Blockade of Endocannabinoid Hydrolytic Enzymes Attenuates Precipitated Opioid Withdrawal Symptoms in Mice^[S]

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ABSTRACT

Δ^9 -Tetrahydrocannabinol (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 CB₁ and CB₂ cannabinoid receptors, the role these receptors play in reducing the variety of opioid withdrawal symptoms remains unknown. The endogenous cannabinoids, *N*-arachidonylethanolamine (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 4-nitrophe-

nyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate (JZL184) dose dependently reduced the intensity of most measures through the activation of CB₁ receptors. JZL184 also attenuated spontaneous withdrawal signs in morphine-dependent mice. The FAAH inhibitor *N*-(pyridin-3-yl)-4-(3-(5-(trifluoromethyl)pyridin-2-yloxy)benzyl)-piperidine-1-carboxamide (PF-3845) reduced the intensity of naloxone-precipitated jumps and paw flutters through the activation of CB₁ 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, although 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.

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Introduction

The risk of opiate dependence from long-term 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 char-

ABBREVIATIONS: THC, [Δ^9]-tetrahydrocannabinol; AEA, *N*-arachidonylethanolamine (anandamide); 2-AG, 2-arachidonylglycerol; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; JZL184, 4-nitrophenyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate; PF-3845, *N*-(pyridin-3-yl)-4-(3-(5-(trifluoromethyl)pyridin-2-yloxy)benzyl)-piperidine-1-carboxamide; LC, locus coeruleus; PAF, periaqueductal gray; EFS, electric field-stimulated; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; MS, mass spectrometry; PG, prostaglandin; JZL195, 4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate.

acterized by a range of aversive effects, including behavioral (e.g., anxiety and shakes), gastrointestinal (e.g., diarrhea, emesis, and dehydration), and other physiological effects (e.g., hypertension, tachycardia, and body aches) (American Psychiatric Association, 1994). 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 than that resulting from heroin but longer lasting (Dyer et al., 1999). Likewise, physical dependence has been reported after long-term buprenorphine treatment (Kuhlman et al., 1998). Given the limitations of these extant replacement therapies, there is a need for nonopioid 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 CB₁ receptor agonists have dampened enthusiasm for their clinical development. On the other hand, 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, 2009; Justinova et al., 2008).

The endocannabinoid system consists of two receptors (CB₁ and CB₂) (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 injection of 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, 2001) and monoacylglycerol lipase (MAGL) (Dinh et al., 2002) limits their therapeutic utility. As an alternative, 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 the central nervous system (Cravatt et al., 2001; Ahn et al., 2009). Likewise, genetic deletion or pharmacological inhibition of MAGL increases brain 2-AG levels by approximately 10-fold (Long et al., 2009a,b; 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 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 with those of THC. Selective CB₁ and CB₂ receptor antagonists were used to assess cannabinoid receptor involvement of the antiwithdrawal effects of JZL184 and PF-3845. In addition, we evaluated whether JZL184 would reduce spontaneous withdrawal in morphine-dependent mice. To evaluate whether compensatory changes in 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 gray (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 naive untreated ilea. Given that genetic deletion or pharmacological inhibition of MAGL leads to increases in 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.

Materials and Methods

Subjects. Male ICR mice (Harlan, Indianapolis, IN) as well as male FAAH(–/–) and FAAH(+ / +) 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 six to eight 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* (Institute of Laboratory Animal Resources, 1996).

Drugs. Morphine pellets (75 mg), placebo pellets, morphine sulfate [(5 α ,6 α)-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol], THC, the CB₂ receptor antagonist *N*-[1*S*-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528), and the CB₁ receptor antagonist rimonabant [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl] were obtained from the National Institute on Drug Abuse (Bethesda, MD). Naloxone hydrochloride [(1*S*,5*R*,13*R*,17*S*)-10,17-dihydroxy-4-(prop-2-en-1-yl)-12-oxa-4-azapentacyclo[9.6.1.0^{1,10}.1,3.0^{5,6}.1,7.0^{8,9}]octadeca-7(18),8,10-trien-14-one] was purchased from Cayman Chemical (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 (sanofi-aventis, Bridgewater, 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/1 g b.wt. THC and naloxone were administered via subcutaneous injection, whereas PF-3845, JZL184, rimonabant, and SR144528 were given via intraperitoneal injection. PF-3845 and JZL184 were given 2 h before testing, to coincide with peak levels of AEA and 2-AG elevations, respectively, after systemic administration (Ahn et al., 2009; Long et al., 2009a). Rimonabant, SR144528, and THC were given 30 min before naloxone treatment. For in vitro isometric tension recording studies, naloxone hydrochloride and morphine sulfate were dissolved

in saline, whereas PF-3845 and rimonabant were dissolved in ethanol and JZL184 was dissolved in dimethyl sulfoxide.

Morphine Pellet Implantation Surgery. To induce opioid dependence, mice were implanted with morphine pellets as described previously (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 were returned to the vivarium until testing.

Naloxone-Precipitated Opioid Withdrawal. Somatic withdrawal signs were scored as described previously (Schlosburg et al., 2009). In brief, mice were placed in white acrylic chambers (20 × 20 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 digital cameras (Unibrain, San Ramon, CA), and the videos were saved using ANY-maze 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 2 days to allow for odors to dissipate. The 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 naive 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 intraperitoneal injection of the ethanol-Emulphor-saline vehicle, and group 3 was given an intraperitoneal 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 after 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 × 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 used 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 of passive tension in 15 ml of siliconized organ baths containing Krebs' solution (118 mM NaCl, 4.6 mM KCl, 1.3 mM NaH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 11 mM glucose, and 2.5 mM CaCl₂), maintained at 37°C and bubbled with 95% O₂ and 5% CO₂. Tissues were allowed to equilibrate for 60 min before the 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 10 μM morphine sulfate at 0 min and incubated with either JZL184 (1 μM) or PF-3845 (1 μM) at 30 min. At 60 min, 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 were used as the reference.

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

Extraction and Quantification of Brain and Ileum Endocannabinoid Levels with Liquid Chromatography-MS. Mice were implanted with placebo or morphine (75-mg) pellets, as described above. On day 3, subjects received a subcutaneous injection of naloxone hydrochloride (1 mg/kg) or saline. Ten minutes 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 LC, PAG, and 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 the adjacent reticulum. The next consecutive section was taken by making a posterior cut at the anterior one-fourth 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 and 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 of chloroform-methanol (2:1, v/v; containing 0.0348 mg of phenylmethylsulfonyl fluoride/ml) after the addition of internal standards to each sample (2 pmol of AEA-d₈ and 1 nmol of 2-AG-d₈). Homogenates were then mixed with 0.3 ml of 0.73% w/v NaCl, vortexed, and then centrifuged for 10 min at 4000 rpm (4°C). The aqueous phase plus debris was collected and extracted two more times with 0.8 ml of chloroform. The organic phases from the three extractions were pooled, and the organic solvents were evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml of chloroform and mixed with 1 ml of ice-cold acetone. The mixtures were then centrifuged for 5 min at 3000 rpm and 4°C to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml of methanol and placed in autosample vials for analysis.

Liquid chromatography-tandem mass spectrometry was used to quantify AEA and 2-AG. The mobile phase consisted of water-methanol (10:90) with 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery HS C18, 4.6 × 15 cm, 3-μm column (Supelco, Bellefonte, PA). The mass spectrometer was run in electrospray ionization 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 AEA-d₈; 379 > 287 and 279 > 269 for 2-AG; and 387 > 96 for 2-AG-d₈. A calibration curve was constructed for each assay on the basis of 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 Liquid Chromatography-MS. 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 100 nM JZL184 (in dimethyl sulfoxide) 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 hexane-ethyl acetate (1:1, v/v) 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 2000g for 5 min, and the organic layer was removed, dried under a stream of N_2 , and resolubilized in chloroform (120 μl), and 10 μl of this resolubilized lipid was injected onto an Agilent G6410B QQQ instrument. Liquid chromatography separation was achieved with a Gemini reverse-phase C18 column (5 μm , 4.6 mm $\times$ 50 mm; Phenomenex, Torrance, CA) together with a precolumn (C18, 3.5 μm , 2 mm $\times$ 20 mm). Mobile phase A was composed of H_2O -methanol (95:5, v/v), and mobile phase B was composed of a 1-propanol-methanol- H_2O (65:35:5, v/v/v). Ammonium hydroxide (0.1%) was included to assist in ion formation in the

negative ionization mode, and 0.1% formic acid was included for the positive ionization mode. The flow rate for each run started at 0.1 $\text{ml} \cdot \text{min}^{-1}$ with 0% B. At 5 min, the solvent was immediately changed to 60% B with a flow rate of 0.4 $\text{ml} \cdot \text{min}^{-1}$ 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 $\text{ml} \cdot \text{min}^{-1}$ before equilibration for 3 min at 0% B at 0.5 $\text{ml} \cdot \text{min}^{-1}$ (23 min total per sample). The following MS parameters were used to measure the indicated metabolites (precursor ion, product ion, collision energy): AEA (348, 62, 11 V), *N*-oleoylethanolamine (326, 62, 11 V), *N*-palmitoylethanolamine (300, 62, 11 V), 2-AG (379, 287, 8 V), C18:1 monoacylglycerol (357, 265, 8 V), phosphatidylglycerol (331, 239, 8 V), dodecylglycerol (261, 261, 0 V), pentadecanoic acid (241, 241, 0 V), arachidonic acid (303, 303, 0 V), PGE_2 (351, 271, 10 V), PGD_2 (351, 271, 10 V), thromboxane B_2 (369, 195, 5 V), and 5,6-epoxyeicosatrienoic acids (319, 191, 3 V). MS analysis was performed with an electrospray ionization 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 $\text{l} \cdot \text{min}^{-1}$, 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 monoacylglycerols, *N*-acylethanolamines, or eicosanoids with the internal standards.

Statistical Analysis. All data are reported as means $\pm$ S.E.M. In the behavioral experiments, noncontinuous behaviors, including jumps and paw tremors, are presented as counted observations. The occurrence of diarrhea was scored as a binary event for the entire

Fig. 1. The primary psychoactive constituent of *C. 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 before naloxone (1 mg/kg s.c.). Rimonabant (Rim; 3 mg/kg i.p.) given 30 min before naloxone antagonized the antiwithdrawal effects of THC (10 mg/kg i.p.). The withdrawal signs measured were number of jumps (A), number of paw flutters (B), weight loss (C), and occurrence of diarrhea (D). Data are expressed as means $\pm$ S.E.M. for A to C. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ versus vehicle; #, $p < 0.05$ versus 10 mg/kg THC; ##, $p < 0.01$ versus 10 mg/kg THC; †, $p < 0.05$ versus vehicle; ‡, $p < 0.05$ versus JZL184. $n = 6$ to 8 mice/group. Veh, vehicle.

30-min period. In addition, the mice were weighed before and after the testing period to determine total body weight loss (grams). AEA, arachidonic acid, PGE₂, and 2-AG levels are reported as picomoles or nanomoles per gram of tissue, where applicable. Data were analyzed using two-way between-measures analysis of variance, 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 was 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

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 a subcutaneous 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 CB₁ 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.

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 CB₁ or CB₂ receptors contribute to the protective effects of THC and JZL184 in the naloxone-precipitated withdrawal paradigm. The CB₁ 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). Rimnabant pretreatment also reversed JZL184-

Fig. 2. The MAGL inhibitor JZL184 attenuated the intensity of naloxone-precipitated morphine withdrawal signs in a CB₁ receptor-dependent manner. Rimnabant (3 mg/kg i.p.) administered 90 min after JZL184 (40 mg/kg i.p.) blocked the antiwithdrawal effects of JZL184. The withdrawal signs measured were number of jumps (A), number of paw flutters (B), weight loss (C), and occurrence of diarrhea (D). Data are expressed as means $\pm$ S.E.M. for A to C. **, *p* < 0.01 versus vehicle; ##, *p* < 0.01 versus JZL184-vehicle; †, *p* < 0.05 versus vehicle; ‡, *p* < 0.05 versus JZL184-vehicle. *n* = 6 to 8 mice/group.

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). Likewise, the antidiarrheal effects of JZL184 were completely reversed by rimonabant treatment (Fig. 2D). In contrast, treatment with the CB₂ 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 antidiarrheal 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 the CB₁ 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) in 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.

FAAH Blockade Attenuates Somatic Signs of Naloxone-Precipitated Morphine Withdrawal through a Mechanism That Requires the Activation of CB₁ 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 versus 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) than 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.

Fig. 3. The reduction in intensity of naloxone-precipitated morphine withdrawal signs is not CB₂ receptor-mediated. The CB₂ antagonist SR144528 (3 mg/kg i.p.) did not reverse the antiwithdrawal effects of JZL184 (40 mg/kg i.p.). The withdrawal signs measured were number of jumps (A), number of paw flutters (B), weight loss (C), and occurrence of diarrhea (D). Data are expressed as means $\pm$ S.E.M. for A to C. ***, $p < 0.001$; **, $p < 0.01$ versus vehicle; †, $p < 0.05$ versus vehicle. $n = 7$ to 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 with those in mice implanted with placebo pellets. The MAGL inhibitor JZL184 attenuated the intensity of the following spontaneous withdrawal signs in morphine-dependent mice: percentage of mice that displayed platform jumping (A); number of paw flutters (B); number of head shakes (C); and weight loss (D). Data are expressed as means $\pm$ S.E.M. for B to D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus placebo; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ versus morphine control; †, $p < 0.05$ versus placebo; ‡, $p < 0.05$ versus morphine control. $n = 6$ to 9 mice/group.

We next evaluated the irreversible FAAH inhibitor PF-3845 (10 mg/kg i.p.) versus vehicle on naloxone-precipitated morphine withdrawal. PF-3845 significantly attenuated the frequency of jumps and paw flutters (Fig. 6, A and B). These effects were reversed by rimonabant as indicated by significant interactions between PF-3845 versus vehicle and rimonabant versus 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) after naloxone challenge. This experiment shows that short-term 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 Ileum. 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 μ M) for 60 min and then naloxone (30 μ M) is used to precipitate contractions. The height of naloxone-precipitated contractions was normalized to contractions elicited by 80 mM KCl. JZL184 (1 μ M) attenuated naloxone-precipitated contractions in morphine-treated tissue [$F(2,15) = 6.6$; $p < 0.01$] (Fig. 7A). However, rimonabant (100 nM) did not reverse the attenuation of naloxone-induced contractions by JZL184 ($p = 0.73$). Representative traces of contractions before and after application of naloxone in the three conditions are shown in Fig. 7B. In contrast, PF-3845 (1 μ M) 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 Naive Ileal Tissue. To further investigate the impact of MAGL and FAAH inhibition on ileum function, we evaluated EFS cholinergic contractions in the longitudinal preparations from mouse ileum. These experiments were performed 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 predrug contraction for each tissue. JZL184 inhibited EFS contractions in the ileum ($p < 0.05$), which was reversed by rimonabant

Fig. 5. FAAH(-/-) mice display attenuated naloxone-precipitated withdrawal signs. The withdrawal signs measured were number of jumps (A), number of paw flutters (B), weight loss (C), and occurrence of diarrhea (D). Data are expressed as means $\pm$ S.E.M. for A to C. **, $p < 0.01$; * $p < 0.05$ versus FAAH(+/+) group. $n = 6$ to 7 mice/group.

[$F(1,8) = 10.2$; $p < 0.05$] (Fig. 8C; see Fig. 8A for a representative trace). In contrast, PF-3845 did not inhibit field-stimulated contractions in the ileum ($p = 0.36$) (Fig. 8C; see Fig. 8B for a representative trace).

Measurement of Endocannabinoid Levels in Brain and Ileum. To determine whether 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 short-term 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 naive 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 the ileum.

Evaluation of Effects of JZL184 on Arachidonic Acid and Prostaglandin Levels in Ileum. The data depicted in Figs. 7C and 8 suggest that FAAH inhibition attenuates ileal contractions during naloxone-precipitated withdrawal in a CB₁-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-CB₁ receptor mechanism. One mechanism by which JZL184 may inhibit naloxone-precipitated contractions is by altering arachidonic acid levels and prostaglandins. It has been shown previously that genetic deletion or pharmacological inhibition of MAGL leads to a decrease in arachidonic acid levels in brain (Long et al., 2009a; Schlosburg 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 μ M) 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

Fig. 6. The FAAH inhibitor PF-3845 attenuated a subset of naloxone-precipitated morphine withdrawal signs in a CB₁ 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 number of jumps (A), number of paw flutters (B), weight loss (C), and occurrence of diarrhea (D). Data are expressed as mean $\pm$ S.E.M. for A to C. **, $p < 0.01$; *, $p < 0.05$ versus vehicle; ###, $p < 0.001$; #, $p < 0.05$ versus PF-3845. $n = 11$ to 12 mice/group.

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 and FAAH, reduces naloxone-precipitated withdrawal in both in vivo and in vitro models of morphine dependence. Although 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 reducing opioid withdrawal. Short-term administered JZL184, which preferentially inhibits MAGL over FAAH and selectively raises brain 2-AG but not anandamide in vivo (Long et al., 2009a,b), 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 CB₁ receptors. In addition, 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 CB₁ receptors are expendable. Although FAAH(−/−) mice showed a reduction in all four measures of naloxone-precipitated withdrawal, PF-3845 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 and naloxone-precipitated contractions in morphine-dependent ilea.

Of note, JZL184 significantly blocked all opioid withdrawal symptoms, whereas 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 2 orders of magnitude higher than those of 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 CB₁ receptors than AEA. Second, 2-AG acts as a full CB₁ receptor agonist, whereas AEA acts as a partial CB₁ 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 com-

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 μ M) attenuated the amplitude of naloxone-precipitated contraction in ileum treated with morphine. Rimonabant (Rim; 100 nM) did not block the antiwithdrawal effects of JZL184 (JZL). **B**, representative traces of contractions in each of the conditions before and after application of naloxone hydrochloride. **C**, the FAAH inhibitor PF-3845 (1 μ M) attenuated the amplitude of naloxone-precipitated contraction. Rimonabant (100 nM) reversed the actions of PF-3845 (PF). **D**, representative traces of contractions in each of the conditions before and after application of naloxone hydrochloride. Data are expressed as means $\pm$ S.E.M. ***, $p < 0.001$; *, $p < 0.05$ versus morphine; ###, $p < 0.001$ versus PF-3845. $n = 4$ to 8 ilea/condition.

partments, which could influence CB₁ receptor signaling in the brain and result in distinct physiological functions for these endocannabinoids. For example, 2-AG drives short-term synaptic plasticity, whereas 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, although 2-AG levels have been reported to be increased in other brain regions of rats treated repeatedly with morphine (Viganò et al., 2003). Likewise, long-term heroin self-administration in rats does not produce overt CB₁ 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 endocannabi-

Fig. 8. Evaluation of JZL184 and PF-3845 on EFS contractions on longitudinal smooth muscle preparations from naive ileum. A, representative trace of EFS contractions before and after addition of JZL184 (1 μ M). B, representative trace of EFS contractions before and after addition of PF-3845 (1 μ M). C, graphical representation of the amplitude of contractions represented as percentage of baseline values. JZL184 reduced the amplitude of EFS-stimulated contractions through the activation of CB₁ receptors, but PF-3845 had no effect. Data are expressed as mean $\pm$ S.E.M. *, $p < 0.05$; #, $p < 0.05$ versus JZL184. $n = 4$ to 5 ilea/group. Rim, rimonabant.

noid 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,b) 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 CB₁ 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 short-term 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 CB₁ and the μ -opioid receptor in the LC and other brain regions as well as in the ileum (Maguma et al., 2010; Scavone

et al., 2010). The observations that short-term CB₁ receptor stimulation inhibits adenylyl cyclase and decreases cAMP production and that μ -opioid and CB₁ receptors have been demonstrated in cell lines to share a common pool of adenylyl cyclase further support this hypothesis (Howlett and Mukhopadhyay, 2000; Levitt et al., 2011).

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 antidiarrheal 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. On the basis of the differential antidiarrheal effects for JZL184 and PF-3845, one would predict a similar association for secretion in an in vitro withdrawal model, but the influence of endocannabinoid catabolic enzyme inhibitors on secretion remains an area for future research. Thus, although 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 the antiwithdrawal effects of JZL184 in vivo, supporting the hypothesis that these actions were mediated by increased CB₁ 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. It is curious that JZL184 did not increase 2-AG levels in ileum. Likewise, JZL184 does not elevate 2-AG in other peripheral tissues (e.g., testes, white adipose tissue, and lung), although it increased this endocannabinoid in other tissues (e.g., liver, kidney, brown adipose tissue, and 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, although in vitro the compound is approximately 500-fold more selective as a MAGL inhibitor ($IC_{50} = 8$ nM) than as a FAAH inhibitor ($IC_{50} = 4000$ nM) (Long et al., 2009a). Short-term 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).

Whereas THC and other direct-acting CB₁ receptor agonists elicit marijuana-like psychoactive effects, endocannabinoid catabolic inhibitors seem to possess fewer cannabimimetic effects than CB₁ 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). Although the MAGL inhibitor

TABLE 1
AEA and 2-AG levels in brain regions and ileum are not altered after treatment with either morphine or placebo and acute challenge with either saline or naloxone (1 mg/kg s.c.)
Data are expressed as means ± S.E.M.

Treatment	AEA Levels				2-AG Levels			
	LC	PAG	Amygdala	Ileum	LC	PAG	Amygdala	Ileum
	pmol/g				nmol/g			
Placebo-saline	3.7 ± 0.3	4.2 ± 0.3	9.5 ± 0.8	1.2 ± 0.3	6.4 ± 0.4	11.1 ± 0.9	11.6 ± 1.3	5.6 ± 0.7
Morphine-saline	5.1 ± 1.0	4.5 ± 0.6	8.2 ± 0.6	1.3 ± 0.2	7.0 ± 0.5	11.8 ± 1.5	10.8 ± 0.9	5.6 ± 1.1
Placebo-naloxone	4.7 ± 0.4	4.8 ± 0.5	9.1 ± 1.1	1.5 ± 0.1	6.9 ± 0.7	11.7 ± 2.4	17.7 ± 4.2	5.8 ± 1.0
Morphine-naloxone	4.3 ± 0.4	4.6 ± 0.5	8.2 ± 0.6	1.7 ± 0.2	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 are expressed as means ± S.E.M. n = 6 mice/group.

Treatment	AEA	2-AG
	pmol/g	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

* p < 0.05 versus vehicle control.

JZL184 produces a broader set of cannabinoid behavioral effects, including hypomotility, it does 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,c). In contrast, dual inhibitors of both FAAH and MAGL, such as 4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate (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 CB₁ receptor function and lacks dependence liability (Schlosburg et al., 2009, 2010). In addition, the observation that FAAH(–/–) mice show an attenuated opioid withdrawal syndrome suggests that the antiopioid withdrawal effects of FAAH inhibition persist even upon genetic deletion of FAAH. On the other hand, long-term treatment with high doses of JZL184 produces CB₁ receptor down-regulation and desensitization accompanied with a mild dependence liability (Schlosburg et al., 2010), although it possible that prolonged partial MAGL blockade may not lead to functional changes in the CB₁ 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.

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Authorship Contributions

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