Blockade of endocannabinoid hydrolytic enzymes attenuates precipitated opioid withdrawal symptoms in mice

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a) **Running Title:** Endocannabinoid modulation of opioid withdrawal

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c) **Article Key Numbers:**

Text pages: 40

Tables: 2

Figures: 8

Supplementary figures: 1

References: 40

Abstract Words: 249

Introduction Words: 740

Discussion Words: 1488

Nonstandard Abbreviations: MAGL, monoacylglycerol lipase; FAAH, fatty acid amide hydrolase; Rimonabant, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl; THC, [Delta]9-tetrahydrocannabinol; ANOVA, analysis of variance; C57, C57BL/6J mouse strain; AEA, N-arachidonylethanolamine (anandamide); 2-AG, 2-arachidonylglycerol; JZL184, 4-Nitrophenyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate; Morphine sulfate, 5α,6α)-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol; Naloxone hydrochloride, (1S,5R,13R,17S)-10,17-dihydroxy-4-(prop-2-en-1-yl)-12-oxa-4-azapentacyclo [9.6.1.01,13.05,17.07,18] octadeca-7(18),8,10-trien-14-one; PF-3845, N-(pyridin-3-yl)-4-(3-(5-(trifluoromethyl)pyridin-2-yloxy)benzyl)- piperdine-1-carboxamide; Rimonabant, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-

(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl; SR144528, N-[(1S)-endo-

1,3,3,-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-

pyrazole-3-carboxamide;

    d) **Recommended Section:** Behavioral Pharmacology

Abstract

Δ^9-tetrahydrocannbinol (THC), the primary active constituent of *Cannabis sativa*, has long been known to reduce opioid withdrawal symptoms. Although THC produces most of its pharmacological actions through the activation of CB_1 and CB_2 cannabinoid receptors, the role these receptors play in reducing the variety of opioid withdrawal symptoms remains unknown. The endogenous cannabinoids, *N*-arachidonoylethanolamine (anandamide; AEA) and 2-arachidonylglycerol (2-AG), activate both cannabinoid receptors, but are rapidly metabolized by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively. The objective of this study was to test whether increasing AEA or 2-AG, via inhibition of their respective hydrolytic enzymes, reduces naloxone-precipitated morphine withdrawal symptoms in *in vivo* and *in vitro* models of opioid dependence. Morphine-dependent mice challenged with naloxone reliably displayed a profound withdrawal syndrome, consisting of jumping, paw tremors, diarrhea, and weight loss. THC and the MAGL inhibitor, JZL184 dose-dependently reduced the intensity of most measures through the activation of CB_1 receptors. JZL184 also attenuated spontaneous withdrawal signs in morphine-dependent mice. The FAAH inhibitor, PF-3845, reduced the intensity of naloxone-precipitated jumps and paw flutters through the activation of CB_1 receptors, but did not ameliorate incidence of diarrhea or weight loss. In the final series of experiments, we investigated whether JZL184 or PF-3845 would attenuate naloxone-precipitated contractions in morphine-dependent ilea. Both enzyme inhibitors attenuated the intensity of naloxone-induced contractions, though this model does not account mechanistically for the autonomic withdrawal responses (i.e., diarrhea) observed *in vivo*. These results indicate that endocannabinoid catabolic enzymes are promising targets to treat opioid dependence.

Introduction

The risk of opiate dependence from chronic use of prescription analgesics and illicit substances remains high, despite growing awareness of the potential for abuse and/or misuse. An important component of opiate dependence is withdrawal, an aversive syndrome that occurs on cessation of drug use and contributes to drug maintenance and thwarts efforts for rehabilitation. In humans, spontaneous opioid withdrawal occurs soon after the last drug use and is characterized by a range of aversive effects, including behavioral (e.g., anxiety, shakes), gastrointestinal (e.g., diarrhea, emesis, dehydration), and other physiological effects (e.g., hypertension, tachycardia, and body aches) (DSM -IV). Current treatment for opiate withdrawal includes maintenance therapy with replacement opiates, such as methadone or buprenorphine. However, these opioid agonists also generate physical dependence. For example, abrupt discontinuation from methadone can trigger a withdrawal syndrome albeit less severe, but longer lasting than that resulting from heroin (Dyer et al., 1999). Similarly, physical dependence has been reported after chronic buprenorphine treatment (Kuhlman et al., 1998). Given the limitations of these extant replacement therapies, there is need for non-opioid drug therapies with reduced associated addiction potential.

A case report from the 19th century suggested that an extract of *Cannabis sativa* may ameliorate opiate addiction (Birch, 1889). Modern studies corroborated this idea by demonstrating that Δ^9-tetrahydrocannabinol (THC), the primary psychoactive constituent of cannabis, attenuates both the intensity of naloxone-precipitated opioid withdrawal signs in morphine-dependent rodents and naloxone-precipitated contractions in ilea given prolonged morphine exposure (Frederickson et al., 1976; Basilico et al., 1999). However, undesirable psychoactive side effects of direct-acting CB_1 receptor agonists have dampened enthusiasm for their clinical development. Conversely, a growing body of literature demonstrates that elevating

endogenous cannabinoids by inhibiting their hydrolytic enzymes offers potential therapeutic benefits, without the undesirable cannabimimetic actions of the exogenous cannabinoids (Solinas et al., 2007; Ahn et al., 2008; Justinova et al., 2008; Ahn et al., 2009).

The endocannabinoid system consists of two receptors (CB_1 and CB_2) (Matsuda et al., 1990; Munro et al., 1993), the endogenous cannabinoids, anandamide (AEA) (Devane et al., 1992) and 2-arachidonylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995) and the enzymes that regulate their synthesis and degradation (Ahn et al., 2008). Although injecting AEA or 2-AG is moderately effective in reducing the intensity of opioid withdrawal signs in mice (Vela et al., 1995; Yamaguchi et al., 2001), their rapid metabolism by the respective enzymes fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996; Cravatt et al., 2001) and monoacylglycerol lipase (MAGL) (Dinh et al., 2002) limits their therapeutic utility. Alternatively, blocking these endocannabinoid catabolic enzymes via chemical inhibition or genetic deletion causes an increase in tissue levels of the appropriate endocannabinoid. Mice treated with FAAH inhibitors, as well as FAAH (-/-) mice, show 10-fold elevations of AEA in CNS (Cravatt et al., 2001; Ahn et al., 2009). Similarly, genetic deletion or pharmacological inhibition of MAGL increases brain 2-AG levels by approximately 10-fold (Long et al., 2009a; Long et al., 2009b; Schlosburg et al., 2010).

In the present study, we evaluated whether elevating endocannabinoids, through the inhibition of their catabolic enzymes, attenuates naloxone-precipitated withdrawal symptoms using *in vivo* and *in vitro* models of morphine dependence. For the *in vivo* studies, we investigated of the efficacy of the respective MAGL and FAAH inhibitors, JZL184 and PF-3845, to reduce naloxone-precipitated jumps, paw flutters, diarrhea and weight loss in mice implanted with morphine pellets. The effects of these enzyme inhibitors were compared to those of THC. Selective CB_1 and CB_2 receptor antagonists were employed to assess cannabinoid receptor

involvement of the anti-withdrawal effects of JZL184 and PF-3845. In addition, we evaluated

whether JZL184 would reduce spontaneous withdrawal in morphine-dependent mice. In order to

evaluate whether compensatory changes of endocannabinoids occur during the state of

withdrawal, AEA and 2-AG levels were quantified in brain regions associated with opioid

dependence (i.e., the locus coeruleus (LC), periaqueductal grey (PAG), and amygdala). For the

in vitro experiments, we evaluated whether JZL184 and PF-3845 inhibit naloxone-precipitated

contractions in morphine-treated ileum. The ileum offers a useful *in vitro* model to investigate

opioid withdrawal (Paton, 1957). Endocannabinoid catabolic enzyme inhibitors were also

assessed for their efficacy in reducing electric field stimulated (EFS) contractions in naïve

untreated ilea. Given that genetic deletion or pharmacological inhibition of MAGL leads to

increases of 2-AG and concomitant decreases in arachidonic acid levels in brain (Long et al.,

2009a; Schlosburg et al., 2010), we quantified whether PF-3845 and JZL184 alter

endocannabinoids, free arachidonic acid, and prostaglandins in ileum.

Methods

Subjects

Male ICR mice (Harlan laboratories; Indianapolis) as well as male FAAH (-/-) and (+/+) mice backcrossed onto a C57BL/6J background for at least 13 generations (Cravatt et al., 2001) served as subjects. The mice weighed between 26 and 30 g and were housed 6-8 per cage in a temperature controlled (20-22°C) environment, in an American Association for the Accreditation of Laboratory Animal Care-approved facility. The mice were kept on a 12 h light/ dark cycle, with all experiments being performed during the light cycle. Food and water were available *ad libitum*. The study was performed with the approval of the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the Guide for the Care and Use of Laboratory Animals.

Drugs

Morphine pellets (75 mg), placebo pellets, morphine sulfate, THC, the CB$_2$ receptor antagonist SR144528, and the CB$_1$ receptor antagonist rimonabant were obtained from the National Institute on Drug Abuse (Bethesda, MD). Naloxone hydrochloride was purchased from Cayman chemicals (Ann Arbor, MI). JZL184 and PF-3845 were synthesized as described previously (Ahn et al., 2009; Long et al., 2009a). THC, PF-3845, JZL184, rimonabant, and SR144528 were dissolved in ethanol, followed by addition of Emulphor-620 (Rhone-Poulenc, Princeton, NJ), and diluted with 0.9% saline to form a vehicle mixture of ethanol:emulphor:saline in a ratio of 1:1:18. Naloxone and morphine were dissolved in 0.9% saline. All injections were administered in volume of 0.01 ml per 1 g body weight. THC and naloxone were administered via subcutaneous (s.c.) injection, whereas PF-3845, JZL184, rimonabant, and SR144528 were given via intraperitoneal (i.p.) injection. PF-3845 and JZL184 were given 2 h before testing, to coincide with peak levels of AEA and 2-AG elevations,

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respectively, following systemic administration (Ahn et al., 2009; Long et al., 2009a). Rimonabant, SR144528, and Δ^9-THC were given 30 min before naloxone treatment. For *in vitro* isometric tension recording studies, naloxone hydrochloride and morphine sulfate were dissolved in saline, while PF-3845 and rimonabant were dissolved in ethanol and JZL184 was dissolved in DMSO.

Morphine pellet implantation surgery

In order to induce opioid dependence, mice were implanted with morphine pellets as previously described (Way et al., 1969). After induction of anesthesia with 2.5% isoflurane, the fur was shaved, the skin was disinfected with a sterile betadine swab (Purdue products, Stamford, CT), and a 1 cm horizontal incision was made in the midscapular region, using sterile surgical scissors. A 75 mg morphine sulfate pellet was inserted subcutaneously, and the incision was closed with a sterile staple. The mice were allowed to recover in heated home cages for 2 h after surgery and then returned to the vivarium until testing.

Naloxone-precipitated opioid withdrawal

Somatic withdrawal signs were scored, as previously described (Schlosburg et al., 2009). In brief, mice were placed in white acrylic chambers (20x20 cm), with a clear acrylic front panel and a mirrored back panel for a 30 min acclimation period. The chambers were enclosed in sound-attenuating cabinets that contained an indirect filtered LED light source and fans for air circulation and white noise. The mice were briefly removed from the chambers for naloxone administration and immediately returned to the chambers for a 30 min observation period. Behavior was recorded using a series of Fire-i[TM] digital cameras (Unibrain, San Ramon, CA), and the videos were saved using the ANY-maze[TM] video tracking software (Stoelting Co., Wood Dale, IL). Chambers were changed between tests and cleaned at the end of testing with an ammonia based cleanser and left to dry for two days, to allow for odors to dissipate. The

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recorded videos were randomized and scored by a trained observer, who was blinded with respect to treatment condition. The primary behavioral signs of interest were frequency of jumps and front paw tremors (including single and double paw flutters and twitches, which are not commonly displayed by naïve mice). The occurrence of diarrhea during the testing period was noted. All behaviors were recorded as new incidences when separated by at least 1 s or interrupted by any other normal behavior. In addition, mice were weighed before and immediately after the 30 min test session to assess body weight loss.

Spontaneous morphine withdrawal

Three groups of mice were used for this experiment. Group 1 was implanted with placebo pellets and Groups 2 and 3 were implanted with 75 mg morphine pellets. The pellets were removed under light isoflurane anesthesia approximately 72 h after implantation. The mice were housed individually in cages that were placed on heating pads for 2 h. At 1 h after pellet removal, Groups 1 and 2 received an i.p. injection of the ethanol:emulphor:saline vehicle, and Group 3 was given an i.p. injection of JZL184 (40 mg/kg). The animals were observed for spontaneous withdrawal signs for 15 min intervals at 2, 4, 6, 8, and 24 h post-pellet removal. Spontaneous withdrawal signs were quantified using a procedure that was adapted from a previous report (Way et al., 1969). The percentage of mice that jumped off a circular platform (15 cm diameter x 70 cm height), the number of paw tremor and head shake incidences, and body weight were recorded at each time point. The mice were housed singly throughout the testing period, and food and water were available *ad libitum*, except during the 15 min observation periods.

Isometric tension recording

Previously described methods to assess effects of opioids on longitudinal ileal muscle were employed in the present study (Ross et al., 2008). One-centimeter segments of ilea were

dissected, flushed of their contents, and trimmed of mesentery. These preparations were suspended in the axis of the longitudinal muscle tied to a glass hook under 1 g passive tension in 15 ml of siliconized organ baths containing Krebs solution (118 mM NaCl, 4.6 mM KCl, 1.3 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose, and 2.5 mM $CaCl_2$), maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. Tissues were allowed to equilibrate for 60 min before start of experiments, with Krebs solution changed every 15 min. Isometric contractions were recorded by a force transducer (GR-FT03; Radnoti, Monrovia, CA) connected to a computer using Acqknowledge 382 software (BIOPAC Systems, Inc., Santa Barbara, CA). The tissues were incubated with 10µM morphine sulfate for 30 min following a 30 min incubation with either JZL184 (1 µM) or PF-3845 (1 µM). Naloxone hydrochloride (30 µM) was then added to the organ bath to precipitate contractions. Contractions to 80 mM potassium chloride buffer at the end of the study was used as the reference.

Neurogenic contractions by transmural field stimulation

Electric field stimulation (FS, 20V, 7.5 Hz) was applied through concentric electrodes over ileal longitudinal muscles. JZL184 (1 µM) or PF-3845 (1 µM) and/or rimonabant (100nM) was added over FS contractions/ relaxations to determine their effects on the neurogenic responses.

Extraction and quantification of brain and ileum endocannabinoid levels with LCMS

Mice were implanted with placebo or morphine (75 mg) pellets, as described above. On day 3, subjects received an s.c. injection of naloxone hydrochloride (1 mg/kg) or saline. Ten min after naloxone injection, the mice were decapitated, and brains and segments of the ilea were harvested. Brains were removed and placed on a glass plate on ice for dissection. A coronal section was taken using the median eminence on the ventral surface as a landmark. Specific

areas of the brain relevant to opioid withdrawal were harvested, including the locus coeruleus (LC), periaqueductal gray (PAG), and the amygdala. The anterior cut was made caudal to the optic chiasm and the posterior cut was made at the caudal extent of the hypothalamus, corresponding to approximately Bregma -0.6 to Bregma -2.6. The amygdala was dissected using the optic tract medially and the external capsule dorsally as landmarks. The sample included the amygdaloid nuclei and adjacent piriform cortex. The brainstem was then placed with the dorsal surface exposed. The PAG section was taken from the anterior superior colliculus to the caudal inferior colliculus, corresponding to approximately Bregma -3.3 to Bregma -5.3. Cortex and hippocampus were discarded and the colliculi were removed. Tissue ventral and lateral to the PAG was removed. The sample included the PAG throughout its rostral-caudal extent, as well as adjacent reticulum. The next consecutive section was taken by making a posterior cut at the anterior 1/4 of the cerebellum, corresponding to approximately Bregma -5.3 to Bregma -5.85. The LC was dissected by removing the cerebellum and the ventral pons, then isolating the tissue lateral to the fourth ventricle. The sample included the locus coeruleus and adjacent parabrachial and tegmental nuclei. The brain areas and ileum segments were snap frozen in liquid nitrogen and stored at -80°C until lipid extraction.

On the day of processing, tissues were weighed and homogenized with 1.4 ml chloroform/methanol (2:1 v/v containing 0.0348 mg PMFS/ml) after the addition of internal standards to each sample (2 pmol AEA-d8 and 1 nmol 2-AG-d8). Homogenates were then mixed with 0.3 ml of 0.73% w/v NaCl, vortexed, and then centrifuged for 10 min at 4,000 rpm (4° C). The aqueous phase plus debris were collected and extracted two more times 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 ice-cold acetone. The mixtures were then centrifuged for 5 min at 3,000 rpm and 4° C to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml methanol and placed in autosample vials for analysis.

LC/MS/MS was used to quantify AEA and 2-AG. The mobile phase consisted of (10:90) water/methanol with 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery HS C18, 4.6×15 cm, 3 μm (Supelco, PA). The mass spectrometer was run in Electrospray Ionization, in positive mode. Ions were analyzed in multiple-reaction monitoring mode, and the following transitions were monitored: (348>62) and (348>91) for AEA; (356>62) for AEAd8; (379>287) and (279>269) for 2-AG; and (387>96) for 2-AG-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 0.03 to 40 pmol for AEA and from 0.05 to 64 nmol for 2-AG.

Extraction and quantification of ileum endocannabinoid and prostaglandin levels via LCMS

In this set of experiments, endocannabinoid, arachidonic acid, and prostaglandin levels were quantified in ileum after either *in vivo* or *in vitro* application of JZL184. In the first experiment, mice were treated with either JZL184 (40 mg/kg) or vehicle and their ilea were harvested 2 h later. In the second experiment, ilea were harvested and the tissue was allowed to equilibrate in Krebs buffer for 30 min followed by incubation with either 100nM JZL184 (in DMSO) or vehicle for 30 min. The tissues were frozen in dry ice and stored at -80° C. Tissues were weighed and subsequently sheered with a tissue disruptor followed by sonication in 6 ml of 1:1 v/v hexane:ethyl acetate and 2 ml of Tris buffer (pH 8.0) containing dodecylglycerol (10 nmol) and pentadecanoic acid (10 nmol) as internal standards for positive and negative mode

ionization, respectively. Samples were centrifuged at 2000 g for 5 min and the organic layer was removed, dried under a stream of N2, resolubilized in chloroform (120 μl), and 10 μl of this resolubilized lipid was injected onto an Agilent G6410B QQQ instrument. LC separation was achieved with a Gemini reverse-phase C18 column (5 μm, 4.6 mm x 50 mm, Phenomonex) together with a pre-column (C18, 3.5 μm, 2 mm x 20 mm). Mobile phase A was composed of a 95:5 v/v H2O:MeOH, and mobile phase B was composed of a 65:35:5 v/v/v i-PrOH:MeOH:H2O. 0.1% ammonium hydroxide was included to assist in ion formation in negative ionization mode and 0.1 % formic acid was included for positive ionization mode.   The flow rate for each run started at 0.1 ml min–1 with 0% B. At 5 min, the solvent was immediately changed to 60% B with a flow rate of 0.4 ml min–1 and increased linearly to 100%B over 10 min. This was followed by an isocratic gradient of 100% B for 5 min at 0.5 ml min–1 before equilibrating for 3 min at 0% B at 0.5 ml min–1 (23 min total per sample). The following MS parameters were used to measure the indicated metabolites (precursor ion, product ion,collision energy in V): AEA (348, 62, 11), OEA (326, 62, 11), PEA (300, 62, 11), 2-AG (379, 287, 8), OG (357, 265, 8), PG (331, 239, 8), dodecylglycerol (261, 261, 0), pentadecanoic acid (241, 241, 0), arachidonic acid (303, 303, 0), PGE2 (351, 271, 10), PGD2 (351, 271, 10), TXB2 (369,195,5), 5,6-EET (319,191,3). MS analysis was performed with an electrospray ionization (ESI) source. The dwell time for each lipid was set to 60 ms. The capillary was set to 4 kV, the fragmentor was set to 100 V, and the delta EMV was set to +300. The drying gas temperature was 350°C, the drying gas flow rate was 11 l min–1, and the nebulizer pressure was 35 psi. Lipids were quantified by measuring the area under the peak in comparison to an external standard curve with MAGs, NAEs, or eicosanoids with the internal standards.

Statistical analysis

All data are reported as mean ± SEM. In the behavioral experiments, non-continuous behaviors, including jumps and paw tremors, are presented as counted observations. The occurrence of diarrhea was scored as a binary event for the entire 30 min period. Additionally, the mice were weighed before and after the testing period to determine total body weight loss (g). AEA, arachidonic acid, PGE2 and 2-AG levels are reported as pmol or nmol per gram of tissue, where applicable. Data were analyzed using two-way between measures analysis of variance (ANOVA), followed by Dunnett's or Scheffe's post hoc test. The percentage of mice between groups presenting with diarrhea or percentage of mice that jumped off platforms were compared by the z test of two proportions. EFS contraction height was compared by paired t-test. Differences of $p<0.05$ were considered significant.

Results

In order to establish the precipitated withdrawal model in our laboratory setting, mice were implanted with placebo or 75 mg morphine pellets and 72 h later, were challenged with an s.c. injection of naloxone. As shown in supplemental Fig. 1, naloxone precipitated somatic and autonomic withdrawal signs, including paw tremors ($p < 0.01$), jumping behavior ($p < 0.01$), diarrhea, and a significant loss in body weight ($p < 0.01$).

The MAGL inhibitor JZL184 and THC attenuate naloxone-precipitated withdrawal signs in morphine-dependent mice through the activation of CB_1 receptors

In the first set of experiments, we replicated previous work showing that THC attenuated naloxone-precipitated withdrawal in morphine-dependent mice (Bhargava, 1976). Seventy-two hours after morphine pellet implantation, mice were treated with vehicle or THC (1, 3, and 10 mg/kg s.c.) and then administered naloxone 30 min later. THC dose-dependently reduced naloxone-precipitated jumping [$F(4,30) = 4.7$; $p < 0.01$; Fig. 1A] and paw fluttering [$F(4,30) = 11.3$; $p < 0.001$; Fig 1B], weight loss [$F(4,30) = 17.9$; $p < 0.001$; Fig. 1C], and diarrhea (Fig 1D), with the highest dose (10 mg/kg) completely abolishing diarrhea in all mice.

In order to examine the role of MAGL inhibition in opioid withdrawal, we treated morphine-pelleted mice with either vehicle or JZL184 (4, 16, and 40 mg/kg, i.p.). JZL184 dose-dependently reduced naloxone-precipitated jumping [$F(3,26) = 18.8$; $p < 0.001$; Fig. 1A], paw fluttering [$F(3,26) = 4.9$; $p < 0.01$; Fig. 1B], diarrhea (Fig. 1D), and weight loss [$F(3, 26) = 6.37$; $p < 0.01$; Fig. 1C]. The highest dose of JZL184 (40 mg/kg) completely prevented the occurrence of diarrhea.

We next examined whether CB_1 or CB_2 receptors contribute to the protective effects of THC and JZL184 in the naloxone-precipitated withdrawal paradigm. The CB_1 receptor

antagonist rimonabant (3 mg/kg, i.p.) blocked the protective effects of THC on naloxone-precipitated jumps ($p < 0.05$), paw flutters ($p < 0.05$), weight loss ($p < 0.01$), and diarrhea (Fig. 1). Rimonabant pretreatment also reversed JZL184-induced attenuation of withdrawal intensity, as indicated by significant interactions between JZL184 and rimonabant on jumps [$F(1, 23) = 8.6$; $p < 0.01$; Fig. 2A], paw flutters [$F(1, 23) = 8.2$; $p < 0.01$; Fig. 2B] and weight loss [$F(1, 23) = 10.5$; $p < 0.01$; Fig. 2C]. Similarly, the anti-diarrheal effects of JZL184 were completely reversed by rimonabant treatment (Fig. 2D). In contrast, treatment with the CB_2 receptor antagonist SR144528 (3 mg/kg, i.p.) did not affect either the intensity of naloxone-precipitated withdrawal signs or JZL184-induced blockade of these withdrawal signs (Fig. 3). The interaction between JZL184 pretreatment and SR144528 failed to achieve statistical significance for jumps [$p = 0.24$; Fig. 3A], paw flutters [$p = 0.76$; Fig. 3B] and weight loss [$p = 0.55$; Fig. 3C]. In addition, SR144528 did not block the anti-diarrheal effects of JZL184 [Fig. 3D]. Taken together, these data indicate that JZL184 attenuates naloxone-precipitated withdrawal symptoms through a mechanism that requires the activation of CB_1 receptor.

JZL184 reduces spontaneous withdrawal signs in morphine-dependent mice

As shown in Fig. 4, morphine pellet removal increased the percentage of mice jumping from the platform (4-8 h), increased the incidences of paw flutters (2-24 h) and head shakes (2-24 h), and resulted in greater weight loss (4-24 h) to mice that had placebo pellets removed. JZL184 (40 mg/kg, i.p.) significantly reduced jumping at 4, 6, and 8 h after morphine pellet removal (Fig. 4A). In addition, JZL184 significantly reduced paw fluttering [$F(10, 100) = 4.3$; $p < 0.001$; Fig. 4B], head shakes [$F(10,100) = 5.2$; $p < 0.001$; Fig 4C], and the amount of weight loss [$F(8,80) = 3.1$; $p < 0.01$; Fig. 4D], as indicated by significant interactions between group and time.

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FAAH blockade attenuates somatic signs of naloxone-precipitated morphine withdrawal through a mechanism that requires the activation of CB$_1$ receptors

To elucidate the role of FAAH in morphine withdrawal, we first examined the impact of genetic deletion of FAAH on naloxone-precipitated morphine withdrawal. FAAH (-/-) mice vs. FAAH (+/+) mice on a C57BL/6 background were implanted with 75 g morphine pellets and challenged with naloxone 72 h later. FAAH (-/-) mice showed significant decreases in jumps (p<0.01; Fig. 5A), paw flutter incidents (p<0.05; Fig. 5B), and weight loss (p<0.05; Fig. 5C) compared with FAAH (+/+) mice. Genetic deletion of FAAH did not affect the occurrence of naloxone-precipitated diarrhea [Fig. 5D]. These data support the idea that FAAH deletion attenuates the expression of some of the primary signs of naloxone-precipitated opioid withdrawal.

We next evaluated the irreversible FAAH inhibitor, PF-3845 (10 mg/kg, i.p.) vs. vehicle on naloxone-precipitated morphine withdrawal. PF-3845 significantly attenuated the frequency of jumps and paw flutters (Fig. 6A and B). These effects were reversed by rimonabant as indicated by significant interactions between PF-3845 vs. vehicle and rimonabant vs. vehicle for jumps [F(1, 41) = 5.9; p <0.05; Fig. 6A] and paw flutters [F(1, 41) = 3.2; p < 0.01; Fig. 6B]. However, PF-3845 did not affect the weight loss during withdrawal (Fig. 6C), or the occurrence of diarrhea (Fig. 6D) following naloxone challenge. This experiment shows that acute FAAH inhibition attenuates a subset of withdrawal signs (i.e., jumps and paw flutters) in a CB$_1$ dependent manner, but does not reduce weight loss or diarrhea.

JZL184 and PF-3845 inhibit naloxone-precipitated isometric muscle contractions in morphine-dependent mouse ilea

The effects of MAGL and FAAH inhibition were evaluated in an *in vitro* model of precipitated withdrawal in which mouse ileal longitudinal muscle is exposed to morphine (10 μM) for 60 min and then naloxone (30 μM) is used to precipitate contractions. The height of naloxone-precipitated contractions was normalized to contractions elicited by 80 mM KCl. JZL184 (1 μM) attenuated naloxone-precipitated contractions in morphine-treated tissue [$F_{(2,15)}$ = 6.6; $p < 0.01$; Fig. 7A]. However, rimonabant (100nM) did not reverse the attenuation of naloxone-induced contractions by JZL184 ($p = 0.73$). Representative traces of contraction before and after application of naloxone in the three conditions are shown in Fig. 7B. In contrast, PF-3845 (1 μM) attenuated the intensity of naloxone-precipitated contractions in a CB_1 dependent manner, as indicated by a significant treatment effect [$F_{(2,16)} = 12.2$; $p < 0.001$; Fig. 7C; see Fig. 7D for representative traces of contractions in the three conditions].

JZL184 but not PF-3845 attenuated neurogenic field-stimulated contractions in naïve ileal tissue

To investigate further, the impact of MAGL and FAAH inhibition on ileum function, we evaluated electric field-stimulated (EFS) cholinergic contractions in the longitudinal preparations from mouse ileum. These experiments were carried out in the absence of morphine and naloxone treatment to study whether the endocannabinoid catabolic enzyme inhibitors directly affected ileal contractility. The height of contractions was normalized to tissue weight and the height of contractions after drug treatment was normalized to percentage of pre-drug contraction for each tissue. JZL184 inhibited EFS contractions in the ileum ($p<0.05$), which was reversed by rimonabant [$F_{(1,8)} = 10.2$; $p < 0.05$, Fig. 8C; Fig 8A for a representative trace]. In contrast, PF-3845 did not inhibit FS contractions in the ileum ($p = 0.36$, Fig. 8C; Fig. 8B for a representative trace).

Measurement of endocannabinoid levels in brain and ileum

To determine if naloxone-precipitated withdrawal in morphine-dependent mice altered levels of endocannabinoids in brain regions associated with opioid dependence, we measured the levels of AEA and 2- AG in the LC, PAG, and amygdala. Endocannabinoid levels were also measured in ileum. None of the treatments altered the levels of either endocannabinoid in any of the brain regions of interest or in the ileum (Table 1).

Although it is well established that acute administration of JZL184 and PF-3845 respectively increases 2-AG and AEA brain levels (Schlosburg et al., 2010), the effects of these inhibitors on endocannabinoid levels have not been measured in gastrointestinal tissue. Thus, in the next experiment, we treated naïve mice with the enzyme inhibitors and quantified endocannabinoids in ileum. As shown in Table 2, PF-3845 (10 mg/kg) significantly increased AEA, but not 2-AG, in the ileum. In contrast, JZL184 did not significantly alter either endocannabinoid in ileum.

Evaluation of effects of JZL184 on arachidonic acid and prostaglandin levels in ileum

The data depicted in Fig. 7C and Fig. 8 suggest that FAAH inhibition attenuates ileal contractions during naloxone-precipitated withdrawal in a CB_1 dependent manner, but does not directly affect ileal function in the absence of morphine. However, the finding that rimonabant did not block the reduction by JZL184 on naloxone precipitated contractions in morphine-dependent ilea (Fig. 7A), suggests a non-CB_1 receptor mechanism. One mechanism by which JZL184 may inhibit naloxone-precipitated contractions is by altering arachidonic acid levels and prostaglandins. It has been previously shown that genetic deletion or pharmacological inhibition of MAGL leads to a decrease in arachidonic acid levels in brain (Long et al., 2009a; Schlosburg

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et al., 2010). Because prostaglandins are a major product of arachidonic acid and also play a role in modulating neurogenic ileal contractions, we examined whether JZL184 would alter levels of prostaglandin E2 and arachidonic acid as well as levels of AEA and 2-AG from ileal tissue. Application of JZL184 (1 µM) to ileum *in vitro* did not significantly alter 2-AG (vehicle: 1.4 ± 0.2 nmol/g; JZL184: 1.3 ± 0.2 nmol/g), AEA (vehicle: 0.9 ± 0.2 pmol/g; JZL184: 2.0 ± 0.5 pmol/g), or PGE2 (vehicle: 97.1 ± 12.1 pmol/g; JZL184: 99.8 ± 15.1 pmol/g), but inexplicably increased levels of arachidonic acid ($p < 0.05$; vehicle: 32.3 ± 3.0 nmol/g; JZL184 66.3 ± 5.3 nmol/g).

Discussion

In the present study, we report that inhibition of the endocannabinoid catabolic enzymes, MAGL or FAAH, reduces naloxone-precipitated withdrawal in both *in vivo* and *in vitro* models of morphine dependence. While previous studies have demonstrated that THC and other cannabinoid agonists reduce precipitated withdrawal in opiate-dependent rodents (Bhargava, 1976; Vela et al., 1995; Yamaguchi et al., 2001), this is the first report demonstrating that elevating endocannabinoids by blocking their hydrolysis represents a viable approach to reduce opioid withdrawal. Acutely administered JZL184, which preferentially inhibits MAGL over FAAH and selectively raises brain 2-AG, but not anandamide in vivo (Long et al., 2009a, Long et al., 2009b), completely blocked all measured behavioral effects of precipitated withdrawal, including paw flutters, jumps, diarrhea, and weight loss. Rimonabant blocked these effects, indicating a necessary role of CB_1 receptors. Additionally, JZL184 reduced spontaneous opioid withdrawal signs. This model possesses particularly good face validity for withdrawal experienced by opioid-dependent individuals. *In vitro*, JZL184 attenuated the intensity of naloxone-precipitated ilea contractions, but rimonabant failed to reverse these effects, suggesting that CB_1 receptors are expendable. While FAAH (-/-) mice showed a reduction in all four measures of naloxone-precipitated withdrawal, PF-3834 reduced only naloxone-precipitated jumping and paw flutters, but did not attenuate diarrhea or body weight loss. Rimonabant blocked the effects of PF-3845 on somatic withdrawal signs naloxone-precipitated contractions in morphine-dependent ilea.

Notably, JZL184 significantly blocked all opioid withdrawal symptoms, while PF-3845 only reduced a subset of these effects. Several explanations may account for the differential effects of these enzyme inhibitors. First, bulk brain levels of 2-AG are at least two orders of magnitude higher than AEA (Ahn et al., 2009; Long et al., 2009a), which was also the case in

LC, PAG, and amygdala. Thus, elevated 2-AG may achieve greater occupancy of brain CB_1 receptors than AEA. Second, 2-AG acts as a full CB_1 receptor agonist, while AEA acts as a partial CB_1 receptor agonist (Sugiura et al., 2002), suggesting that 2-AG produces greater intrinsic effects at the receptor than AEA. Third, MAGL and FAAH are found in different neuronal populations and subcellular compartments, which could influence CB_1 receptor signaling in the brain and result in distinct physiological functions for these endocannabinoids. For example, 2-AG drives short-term synaptic plasticity, while AEA is quiescent (Pan et al., 2009). Of course, it is possible that the efficacy of PF-3845 could be enhanced by increasing the drug pretreatment time or administering multiple injections of this FAAH inhibitor. Thus, comparison of results with JZL184 and PF-3845 suggests that AEA and 2-AG play different roles in modulating opiate withdrawal.

In our studies, induction of withdrawal in morphine-dependent mice did not alter AEA or 2-AG levels in brain areas associated with opiate withdrawal, including LC, PAG, and amygdala, though 2-AG levels have been reported to be increased in other brain regions of rats treated repeatedly with morphine (Vigano et al., 2003). Likewise, chronic heroin self-administration in rats does not produce overt CB_1 receptor functional changes in LC, PAG, amygdala, and other brain structures (Sim-Selley et al., 2000). This pattern of results argues against direct activation of the endocannabinoid system during opioid withdrawal. On the other hand, inhibitors of endocannabinoid hydrolysis elevate AEA and 2-AG levels in whole brain (Ahn et al., 2009; Long et al., 2009a; Long et al., 2009b) as well as in brain regions associated with opiate withdrawal. Here, we report that MAGL and FAAH inhibitors attenuate the expression of withdrawal signs by extending the activity of endocannabinoids at the CB_1 receptor. Although determining the molecular mechanisms for endocannabinoid dampening of opioid withdrawal is beyond the scope of the present study, findings from previously published reports provide

insight. It is well established that acute morphine inhibits adenylyl cyclase at the cellular level, and opiate withdrawal leads to increased production of adenylyl cyclase and downstream signaling molecules in the LC and PAG (Nestler and Tallman, 1988; Punch et al., 1997). These signaling events are associated with the expression of behavioral withdrawal signs (Shaw-Lutchman et al., 2002). Thus, increasing endocannabinoid activity may counteract the consequences of cAMP overshoot during opioid withdrawal. This notion is consistent with the colocalization of CB_1 and MOR in the LC and other brain regions as well as in the ileum (Maguma et al., 2010; Scavone et al., 2010). The observations that acute CB_1 receptor stimulation inhibits adenylyl cyclase and decreases cAMP production and that MOR and CB_1 receptors have been demonstrated in cell lines to share a common pool of adenylyl cyclase further supports this hypothesis (Howlett and Mukhopadhyay, 2000; Levitt et al., 2010).

Two observations from the present study indicate that the *in vitro* ileum model does not account for naloxone-precipitated diarrhea in morphine-dependent mice. First, both JZL184 and PF-3845 attenuated naloxone-precipitated contractions in morphine-treated ilea, but only JZL184 blocked the occurrence of diarrhea. Second, rimonabant blocked the anti-diarrheal effects of JZL184, but did not reduce naloxone-precipitated contractions in ileum. The antecedents for diarrhea include enhanced motility and increased secretion of fluids and electrolytes. Based on the differential anti-diarrheal effects between JZL184 and PF-3845, one would predict a similar association on secretion in an *in vitro* withdrawal model, but the influence of endocannabinoid catabolic enzyme inhibitors on secretion remains an area for future research. Thus, while naloxone-precipitated contractions in morphine-treated ileum reflect a useful *in vitro* model of opioid withdrawal, this model does not account mechanistically for the autonomic withdrawal responses (i.e., diarrhea) observed *in vivo*.

MAGL blockade also reduces free arachidonic acid levels in brain (Long et al., 2009a), suggesting that the actions of JZL184 could be mediated in part by a blunting in the production of prostaglandins and other eicosanoids. However, rimonabant reversed all of JZL184's anti-withdrawal effects *in vivo*, supporting the hypothesis that these actions were mediated by increased CB_1 receptor activity via elevated levels of 2-AG. Moreover, JZL184 did not alter prostaglandins and actually increased arachidonic acid in ileum, which further argues against this alternative mechanism of action. Curiously, JZL184 did not increase 2-AG levels in ileum. Similarly, JZL184 does not elevate 2-AG in other peripheral tissues (e.g., testes, white adipose tissue, and lung), though it increased this endocannabinoid in other tissues (e.g., liver, kidney, brown adipose tissue, spleen) (Long et al., 2009b). Nonetheless, it is possible in the present study that increases in 2-AG occurred in key synapses of enteric neurons obscured by bulk 2-AG in the whole tissue or that other enzymes regulate 2-AG in ileum, as has been observed for other non-neuronal cells/tissues (Marrs et al., 2010).

It should be noted that JZL184 can also inhibit FAAH, though *in vitro* the compound is approximately 500-fold more selective as a MAGL inhibitor (IC_{50} = 8 nM) than as a FAAH inhibitor (IC_{50} = 4,000 nM) (Long et al., 2009a). Acute administration of 40 mg/kg JZL184 in the ethanol:emulphor:saline (1:1:18) vehicle, used in the present study, inhibits FAAH by approximately 50%, which is insufficient to increase brain AEA levels (Long et al., 2009b).

While THC and other direct-acting CB_1 receptor agonists elicit marijuana-like psychoactive effects, endocannabinoid catabolic inhibitors appear to possess fewer cannabimimetic effects than CB_1 receptor agonists. In particular, FAAH inhibitors do not substitute for THC in the drug discrimination paradigm, do not produce hypothermia, hypomotility, catalepsy or memory impairment, and lack reinforcing properties (Ahn et al., 2008). While the MAGL inhibitor JZL184 produces a broader set of cannabinoid behavioral effects, including hypomotility, it does

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not cause the full spectrum of THC-like effects. In particular, JZL184 does not substitute for THC in the drug discrimination paradigm and does not produce catalepsy (Long et al., 2009b; Long et al., 2009c). In contrast, dual inhibitors of both FAAH and MAGL, such as JZL195, produce much greater THC-like effects, including catalepsy, increased antinociceptive efficacy, and substitution in the THC discrimination paradigm (Long et al., 2009c) than single enzyme inhibitors, underscoring the importance of maintaining selectivity for individual endocannabinoid pathways in the development of FAAH and MAGL inhibitors. Moreover, sustained FAAH blockade does not alter CB_1 receptor function and lacks dependence liability (Schlosburg et al., 2009; Schlosburg et al., 2010). Additionally, the observation that FAAH (-/-) mice show an attenuated opioid withdrawal syndrome suggests that the anti-opioid withdrawal effects of FAAH inhibition persist even upon genetic deletion of FAAH. Conversely, chronic treatment of high doses of JZL184 produces CB_1 receptor downregulation and desensitization accompanied with a mild dependence liability (Schlosburg et al., 2010), though it possible that prolonged partial MAGL blockade may not lead to functional changes in the CB_1 receptor.

Cannabinoids and morphine act at similar brain regions and affect similar signal transduction pathways. The *in vitro* and *in vivo* studies presented here establish that endocannabinoid catabolic enzymes may represent attractive targets to treat severe withdrawal signs associated with opioid dependence. The results from the present study are the first to show that elevation of endocannabinoid levels ameliorates the expression of opioid withdrawal. Thus, the enzymes responsible for endocannabinoid degradation offer promising targets for possible new treatments for opioid dependence.

JPET # 181370

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Footnotes

Legends for figures

Fig. 1. The primary psychoactive constituent of *Cannabis sativa* THC and the irreversible MAGL inhibitor JZL184 attenuate the intensity of naloxone-precipitated withdrawal signs in a dose-dependent manner. THC (30 min pretreatment) or JZL184 (2 h pretreatment) was given prior to naloxone (1 mg/kg, s.c.). Rimonabant (3 mg/kg, i.p.) given 30 min before naloxone challenge antagonized the anti-withdrawal effects of THC (10 mg/kg, i.p.). The withdrawal signs measured included: **A.** number of jumps, **B.** number of paw flutters, **C.** weight loss and **D.** occurrence of diarrhea. Data expressed as mean ± SEM for panels A-C. ***p<0.001, **p<0.01, *p<0.05 vs. vehicle; # p<0.05 vs. 10mg/kg THC; ## p<0.01 v/s 10mg/kg THC; † p<0.05 vs. vehicle; ‡ p < 0.05 vs. JZL184; n = 6-8 mice/group.

Fig. 2. The MAGL inhibitor JZL184 attenuated the intensity of naloxone-precipitated morphine withdrawal signs in a CB_1 receptor dependent manner. Rimonabant (3 mg/kg, i.p.) administered 90 min after JZL184 (40 mg/kg, i.p.) blocked the anti-withdrawal effects of JZL184. The withdrawal signs measured were: **A.** number of jumps, **B.** number of paw flutters, **C.** weight loss and **D.** occurrence of diarrhea. Data expressed as mean ± SEM for panels A-C. **p<0.01 vs. vehicle; ## p<0.01 vs. JZL184-vehicle; † p<0.05 vs. vehicle; ‡ p<0.05 vs. JZL184-vehicle; n = 6-8 mice/group.

Fig. 3. The reduction of intensity of naloxone-precipitated morphine withdrawal signs is not CB_2 receptor mediated. The CB_2 antagonist SR144528 (3 mg/kg, i.p.) did not reverse the anti-withdrawal effects of JZL184 (40 mg/kg, i.p.). The withdrawal signs measured were: **A.** number of jumps, **B.** number of paw flutters, **C.** weight loss and **D.** occurrence of diarrhea. Data

expressed as mean ± SEM for panels A-C. ***p<0.001, **p<0.01 vs. vehicle; † p<0.05 vs. vehicle; n = 7-8 mice/group.

Fig. 4. JZL184 reduces spontaneous withdrawal signs in morphine-dependent mice. Removal of morphine pellets leads to increased spontaneous withdrawal signs compared to mice implanted with placebo pellets. The MAGL inhibitor JZL184 attenuated the intensity of the following spontaneous withdrawal signs in morphine-dependent mice: **A.** Percentage of mice that displayed platform jumping, **B.** number of paw flutters, **C.** number of head shakes, and **D.** weight loss. Data expressed as mean ± SEM for panels B-D. *p<0.05,**p<0.01,***p<0.001 vs. placebo; #p<0.05, ## p<0.01, ###p<0.001 vs. morphine control; † p<0.05 vs. placebo; ‡ p<0.05 vs. morphine control; n = 6-9 mice/group.

Fig. 5. FAAH (-/-) mice display attenuated naloxone-precipitated withdrawal signs. The withdrawal signs measured were **A.** number of jumps, **B.** number of paw flutters, **C.** weight loss and **D.** occurrence of diarrhea. Data expressed as mean ± SEM for panels A-C. **p<0.01, *p<0.05 vs. FAAH (+/+) group; n=6-7 mice/group.

Fig. 6. The FAAH inhibitor PF-3845 attenuated a subset of naloxone-precipitated morphine withdrawal signs in a CB_1 receptor dependent manner. PF-3845 (10 mg/kg, i.p.) reduced the intensity of jumps and paw flutters, which was reversed by rimonabant (3 mg/kg, i.p.). The withdrawal signs measured were **A.** number of jumps, **B.** number of paw flutters, **C.** weight loss and **D.** occurrence of diarrhea. Data expressed as mean ± SEM for panels A-C. **p<0.01, *p<0.05 vs. vehicle; ### p<0.001, # p < 0.05 vs. PF-3845; n=11-12 mice/group.

Fig. 7. Inhibitors of endocannabinoid catabolic enzymes reduced naloxone-precipitated contractions of morphine-treated ilea, as measured by isometric tension recording. **A.** The MAGL inhibitor JZL184 (1 µM) attenuated the amplitude of naloxone-precipitated contraction in ileum treated with morphine. Rimonabant (100nM) did not block the anti-withdrawal effects of JZL184. **B.** Representative traces of contractions in each of the conditions before and after application of naloxone hydrochloride. **C.** The FAAH inhibitor PF-3845 (1 µM) attenuated the amplitude of naloxone-precipitated contraction. Rimonabant (100nM) reversed the actions of PF-3845. **D.** Representative traces of contractions in each of the conditions before and after application of naloxone hydrochloride. . Data are expressed as mean ± SEM. ***$p<0.001$, * $p<0.05$ vs. morphine; ###$p<0.001$ vs. PF-3845; n= 4-8 ilea/condition.

Fig. 8. Evaluation of JZL184 and PF-3845 on field stimulated (EFS) contractions on longitudinal smooth muscle preparations from naïve ileum. **A.** Representative trace of EFS contractions before and after addition of JZL184 (1µM). **B.** Representative trace of EFS contractions before and after addition of PF-3845 (1µM). **C.** Graphical representation of the amplitude of contractions represented as % of baseline values. JZL184 reduced the amplitude of EFS-stimulated contractions though the activation of CB_1 receptors, but PF-3845 had no effect. Data expressed as mean ± SEM. *$p<0.05$, # $p<0.05$ vs. JZL184; n = 4-5 ilea/group.

TABLES

Table 1. AEA (top) and 2-AG (bottom) levels in brain regions and ileum are not altered following treatment with either morphine or placebo and acute challenge with either saline or naloxone (1 mg/kg, s.c.). Data expressed as mean ± SEM

Treatment	AEA levels (pmol/g)			
	LC	PAG	Amygdala	Ileum
Placebo-saline	3.7 ± 0.3	4.2 ± 0.3	9.5 ± 0.8	1.2 ± 0.3
Morphine- saline	5.1 ± 1.0	4.5 ± 0.6	8.2 ± 0.6	1.3 ± 0.2
Placebo- naloxone	4.7 ± 0.4	4.8 ± 0.5	9.1 ± 1.1	1.5 ± 0.1
Morphine- naloxone	4.3 ± 0.4	4.6 ± 0.5	8.2 ± 0.6	1.7 ± 0.2
	2-AG levels (nmol/g)			
	LC	PAG	Amygdala	Ileum
Placebo-saline	6.4 ± 0.4	11.1 ± 0.9	11.6 ± 1.3	5.6 ± 0.7
Morphine- saline	7.0 ± 0.5	11.8 ± 1.5	10.8 ± 0.9	5.6 ± 1.1
Placebo- naloxone	6.9 ± 0.7	11.7 ± 2.4	17.7 ± 4.2	5.8 ± 1.0
Morphine- naloxone	6.3 ± 0.6	10.2 ± 0.8	17.0 ± 3.6	6.6 ± 1.9

Table 2. Effects of FAAH and MAGL inhibitors on AEA and 2-AG in the ileum. Data expressed as

mean ± SEM. n = 6 mice/group; *p < 0.05 vs. vehicle control.

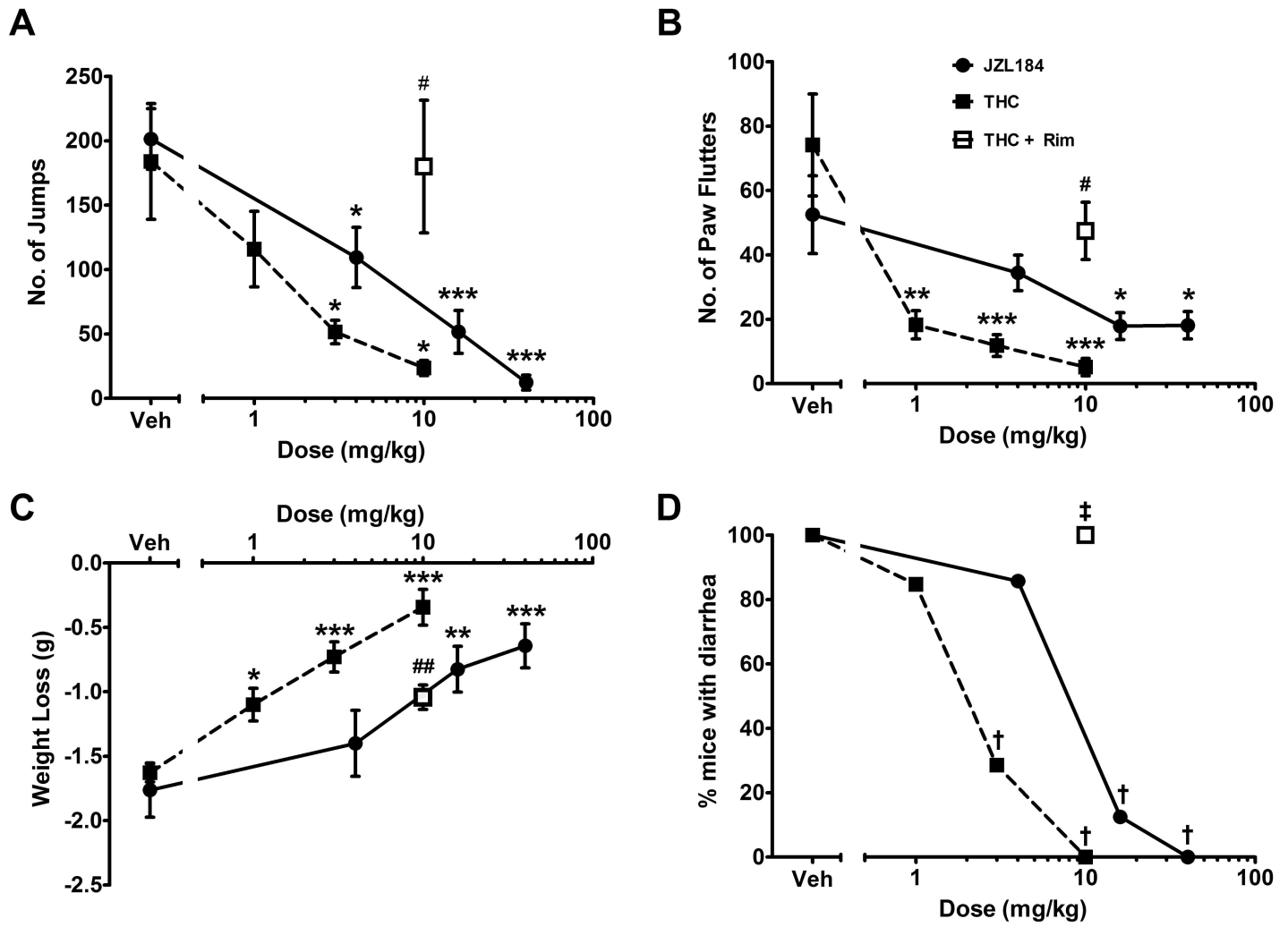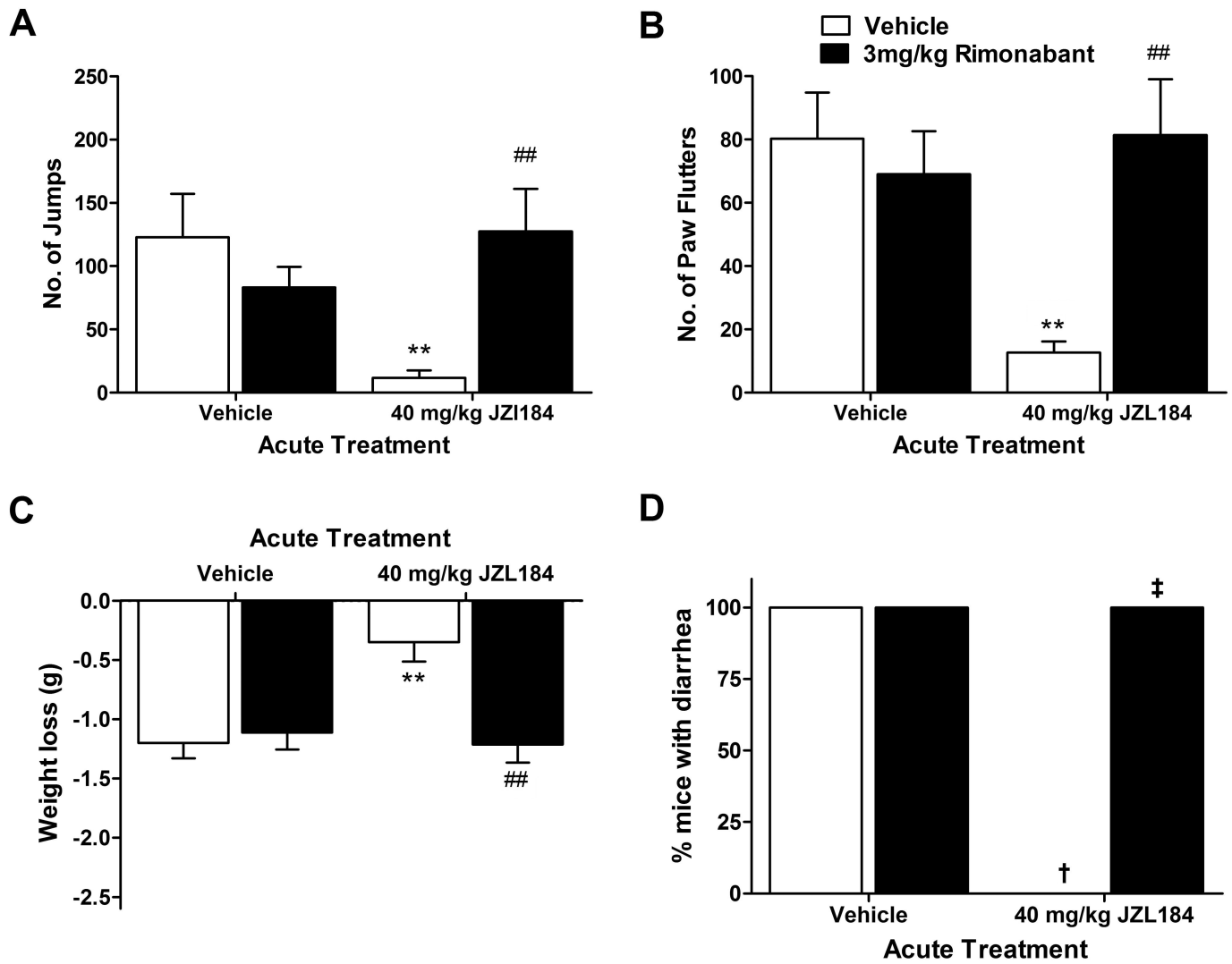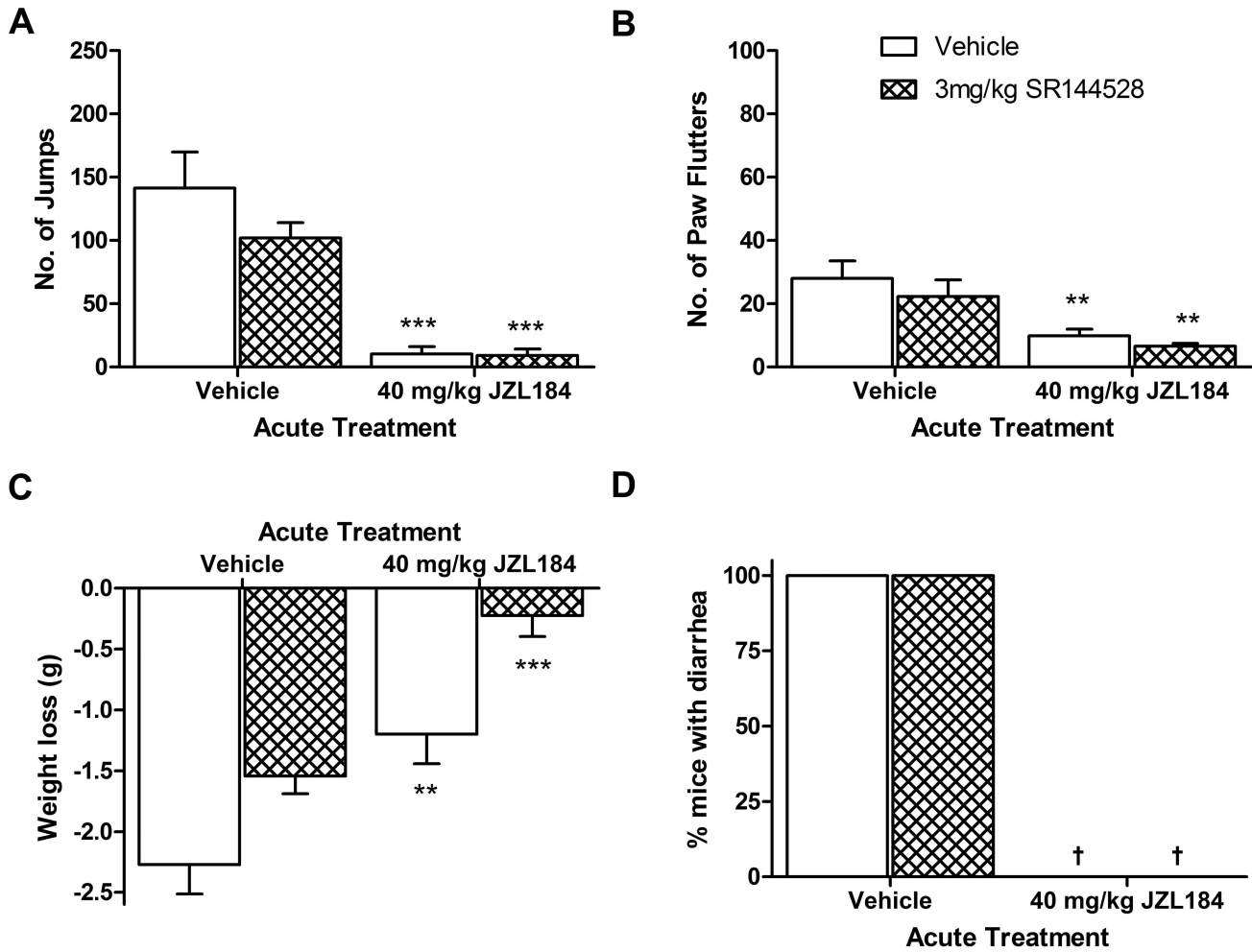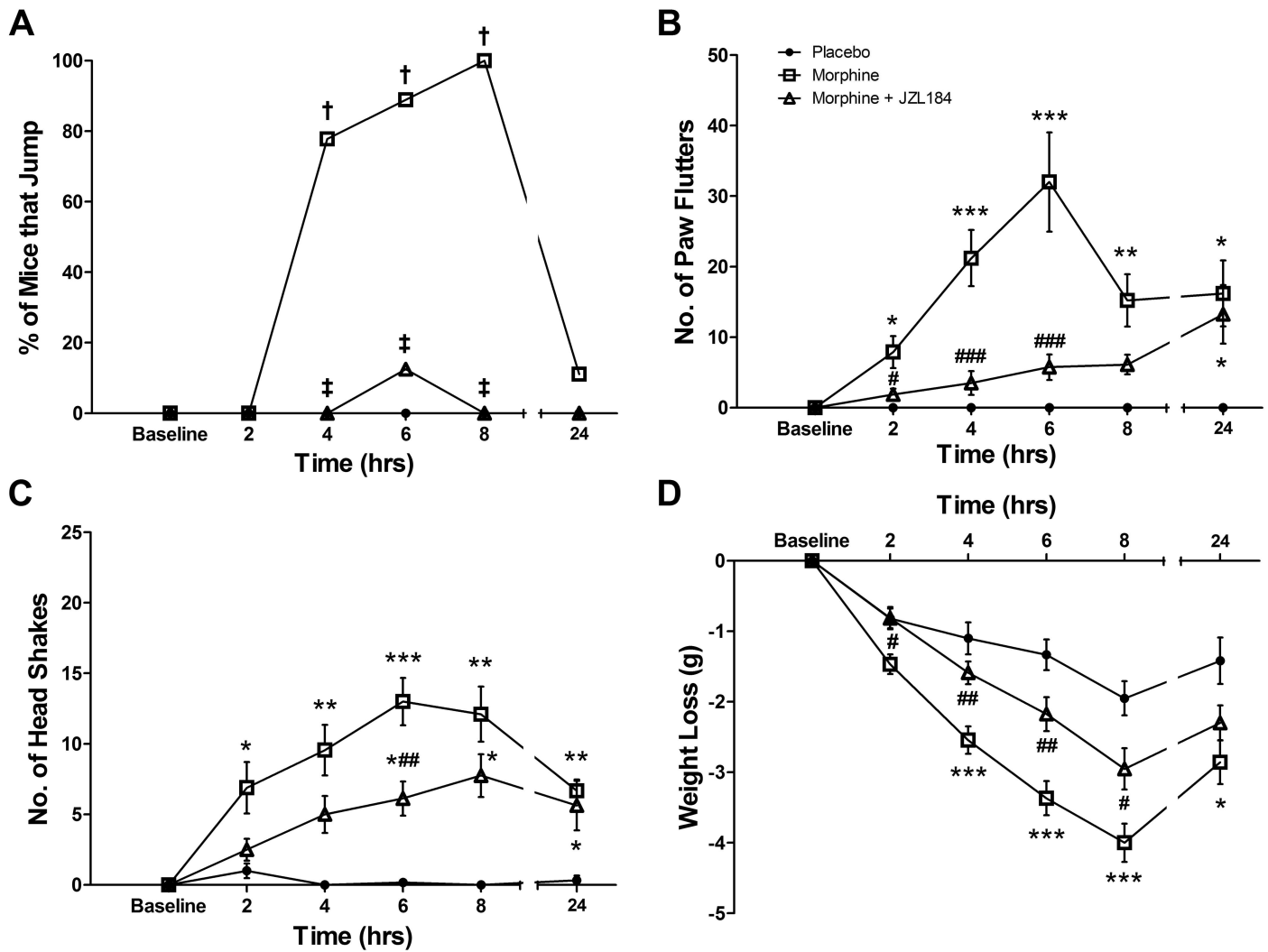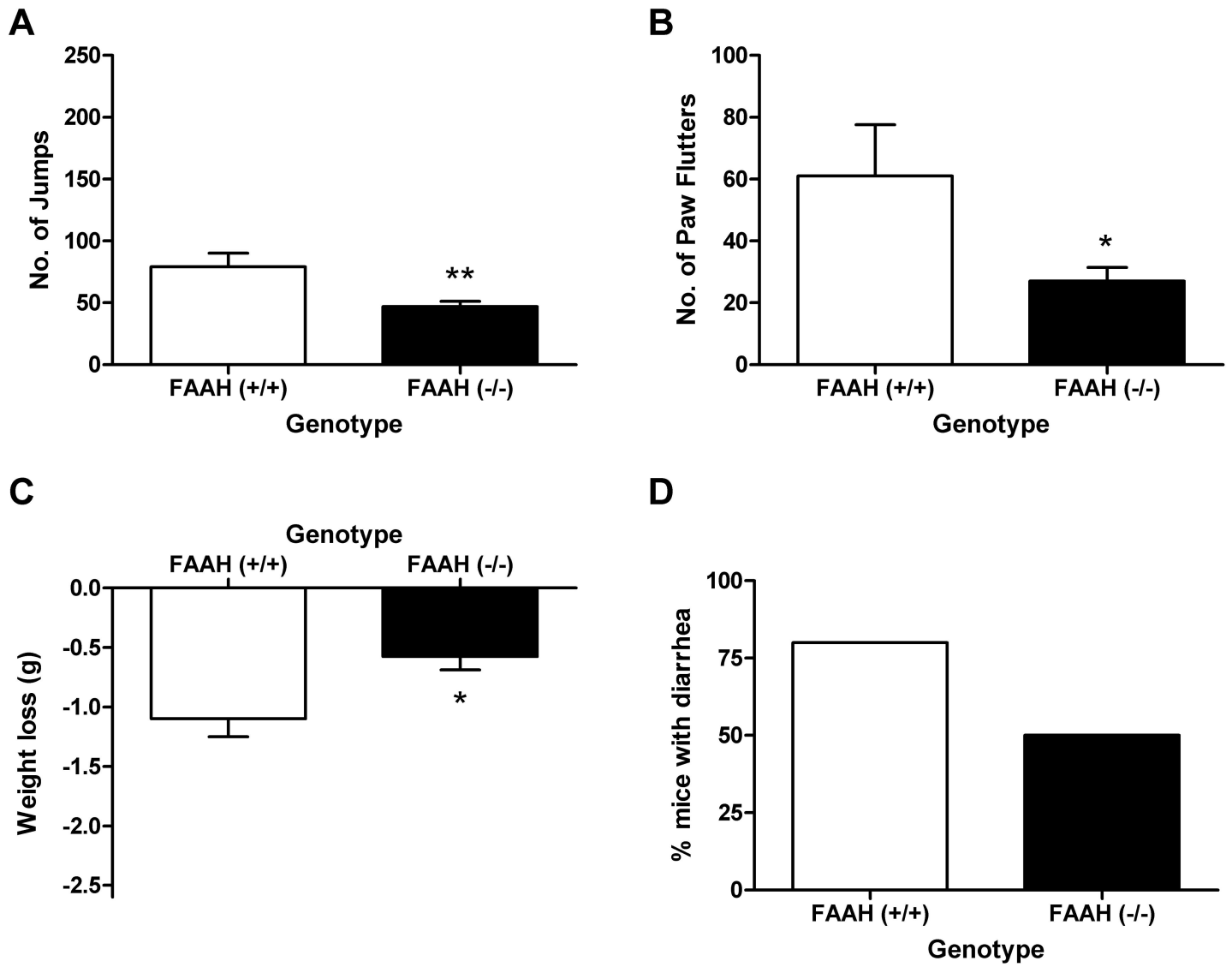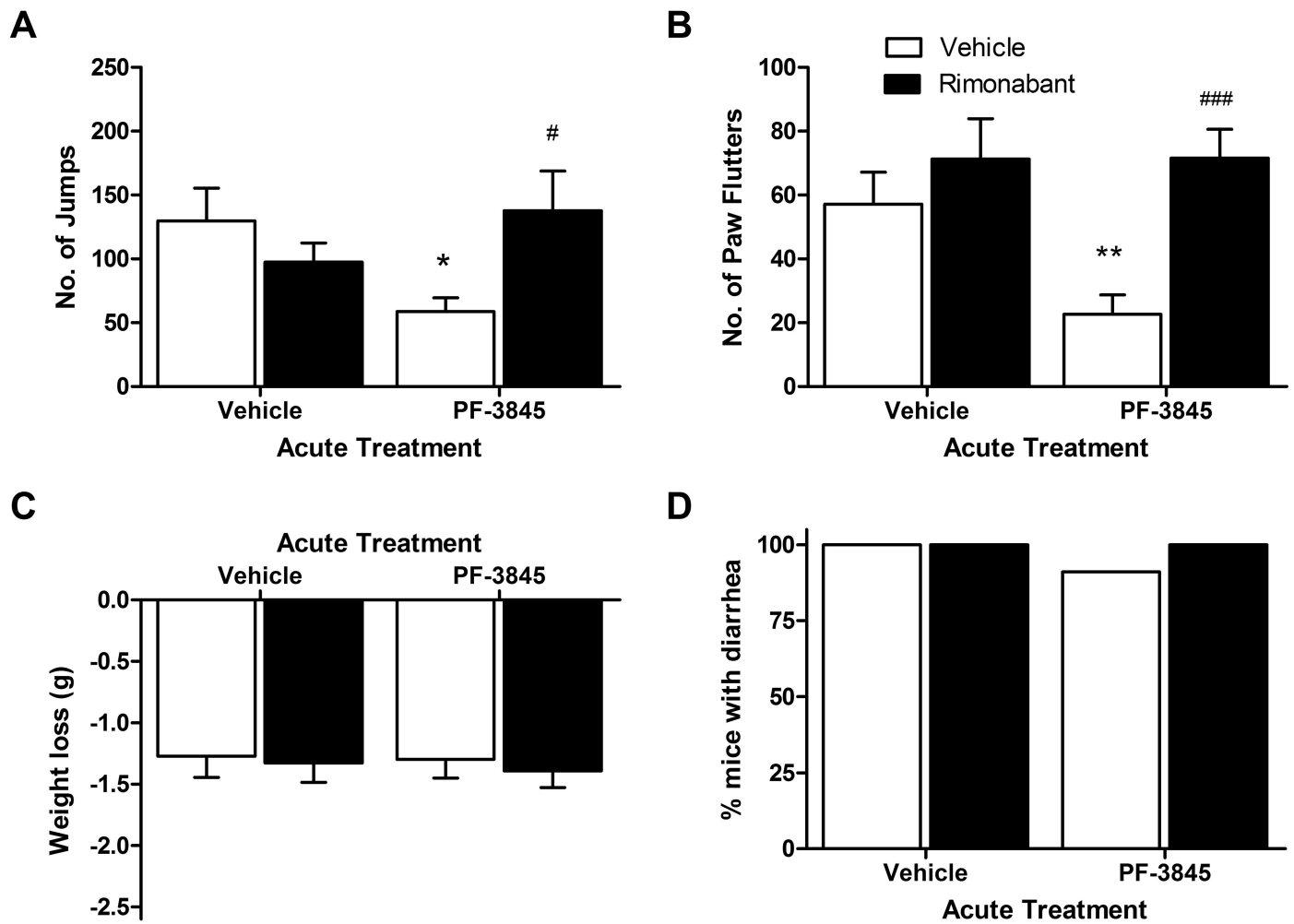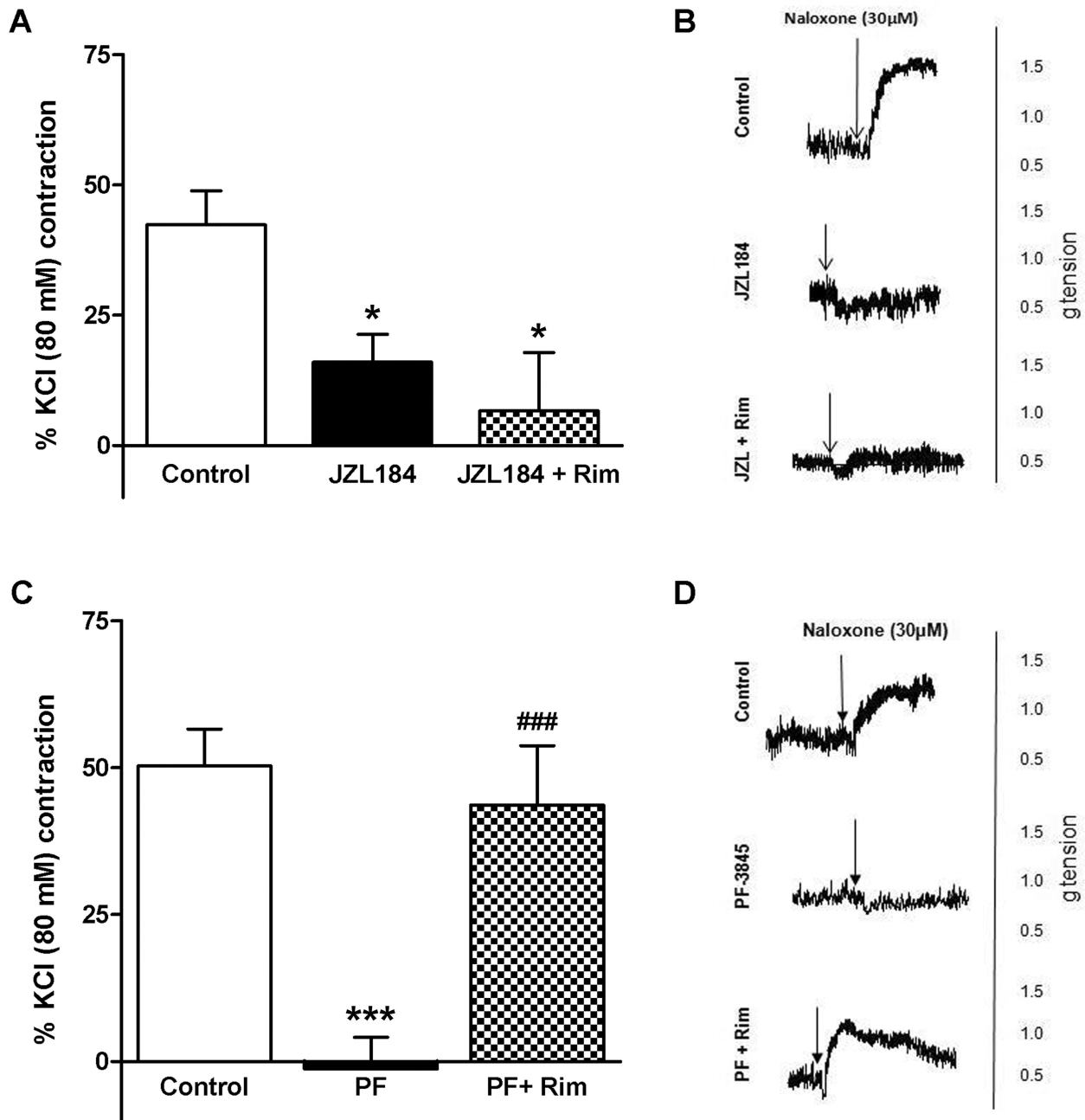| Treatment	AEA pmol/g	2-AG nmol/g
Vehicle	3.2 ± 0.5	13.2 ± 3.2
PF-3845 (10 mg/kg)	7.9 ± 1.1*	8.8 ± 2.3
JZL184 (40 mg/kg)	6.6 ± 1.6	19.5 ± 4.8

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8