>3</sup>H]SR141716A (rimonabant) in 50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 100 mM NaCl, pH 7.4 (assay buffer) including BSA (0.5 g/100 ml) for 90 min at 30°C. Non-specific binding was

determined using 2  $\mu\text{M}$  of 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 pre-soaked in Tris buffer containing 5 g L-1 BSA (Tris-BSA) and three washes with 4  $^{\circ}\text{C}$  Tris-BSA. Bound radioactivity was evaluated by liquid scintillation spectrophotometry at 45% efficiency after extraction in Budget-Solve TM scintillation fluid.

**Agonist-stimulated [ $^{35}\text{S}$ ]GTP $\gamma$ S binding.** The other half of each brain was processed for CP55, 940-stimulated [ $^{35}\text{S}$ ]GTP $\gamma$ S binding. Tissue was centrifuged at 50,000 x g for 10 min at 5 $^{\circ}\text{C}$ . The supernatant was removed and samples were resuspended in 15 ml of TME membrane buffer. Centrifugation was repeated, the pellet re-suspended in assay buffer, and the protein concentration determined. Membranes then were pretreated with adenosine deaminase (10 mU/ml) for 15 min at 30  $^{\circ}\text{C}$ . Membrane protein (10  $\mu\text{g}$ ) was incubated in assay buffer with 100 mM NaCl, 0.1% BSA, 30  $\mu\text{M}$  GDP, 0.1 nM [ $^{35}\text{S}$ ]GTP $\gamma$ S, and varying concentrations of CP55, 940 (0.01–10  $\mu\text{M}$ ) for 2 h at 30 $^{\circ}\text{C}$ . Non-specific binding was determined using 20  $\mu\text{M}$  unlabeled GTP $\gamma$ S. 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 TM (Research Products International Corp., Mount Prospect, IL) scintillation fluid.

**Electrophysiology.** C57BL/6J mice were given daily i.p. injections for six 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 h after the final injection, the mice were anesthetized under isoflurane inhalation and decapitated. In order to determine the involvement of CB<sub>1</sub> and CB<sub>2</sub> receptors in this assay, we tested whether the respective receptor antagonists AM251 (2  $\mu\text{M}$ ) or AM630 (2  $\mu\text{M}$ ) would block WIN55,212-2-depressed IPSCs. Cingulate cortex slices (300  $\mu\text{m}$  thick) were cut using a vibratome (Leica) 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<sub>2</sub>PO<sub>4</sub>, 0.5 CaCl<sub>2</sub>, 7 MgSO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 10 glucose, and 1 sodium ascorbate. The slices were transferred into artificial cerebrospinal fluid (ACSF) containing (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 10 glucose and were allowed to recover for at least 1 h at room temperature. All solutions were saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

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). Pyramidal neurons in layer V of the anterior cingulate cortex were identified visually based upon 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<sub>2</sub>, 4 MgATP, 0.3 Na<sub>2</sub>GTP, and 10 Na<sub>2</sub>-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 h and pre-injection measures. Paw withdrawal thresholds to the von Frey filaments in the carrageenan-injected and contralateral (i.e., control) paws at the 5 h time point were used to assess mechanical allodynia and at

least 10 days after CCI surgery. The magnitude of the anti-allodynic effects of the enzyme inhibitors alone and in combination were calculated as percent maximum possible effect (% MPE) by the following formula:  $\%MPE = 100 * (\text{enzyme inhibitor von Frey threshold value} - \text{vehicle von Frey threshold value}) / (\text{sham von Frey value} - \text{vehicle von Frey threshold value})$ . All data are depicted as mean  $\pm$  standard error of the mean (SEM). 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. [<sup>3</sup>H]SR141716A and [<sup>35</sup>S]GTP $\gamma$ S binding data were fit by nonlinear regression analysis to obtain B<sub>max</sub> and K<sub>D</sub> or E<sub>max</sub> values, respectively. The pharmacological 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, and 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 the baseline. The depression (percentage) of IPSCs by WIN55,212-2 was calculated as follows:  $100 \times [\text{mean amplitude of IPSCs during the last 5 min treatment} / \text{mean amplitude of baseline IPSCs}]$ . 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 prior to intraplantar administration of carrageenan (**Figure 1A**). As shown in **Figure 1B**, combined administration of these enzyme inhibitors fully prevented carrageenan-induced allodynia (79 + 14% MPE), while 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 anti-edematous effects [ $F(3, 28) = 51.2, p < 0.001$ ; **Figure 1C**]. Immediately following 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 [ $F(4, 35) = 389.0, p < 0.001$ ; **Figure 1D**]. JZL184 (4 mg/kg) given alone or in combination with PF-3845 produced a 3-fold elevation in 2-AG levels [ $F(4, 35) = 99, p < 0.001$ ; **Figure 1E**]. The elevation in brain 2-AG was not accompanied by significant reductions in the levels of arachidonic acid [ $p > 0.41$ ] **Figure 1F**. On the other hand, full blockade of MAGL produced by JZL184 (40 mg/kg) elevated 2-AG levels eight fold [**Figure 1E**] and reduced brain arachidonic acid levels by two fold [ $F(4,35) = 18.92, p < 0.001$ ; **Figure 1F**].

In order to determine whether cannabinoid receptors mediate the anti-allodynic and anti-edematous effects of combined FAAH and MAGL inhibition, we employed the respective CB<sub>1</sub> and CB<sub>2</sub> receptor antagonists rimonabant (1 mg/kg) and SR144528 (3 mg/kg). As shown in **Figure 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 anti-allodynic effects of combined administration PF-3845 and JZL184. In contrast SR144528 [ $F(1, 21) = 23.0, p < 0.001$ ], but not rimonabant [ $p = 0.73$ ], significantly prevented the anti-edematous effects of the

combination (**Figure 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 anti-allodynic effects of PF-3845 and JZL184 require both CB<sub>1</sub> and CB<sub>2</sub> receptors, but the edematous effects of these enzyme inhibitors require only CB<sub>2</sub> 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 (**Figure 3A**). As shown in **Figure 3B**, combined administration of high dose PF-3845 (10 mg/kg) and low dose JZL184 (4 mg/kg) produced greater anti-allodynic 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 anti-allodynic 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 anti-edematous effects compared with single enzyme inhibition (**Figure 3C**). Combination of PF-3845 and JZL184 also produced enhanced anti-allodynic 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; **Figure 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 carrageenan-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 pharmacological 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 (**Figure 4A and 4B**). Assessment of JZL184 (0, 4, 16, 40, or 100 mg/kg) in combination with 10 mg/kg PF-3845 on locomotor activity (**Figure 4C**), catalepsy (**Figure 4D**), and hypothermia (**Figure 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 anti-allodynic and anti-edematous actions, without CB<sub>1</sub> receptor down-regulation or desensitization.**

As shown in **Figure 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 anti-edematous effects of dual FAAH-MAGL inhibition were virtually identical in magnitude following acute and repeated administration [ $F(2, 22) = 8.47, p < 0.01$ ; **Figure 5B**].

As previously reported, repeated treatment of high dose JZL184 (40 mg/kg) reduced whole brain CB<sub>1</sub> receptor levels by 33.3% and produced a 56.3% reduction in maximal CP55,940-stimulated G-protein activity. However, simultaneous repeated administration of high dose PF-3845 (10 mg/kg) + low dose JZL184 (4 mg/kg) as well as each treatment alone did not significantly alter CB<sub>1</sub> receptor levels (**Figure 5C**) or CP55,940-stimulated G-protein activity (**Figure 5D**) as compared to the vehicle + vehicle control group.

Finally, we examined whether repeated administration of the combination treatment for six 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 following repeated administration of 40 mg/kg JZL184 (Schlosburg et al., 2010). In order to infer which cannabinoid receptors mediate the acute actions of WIN55,212-2 on IPSCs, we employed the selective CB<sub>1</sub> receptor antagonist AM251 (2  $\mu$ M)

and the selective CB<sub>2</sub> 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 was blocked by the CB<sub>1</sub> receptor selective antagonist AM251 (91.3 ± 4.1% of baseline;  $p < 0.01$  vs. control), but was not significantly affected by AM630 (67.8 ± 4.1% of baseline;  $p = 0.40$  vs. control; **Figure 6A**). These findings indicate that CB<sub>1</sub> receptors, not CB<sub>2</sub> receptors, mediate WIN55,212-2-induced depression of IPSCs.

We next examined cingulate slices from mice given a daily injection for six 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;  $p = 0.48$ ; **Figure 6A,B**), suggesting that repeated JZL184 at this low dose did not induce significant CB<sub>1</sub> receptor desensitization. In a previous study, we reported no apparent CB<sub>1</sub> receptor desensitization in mice given a daily injection of PF-3845 (10 mg/kg) for 6 days (Schlosburg *et al.*, 2010). Consistent with this finding, we found that the magnitude of WIN55,212-2-induced depression of IPSCs in the cingulate cortex was not significantly different 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,  $n = 8$ ;  $p = 0.90$ ; **Figure 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) ( $p < 0.05$ ; **Figure 6B**).

## Discussion

Simultaneous inhibition of the primary endocannabinoid catabolic enzymes FAAH and MAGL produces augmented antinociceptive effects as compared to 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; Hrubá et al., 2015; Walentiny et al., 2015). Moreover, prolonged and complete MAGL inactivation results in tolerance accompanied with CB<sub>1</sub> 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<sub>1</sub> receptor functional tolerance (Kinsey et al., 2013). Thus, the present study tested whether full FAAH inhibition combined with partial MAGL inhibition would produce enhanced anti-allodynic 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 does not elicit cannabimimetic side effects commonly associated with CB<sub>1</sub> receptor agonists. Finally, repeated daily administration of this combination of inhibitors over six days continued to block carrageenan-induced allodynia, with no evidence of CB<sub>1</sub> receptor functional tolerance.

In order to infer the separation between the anti-allodynic 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. While 4 mg/kg JZL184 plus PF-3845 completely reversed carrageenan-induced allodynia, significant cataleptic, hypothermic and locomotor depressant effects emerged only following 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, combination of 10 mg/kg PF-3845 and 40 mg/kg JZL184 partially substituted and 120 mg/kg JZL184 fully substituted (Hruba et al., 2015). However, the different vehicles employed in the drug discrimination experiment (i.e., 1:1:18 mixture of propylene glycol, Tween 80, and saline) versus the other experiments (i.e., 1:1:18 mixture of ethanol, alkamuls-620, and saline) limits direct comparison. Nonetheless, the observations that the dose of JZL184 in combination with PF-3845 that elicited significant anti-allodynic effects in CCI and carrageenan assays was 25 fold lower than the dose required to produce significant cataleptic, hypothermic, and motor depressive effects represents a reasonable separation between anti-allodynic 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 anti-edematous and anti-allodynic effects in the carrageenan model, we did not examine the consequences of repeated administration in the CCI model of neuropathic pain. However, previous studies show that the anti-allodynic effects of low dose JZL184 (i.e.,  $\leq 8$  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<sub>1</sub> receptor downregulation or desensitization in whole brain. Because repeated injections of this JZL184 dose resulted in brain region-dependent CB<sub>1</sub> 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<sub>1</sub> receptor mediated effect (Fig. 6A). Repeated administration of PF-3845 (10 mg/kg) in combination with high dose of 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). However, repeated administration of PF-3845 (10 mg/kg) in combination with low dose of JZL184 (4 mg/kg) did not lead to CB<sub>1</sub> receptor functional tolerance in the cingulate cortex.

Rimonabant as well as SR144528 completely prevented the anti-allodynic actions of combined PF-3845 and JZL184 in the carrageenan model of inflammatory pain, indicating that CB<sub>1</sub> and CB<sub>2</sub> receptors play necessary roles in this response. Likewise, the anti-allodynic effects JZL195 in the CFA 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 anti-allodynic effects of MAGL inhibitors requiring activation of both cannabinoid receptors, while the anti-edematous effects of the drugs are mediated via CB<sub>2</sub> receptors (Ghosh et al., 2013; Ignatowska-Jankowska et al., 2014). Similarly, CB<sub>2</sub> receptors, but not CB<sub>1</sub> receptors, mediate the anti-edematous effects of the FAAH inhibitor URB597 in the carrageenan model (Holt et al., 2005). The lack of further anti-edematous 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 anti-allodynic and anti-edematous 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<sub>1</sub> and CB<sub>2</sub> 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 anti-allodynic effects remains unanswered. Several non-mutually 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 three-fold increase in brain 2-AG levels, irrespective of co-administration of

PF-3845, and PF-3845 produced greater than a 10-fold increase of brain AEA levels, regardless of the presence of JZL184. However, the fact that 2-AG levels are approximately three orders of magnitude higher than AEA levels in whole brain negates this idea of mass action. Alternatively, it is plausible that simultaneous inhibition of MAGL and FAAH produces augmented anti-allodynic effects because the respective endocannabinoids activate distinct CB<sub>1</sub> and CB<sub>2</sub> 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 anti-allodynic effects. In particular, FAAH metabolizes other bioactive fatty acid amides besides AEA, including N-palmitoylethanolamine, N-oleoylethanolamine, and oleamide (Cravatt et al., 1996; Cravatt et al., 2001), as well as N-acyl taurines (Saghatelian et al., 2006), which activate various TRP channels and PPAR- $\alpha$  receptors. In addition, MAGL plays an important role in the biosynthesis of arachidonic acid in brain and its blockade results in reduced arachidonic acid, and concomitant reductions in prostaglandins (Nomura et al., 2011). The observation that 4 mg/kg JZL184 did not alter arachidonic acid 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 arachidonic acid as well as endocannabinoids in regions associated with pain (e.g., cingulate cortex, PAG, rostral ventral medulla, spinal cord, dorsal root ganglia, etc.). 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 anti-allodynic 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 and catalepsy) and THC-like subjective effects represent challenges for translational implications. Moreover, development of tolerance and CB<sub>1</sub> 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 anti-allodynic efficacy, but also circumvents occurrence of cannabimimetic side effects and CB<sub>1</sub> 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 anti-allodynic effects compared with single enzyme inhibition, but with reduced untoward side effects and no evidence of CB<sub>1</sub> receptor functional tolerance.

### **Authorship Contributions.**

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

Conducted experiments: Ghosh, Kinsey, Liu, Hrubá, 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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## Footnotes

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## Figure Legends

**Figure 1.** Combined administration of high dose PF-3845 (10 mg/kg) and low dose JZL184 (4 mg/kg) completely prevented carrageenan-induced mechanical allodynia (**Panel A**), but did not further reduce carrageenan-induced paw edema (**Panel B**), compared with single enzyme inhibition. **Panel 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. **Panel 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. **Panel E.** High dose JZL184 (40 mg/kg) significantly decreased arachidonic acid (AA) brain levels, but none of the other treatments had an effect. **Panel F.** Experimental time line. Drugs were administered 2 h before the intraplantar injection of carrageenan. Mechanical allodynia was measured 5 h after carrageenan and paw thickness was measured prior to and 5 h after carrageenan administration. Mice were euthanized immediately after behavioral testing (i.e., approximately 7 h 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 presented as mean  $\pm$  SEM,  $n = 7-8$  mice/group.

**Figure 2.** Assessment of cannabinoid receptors mediating the anti-allodynic and anti-edematous effects produced by combined administration of JZL184 (4 mg/kg) and PF-3845 (10 mg/kg). **Panel A.** Rimonabant (1 mg/kg) or SR144528 (3 mg/kg) blocked the anti-allodynic effects of the inhibitors given in combination. **Panel B.** SR144528 (3 mg/kg), but not rimonabant (1 mg/kg), blocked the anti-edematous effects of the inhibitors given in combination. Mechanical allodynia was measured 5 h after

carrageenan and paw thickness was measured prior to and 5 h after carrageenan administration. JZL184 + PF-3845 or vehicle was injected 2 h before carrageenan. Rimobabant or SR144528 was injected 30 min prior to vehicle or JZL184 + PF-3845. \*\*\* $p < 0.001$  versus vehicle/vehicle. ##  $p < 0.05$  versus vehicle/JZL184 + PF-3845 (Bonferroni test). Data presented as mean  $\pm$  SEM,  $n = 6$  mice/group.

**Figure 3.** Simultaneous full FAAH inhibition and partial MAGL inhibition fully reverses inflammatory and neuropathic nociception. **Panel A.** Experimental timeline. Drugs were administered 3 h post carrageenan. Paw thickness was measured prior to and 5 h after carrageenan administration and mechanical allodynia was measured 5 h after carrageenan. Combined administration of high dose PF-3845 (10 mg/kg) and low dose JZL184 (4 mg/kg) significantly reversed mechanical allodynia (**Panel B**), but did not further reduce paw edema (**Panel C**), compared with single enzyme inhibition in the carrageenan model of inflammatory pain. **Panel D.** Combined administration of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) produced enhanced anti-allodynic effects compared to either JZL184 or PF-3845 alone in the CCI model of neuropathic pain. Mechanical allodynia was assessed 2 h post 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  $\pm$  SEM,  $n = 6$  mice/group (Panels B and C) and  $n = 20$  mice/treatment (Panel D).

**Figure 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 (**Panel A**) or affect response rates (**Panel 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 (**Panel C**), cataleptic

(**Panel D**), and hypothermic (**Panel E**) effects. Baseline rectal temperature was  $36.3 \pm 0.1^\circ \text{C}$  (mean  $\pm$  SEM) \* $p < 0.5$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  vs. vehicle-vehicle.

Values reflect mean  $\pm$  SEM,  $n = 10$  mice.

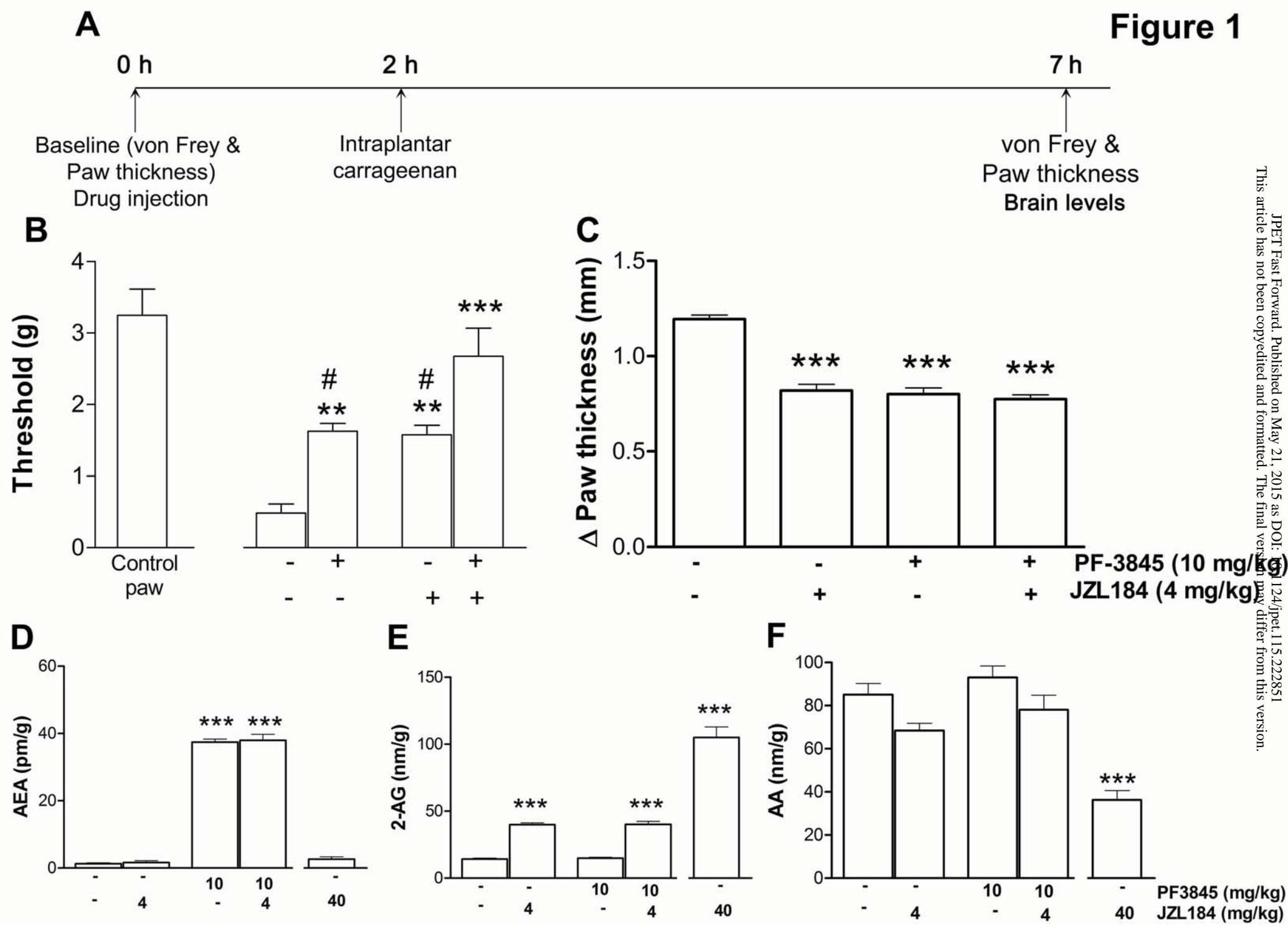
**Figure 5.** Repeated administration of combined JZL184 and PF-3845 produces sustained anti-allodynic (**Panel A**) and anti-edematous (**Panel B**) effects in the carrageenan model of inflammatory pain. Acute administration or repeated (six days daily) administration of low dose high dose PF-3845 (10 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 h after drug administration.

Mechanical allodynia was tested 5 h after carrageenan and paw thickness was measured prior to and 5 h after carrageenan administration.  $\text{CB}_1$  receptor binding (**Panel C**) and receptor-mediated G-protein activation (**Panel D**) are maintained following repeated injections of high dose PF-3845 and low dose JZL184 administered separately or in combination. However, six days of daily injections of high dose JZL184 (40 mg/kg) reduced both the [ $^3\text{H}$ ]SR141716A  $B_{\text{max}}$  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. Data presented as mean  $\pm$  SEM,  $n = 8-9$  mice/group.

**Figure 6.** Effects of repeated *in vivo* administration of JZL184 alone or in combination with PF-3845 (10 mg/kg) on WIN55,212-2 (2  $\mu\text{M}$ )-induced depression of IPSCs in the cingulate cortex. **Panel A.** The  $\text{CB}_1$  receptor antagonist AM251 (2  $\mu\text{M}$ ) blocked WIN55,212-2-induced depression of IPSCs ( $p < 0.01$ ), whereas the  $\text{CB}_2$  receptor antagonist AM630 (2  $\mu\text{M}$ ) had no significant effect ( $p = 0.4$ ). **Panel B.** Mice treated for six 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

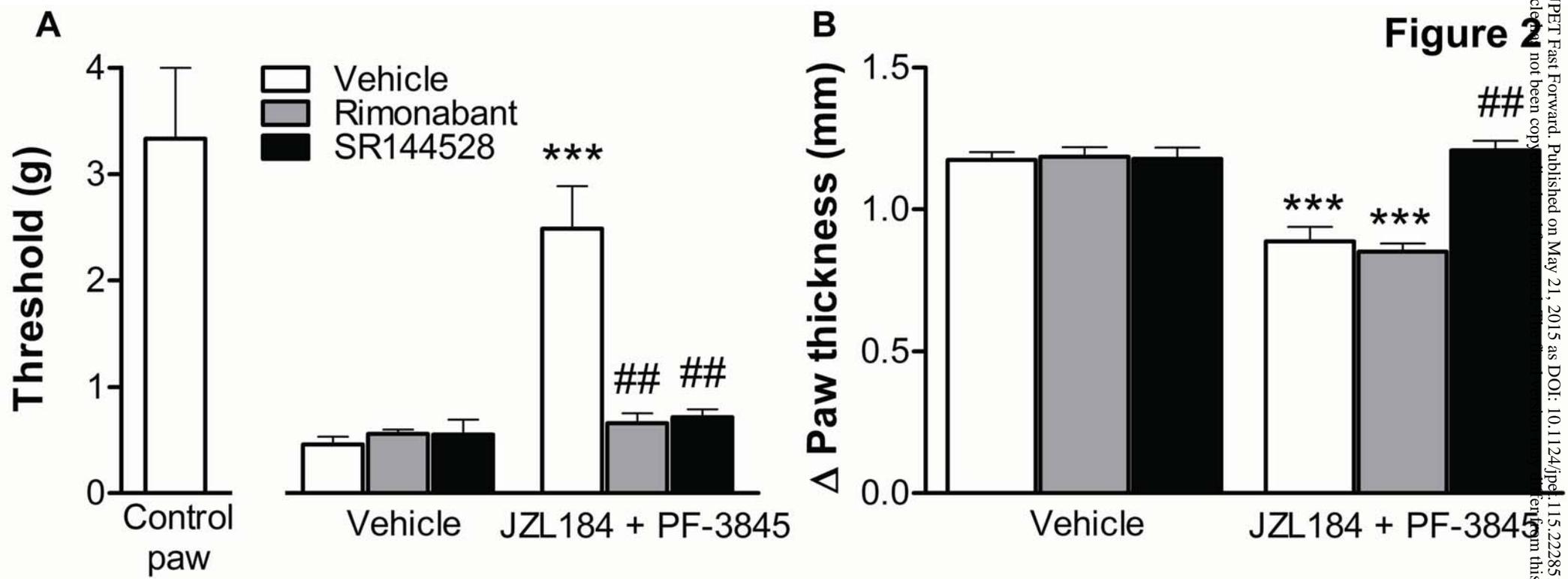
with that in mice treated with repeated vehicle shown in Panel A ( $p = 0.48$ ). However, WIN55,212-2 induced significantly less depression of IPSCs in mice treated with high dose JZL184 (40 mg/kg) plus PF-3845 than in mice in each other group ( $p < 0.05$ ). Data presented as mean  $\pm$  SEM, and were obtained from multiple slices ( $n = 6-8$ ) of three mice per group.

**Figure 1**



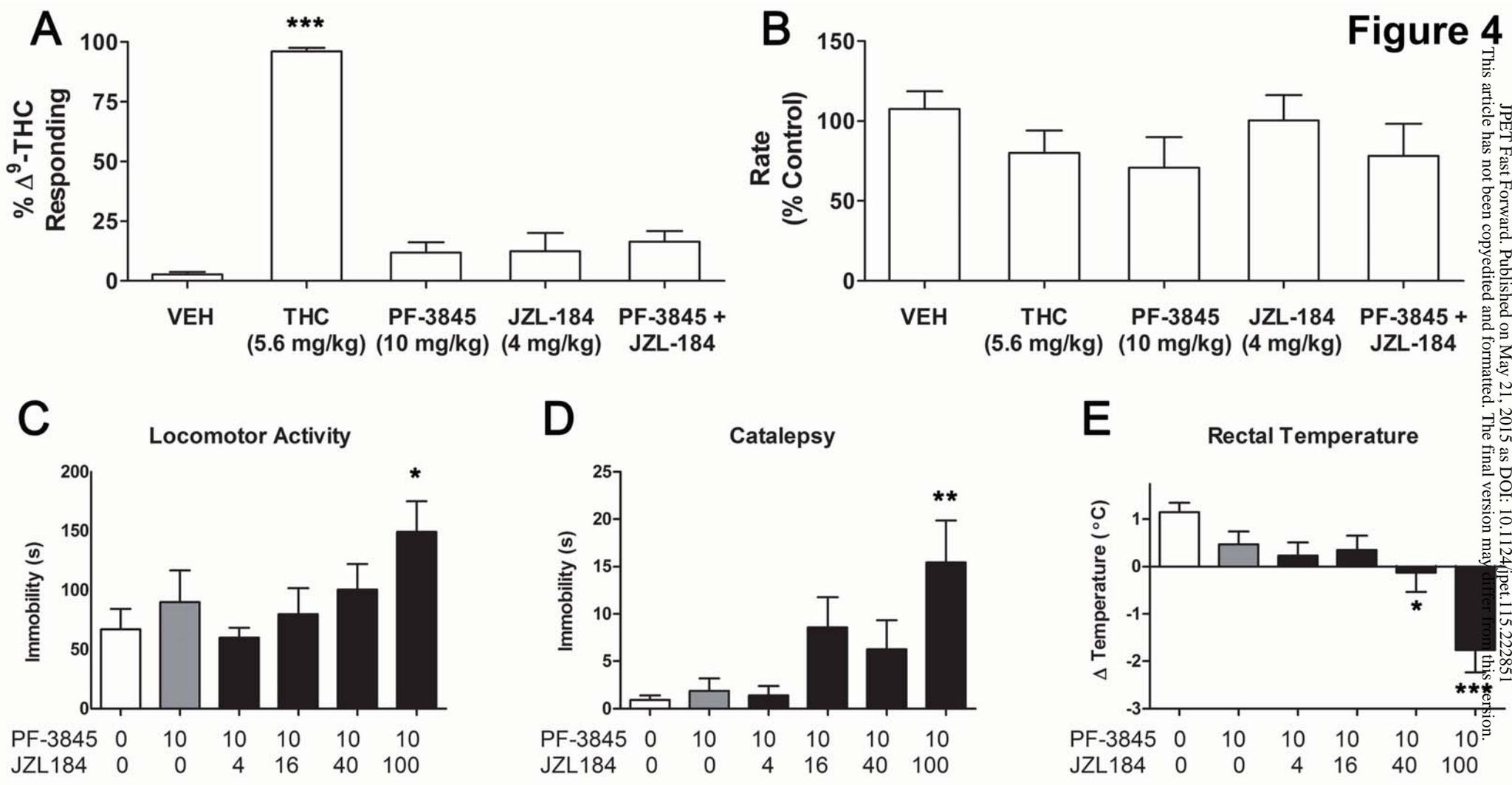
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Figure 2



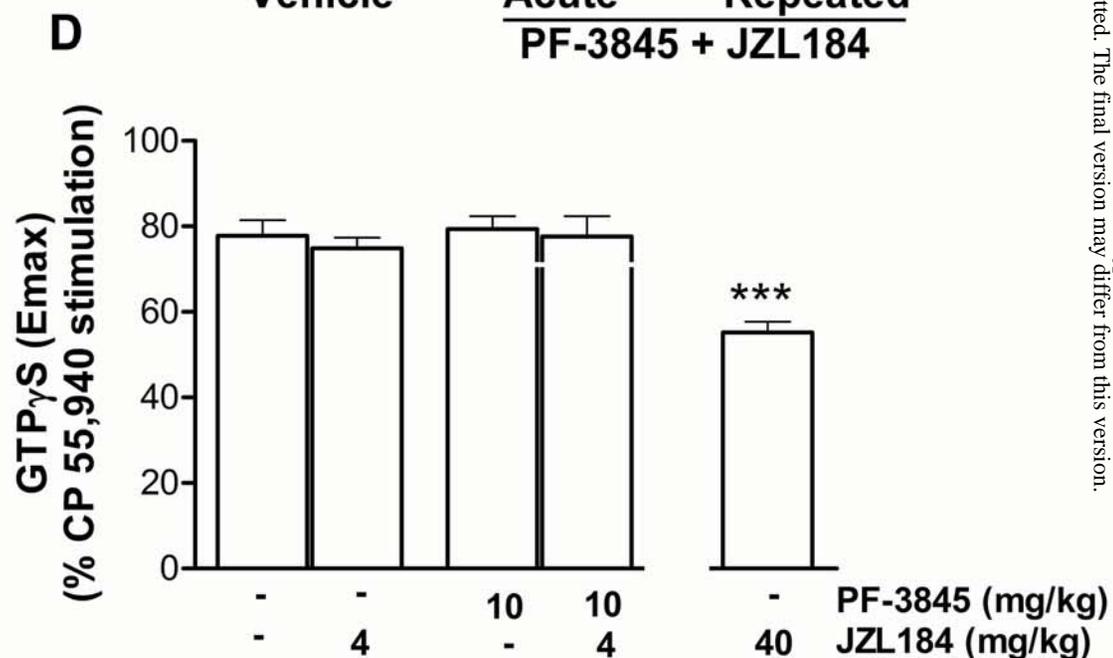
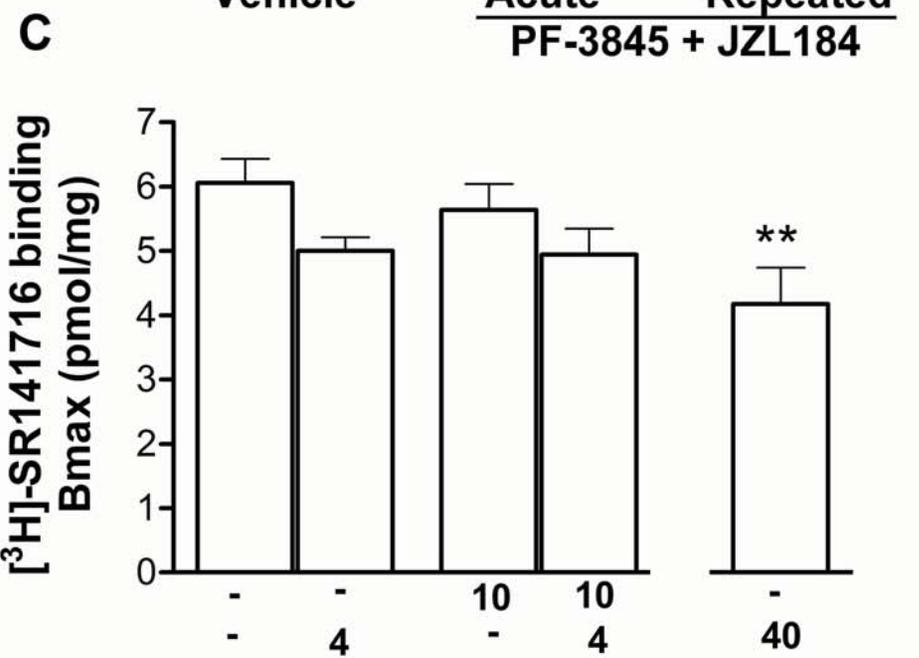
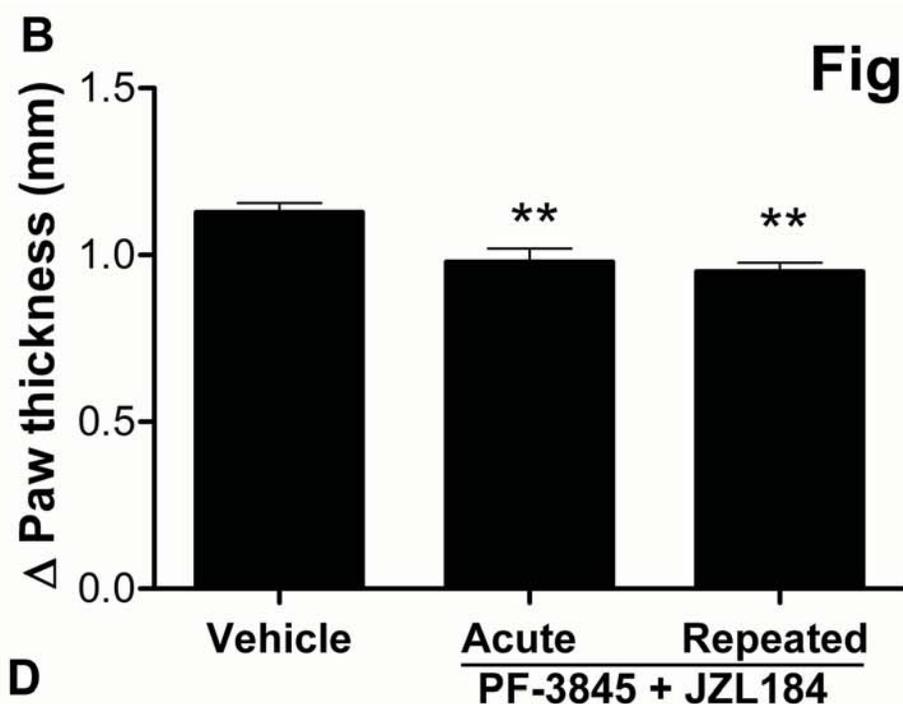
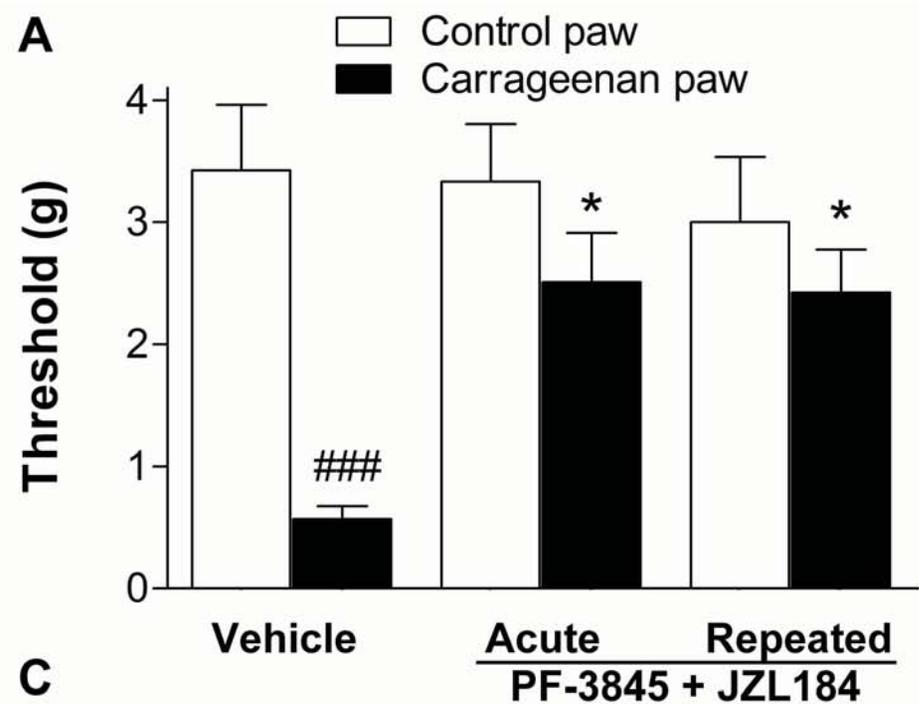


**Figure 4**

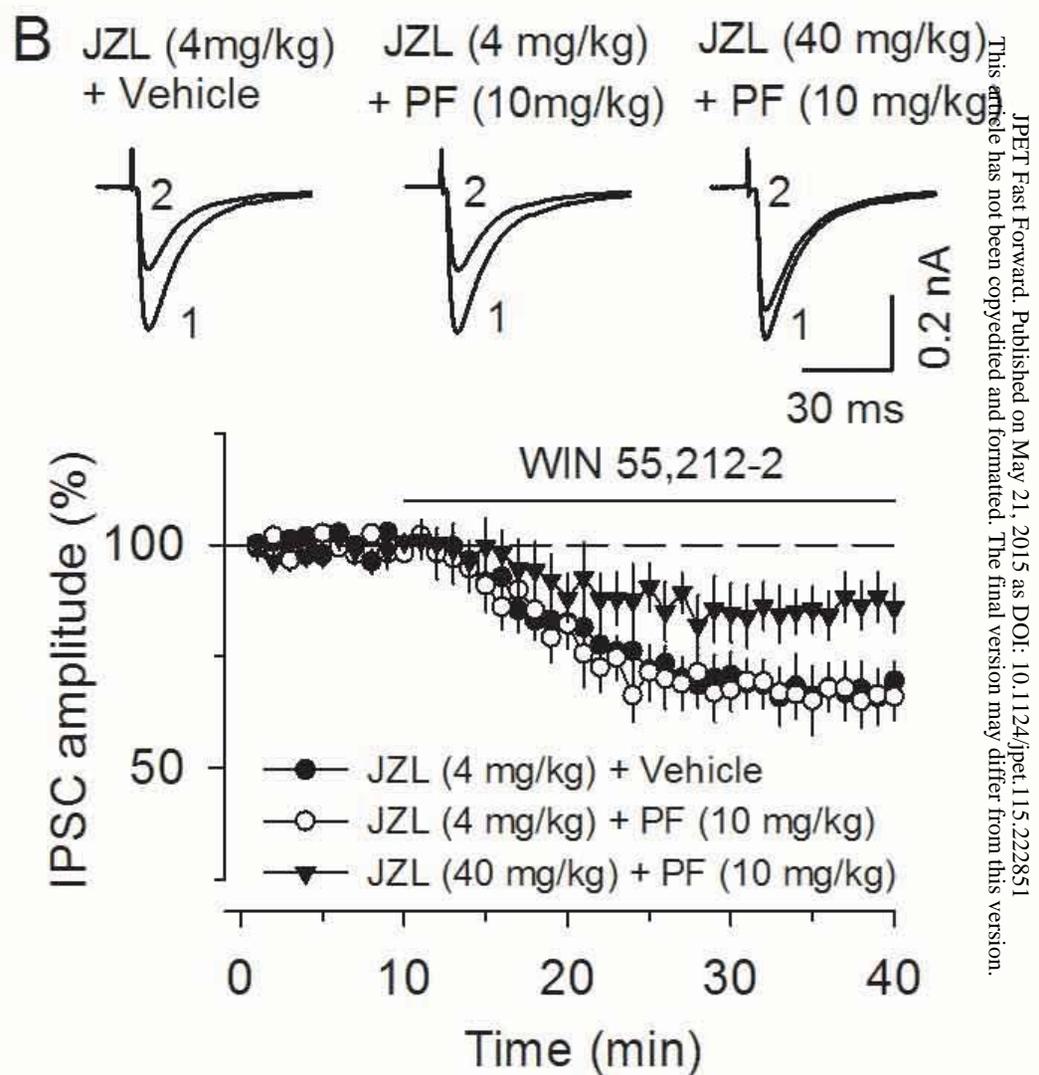
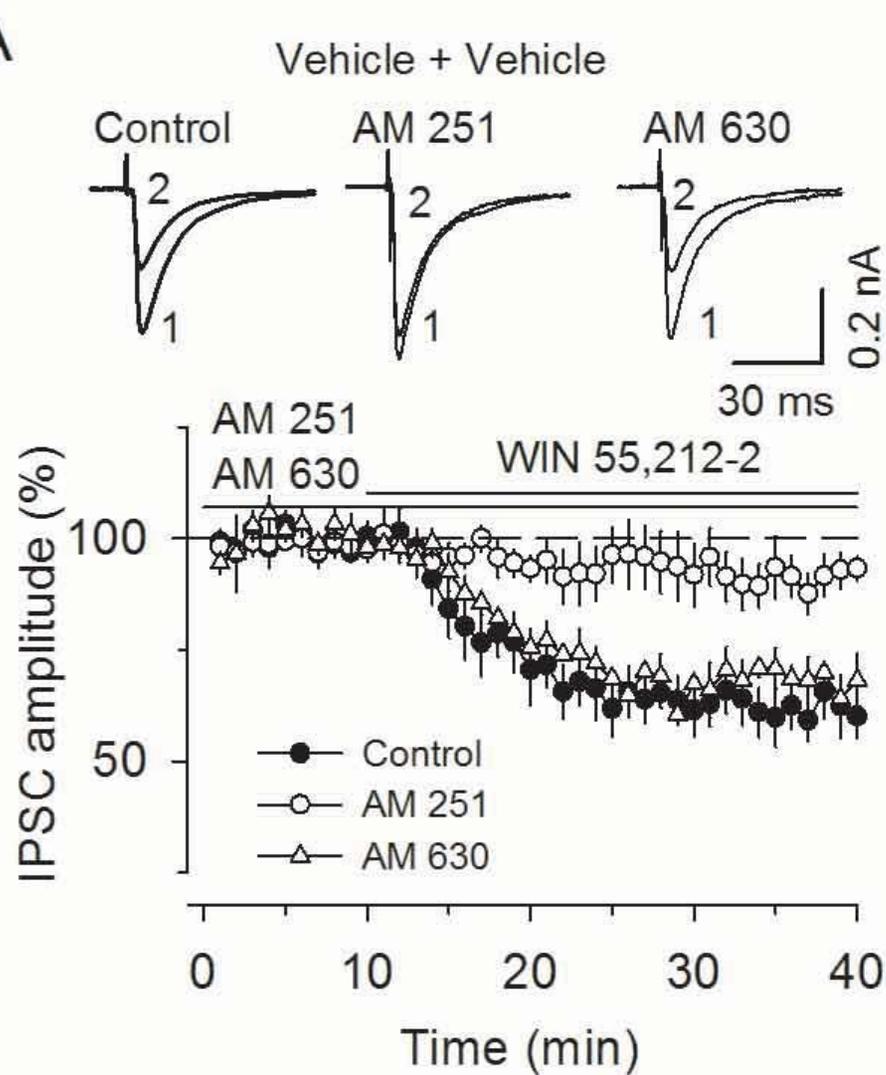


**Figure 5**

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**Figure 6**



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