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Elevating Endogenous Cannabinoids Reduces Opioid Withdrawal in Mice

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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2-AG	2-arachidonoyl glycerol
[3H]CP55,940	tritium labeled 2-[(1S,2R,5S)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol
[35S]GTPγS	guanosine 5'-O-[gamma-thio]triphosphate
%MPE	maximal percent effect
AA	arachidonic acid
ABHD	α/β hydrolase
AC	adenylyl cyclase
Ach	acetylcholine
AEA	anandamide
AMYG	amygdala
ANOVA	analysis of variance
Bmax	maximal specific binding sites
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CB_1	cannabinoid receptor, subtype 1
CB_2	cannabinoid receptor, subtype 2
CBLM	cerebellum
DAGL	diacylglycerol lipase
DOR	∂ (delta) opioid receptor
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
eCB	endocannabinoid

Emax	maximum possible effect of an agonist
FAAH	fatty acid amide hydrolase
G-protein	guanine nucleotide binding protein
Gi	cAMP inhibitory G-protein
Gs	cAMP stimulatory G-protein
GPCR	G-protein coupled receptor
i.p.	intraperitoneal
JZL184	4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl) piperidine-1-carboxylate
JZL195	4-nitrophenyl 4-(3-phenoxybenzyl) piperazine-1-carboxylate
KOR	κ (kappa) opioid receptor
LC	locus coereleus
LC-MS-MS	liquid chromatography tandem mass spectrometry
MAPK	mitogen activated protein kinase
MAGL	monoacylglycerol lipase
MOR	μ (Mu) opioid receptor
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D
OEA	oleylethanolamine
PAG	periaqueductal gray
PEA	palmitoylethanolamine
PF-3845	N-(pyridin-3-yl)-4-(3-(5-(trifluoromethyl)pyridin-2-yloxy)benzyl) piperdine-1-carboxamide
PG	prostaglandin

Rim	rimonabant (SR141716A)
SAMHSA	Substance Abuse and Mental Health Services Administration
s.c.	subcutaneous
SR2	SR144528
THC	Δ^9-tetrahydrocannabinol
Tris	tris(hydroxymethyl)aminomethane
URB597	[3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate
WIN-55,212	(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de)-1,4-benzoxazin-6-yl]-1-napthalenylmethanone

Abstract

ELEVATING ENDOGENOUS CANNABINOIDS REDUCES OPIOID WITHDRAWAL IN MICE

By **Divya Ramesh**

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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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 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 dissertation was to test whether increasing AEA or 2-AG, via inhibition of their respective hydrolytic enzymes, reduces morphine withdrawal symptoms in *in vivo* and *in vitro* models of opiate dependence. Morphine-dependent ICR mice subjected to acute naloxone challenge or abrupt withdrawal (via pellet removal) reliably displayed a profound withdrawal syndrome, consisting of jumping, paw tremors, head shakes, diarrhea, and weight loss. THC and the MAGL inhibitor, JZL184 dose-dependently reduced the intensity of precipitated withdrawal measures through the activation of CB_1 receptors. The FAAH inhibitor, PF-3845, reduced the intensity of a subset of precipitated signs through the activation of CB_1 receptors,

but did not ameliorate the incidence of diarrhea or weight loss. In the next set of experiments, MAGL inhibition dose-dependently reduced the intensity of all spontaneous withdrawal signs (i.e jumps, paw flutters, head shakes, weight loss and diarrhea) in a CB_1 receptor dependent manner. However, FAAH inhibition reduced the intensity of head shakes and paw flutters, but did not affect other signs. Strikingly, a combination of low-dose JZL184 and high-dose PF-3845 reduced abrupt withdrawal signs in a manner similar to complete MAGL inhibition, which suggests potential therapeutic advantages of dual enzyme inhibition. This combination elevated appropriate eCB levels and caused moderate CB_1 receptor desensitization, but did not affect receptor number in whole brain. Since MAGL, but not FAAH inhibition, blocked diarrhea during opioid withdrawal *in vivo*, we investigated whether inhibitors of each enzyme would differentially attenuate naloxone-precipitated contractions and secretion in morphine-dependent ilea *in vitro*. Both enzyme inhibitors attenuated the intensity of naloxone-induced contractions, and blocked naloxone-precipitated hypersecretion. Although these models offer useful tools for investigating in vitro end-points of withdrawal, further research is needed to elucidate the anti-diarrheal mechanism of these inhibitors. If targeting endocannabinoid catabolic enzymes is indeed a viable approach to treat other abuse disorders, it is important to know whether these inhibitors would themselves have abuse or dependence liability. In the final series of experiments we tested whether prolonged elevation of endocannabinoid leads to the development of cannabinoid dependence, based on the occurrence of somatic withdrawal signs upon challenge with rimonabant, a CB_1 receptor antagonist. Repeated treatment with high doses, but not low doses, of JZL184 led to cannabinoid dependence. These results indicate that the strategy of increasing endogenous cannabinoids through the inhibition of their catabolic enzymes represents a promising approach to ameliorate opioid withdrawal symptoms.

Chapter 1: Introduction

Opiate Dependence

Opioids have been used for centuries for their acute analgesic, antitussive, antidiarrheal and euphoric effects. Importantly, opiates are the mainstay in the management of chronic pain conditions. Tolerance manifests clinically as the need for increasing drug doses over time to produce equivalent therapeutic effects and is demonstrated in the laboratory as decreased potency and/or efficacy of a drug to produce its acute pharmacological effects. Dependence is revealed upon cessation of opioid treatment and presents as a withdrawal syndrome that includes both physiological and motivational signs. 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; thus thwarting 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.

Natural Opiates and Opioid Receptors

The naturally occurring opiates such as morphine and codeine are obtained from the poppy plant *Papaver somniferum*. The endogenous opioid system consists of several families of related neuropeptides and the three primary opioid receptor subtypes mu, delta and kappa. The opioid peptides as well as natural and synthetic opioids act via interactions with these receptors (Akil et al., 1984; Thompson et al., 1993; Mansour et al., 1995). Opioid receptors were first identified in 1973 using stereospecific radioligand binding (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973) and subsequently classified into three types, mu, delta and kappa, based on results of behavioral and bioassay studies (Martin et al., 1976; Lord et al., 1977). Further pharmacological characterization has revealed that mu opioid receptors mediate the effects of most clinically relevant opioids, such as morphine and heroin. Cloning of the opioid receptors confirmed the existence of mu (MOR), kappa (KOR) and delta (DOR) opioid receptors and confirmed their heptahelical structure (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993a; Chen et al., 1993b; Meng et al., 1993; Thompson et al., 1993). The generation of genetically altered (transgenic) mice lacking each of the opioid receptors has confirmed that MOR mediates the acute effects of most clinically relevant opioid drugs, such as analgesia, respiratory depression, locomotor stimulation and reinforcement, as well as chronic effects such as physiological withdrawal signs (Matthes et al., 1996; Sora et al., 1997; Loh et al., 1998; Becker et al., 2000). Heroin, a derivative of morphine, which is rapidly metabolized to 6-acetyl morphine and subsequently converted morphine, also acts primarily via MOR (Kitanaka et al., 1998; Contarino et al., 2002).

Opioid Withdrawal

Dependence has been defined in DSM-IV as a maladaptive pattern of substance abuse including tolerance, withdrawal and a persistence desire to take the drug. Chronic use of opiates such as morphine and heroin leads to tolerance and dependence. Opiate drug abuse affects about 0.6% of the US population annually (NSDUH) and repeated use of opiates leads to abstinence signs in addicts.

In the preclinical setting the opiate withdrawal syndrome is well characterized in rodents. Both the precipitated and spontaneous withdrawal model are commonly used to study opiate abstinence signs. Mice implanted with either a subcutaneous morphine pellet or exposed to repeated injections of increasing doses of morphine over a period of few days express overt quantifiable withdrawal signs upon administration of an opiate receptor antagonist, such as naloxone or abrupt cessation of morphine treatment (Way et al., 1969). The most commonly observed signs are jumps, paw tremors, head shakes, ptosis, teeth chattering, weight loss, and production of diarrhea.

Neuroadaptive changes

The most prominent neuroadaptive changes during morphine induced dependence include desensitization of MORs and upregulation of the cAMP pathway. The mitogen activated protein kinase (MAPK) pathway as well as Ca^{2+} signaling are also affected during morphine dependence with MAPK levels being modulated differentially by morphine treatment in different parts of the brain. The primary consequence of morphine withdrawal is 'superactivation' of adenylyl cyclase (AC) and a subsequent overproduction of its downstream signaling molecule, cAMP. MOR signal transduction is $G_{\alpha\ i/o}$ coupled and chronic stimulation

of μOR by morphine leads to suppression of AC activity over a prolonged period of time. Concurrently, chronic morphine treatment also causes compensatory increases in activity of other subforms of AC thus restoring cAMP levels back to normal over the time course of chronic morphine treatment. When morphine treatment is abruptly halted or an opiate receptor antagonist such as naloxone is administered, causing disinhibition of AC activity and leading to massive overstimulation of AC coupled with increased AC production. This process leads to an overactivation of other downstream effectors of AC such as cAMP, PKA and transcription factors such as cAMP response element binding protein (CREB) (Nestler and Tallman, 1988; Guitart et al., 1992) (Fig. 1). CREB is also a downstream target of MAPK and Ca^{2+} (Eitan et al., 2003). Other cAMP actions during withdrawal include PKA-mediated enhanced GABAergic synaptic transmission in areas such as periaqueductal grey (PAG), ventral tegmental area (VTA), nucleus accumbens (N Acc.) and dorsal raphe. [For complete review see (Williams et al., 2001; Bailey and Connor, 2005; Sim-Selley, 2005)]

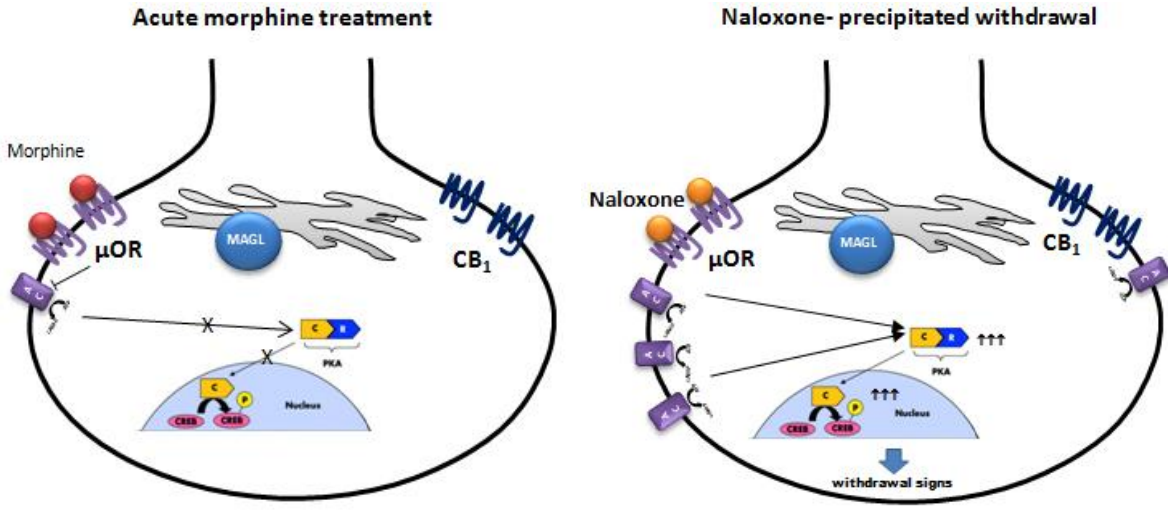

Figure 1: Effects of acute and repeated exposure to opiates
(Rt-left) Chronic morphine exposure leads to upregulation of AC and normalization of downstream signaling; Induction of withdrawal following chronic morphine exposure causes cAMP "overshoot" and increasing signaling of cAMP cascade resulting in expression of withdrawal signs

The cAMP superactivation and changes in CREB activation during withdrawal is most prominent in the LC as well as in N Acc, amygdala, DRN and VTA and is associated with the expression of external somatic signs during withdrawal (Widnell et al., 1994; Lane-Ladd et al., 1997; Shaw-Lutchman et al., 2002; Han et al., 2006). CREB mediates the action of opioids on the expression of several genes in brain regions responsible for drug-seeking behavior and manifestation of withdrawal mediated changes in synaptic plasticity (Bilecki and Przewlocki, 2000). CREB binds to cAMP response element (CRE) in the promoter region of target genes and enhances the transcription of these genes. The phosphorylation of CREB is determined by cAMP in the LC and its function is regulated by the MOR. Further evidence for a role of CREB in mediating cellular adaptation in response to chronic opioid treatment is that LC neurons show decreased hyperexcitability following morphine withdrawal in CREB1(-/-) mice and in mice with a conditional mutation in CREB (Valverde et al., 2004). It is likely that adaptations to MORs in this brainstem region contribute to dependence and withdrawal.

Cannabinoids

Marijuana or *Cannabis sativa* has been used and cultivated for a variety of purposes as archaeological evidence dates back to 2350 B.C (Cannabinoids in nature and medicine, 2009). Δ^9-tetrahydrocannabinol (THC) is the primary psychoactive constituent of marijuana. In addition, more than 70 other phytocannabinoids have been discovered (Elsohly and Slade, 2005), and hundreds, if not thousands, of cannabinoids have been synthesized. The first constituent of marijuana was isolated by Roger Adams in 1940; which came to known as cannabidiol, lacking psychoactive properties and whose structure was later completely determined by Raphael Mechoulam (Adams et al., 1940; Mechoulam and Gaoni, 1965). In 1964, Raphael Mechoulam was the first to report on chemical compounds extracted and isolated

from *cannabis sativa* which contributed to the biological activity of the plant. While discovering several active compounds of similar lipid structural class, he isolated Δ^9-tetrahydrocannabinol (THC) as the primary psychoactive compound responsible for marijuana's activity (Gaoni and Mechoulam, 1964). The FDA has since approved Marinol® (synthetic THC) for the treatment of AIDS related anorexia and emesis induced by chemotherapy. In Canada and Europe, Sativex® (THC:cannabidiol; 1:1) is approved for the management of cancer pain and neuropathic pain associated with multiple sclerosis. However, marijuana and THC have several non-approved beneficial applications such as anxiolysis, management of chronic pain and treatment of abuse disorders. However, dependence, particularly withdrawal from prolonged cannabinoid use, which limits clinical utility of cannabis and its psychoactive ingredients as well as direct acting CB_1 receptor agonists.

Cannabinoid dependence

Studies of cannabis dependence and withdrawal in humans

While classical cannabinoids such as THC have many promising clinical applications, including the treatment of opiate dependence, the therapeutic potential of marijuana as well as THC is limited by their dependence liability. While the current revisions of the DSM-IV and ICD-10, the two most common diagnostic guidebooks used by medical professionals today to diagnose substance-use disorders, include criteria and symptomology for diagnosing cannabis abuse and dependence, only the latter recognizes a withdrawal syndrome as a component of a cannabis dependence disorder (American Psychiatric and American Psychiatric Association. Task Force on, 2000). The DSM-IV reflects a common attitude of many in medicine and the general public that cannabis is not a physically addictive substance in which a withdrawal

syndrome can produce clinically relevant symptoms of any severity and duration to impact subsequent substance-use behavior. The source of this belief is likely due to a multitude of factors, such as the relatively slow onset and unique constellation of the withdrawal syndrome, and is further described below.

Cannabis is the most commonly used illicit drug worldwide, with approximately 56% of young adults (i.e., 19-28 years) in America having tried cannabis in their lifetime (Johnston, 2010) . This high prevalence of marijuana use allows for many people to have personal and/or anecdotal experience with marijuana without necessarily having personal interactions with dependent users. So while only about 3-4% of individuals who have ever tried cannabis meet criteria for a cannabis-use disorder (compared with 15-25% for cocaine), the total number of Americans classified with such disorders is 4.3 million, more than twice that of cocaine and heroin combined (SAMHSA and Studies, 2008). In addition, the severity of cannabis withdrawal is not generally associated with any symptoms requiring hospitalization or viewed as potentially life-threatening (Wallace and Cunningham, 1944). Furthermore, only a subset of regular marijuana users experience a clustering of symptoms upon cessation of use, with reports of frequency ranging from 1/6 up to 1/2 (Wiesbeck et al., 1996; Budney et al., 1999). Common symptoms observed during cannabis withdrawal include anger, aggression, irritability, anxiety/nervousness, decreased appetite or weight loss, restlessness, and sleep difficulties with strange dreams (Budney and Hughes, 2006). Marijuana withdrawal has been compared to that of tobacco, and is reported to increase craving and desire to resume use (Budney et al., 2008; Vandrey et al., 2008). A similar abstinence syndrome has also been shown upon cessation of repeated oral THC, the primary psychoactive component of marijuana, in human studies (Haney et al., 1999). While the immediate physical impact of these symptoms are mild when compared

to certain other drugs of abuse the comprehensive impact of the cannabis withdrawal syndrome is becoming better understood. However, any abstinence syndrome may increase the desire to continue drug use and thus, represents a complication in treating dependence from other drugs of abuse.[For a complete review see (Ramesh et al., 2011b)]

Neurobiological adaptations during cannabinoid dependence

In addition to the increased characterization of a behavioral profile during cannabinoid withdrawal, preclinical studies have investigated the molecular changes that result from repeated exposure, and cessation, of cannabinoids. Such studies provide indicators of adaptations that drive withdrawal symptomology, and improve strategic targeting of therapeutics. Chronic administration of cannabinoid agonists results in down-regulation of the CB_1 receptor in several brain regions (e.g., cerebellum, hippocampus and striatum with globus pallidus) as measured by radioligand binding (Breivogel et al., 1999). At the level of the G-protein, chronic treatment with THC produces a time and region-dependent desensitization of CB_1 receptor activity (Romero et al., 1997; Breivogel et al., 1999). Other evidence of dysregulation within the endocannabinoid system includes region-specific alterations in endocannabinoid content following precipitated withdrawal in rats and mice in the cerebellum, cortex, limbic forebrain and brainstem (Gonzalez et al., 2004).

Alterations in numerous other neurotransmitter systems are also associated with withdrawal from THC in rodents. Following chronic administration of THC, brain levels of serotonin are decreased, concurrent with increases in its primary metabolite (Taylor and Fennessy, 1978; Taylor and Fennessy, 1982). The finding that various serotonin uptake inhibitors elicited writhing behavior, backward kicks, jumps, and wet shakes in rats treated repeatedly with THC, suggests serotonergic involvement in cannabinoid withdrawal-like

behavior (Verberne et al., 1980). In addition, histamine levels in the brain decrease both during initial exposure to THC, as well as during somatic withdrawal induced by a serotonin reuptake inhibitor clomipramine (Verberne et al., 1985). Several studies have shown evidence of upregulation and release of the stress-related peptide corticotropin-releasing factor upon precipitated withdrawal, a phenomenon common during withdrawal to many drugs of abuse (Rodriguez de Fonseca et al., 1997; Gonzalez et al., 2004).

At the intracellular level, the cAMP second messenger signaling system appears to modulate cannabinoid withdrawal. Rimonabant administered to THC-dependent mice resulted in significant increases in both basal and forskolin-stimulated adenylyl cyclase activity in the mouse cerebellum, but not in other regions (Hutcheson et al., 1998). Similar results were obtained with calcium-calmodulin stimulated cyclase activity from cerebella of THC-dependent rats undergoing precipitated withdrawal (Rubino et al., 2000). Rimonabant-precipitated cannabinoid withdrawal also results in upregulation of protein kinase A (PKA) activity, which is downstream of cAMP, in the cerebella of THC dependent rats (Tzavara et al., 2000). Additionally, infusions of the cAMP blocker Rp-8Br-cAMPs into the cerebellum attenuated expression of precipitated withdrawal signs and PKA activity in rats undergoing precipitated withdrawal. Conversely, infusion of Sp-8Br-cAMPs, a cAMP analog, into the cerebellum elicited cannabinoid withdrawal somatic signs in drug naïve mice. These findings provide strong evidence for the functional role of the cAMP cascade, particularly in the cerebellum, in modulating withdrawal from cannabinoids.

The endocannabinoid system

Cannabinoid receptors

THC, as well as many other cannabinoids, has been demonstrated to bind to and activate two types of cannabinoid receptors that have been cloned, CB_1 (Matsuda et al., 1990) and CB_2 (Gerard et al., 1991). Both cannabinoid receptors are G-protein coupled receptors, which are associated with $G_{i/o}$ G-proteins [for a complete review see (Howlett, 2002)]. Activation of these receptors decreases cAMP production via blockade of adenylyl cyclase (Howlett et al., 1990), activation of inwardly rectifying potassium (GIRK) channels via Gβγ subunits (Mackie et al., 1995; McAllister et al., 1999). Furthermore activation of the cannabinoid receptors inhibits N- and P/Q-type calcium channels, which reduces synaptic vesicle fusion to the nerve terminal thereby inhibiting the release of excitatory and inhibitory neurotransmitters. The CB_1 receptor is heterogeneously expressed throughout the central nervous system (CNS) and periphery (Herkenham et al., 1990; Matsuda et al., 1993; Felder and Glass, 1998) and has been demonstrated to be responsible for most of the pharmacological actions of THC, particularly within the CNS. In support of this notion, cannabinoids induce tetrad effects (decrease in locomotor activity, hypothermia, catalepsy, and analgesia) which are reversed by a CB_1 antagonist and do not occur in CB_1 (-/-) mice (Rinaldi-Carmona et al., 1994; Compton et al., 1996b). The CB_2 receptor is tightly associated with immune cells (Klein et al., 2003) and was initially believed to be expressed solely in the periphery. However, more recently this receptor has been found to be expressed in microglial cells (Cabral and Marciano-Cabral, 2005) and in neurons (Van Sickle et al., 2005) within the brain. CB_1 and CB_2 receptors share approximately 44% homology with each other (Munro et al., 1993). Cannabinoid receptors have been localized on presynaptic terminals of both GABAergic (Katona et al., 1999) and glutamatergic neurons

(Huang et al., 2001; Szabo and Schlicker, 2005). Transient suppression of the inhibitory transmission (i.e. GABA) is termed depolarization-induced suppression of inhibition (DSI). Conversely transient suppression of the stimulatory neurotransmitter (e.g. glutamate) is called depolarized-induced suppression of excitation (DSE). Both result in cannabinoid receptor mediated hyperpolarization of a repetitively depolarized neuron, which suppresses subsequent vesicular fusion and release of glutamate or GABA.

Endogenous ligands and their regulatory pathways

A major breakthrough in cannabinoid pharmacology came with the discovery of the endogenous cannabinoids (endocannabinoids), which activate and bind to cannabinoid receptors (Di Marzo and Fontana, 1995). These endocannabinoids are derived from phospholipid precursors in the postsynaptic neuron. Unlike classical neurotransmitters, endocannabinoids are not stored in vesicles, but are released on demand and travel in a retrograde manner from post-synaptic terminals to act on pre-synaptic cannabinoid receptors (Ahn et al., 2008). The most noteworthy endocannabinoids discovered are N-arachidonylethanolamine (anandamide, AEA) (Devane et al., 1992) and 2-arachidonylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). Additionally, three other endocannabinoids have been discovered, which include nolandin ether (Hanus et al., 2001), virodhamine (Porter et al., 2002) and N-arachidonyl dopamine (Huang et al., 2002). Despite sharing common receptors and considerable structural similarity, AEA and 2-AG can be distinguished by multiple factors. First, these endocannabinoids activate cannabinoid receptors to different degrees *in vitro*, with anandamide acting as a partial agonist whereas 2-AG acts a full agonist at the CB_1 receptor (Gonsiorek et al., 2000). Mice repeatedly treated with FAAH inhibitors as well as FAAH(-/-) mice show normal CB_1 function and display CB_1 desensitization following treatment with exogenous AEA. On the

other hand, prolonged exposure to the MAGL inhibitor JZL184 as well as MAGL(-/-) results in significant downregulation and desensitization of CB_1 receptors as well as physical cannabinoid withdrawal (Falenski et al., 2010; Schlosburg et al., 2010). Second, the endogenous quantities of anandamide and 2-AG differ dramatically, with the latter being found at more than 100-fold higher concentrations in the nervous system (Ahn et al., 2009; Long et al., 2009a). Third, while AEA and 2-AG both activate CB_1 and CB_2, AEA has been has also been shown to activate vannilloid (TRPV1) and PPARα receptors (Ross, 2003; Jhaveri et al., 2008). Finally, the available signaling pool for each lipid is regulated by distinct synthetic and catabolic enzymatic pathways [(Ahn et al., 2008) summarized in Fig 2].

AEA synthesis is regulated by multiple pathways, and was initially believed to be formed via cleavage of N-arachidonylphosphatidylethanolamine (NAPE) by NAPE-phospholipase D. However, it has been shown that NAPE-PLD knock-out mice possess wild-type levels of AEA (Leung et al., 2006) suggesting that the existence of other biosynthetic pathways. Other pathways of AEA synthesis include via phosphodiesterase (PDE), or synthesis via cleavage of phospholipase C (PLC) and a phosphatase (Liu et al., 2006). On the other hand, 2-AG is synthesized by the cleavage of diacylglycerol (DAG) by DAG lipase-alpha (DAGLα) (Gao et al., 2010; Tanimura et al., 2010). This is evidenced by the observation that DAGLα (-/-) mice when compared to DAGLβ (-/-) mice exhibit a significantly decreased production of 2-AG levels.

As with most signaling messengers, AEA and 2-AG are rapidly inactivated soon after they are released. AEA is taken back into the post-synaptic terminal and degraded by the enzyme fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996). FAAH is located within the post-synaptic terminal (Gulyas et al., 2004) and is responsible for the degradation of other fatty

acid amides (FAAs) such as oleamide, the sleep agent, palmitoylethanolamide (PEA), the anti-inflammatory agent, and oleylethanolamide (OEA) the satiety lipid (Cravatt et al., 1995). Conversely, approximately 85% of 2-AG is degraded within the presynaptic terminal by the enzyme monoacylglycerol lipase (MAGL), while the remaining 2-AG is degraded by enzymes alpha/beta hydrolase 6 and 12 (ABHD) (Blankman et al., 2007). Although 2-AG is present at levels 170-1000 fold greater than AEA (Sugiura et al., 2002), both endocannabinoids produce some similar cannabinoid effects *in vitro*. Administration of THC or other synthetic cannabinoids do not elicit effects mimicking the physiological effects of endocannabinoids because the former causes a persistent inhibition of neurotransmitter release, while the latter elicits localized and transient effects (Vaughan and Christie, 2005).

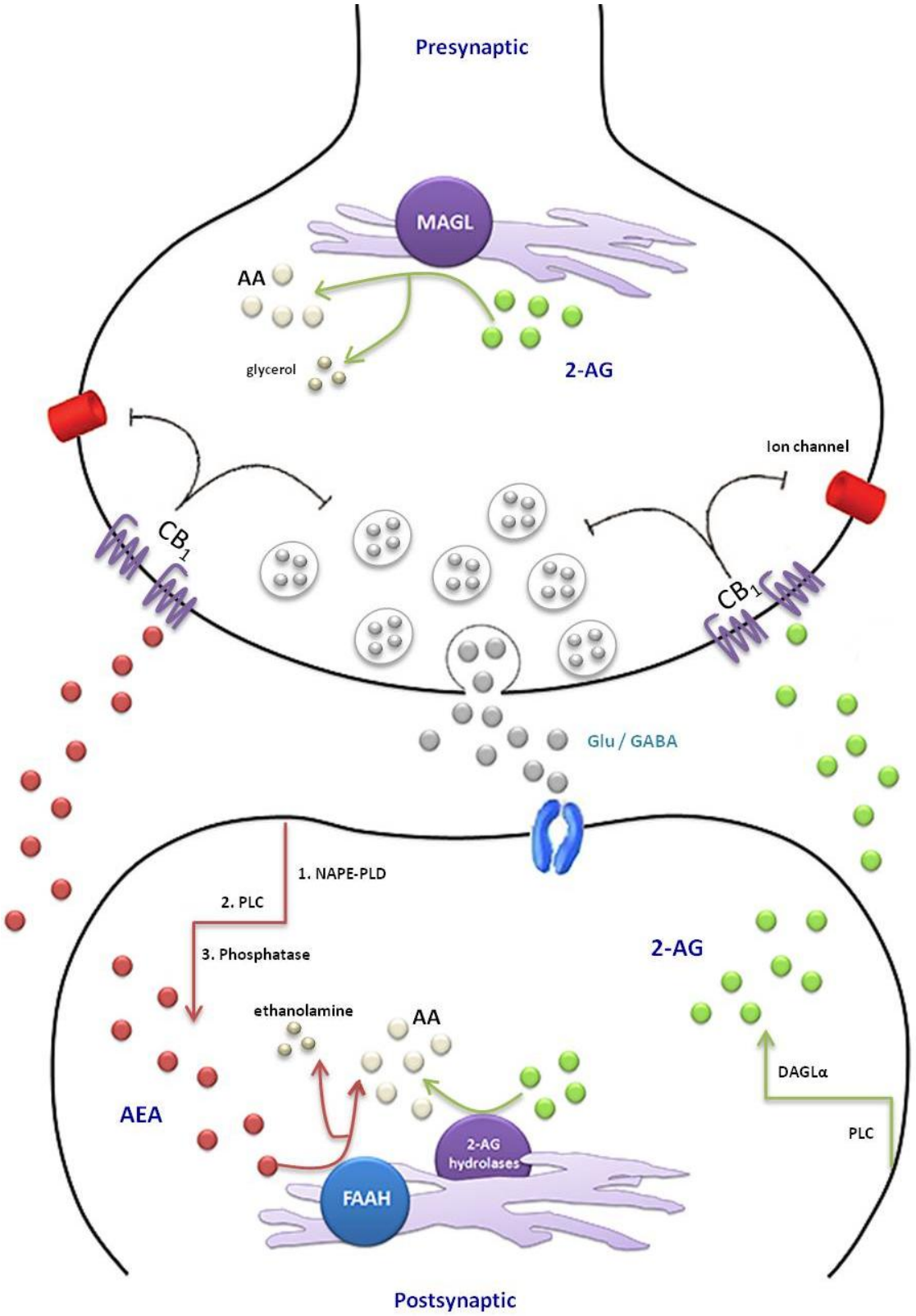

Figure 2. The endocannabinoid system (CNS): receptors, ligands and their regulatory pathways [Adapted from (Ahn et al., 2008)]

Inhibitors of endocannabinoid catabolic enzymes: second generation inhibitors

It is noteworthy that blockade of the enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) or their genetic deletion elevates brain levels of the respective endocannabinoids, AEA (Kathuria et al., 2003; Lichtman et al., 2004) and 2-AG (Long et al., 2009a). FAAH (-/-) mice have been generated by inducing a targeted disruption of the FAAH gene by removing the first exon of the gene by homologous recombination. These mice show 10-fold elevations of AEA, other FAAs (e.g., OEA, PEA) in CNS and display an antinociceptive phenotype (Cravatt et al., 2001). Irreversible (PF-3845, URB597) as well as reversible inhibitors of FAAH elevate AEA levels multi-fold in the brain (Fegley et al., 2005; Ahn et al., 2009). Importantly, PF-3845 is highly selective for FAAH, unlike URB597 that also binds to other serine hydrolases. Acute treatment with PF-3845 results in FAAH inhibition up to 24 h and maximal AEA elevation up to 12 h in mice. In pharmacological assays, PF-3845 has analgesic effects in the lipopolysaccharide (LPS), chronic constrictive injury (CCI) and acetic acid stretching models of pain as well as anxiolytic-like effects in marble-burying assay (Kinsey et al., 2010; Booker et al., 2011; Kinsey et al., 2011b).

Similarly MAGL, the primary degradative enzyme for 2-AG, represents a target for the development of selective inhibitors to treat pain and inflammatory, anxiety-related, and cannabis-related and other abuse disorders. Genetic deletion or complete pharmacological inhibition of MAGL with JZL184 increases brain 2-AG levels by approximately 10-fold (Long et al., 2009a; Long et al., 2009b; Schlosburg et al., 2010). The pharmacological actions of JZL184 have been summarized in Table 1. 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).

Although acute administration of 40 mg/kg JZL184 in ethanol:emulphor:saline (1:1:18) vehicle inhibits FAAH activity by approximately 50%, this inhibition is insufficient to increase brain AEA levels (Long et al., 2009b).

Table 1. Pharmacological effects of JZL184 in preclinical assays

Species	**Type of Assay**	**Dosing Regimen**	**Effect**	**Reference**
Mouse	Tetrad	JZL184 (40 mg/kg, acute ;i.p.)	Hypothermia, hypomotility, catalepsy, hyper-reflexia	(Long et al., 2009a; Long et al., 2009b)
Mouse	Acetic acid stretching	JZL184 (8, 16 mg/kg; acute & 8 mg/kg repeated; i.p.)	Antinociception	(Long et al., 2009a; Busquets-Garcia et al., 2011)
Mouse	CCI (mechanical & cold allodynia)	JZL184 (16,40 mg/kg; acute; i.p.)	Anti-allodynic	(Kinsey et al., 2009; Kinsey et al., 2010)
Mouse; Rat	Carrageenan-induced paw inflammation	JZL184 (4-40 mg/kg; i.p.)	Partial anti-allodynic and anti-edema	(Ghosh et al., 2012; unpublished)
Mouse	Bone cancer pain	JZL184 (10 µg; i.p.l)	Anti-hyperalgesic	(Khasabova et al., 2011)
Mouse; Rat	Formalin pain test	JZL184(16 mg/kg i.p.); (0.001-300 µg; i.pl)	Antinociceptive at both early and late phase	(Long et al., 2009a; Guindon et al., 2011)
Rat	Capsaicin-induced	JZL184 (100µg;	Antinociception	(Spradley et al.,

	behavioral sensitization	i.pl)	and anti-hyperalgesia	2010)
Mouse	Marble burying	JZL184 (16, 40 mg/kg; i.p.)	Anxiolysis	(Kinsey et al., 2011b)
Mouse	Elevated plus-maze; zero maze	JZL184 (8 mg/kg; acute & repeated; i.p.)	Anxiolysis:	(Busquets-Garcia et al., 2011)
Rat	Elevated plus-maze	JZL184 (4- 8 mg/kg; acute & repeated; i.p.)	Anxiolysis under high-stress	(Sciolino et al., 2011)
Mouse	NSAID induced gastric ulcers	JZL184 (4 mg/kg; acute & repeated; i.p.)	Prevents gastric hemorrhage	(Kinsey et al., 2011a)
Mouse	(TNBS)-induced colitis	JZL184 (16 mg/kg; i.p.)	Restoration of intestinal barrier integrity; inhibition of pro-inflammatory cytokine release	(Alhouayek et al., 2011)
Shrew	LiCl- induced vomiting	JZL184 (16,40 mg/kg; i.p.)	Anti-emetic	(Sticht et al., 2011)
Mouse	THC physical withdrawal	JZL184 (16 mg/kg; i.p.)	Attenuation of paw flutters	(Schlosburg et al., 2009)
Mouse	Opioid withdrawal	JZL184 (4-16 mg/kg; i.p.)	Attenuation of somatic withdrawal signs	(Ramesh et al., 2011a)

Mouse; rat	DSE from Purkinje neurons in cerebellar slices and DSI from CA1 pyramidal neurons in hippocampal slices	JZL184 (1µM)	Prolongation of DSE and DSI	(Pan et al., 2009; Schlosburg et al., 2010)
Rat	Aβ induced cytotoxicity and IHC in hippocampal neurons	JZL184 (3µM)	reduces Aβ-induced neurodegeneration and apoptosis	(Chen et al., 2011)

Rationale and hypothesis

Overall hypothesis

Inhibition of the endocannabinoid catabolic enzymes FAAH and MAGL will reduce morphine withdrawal somatic signs and diarrhea in mice through the activation of cannabinoid receptors by anandamide and 2-AG, respectively.

Selection of MAGL and FAAH inhibitors

We specifically chose the respective irreversible MAGL and FAAH inhibitors, JZL184 and PF-3845, since they are amongst the most selective inhibitors presently available (Ahn et al., 2009; Long et al., 2009b). The inhibitors are highly selective for either enzyme, have a long half-life (up to 24 h), produce profound (10-fold) elevations of the appropriate endocannabinoid (up to 12 h) and have few off-site targets. 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} = 4,000 nM) (Long et al., 2009a). In vivo, acute treatment with JZL184 leads to partial FAAH inhibition without elevation of AEA. However, repeated treatment with JZL184 leads to augmented levels of AEA in mice (Schlosburg et al., 2010).

Chapter 2. Dependence liability of MAGL and FAAH inhibitors

In initial studies, we examined the dependence liability of FAAH and MAGL inhibitors in the precipitated cannabinoid withdrawal model. We employed FAAH (-/-) mice, FAAH inhibitors, and MAGL inhibitors to examine the role of endocannabinoid elevations in modulating established CB_1-mediated responses (see Chapter 2). We expected that mice that had been subjected to prolonged FAAH inhibition would show no signs of precipitated physical

withdrawal. This prediction is based on previous studies demonstrating minimal drug abuse potential and adaptations of cannabinoid receptors following prolonged FAAH inhibition (Schlosburg et al., 2009; Falenski et al., 2010; Schlosburg et al., 2010). MAGL inhibition may have a greater potential of physical dependence than FAAH, as 2-AG is a full agonist and its concentration in brain is approximately 200 fold greater than that of anandamide. Simultaneous prolonged inhibition of both MAGL and FAAH is expected to lead to cannabinoid dependence, as dual inhibition acutely has shown numerous characteristics similar to THC not seen under conditions with either enzyme inhibited alone, notably catalepsy and THC substitution in discriminative stimulus testing. Finally, we evaluated the abuse liability of partial MAGL inhibition by using a low dose of JZL184 alone in combination with high dose of the FAAH inhibitor PF-3845. We predicted that development of cannabinoid dependence from prolonged MAGL inhibition is dose-dependent with rimonabant precipitating physical withdrawal in mice treated repeatedly with high doses of JZL184.

Chapter 3. Effects of inhibitors on naloxone-precipitated opioid withdrawal – Somatic signs

In Chapter 3, we described experiments that evaluated whether elevating endocannabinoids, through the inhibition of their catabolic enzymes, attenuates naloxone-precipitated withdrawal symptoms in morphine-dependent mice. 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. We investigated the efficacy of these inhibitors 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 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). We hypothesize that MAGL and FAAH inhibitors as well as THC will attenuate signs of precipitated opioid withdrawal through the activation of CB_1 receptors. We suggest this hypothesis based on observations that THC and other exogenous cannabinoid agonists such as AEA and 2-AG have been demonstrated to reduce signs of naloxone-precipitated withdrawal signs in rodents (Hine et al., 1975; Bhargava, 1976; Vela et al., 1995; Yamaguchi et al., 2001).

Chapter 4. Effects of MAGL and FAAH inhibitors on spontaneous opioid withdrawal signs

In Chapter 4, we used the spontaneous withdrawal model since it represents high face validity for withdrawal typically experienced by opioid-dependent individuals. Accordingly, we investigated the dose response relationship of the MAGL inhibitor JZL184 to reduce spontaneous withdrawal induced jumps, paw flutters, head shakes, diarrhea and weight loss in mice implanted with morphine pellets. Next, we tested the effectiveness of a novel combination of low-dose JZL184 and high dose of the FAAH inhibitor PF-3845 in reducing abrupt morphine withdrawal signs. This combination was selected in an attempt to produce maximal morphine anti-withdrawal effects, while minimizing side effects of JZL184 (see Chapter 4). The selective CB_1 receptor antagonist, rimonabant was employed to assess cannabinoid receptor involvement of the anti-withdrawal effects of JZL184 and the combination. In order to evaluate whether compensatory changes of endocannabinoid system occurs following treatment with MAGL and FAAH inhibitors in ICR mice, endocannabinoid and arachidonic acid (AA) levels were quantified in whole brain. In addition, we measured changes in CB_1 receptor binding and G-

protein activation from brains of mice repeatedly treated with MAGL and FAAH inhibitors. We hypothesize that the combination will also reduce all signs of spontaneous withdrawal without inducing cannabimimetic behavioral effects and receptor adaptations. We predict these results based on the observation that the combination produces augmented analgesic efficacy and lacks dependence potential (Kinsey, Wise et al., unpublished data; Chapter 2 results).

Chapter 5. Anti-diarrheal mechanism of action of MAGL inhibition: In vitro naloxone-precipitated withdrawal - ileum

Given the differential effects of MAGL and FAAH inhibition on diarrhea during morphine withdrawal we investigated the effects of JZL184 and PF-3845 on naloxone-precipitated contractions *in vitro* in the ileum (Chapter 5). 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. Since it is known that cannabinoids such as THC and WIN-55212 reduce naloxone-precipitated contractions in morphine-treated ilea, we hypothesize that MAGL and FAAH inhibitors will reduce naloxone-precipitated neurogenic contractions in a CB_1-dependent manner. The precursors for diarrhea include both hyper-contractility as well as increased secretion of fluids and electrolytes. Hence, we also evaluated whether PF-3845 and JZL184 inhibit naloxone-precipitated hypersecretion of electrolytes in morphine-treated ileum in the Ussing's chamber. To study the involvement of CB_1 receptors we investigated the effects of JZL184 in morphine-treated ilea from CB_1(-/-) mice. We hypothesized that both MAGL and FAAH inhibition will reverse naloxone-precipitated hypersecretion via CB_1 mechanism since its known that cannabinoid agonists have anti-secretory effects in small intestine. To verify that pharmacological actions of MAGL inhibition are mediated by increases of 2-AG and/or

concomitant decreases in arachidonic acid levels, we quantified whether PF-3845 and JZL184 alter endocannabinoids, free arachidonic acid, and prostaglandins in ileum.

Chapter 2: Evaluation of rimonabant-precipitated withdrawal in mice subjected to prolonged inhibition of eCB catabolic enzymes

In these experiments, we asked whether prolonged MAGL or FAAH blockade produces cannabinoid physical dependence, a phenotype that has been observed in rodents that have been exposed to repeated treatments with direct CB_1 agonists.

Preclinical Studies of Cannabinoid Dependence

Repeated administration of THC or other cannabinoid agonists leads to dependence in a variety of laboratory animals. Preclinical models for assessing dependence include those for measuring reinforcing and rewarding properties, such as self-administration, conditioned place preference, and intracranial self-stimulation (for a complete review see Panagis et al., 2008) as well as withdrawal signs. The withdrawal can include both physiological signs, as well as indicators of emotional state.

a. Characterization of cannabinoid withdrawal

The two general procedures used to induce a state of drug withdrawal in preclinical drug dependence studies are spontaneous withdrawal and precipitated withdrawal. Spontaneous withdrawal occurs following abrupt cessation of the drug, which is metabolized and cleared from the body. In precipitated withdrawal, an appropriate selective receptor antagonist is used to displace the agonist from the receptor resulting in the rapid onset of withdrawal symptoms. The specific withdrawal symptoms, intensity, and duration depend on the pharmacologic characteristics of the compound, with drugs from the same class generally sharing similar withdrawal syndromes. The precipitated withdrawal model is employed more often than the spontaneous withdrawal model to investigate cannabinoid dependence in laboratory animals

because of the long half-life of THC and subtle withdrawal effects make it difficult to observe and quantify upon abrupt cessation. The results of preclinical studies employing precipitated and spontaneous withdrawal procedures are shown in Table 2.

b. Spontaneous Cannabinoid Withdrawal in Laboratory Animals

The quantification of abrupt withdrawal signs in laboratory animals is very challenging as THC has a long half-life (Huestis, 2005) resulting in delayed onset and longer duration of withdrawal symptoms. The results from laboratory studies have often been mixed. The study of somatic withdrawal signs from repeated administration of THC and other cannabinoids has been examined in different animal species. While a variety of abnormal behavior signs, such as tremors, wet dog shakes and hyperirritability, have been reported in rats (Kaymakcalan et al., 1977), other studies failed to find significant abrupt withdrawal signs in rodents (Leite and Carlini, 1974; Aceto et al., 1996). Similarly, spontaneous withdrawal signs were not observed in pigeons following chronic exposure to THC (McMillan et al., 1973). Rhesus monkeys chronically infused with intravenously administered THC (0.5 mg/kg q.i.d. for 3 weeks) displayed substantial increases in gross movement, eye contact, and tooth baring during the first week of abstinence (Fredericks and Benowitz, 1980). These behaviors reflect rebound withdrawal symptoms, as they were initially suppressed by acute THC and subsequently underwent tolerance. In another study, food reinforced operant responding was decreased in monkeys following upon abrupt cessation of drug infusions (Beardsley et al., 1986). Spontaneous withdrawal responses also occur following discontinuation from chronic administration of the full cannabinoid receptor agonists WIN-55, 212 in rats (Aceto et al., 2001) and CP-55,940 in mice (Oliva et al., 2003). The data from these studies suggest that while spontaneous withdrawal from cannabinoids can be observed in experimental animals, the ability

to observe and quantify these withdrawal effects depends on various factors such as: the species, cannabinoid selected, duration of drug administration, the time point at which withdrawal is assessed, and the specific end points. While the spontaneous withdrawal procedure presents with considerable challenges and may be prone to false negatives because of the slow elimination of THC and its metabolites (Huestis, 2005), it is considered to possess more face validity than precipitated withdrawal procedure for modeling human cannabis withdrawal.

c. Precipitated Cannabinoid Withdrawal in Laboratory Animals

The development of the CB_1 receptor antagonist rimonabant (Rinaldi-Carmona et al., 1994) and other CB_1 receptor antagonists has provided highly useful tools to investigate precipitated withdrawal in cannabinoid-dependent animals. Rimonabant binds with high affinity to the CB_1 receptor and antagonizes the pharmacological effects of many cannabinoid receptor agonist activities in laboratory animals and humans (Rinaldi-Carmona et al., 1994; Compton et al., 1996a; Lichtman et al., 1998b; Winsauer et al., 1999; Huestis et al., 2001; Huestis et al., 2007).

Somatic Withdrawal Signs: Soon after the discovery of rimonabant, two separate groups independently used this antagonist to demonstrate somatic precipitated withdrawal signs in rats. These withdrawal signs include wet-dog shakes, forepaw fluttering, chewing, increased horizontal and vertical activity, retropulsion, and ptosis (Aceto et al., 1995; Tsou et al., 1995). Rimonabant also precipitates a profound withdrawal syndrome in mice that are chronically exposed to either THC or marijuana smoke (Cook et al., 1998; Wilson et al., 2006). The withdrawal signs commonly observed in mice, such as paw tremors and wet dog shakes, are observed consistently across all strains (Cook et al., 1998; Huang et al., 2009). Other signs such

as mastication, sniffing, and pilerection are of low frequency, but are scored in a cannabinoid composite withdrawal index (Hutcheson et al., 1998; Ledent et al., 1999; Tzavara et al., 2000; Lichtman et al., 2001b). It should be noted that rimonabant can also produce pharmacological effects in naïve animals that resemble withdrawal symptoms [for a review see(Lichtman and Martin, 2005)], including increases in ear scratching, head-shakes, and increased grooming behavior (Cook et al., 1998; Darmani and Pandya, 2000). Thus, it is critical to include appropriate vehicle-treated control groups in studies employing a CB_1 receptor antagonist to precipitate cannabinoid withdrawal to control for intrinsic effects of the drug at testing. Nonetheless, the observation that rimonabant elicits a far greater magnitude of withdrawal-like behavior (e.g., head shakes and paw tremors) in subjects receiving repeated administration of THC or repeated exposure to marijuana smoke than control animals (Cook et al., 1998; Wilson et al., 2006) supports the utility of this precipitated withdrawal model.

Aversive and Subjective Signs: There are few reports examining aversive or emotional responses in rodents undergoing cannabinoid withdrawal. Rimonabant challenge to THC-dependent mice elicited decreased time in open arm time in the elevated plus maze test (Huang et al., 2010), suggesting the occurrence of an anxiogenic-like state in subjects undergoing cannabinoid withdrawal, but failed to elicit aversive/dysphoric effects in the conditioned place aversion test (Hutcheson et al., 1998). In THC-dependent dogs, rimonabant precipitates a withdrawal syndrome that includes distinct gastrointestinal signs such as diarrhea, vomiting, excessive salivation as well as decreases in social behavior and increases in restless behavior and trembling (Lichtman et al., 1998a). Other studies are focusing on subjective signs of cannabinoid withdrawal. Monkeys chronically treated with THC demonstrated robust discrimination of the CB_1 antagonist rimonabant (McMahon and France, 2003; McMahon,

2006; Stewart and McMahon, 2010). THC discontinuation produced a similar discriminative stimulus to rimonabant in THC-treated animals. The data suggest that the interoceptive cues of THC cessation-induced abstinence are mediated by the CB_1 receptor.

Precipitated Withdrawal with Other Cannabinoid Agonists: Rimonabant also precipitates withdrawal signs following chronic administration of other cannabinoid agonists such as anandamide, methanandamide, WIN-55,212, CP-55,940 and HU-210 (Rodriguez de Fonseca et al., 1997; Aceto et al., 1998; Rubino et al., 1998; Aceto et al., 2001). The withdrawal syndrome precipitated following repeated anandamide administration is not as robust as with other cannabinoids (Costa et al., 2000; Falenski et al., 2010).

Table 2. Preclinical studies investigating cannabinoid withdrawal employing precipitated and spontaneous withdrawal procedures

Species	**Type of Withdrawal**	**Agonist, Dosing Regimen, Route of Administration**	**Dependent Variable**	**Reference**
Mouse	Precipitated	THC, 6.5 days: 10 mg/kg s.c. (b.i.d.)	Somatic signs	(Cook et al., 1998)
Mouse	Precipitated	THC, 5.5 days: 10 20 mg/kg i.p. (b.i.d.)	Somatic signs, adenylyl cyclase overshoot in cerebellum	(Hutcheson et al., 1998)
Mouse	Precipitated	THC, 5.5 days: 20 mg/kg i.p. (b.i.d.)	Somatic signs	(Valverde et al., 2000)
Mouse	Precipitated	THC, 6.5 days: 10 mg/kg	Somatic signs	(Lichtman et al.,

		s.c. (b.i.d.)		2001a)
Mouse	Precipitated	THC, 5.5 days: 20 mg/kg i.p. (b.i.d.)	Somatic signs, body wt.	(Anggadiredja et al., 2003)
Mouse	Spontaneous	CP, 6.5 days: 0.5 mg/kg i.p. (b.i.d.)	Increased locomotor activity, endocrine gene transcription levels	(Oliva et al., 2003)
Mouse	Precipitated	THC, MAR, 5 days: 200 mg; 5 or 10 mg/kg/ i.v. (s.i.d.)	Somatic signs	(Wilson et al., 2006)
Mouse	Precipitated	THC, 5.5 days: 20 mg/kg i.p. (b.i.d.)	Somatic signs	(Tourino et al., 2007)
Mouse	Precipitated	THC, 4.5 days: 25 mg/kg s.c. (b.i.d.)	Somatic signs, activity	(Huang et al., 2009)
Mouse	Precipitated	THC, 5.5 days: 50 mg/kg s.c. (b.i.d.); 10 mg/kg (s.i.d.)	Somatic signs	(Schlosburg et al., 2009)
Mouse	Precipitated	THC, AEA, 5.5 days: 50 mg/kg s.c. (b.i.d.)	Somatic signs	(Falenski et al., 2010)
Mouse	Precipitated	THC, 10 days: 10 mg/kg s.c. (s.i.d.)	Anxiogenic-like effects (plus maze)	(Huang et al., 2010)
Mouse	Precipitated	JZL184 (40 mg/kg), THC (10,50 mg/kg) 6 days	Somatic signs	(Schlosburg et al., 2010)

Rat	Precipitated	THC, 4 days: 0.5-4; 2.5-20; 12.5-100 mg/kg/hr. i.p.	Somatic signs	(Aceto et al., 1995)
Rat	Precipitated	THC, 6.5 days: 15 mg/kg i.p. (b.i.d.)	Activity	(Tsou et al., 1995)
Rat	Precipitated, spontaneous	`THC, 4 days: 12.5-100; 2.5-20; 0.5-4 mg/kg/ 24hr. i.p. (cont.)	Somatic signs with precipitated only	(Aceto et al., 1996)
Rat	Precipitated	THC, 6.5 days: 15 mg/kg i.p. (b.i.d.)	Somatic signs	(Diana et al., 1998)
Rat	Precipitated	CP, 6.5 days: 0.4 mg/kg i.p. (b.i.d.)	Somatic signs, activity	(Rubino et al., 1998)
Rat	Precipitated	THC, 6 days: 10-30; 10-40; 10-50 mg/kg s.c. (b.i.d.)	Operant rate	(Beardsley and Martin, 2000)
Rat	Precipitated, spontaneous	WIN, 4 days: 1-8; 2-16; 4-16 mg/kg/24 hr. i.p. (cont.)	Somatic signs, body weight loss	(Aceto et al., 2001)
Rat	Precipitated	THC, 4 days: 12.5-100 mg/kg/24 hr. i.p. (cont.)	Somatic signs	(Breivogel et al., 2003)
Rat	Precipitated	HU210, 5.5 days: 100 µg/kg i.p. (b.i.d.)	Somatic signs	(Cui et al., 2001)
Rat	Precipitated	THC, 8 days: 10 mg/kg i.p. (b.i.d.)	Somatic signs, activity	(Gonzalez et al., 2004)
Dog	Precipitated	THC, 10 days: 0.6-1 mg/kg	Somatic signs	(Lichtman et al.,

		i.v. (b.i.d.)		1998a)
Monkey	Spontaneous	THC, 10 days 0.05 mg/kg/hour i.v.	Operant rate suppression	(Beardsley et al., 1986)
Monkey	Precipitated, spontaneous	THC, Continuous (drug discrimination study): 1 mg/kg (b.i.d.)	Somatic signs, operant rate suppression	(Stewart and McMahon, 2010)

AEA, anandamide; b.i.d., twice a day; HU-210, a synthetic cannabinoid; i.p., intraperitoneally; i.v., intravenously; CP, CP 55940; MAR, marijuana; s.c., subcutaneously; s.i.d, once a day; cont., continuous infusion;THC, Δ^9-tetrahydrocannabinol; WIN, WIN 55212-2; (Ramesh et al., 2011b).

If targeting endocannabinoid catabolic enzymes is indeed a viable approach to treat other abuse disorders, it is important to know whether these inhibitors would themselves have abuse or dependence liability. FAAH inhibitors have been extensively investigated in a variety of such paradigms. Importantly, mice with persistently elevated anandamide levels following inhibition of FAAH, with the inhibitor URB597 and mice lacking the FAAH enzyme, do not show any withdrawal symptoms following treatment with rimonabant (Schlosburg et al., 2009; Falenski et al., 2010). Additionally, it has been demonstrated that URB597 does not produce rewarding effects in the rat conditioned place preference paradigm, does not substitute for THC in the drug discrimination paradigm, and is not self-administered in nonhuman primates (Gobbi et al., 2005; Justinova et al., 2008). There is also supporting biochemical evidence that chronic treatment with FAAH inhibitors does not lead to long-term neural adaptations. Once-daily dosing with URB597 for 5 weeks exerted anxiolytic effects without altering CB_1 messenger RNA levels (Bortolato et al., 2007). Likewise, repeated dosing of PF-3845 did not lead to desensitization or downregulation of CB_1 receptors and did not alter CB_1 receptor-mediated synaptic plasticity of hippocampal neurons. In contrast, sustained inactivation of MAGL, by

repeated treatment with high doses of JZL184 results in loss of its analgesic efficacy and produces cross-tolerance to the effects of other CB_1 agonists in mice. Prolonged MAGL blockade with JZL184 also results in desensitization and downregulation of CB_1 receptors as well as impaired endocannabinoid dependent synaptic plasticity (Schlosburg et al., 2010). To support the preceding literature, we investigated the ability of prolonged MAGL and FAAH inhibition, using both pharmacologic and genetic approaches, to elicit rimonabant-precipitated cannabinoid withdrawal in C57 mice.

Methods

Subjects

Male C57BL/6J mice (Jackson laboratories) 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^{o}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

THC and CB_1 receptor antagonist rimonabant were obtained from the National Institute on Drug Abuse (Bethesda, MD). URB597 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 URB597 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. All injections were administered in volume of 0.01 ml per 1 g body weight. THC was administered via subcutaneous (s.c.) injection, whereas PF-3845, JZL184, rimonabant, and URB597 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, respectively, following systemic administration (Ahn et al., 2009; Long et al., 2009a). THC was given 30 min before rimonabant treatment.

Rimonabant-precipitated cannabinoid withdrawal

The mice were given a once daily injection of PF-3845 (10mg/kg, i.p.), JZL184 (4/8/16/40mg/kg, i.p.) or Δ^9–THC (10mg/kg s.c.) for 6 days. Under all conditions mice were give an i.p. injection of rimonabant 2 hr after the last injection of PF-3845, JZL184 and JZL195 or 30 mins after last THC injection.

Behavior was then observed and recorded for 1 hr, using the ANY-maze™ software after the rimonabant injection. The videos were then scored by time-sampling examining periods of 5 min intervals, and then moving 5 min ahead on the video starting at minute 5 post-rimonabant injection (i.e. 5-10 min, 15-20 min, etc). At the end of the hour video, each animal had a similar sampled 30 min period observed and scored from their recordings. While several behavioral endpoints were observed that have been previously described in the literature as common in mice going through cannabinoid withdrawal (i.e. ptosis, retropulsion, piloerection, etc.), behaviors scored and presented are the most common, quantifiable, and with the highest inter-rater reliability (Cook et al., 1998). The primary behavior observed was front paw flutters that included a range of behavior from single-paw tremors to full fluttering/shaking of both

paws simultaneously. These motions of the paws are not typical of normal behavior. Also recorded were head twitches, which generally manifest as rotational shakes of the head, similar to what is described as “wet dog shakes” in rat.

Results

2.1. Rimonabant-precipitated withdrawal potential following repeated FAAH inhibition

The aim of this experiment was to examine whether repeated administration of FAAH inhibitors produces cannabinoid physical dependence. Mice were treated with URB597 (10 mg/kg) or vehicle twice daily for 5.5 days and were challenged with rimonabant 1 h after their final injection. Rimonabant produced no significant differences between mice that were given repeated injections of URB597 and vehicle on paw tremors [$p = 0.44$; Figure 3A] as well as head twitching [$p = 0.68$; Fig. 3B]. We also tested the longer-acting, more selective second generation FAAH inhibitor, PF-3845. Once-daily treatment with PF3845 (10 mg/kg) elicited minimal signs following rimonabant precipitation, with no significant differences in the intensity of paw flutters [$p = 0.30$; Fig. 3C] and head twitches [$p = 0.39$; Fig. 3D] in comparison to vehicle treated mice.

2.2. Rimonabant precipitates withdrawal in mice treated repeated with high dose, but not low dose, JZL184

In order to examine withdrawal potential from sustained blockade of MAGL, we evaluated rimonabant-precipitated withdrawal signs in mice treated with either vehicle or JZL184 (4, 8, 16 or 40 mg/kg, i.p./once a day/ 6 days). JZL184 treatment led to an overall significant increase in the intensity of paw flutters [$F(4,42) = 7.551$; $p<0.001$; Fig. 4A]. Post-hoc analysis with Dunnett's test showed that repeated treatment with high doses of JZL184 (viz. 16 and 40 mg/kg) resulted in significant increases in incidence of paw flutters, and that withdrawal is not observed following repeated treatment with low doses of JZL184 (4 and 8 mg/kg). In addition, JZL184 treatment had no effect on the intensity of head twitches at any of the doses tested (Fig.

4B). This pattern of findings suggests that sustained and complete blockade of MAGL is necessary to elicit cannabinoid physical withdrawal.

2.3. Rimonabant precipitates withdrawal in mice with simultaneous complete MAGL and FAAH inhibition

In order to examine additional withdrawal potential due to simultaneous elevations in AEA and 2-AG, we treated both FAAH (+/+) and (-/-) mice treated repeatedly with the MAGL inhibitor JZL184. Upon precipitation with the CB_1 antagonist rimonabant, fluttering and head twitching incidences were recorded as representative of cannabinoid withdrawal. As shown in Figure 5A, paw tremors were increased in mice treated with repeated JZL184 (40 mg/kg). Accordingly, there was a significant treatment effect [$F(1,69)=45.0$, $p<0.001$] for JZL184, however there was no effect of genotype on the expression of paw flutters ($p=0.29$) nor an interaction between treatment and genotype ($p=0.36$). When comparing to low-dose (10mg/kg daily) THC treated mice, JZL184 treated mice showed comparable elevations in fluttering versus vehicle treated mice (THC vs. JZL184 in FAAH (+/+): $p=0.99$; Scheffe's post-hoc; Fig 5A). When examining head twitching response, shown in Figure 5B, there was an effect of treatment [$F(4,77)=3.6$, $p<0.01$]. Post-hoc analysis showed that the THC-treated mice were the only group to show elevated head twitching above that of vehicle treated groups ($p<0.05$; Scheffe's post-hoc).

To control for the possibility of developmental adaptations in FAAH (-/-) mice, follow-up experiments were performed using pharmacological means of dual MAGL-FAAH inhibition. This experiment tested combined administration of JZL184 (40 mg/kg) and PF3845 (10 mg/kg). Repeated treatment with PF-3845 did not lead to physical withdrawal signs following

rimonabant challenge. In addition, PF-3845 did not alter the magnitude of paw flutters following rimonabant challenge in mice given repeated JZL184 [$F(3, 28) = 10.0$, $p < 0.001$; Figure 6A]. While there was an overall treatment effect on head twitches [$F(3, 28) = 3.5$, $p < 0.05$; Figure 6B], no individual group was significantly elevated from vehicle.

2.4. Sustained simultaneous partial MAGL and complete FAAH inhibition does not lead to physical withdrawal on rimonabant challenge

Finally, we evaluated whether repeated treatment with a combination of low-dose JZL184 and high-dose PF-3845 would also lead to cannabinoid physical withdrawal. This combination was selected to retain beneficial therapeutic effects of MAGL and FAAH inhibition while avoiding side-effects of complete MAGL inhibition. In this experiment, mice were repeatedly treated with either vehicle, JZL184 (40mg/kg) or JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) for 6 days and followed by rimonabant challenge 2hr after the last injection. While rimonabant precipitated paw flutters in mice treated with the high dose JZL184, mice treated with the combination of low dose JZL184 and high dose PF-3845 did not exhibit elevated paw flutters [$F(2,21) = 14.101$; $p<0.001$; Fig. 7A]. Neither treatment elicited significantly elevated head twitches in comparison to vehicle treated mice (Fig. 7B).

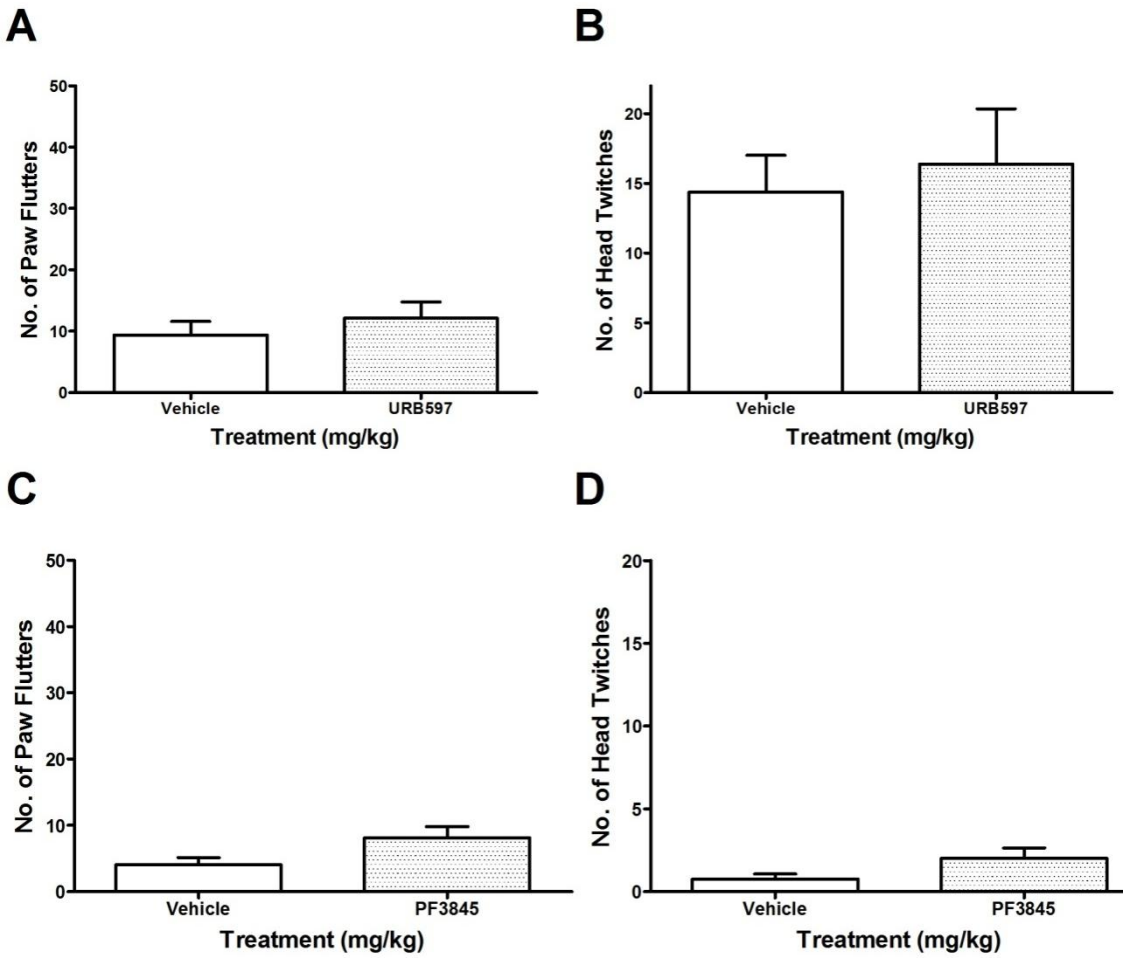

Figure 3. Evaluation of rimonabant-precipitated withdrawal signs following repeated high-dosing of FAAH inhibitors.

The first generation, short-acting inhibitor URB597 was given at 10 mg/kg twice daily, while the more potent and longer-acting inhibitor PF3845 was given daily at a dose of 10 mg/kg. Neither FAAH inhibitor given repeatedly elicited (A) and (C) paw flutters or (B) and (D) head twitches compared to vehicle. Data represented as mean ± SEM; n = 8 mice/ group.

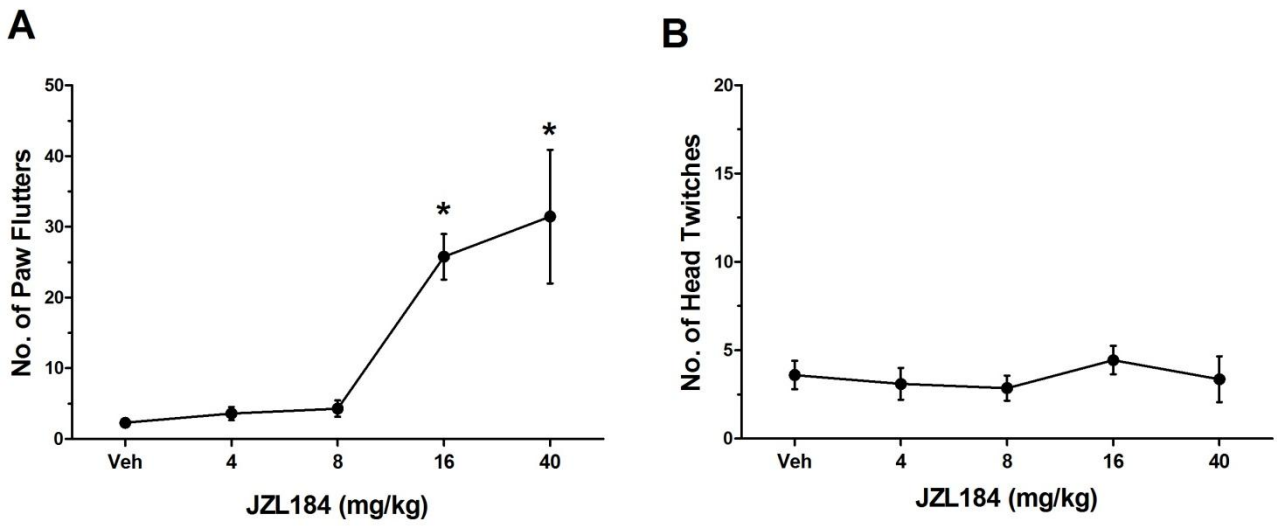

Figure 4. Rimonabant-precipitated withdrawal in mice treated once a day for 6 days with the MAGL inhibitor JZL184 (4,8,16 or 40 mg/kg) or vehicle.

(A) Rimonabant precipitated a significantly increased intensity of paw flutters in mice treated with 16 mg/kg and 40 mg/kg JZL184 in comparison to vehicle treated mice. In contrast, mice treated with 4 mg/kg and 8 mg/kg JZL184 did not exhibit elevated incidences of paw flutters. (B) Neither dose of JZL184 precipitated a siginificantly altered intensity of head twitches. Data represented mean ± SEM; n=7-11 mice/ group; *$p < 0.05$ v/s vehicle control.

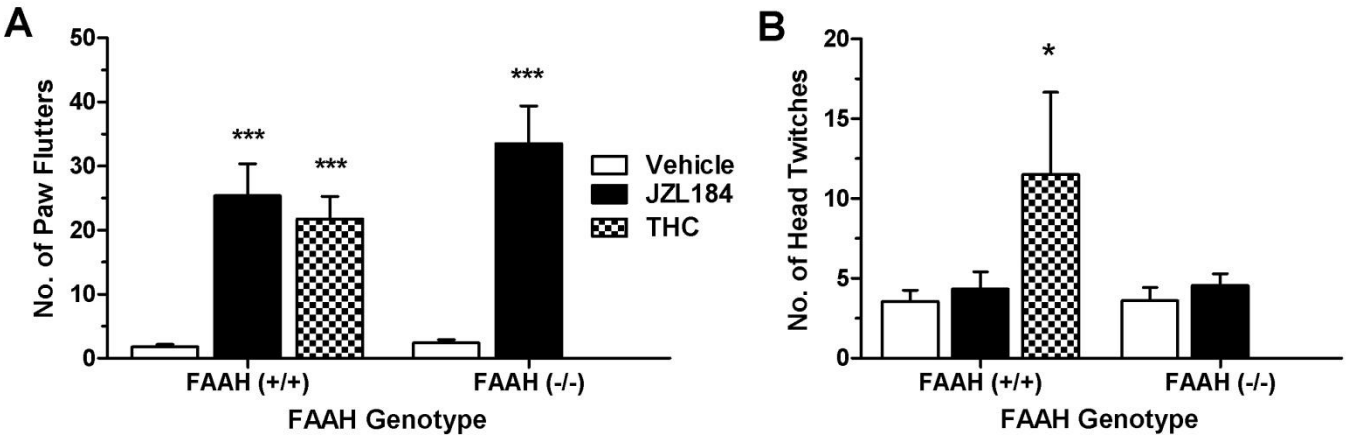

Figure 5. Prolonged elevation of 2-AG, by repeated JZL184 administration, leads to signs of cannabinoid physical dependence in both FAAH (+/+) and (-/-) mice

(A) Mice treated with 6-day JZL184 (40 mg/kg) show significant paw fluttering withdrawal behavior upon precipitation with the cannabinoid antagonist rimonabant (10 mg/kg) equally in both FAAH (+/+) and (-/-) mice. The level of fluttering is comparable to that of a 6-day moderate dose of THC (10 mg/kg). (B) JZL184 did not elicit head twitch behavior in a manner similar to THC treatment. Data represented as mean ± SEM; n = 8-15 per group, ***$p < 0.001$ v/s respective vehicle controls.

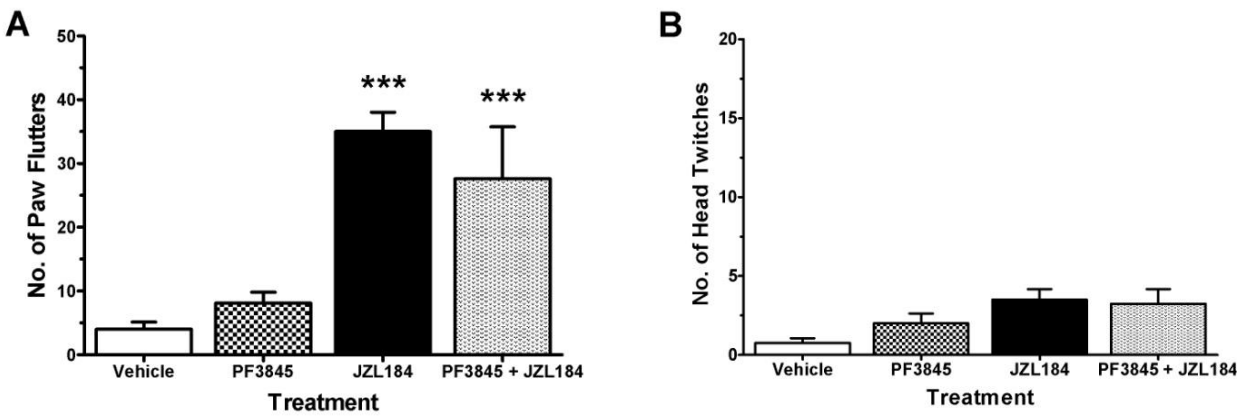

Figure 6. Cannabinoid precipitated withdrawal elicited by prolonged MAGL inhibition is not altered by simultaneous inhibition of FAAH.

(A) Mice treated with 6-day JZL184 (40 mg/kg) show significant paw fluttering withdrawal behavior upon precipitation with the cannabinoid antagonist rimonabant (10 mg/kg), which is absent following 6-day FAAH inhibition via PF-3845 (10 mg/kg), nor enhanced by co-administration of both enzyme inhibitors simultaneously. (B) None of the combination of enzyme inhibitors elicited altered head twitching behavior. n = 8 per group, ***$p < 0.001$ v/s respective vehicle controls.

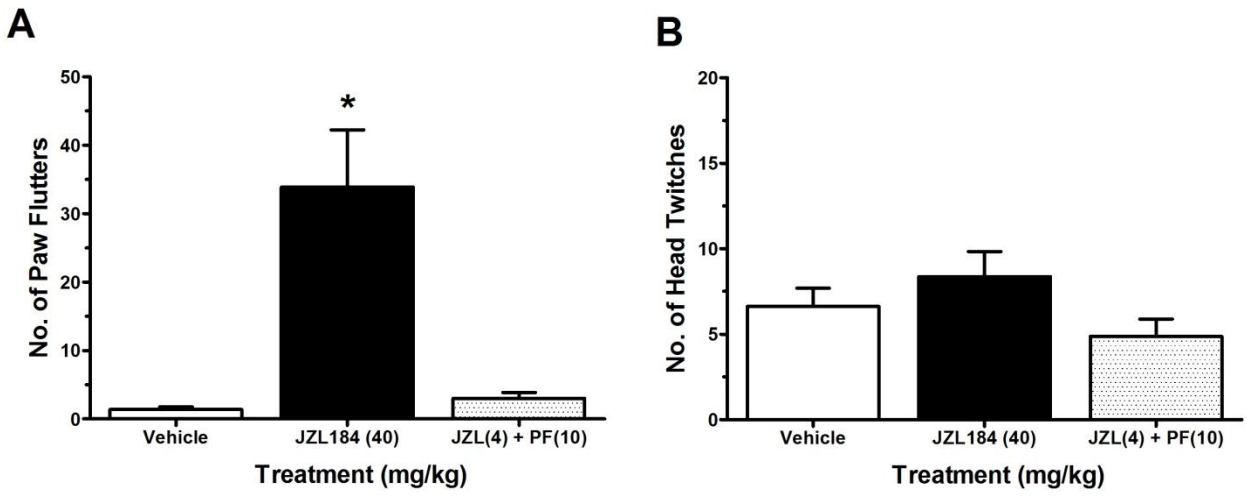

Figure 7. Rimonabant does not precipitate withdrawal signs in mice treated repeatedly with a combination of low dose JZL184 and high dose FAAH inhibitor PF-3845

(A) Rimonabant precipitated significantly elevated paw flutters in mice treated for 6 days with a high dose of JZL184 (40 mg/kg) but not in those treated with a combination of low-dose JZL184(4 mg/kg) and PF-3845(10 mg/kg) (B) Head twitching behavior is not altered by either treatment. Data represented as mean ± SEM; n=8 mice/group; *$p<0.05$ v/s vehicle control.

2.5. Discussion

In these experiments we have examined the potential of repeated administration of MAGL and FAAH inhibitors to produce physical cannabinoid withdrawal in mice. Repeated treatment with the FAAH inhibitors, URB597 and PF-3845, did not lead to rimonabant-precipitated cannabinoid withdrawal. On the contrary, prolonged treatment with high doses of the MAGL inhibitor JZL184 by itself or in combination with FAAH inhibition/deletion led to physical withdrawal on rimonabant challenge. However, repeated treatment with low doses of JZL184 or a combination of low dose JZL184 with PF-3845 did not lead to cannabinoid withdrawal.

The ability of prolonged complete MAGL inhibition to elicit precipitated cannabinoid withdrawal represents a drawback of using these inhibitors in treatment of abuse disorders. It should be noted that the severity of withdrawal is comparable in magnitude to that of the lowest level of quantifiable withdrawal from repeated THC treatment. This may indicate that while the risk of physical dependence may exist, it is minimal compared to exogenous cannabinoid agonists. Also promising is the observation that though simultaneous complete FAAH and MAGL inhibition produces enhanced acute cannabinoid-mediated activity (Long et al., 2009c), there appears to be little enhanced magnitude of precipitated withdrawal compared to MAGL inhibition alone. This observation was replicated using both genetic and pharmacological tools.

The lack of rimonabant-precipitated cannabinoid withdrawal signs by repeated URB597 or PF3845 treatment adds to a growing body of literature demonstrating that FAAH inhibitors lack dependence potential (Solinas et al., 2007; Justinova et al., 2008) that are typical of exogenous cannabinoids. While prolonged treatment with high doses of JZL184 leads to cannabinoid dependence; repeated treatment with low, pharmacologically active doses of

JZL184 at 4 and 8 mg/kg did not lead to physical withdrawal. JZL184 at these doses also does not undergo tolerance to its analgesic effects in the CCI and carrageenan pain assays (Kinsey et al., Ghosh et al., unpublished data). Finally, rimonabant did not precipitate withdrawal in mice repeatedly treated with a combination of low-dose JZL184 (4 mg/kg) and PF-3845 (10 mg/kg). This combination produces augmented anti-allodynic effects in both the CCI and carrageenan pain assays and repeated treatment does not lead to tolerance or functional CB_1 receptor adaptations in C57BL6/J mice (Wise et al, unpublished data). The lack of dependence and analgesic tolerance following repeated administration of FAAH inhibitors and low doses of JZL184 indicates that they are a viable target for the treatment of chronic pain conditions and abuse disorders.

Thus, elevation of the two primary endocannabinoids in brain leads to different consequences on physical dependence. These differential actions of prolonged MAGL and FAAH inhibition are not fully understood and may be partly attributed to (1) higher concentrations of 2-AG compared with anandamide after sustained blockade of their degradative enzymes and (2) differences in the efficacy of these endocannabinoids at the CB_1 receptor (Howlett and Mukhopadhyay, 2000) or (3) the result of regionally or neuron selective production and release of eCBs.

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Chapter 3: Effects of inhibition of eCB catabolic enzymes on precipitated somatic opioid withdrawal signs

A case report from the 19th century suggested that an extract of *Cannabis sativa* may ameliorate opiate addiction (Birch, 1889). In this report, a tincture of cannabis extract alleviated signs of opioid abstinence such as lack of appetite, anorexia, disordered bowels and conscious delusions in an opium poppy addict. Modern studies corroborated this idea by demonstrating that THC, the primary psychoactive constituent of cannabis, attenuates the intensity of naloxone-precipitated opioid withdrawal signs in morphine-dependent rodents (Hine et al., 1975; Bhargava, 1976). A low oral dose of THC when co-administered with high-dose oral morphine attenuated the development of dependence and expression of withdrawal signs in mice (Cichewicz and Welch, 2003). A 30-day pretreatment with THC also resulted in decreased withdrawal signs in morphine-dependent mice (Jardinaud et al., 2006). The CB_1 receptor appears to play a significant role in modulating dependence to morphine and other opiates. CB_1 (-/-) mice show significantly reduced self-administration of morphine and attenuated naloxone-precipitated morphine withdrawal signs (Ledent et al., 1999; Lichtman et al., 2001b). Other exogenous cannabinoid agonists such as AEA and 2-AG have been demonstrated to reduce signs of naloxone-precipitated withdrawal signs in rodents (Vela et al., 1995; Yamaguchi et al., 2001). 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). It has been reported that there are significant changes in brain levels of endocannabinoids following chronic morphine treatment (Gonzalez et al., 2003; Vigano et al., 2004; Caille et al., 2007). These findings support the notion that

endocannabinoids and the CB_1 receptor play an important role in modulating the expression of opioid withdrawal. In this section, we describe experiments that evaluated whether elevating endocannabinoids, through the inhibition of their catabolic enzymes, attenuates naloxone-precipitated withdrawal symptoms in morphine dependent mice. The precipitated withdrawal model will enable a relatively high throughput initial screen of the inhibitors to assess their anti-withdrawal effects. We investigated the efficacy of the respective MAGL and FAAH inhibitors, JZL184 and PF-3845, as well as THC to reduce naloxone-precipitated withdrawal signs. Additionally, we investigated whether the effects of these drugs in modulating opioid withdrawal are mediated through the CB_1 receptor.

Methods

Subjects

Selection of Strain: Initially C57BL6/J (25g; M/F) mice were used for studying withdrawal. However, morphine (75 mg) pellet implantation led to fatalities in approximately 40% of the mice, presumably due to respiratory depression or other consequences of overdose. Moreover, the surviving mice lost a significant portion of body weight during the 3 day period after pellet implantation. ICR mice (Harlan), on the other hand, did not exhibit significant fatalities and survived the 3 day pellet implantation without losing body weight. For the FAAH (-/-) studies, we found that using male mice weighing over 30 g increased survival rate to about 80% in this study. Given the increased mortality and morbidity observed following morphine pellet implantation in C57 mice, we selected ICR mice for the rest of the *in vivo* studies.

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^{o}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, 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.

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 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.

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.

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, and 2-AG levels are reported as pmol or nmol per gram of tissue, where applicable. Data were analyzed using t-test or two-way between measures analysis of variance (ANOVA), followed by Dunnett's or Scheffe's post hoc test. Differences of $p<0.05$ were considered significant.

Results

3.1. Naloxone precipitates a profound withdrawal syndrome in morphine-dependent mice

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 Fig. 8, 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 ($p<0.01$).

3.2. The MAGL inhibitor JZL184 and THC dose-dependently 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. 9A] and paw fluttering [$F(4,30) = 11.3$; $p < 0.001$; Fig 9B], weight loss [$F(4,30) = 17.9$; $p < 0.001$; Fig. 8C], and diarrhea (Fig 9D), 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. 9A], paw fluttering [$F(3,26) = 4.9$; $p < 0.01$; Fig.9B], diarrhea (Fig. 9D), and weight loss [$F(3, 26) = 6.37$; $p < 0.01$; Fig. 9C]. The highest dose of JZL184 (40 mg/kg) completely prevented the occurrence of diarrhea (Fig. 9D).

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. 8). 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. 10A], paw flutters [$F(1, 23) = 8.2$; $p < 0.01$; Fig. 10B] and weight loss [$F(1, 23) = 10.5$; $p < 0.01$; Fig. 10C]. Similarly, the anti-diarrheal effects of JZL184 were completely reversed by rimonabant treatment (Fig. 10D). 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. 11). The interaction between JZL184 pretreatment and SR144528 failed to achieve statistical significance for jumps [$p = 0.24$; Fig. 11A], paw flutters [$p = 0.76$; Fig. 11B] and weight loss [$p = 0.55$; Fig. 11C]. In addition, SR144528 did not block the anti-diarrheal effects of JZL184 [Fig. 11D]. Taken together, these data indicate that JZL184 attenuates naloxone-precipitated withdrawal symptoms through a mechanism that requires the activation of CB_1 receptor.

3.3. JZL184 maintains its anti-withdrawal effects after repeated treatment.

In order to determine whether prolonged MAGL inhibition maintains anti-opioid withdrawal efficacy, we treated the mice for six days with the MAGL inhibitor JZL184 (4 or 40 mg/kg, i.p.) during which time a 75mg morphine pellet was implanted on day 3. Withdrawal was precipitated with an acute treatment of naloxone (1 mg/kg, s.c.) 72 h after pellet implantation. JZL184 (40mg/kg) reduced naloxone-precipitated jumping [$F(2,20) = 5.422$; $p < 0.01$; Fig. 12A] but did not significantly reduce the number of paw flutters (Fig. 12B) ($p=0.58$).

There was however, a significant reduction in the amount of weight loss [$F(2,20) = 6.871$; $p < 0.001$; Fig. 12C] and percent of mice with diarrhea (Fig. 12D). In contrast, repeated treatment with JZL184 (4mg/kg) did not affect any of the measured withdrawal symptoms.

3.4. 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. 13A), paw flutter incidents ($p<0.05$; Fig. 13B), and weight loss ($p<0.05$; Fig. 13C) compared with FAAH (+/+) mice. Genetic deletion of FAAH did not affect the occurrence of naloxone-precipitated diarrhea [Fig. 13D]. 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. 14A 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. 14A] and paw flutters [$F(1, 41) = 3.2$; $p < 0.01$; Fig. 14B]. However, PF-3845 did not affect the weight loss during withdrawal (Fig. 14C), or the occurrence of diarrhea (Fig. 14D) 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.

3.5. Measurement of endocannabinoid levels in brain following naloxone-precipitated withdrawal

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 3, 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.

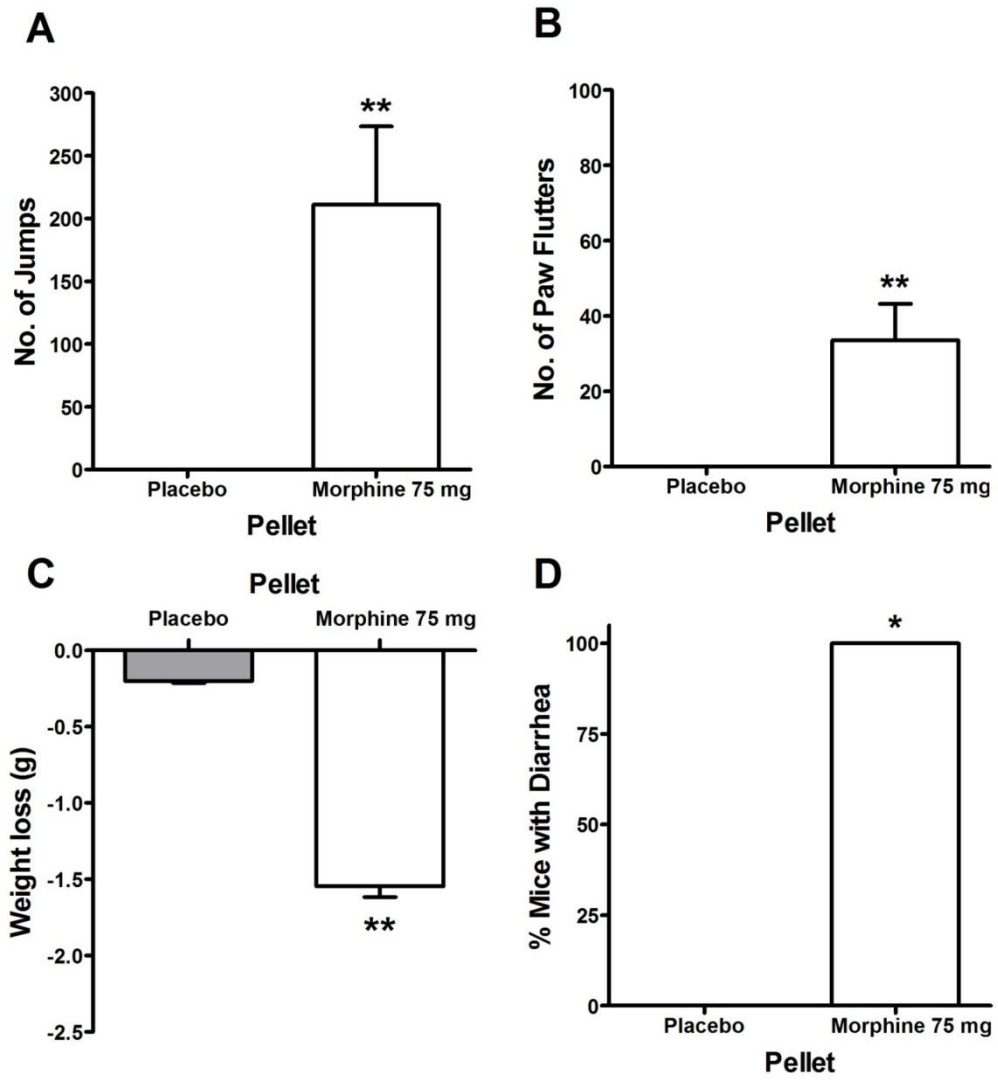


Figure 8. Naloxone precipitates somatic withdrawal signs in mice implanted with a subcutaneous morphine pellet (75 mg) for 3 days but not in mice implanted with a placebo pellet.

The withdrawal signs measured included: (A**)** jumps, (B) paw flutters, (C) weight loss and (D) diarrhea. Data expressed as mean ± SEM. *p,0.05; **p < 0.01 v/s placebo control; n = 6-8 mice/group

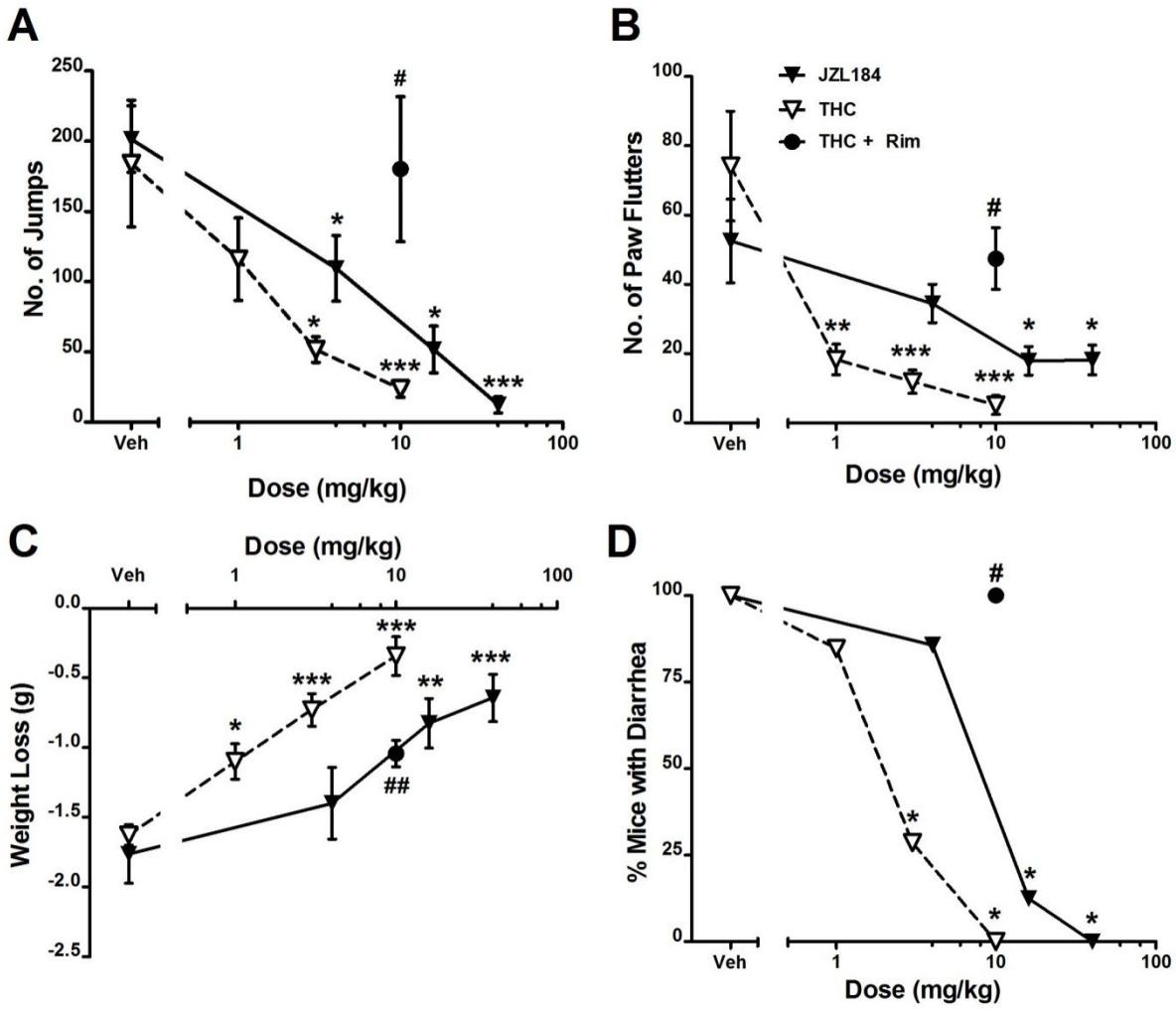


Figure 9. The primary psychoactive constituent of *cannabis sativa* THC and the irreversible MAGL inhibitor JZL184 attenuate the intensity of naloxone-precipitated withdrawal signs in a dose-dependent manner.

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**)** jumps, (B) paw flutters, (C) weight loss and (D) 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 THC(10mg/kg); n = 6-8 mice/group.

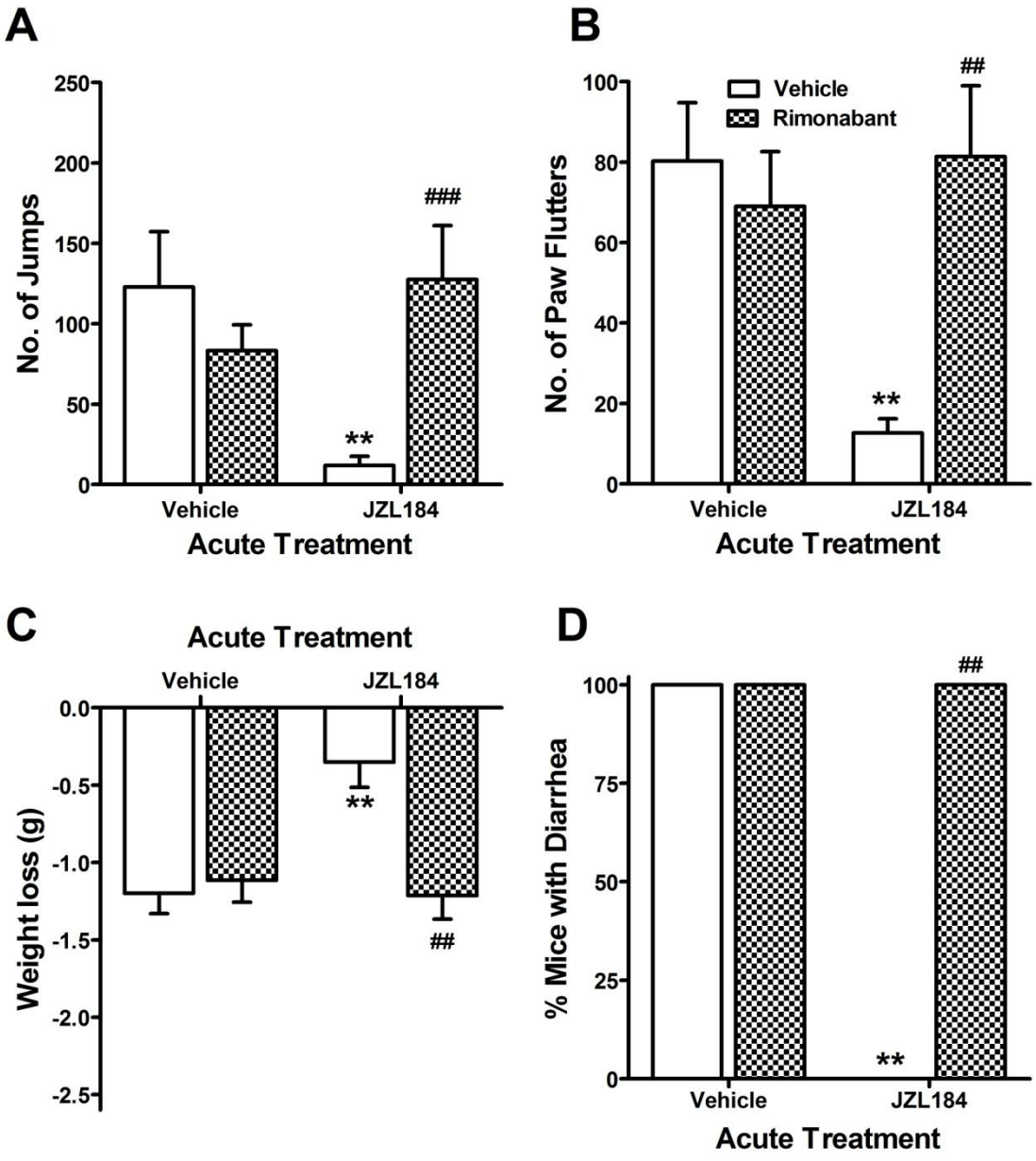


Figure 10. The MAGL inhibitor JZL184 attenuates the intensity of naloxone-precipitated morphine withdrawal signs in a CB_1 receptor dependent manner

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**)** jumps, (B) paw flutters, (C) weight loss and (D) diarrhea. Data expressed as mean ± SEM for panels A-C. **p<0.01 vs. vehicle; ## p<0.01 vs. JZL184-vehicle; n = 6-8 mice/group.

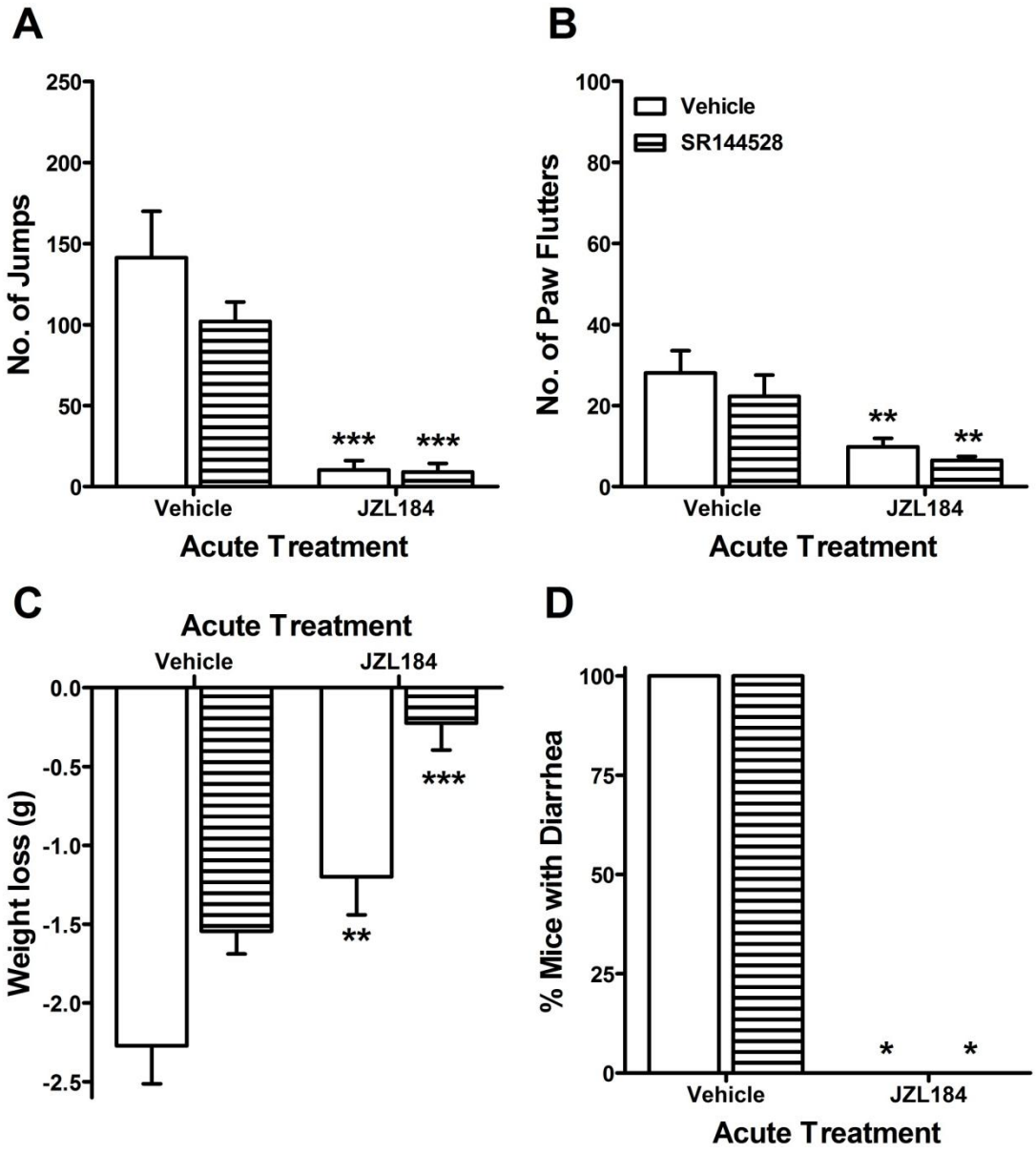


Figure 11. The reduction of intensity of naloxone-precipitated morphine withdrawal signs is not CB_2 receptor mediated.

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**)** jumps, (B) paw flutters, (C) weight loss and (D) diarrhea. Data expressed as mean ± SEM for panels A-C. ***$p<0.001$, **$p<0.01$ vs. vehicle; n = 7-8 mice/group.

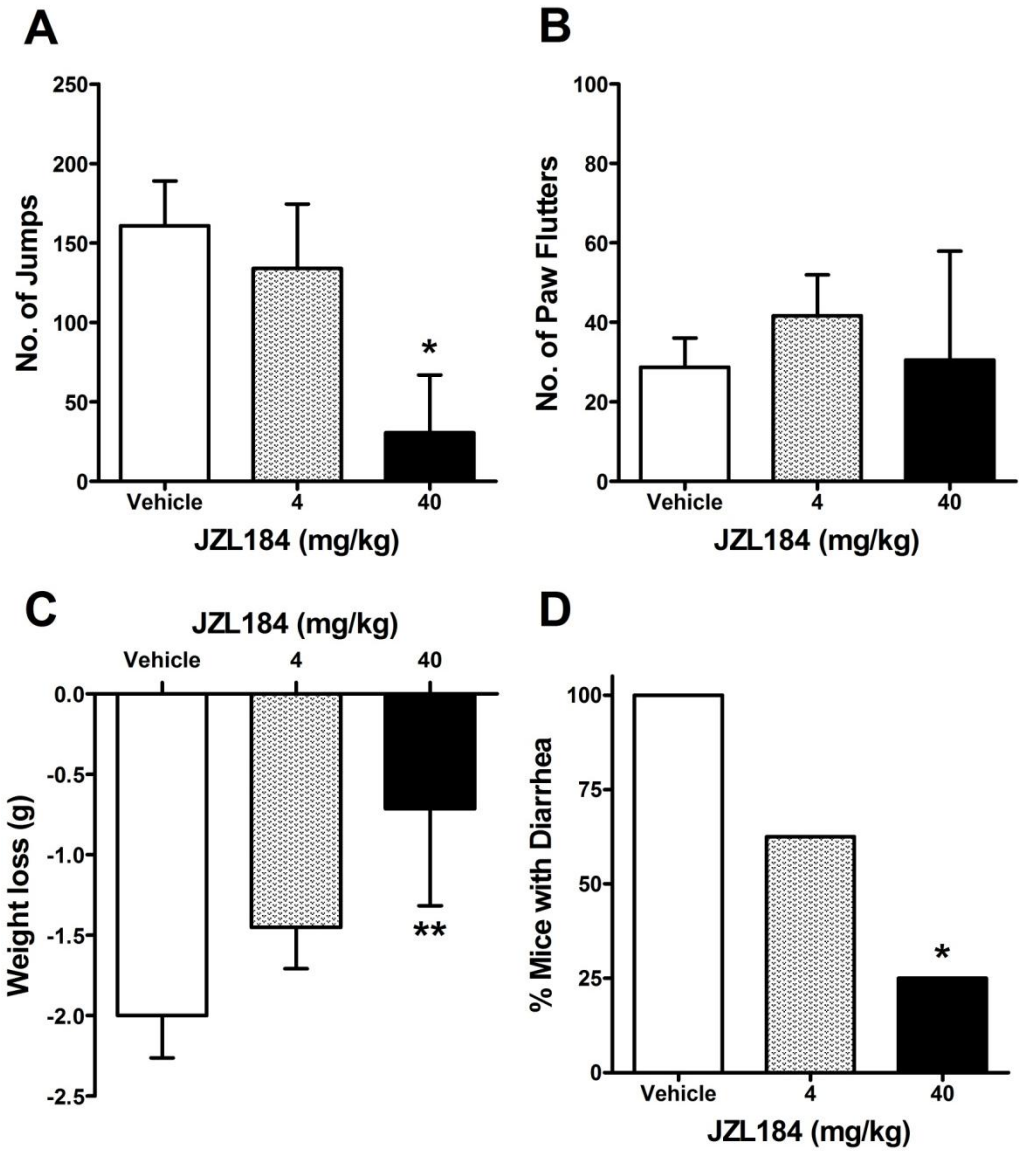


Figure 12: JZL184 maintains it anti-withdrawal effects following repeated treatment in morphine-dependent mice

Repeated treatment with JZL184 (40mg/kg, once a day, 6 d) reduced the intensity of jumps, weight loss and occurrence of diarrhea, but repeated treatment with a low (4mg/kg) dose did not have anti-withdrawal effects. The withdrawal signs measured were (A**)** jumps, (B) paw flutters, (C) weight loss and (D) 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.

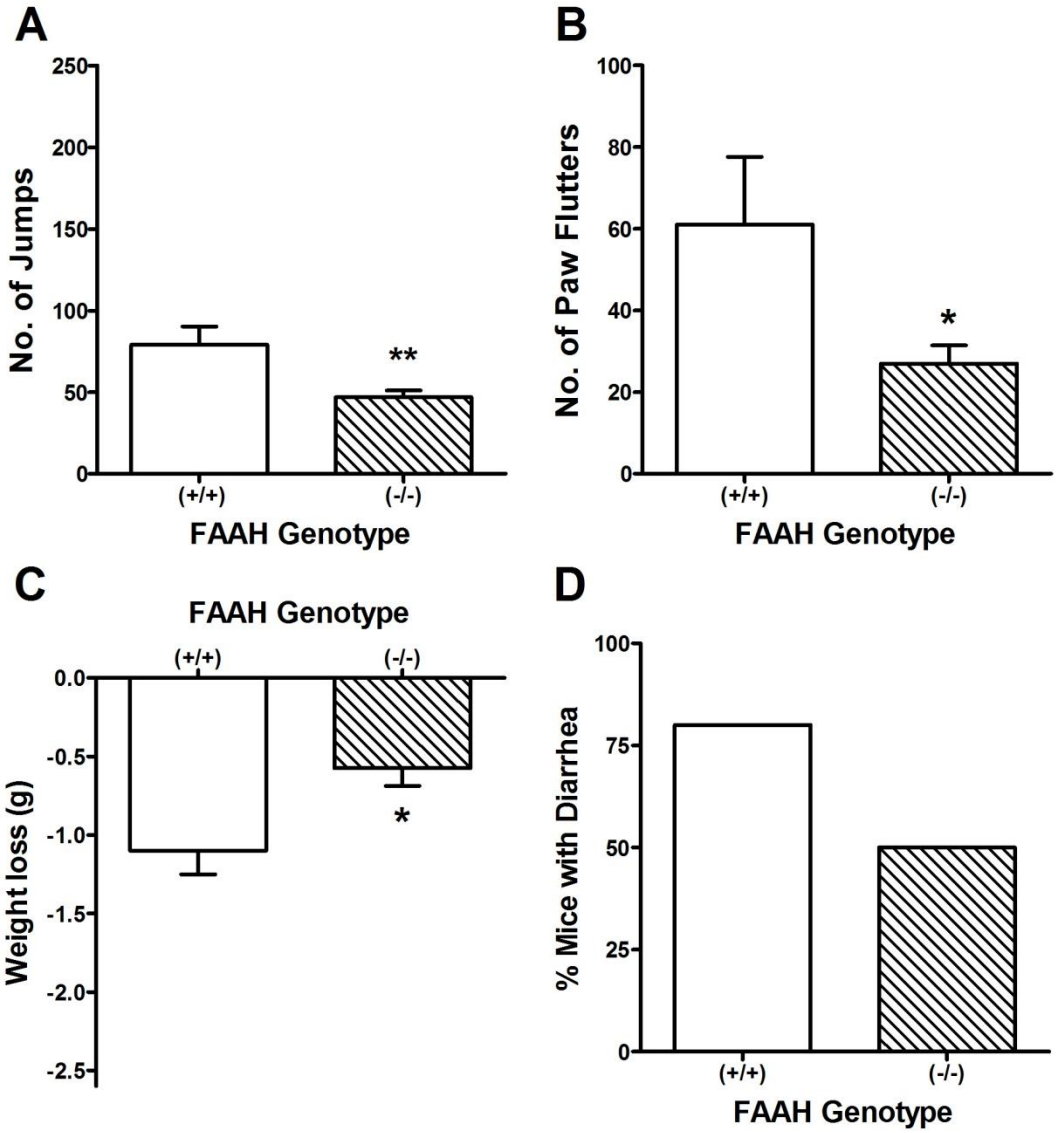


Figure 13. FAAH (-/-) mice display attenuated naloxone-precipitated withdrawal signs.

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

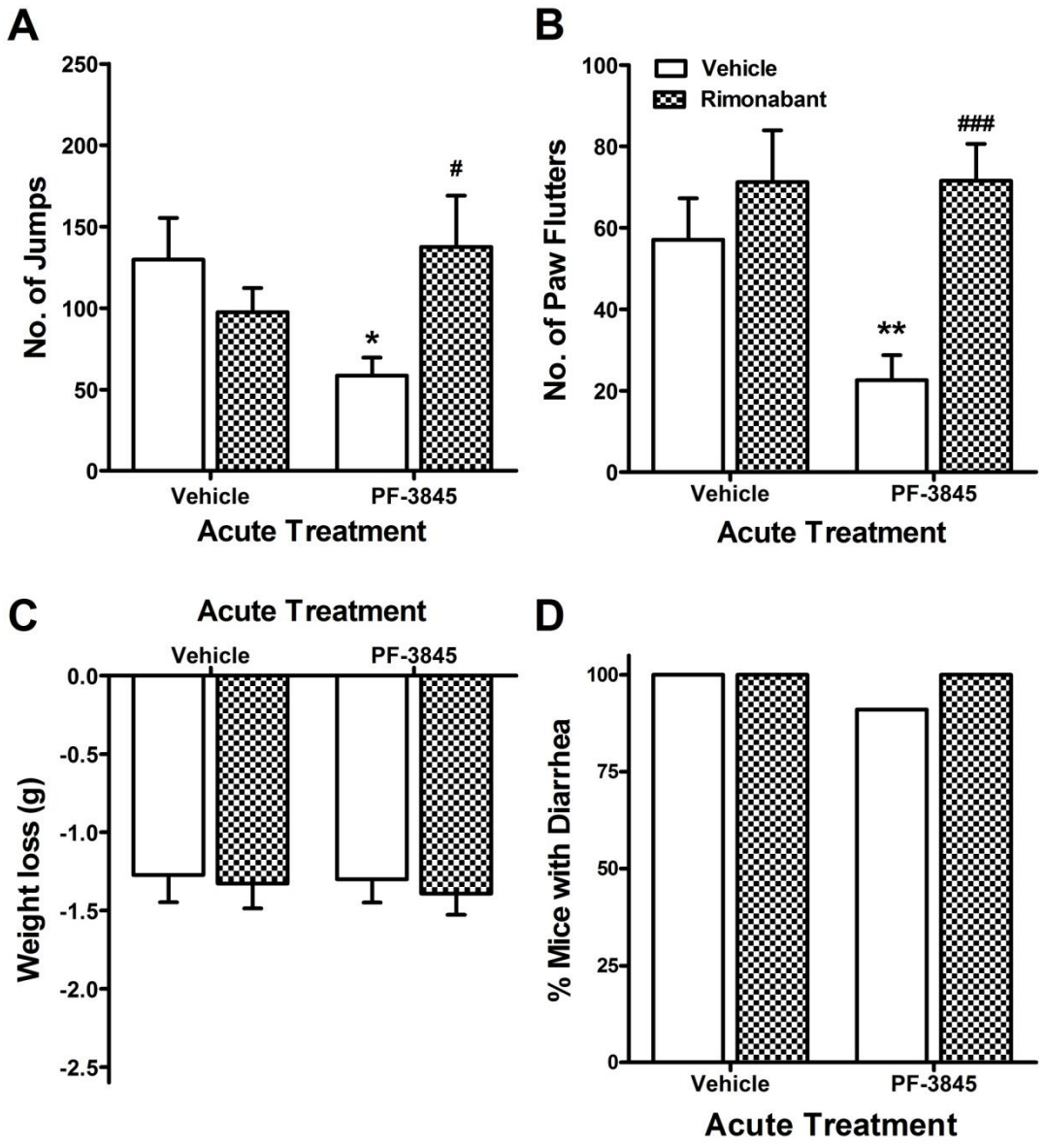


Figure 14. The FAAH inhibitor PF-3845 attenuates a subset of naloxone-precipitated morphine withdrawal signs in a CB_1 receptor dependent manner.

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**)** jumps, (B) paw flutters, (C) weight loss and (D) 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.

Table 3. Endocannabinoid levels in brain regions associated with opioid withdrawal

AEA (top) and 2-AG (bottom) levels 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; $p<0.05$ v/s Placebo-saline

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

3.6. Discussion

In these experiments, we report that inhibition of the endocannabinoid catabolic enzymes, MAGL or FAAH, reduces naloxone-precipitated withdrawal in an i*n vivo* model 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. On the other hand, the CB_2 receptor antagonist SR144528 did not block the effects of JZL184 indicating that CB_2 receptors are not required for its anti-withdrawal effects. Repeated treatment with high doses of JZL184 led to maintenance of anti-withdrawal effects. While FAAH (-/-) mice showed a reduction in all three measures of naloxone-precipitated withdrawal, PF-3834 reduced only naloxone-precipitated jumping and paw flutters, but did not attenuate diarrhea or body weight loss.

In this set of studies, we have examined the effects of FAAH and MAGL inhibitors on naloxone-precipitated opioid withdrawal signs. The precipitated withdrawal model uses an antagonist (i.e naloxone) to abruptly halt MOR activation by morphine allowing expression of symptoms mimicking that of natural withdrawal. This model offers potential advantages that the syndrome is short in duration and the symptoms observed are reliably and overtly quantifiable.

The pharmacological effects of JZL184 undergo tolerance in a variety of pain assays after repeated treatment at high doses (Schlosburg et al., 2010). Here we report that the anti-withdrawal effects of high dose JZL184 do not undergo tolerance. This may be due to different signaling pathways at the CB_1 receptor or distinct neural substrates mediating withdrawal, different from those involved in mediating analgesia.

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. Thus, MAGL and FAAH inhibitors attenuate the expression of withdrawal signs by extending the activity of endocannabinoids at the CB_1 receptor.

Chapter 4: Effect of inhibition of eCB catabolic enzymes on spontaneous opioid withdrawal signs

In Chapter 3, we have established that inhibitors of endocannabinoid catabolic enzymes reduce naloxone-precipitated opioid withdrawal signs. The precipitated withdrawal model is widely used because it produces a severe withdrawal response with a quick onset in a short duration of time. The spontaneous withdrawal procedure, on the other hand, more closely models the antecedents of withdrawal occurring in opioid addicts, though it has a slower throughput than the precipitated withdrawal model. Thus, potential treatments for opioid withdrawal are not evaluated for their efficacy in attenuating spontaneous withdrawal signs as often as in precipitated withdrawal models. In mice, abrupt cessation of morphine administration leads to expression of withdrawal signs similar to that elicited by an opioid antagonist. Particularly, platform jumping is commonly observed and considered to be a reliable indicator of the induction of withdrawal (Way et al., 1969). These somatic signs last for up to 48 h, with peak intensity of withdrawal occurring between 6-8 h following cessation of opioid administration. This model also allows for examining the anti-withdrawal effects and selecting agents that have a sustained half-life throughout the time course of withdrawal. Despite the fact that the spontaneous withdrawal procedure presents with considerable challenges, it is considered to possess more face validity than precipitated withdrawal procedure for modeling human opioid withdrawal.

In this chapter, we investigated the dose-response relationship of the MAGL inhibitor JZL184 to reduce spontaneous withdrawal induced jumps, paw flutters, head shakes, diarrhea and weight loss in mice implanted with morphine pellets. In addition, we tested the

effectiveness of a combination of low-dose JZL184 and high dose of the FAAH inhibitor PF-3845 in reducing abrupt morphine withdrawal signs. The selective CB_1 receptor antagonist, rimonabant was employed to assess cannabinoid receptor involvement of the anti-withdrawal effects of JZL184 and the combination. In order to evaluate whether compensatory changes of endocannabinoid system occurs following treatment with MAGL and FAAH inhibitors in ICR mice, endocannabinoid and arachidonic acid (AA) levels were quantified in whole brain. In addition, we measured changes in CB_1 receptor number and G-protein activation following repeated treatment with these inhibitors.

Methods

Subjects

Male ICR mice (Harlan laboratories; Indianapolis) weighing between 26 and 30 g served as subjects. The mice were housed 4-5 per cage in a temperature controlled (20-22^{o}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, and 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 by Organix Inc. (Woburn, MA) (Ahn et al., 2009; Long et al., 2009a). THC, PF-3845, JZL184 and rimonabant 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 a volume of 0.01 ml per 1 g body weight. THC and naloxone were administered via subcutaneous (s.c.) injection, whereas PF-3845, JZL184 and rimonabant were given via intraperitoneal (i.p.) injection. For spontaneous withdrawal studies all treatments were given 1 h before the first test time-point.

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 1 h after surgery and then returned to the vivarium until testing.

Spontaneous morphine withdrawal

In withdrawal experiments, mice were implanted with 75 mg morphine pellets. Approximately, 71 h after pellet implantation, mice were weighed and assessed for baseline withdrawal behavior. 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 received an injection of drug or vehicle. The animals

were observed for spontaneous withdrawal signs for 15 min intervals at 2, 4, 6 and 8 h post-pellet removal. The mice continued to be housed singly throughout the testing period, and food and water were available *ad libitum*, except during the 15 min observation periods. Spontaneous withdrawal signs were quantified using a procedure that was adapted from Way et a. (1969). The percentage of mice that jumped off a circular platform (15 cm diameter x 70 cm height), the total number of paw tremor and head shake incidences, and body weight were recorded at each time point. Paw flutters and head shakes were each pooled across all time points to represent the total number of incidences of the behaviors observed. The percentage of mice presenting with diarrhea across the 8 h test session was also recorded. Finally, the mice were weighed at the end of each testing period.

Behavioral assessment of cannabinoid activity

For this study mice were injected with either vehicle (JZL184 40 mg/kg or PF3845 10 mg/kg, i.p.) on the sixth day (single groups), or drug (JZL184 40 mg/kg, JZL184 4mg/kg, PF3845 10 mg/kg, or JZL184 4 mg/kg + PF-3845 10 mg/kg i.p.). Catalepsy was evaluated using the bar test, in which the front paws of each subject were placed on a rod (0.75 cm diameter) that was elevated 4.5 cm above the surface. Mice were timed if they remained motionless with their paws on the bar (with the exception of respiratory movements), and the time motionless from 3 attempts to place on the bar were totaled with a cutoff of 60 s. Hyper-reflexive popping and jumping away from the bar was also scored. In the tail immersion test, each mouse was placed head first into a small bag fabricated from absorbent under pads (VWR Scientific Products; 4 cm diameter, 11 cm length) with the tail out of the bag. The experimenter gently held the mouse and immersed approximately 1 cm of the tip of the tail into a water bath maintained at 52.0°. The latency for the animal to withdraw its tail from the water within a 10s cutoff time was

scored. Rectal temperature was determined by inserting a thermocouple probe 2.0 cm into the rectum and temperature was obtained from a telethermometer. Before any injections, baseline tail nociceptive latencies and rectal temperatures were assessed for all tests.

Agonist-stimulated [^{35}S]GTPγS binding

Mice were sacrificed by decapitation, and the whole brain, minus olfactory bulbs, was removed. Tissues were stored at -80°C until use. Samples were placed in 5 ml of cold membrane buffer (50 mM Tris-HCl, 3 mM MgCl2, 1 mM EGTA, pH 7.4) and homogenized. Homogenized samples were centrifuged at 50,000g at 4°C for 10 min. The supernatant was removed and samples were resuspended in 5 ml of assay buffer A (50 mM Tris-HCl, 3 mM MgCl2, 0.2 mM EGTA, 100 mM NaCl, pH 7.4). Protein concentration was determined by the Bradford method (Bradford, 1976). To reduce basal activation by adenosine receptors, preincubation for 15 min at 30°C with adenosine deaminase (3 mU/ml) in assay buffer was performed before addition to the final buffer mixture. Concentration-effect curves were generated by incubating 5 μg of membrane protein in assay buffer B (assay buffer A plus 1.25 g/l BSA), with 3 nM to 3 μM CP55,940, 30 μM GDP, and 0.1 nM [^{35}S]GTPγS in 0.5-ml total volume. Solutions were vortexed and to incubated for 2 h at 30°C. Basal binding was measured in the absence of agonist, and nonspecific binding was measured in the presence of 20 μM unlabeled GTPγS. The reaction was terminated by vacuum filtration though Whatman GF/B glass fiber filters, followed by three washes with 4°C Tris buffer (50 mM Tris-HCl, pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency after 10-h extraction in ScintiSafe Econo 1 scintillation fluid.

[^{3}H]-SR141716A binding

Membranes were prepared as described above. Membrane proteins (10 μg) were incubated with 0.1-2.5 nM [^{3}H]-SR141716A in 50 mM Tris-HCl, 3 mM MgCl2 , 0.2 mM EGTA, 100 mM NaCl, 1.25 g/L BSA, pH 7.4 in the presence or absence of 5 μM unlabeled rimonabant (to determine non-specific binding) for 90 min at 30°C. The reaction was terminated by vacuum filtration though Whatman GF/B glass fiber filter that was pre-soaked in Tris buffer containing 5 g/L BSA (Tris-BSA), followed by three washes with 4°C Tris-BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency after extraction in ScinitSafe Econo 1 scintillation fluid.

Statistical analysis

All data are reported as mean ± SEM. In the behavioral experiments, non-continuous behaviors, including head shakes and paw tremors, are presented as counted observations. The occurrence of jumps and diarrhea was scored as a binary event for the entire 15 min period for each time point. Weight loss (g) was calculated by subtracting the body weight at the conclusion of each 15 min observation period from the pre-pellet removal weight. Data were analyzed using one-way or 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 and percentage of mice that jumped off platforms were compared by the z test of two proportions.

Results

4.1. Abrupt removal of morphine pellet induces opiate withdrawal signs in mice

As shown in Figure 15, morphine pellet removal increased the percentage of mice jumping from the platform (4-8 h; $p<0.05$; Fig 15A), increased the incidences of paw flutters (2-24 h; $p<0.05$; Fig 15B) and head shakes (2-24 h; $p<0.05$; Fig 15C), and resulted in greater weight loss (4-24 h; $p<0.05$; Fig 15 D) compared to mice that had placebo pellets removed. Morphine pellet removal also resulted in diarrhea (Fig 15E).

4.2. JZL184 dose-dependently attenuates spontaneous withdrawal signs in a CB_1-dependent manner

In order to examine whether MAGL inhibition reduces spontaneous withdrawal signs, we treated morphine-pelleted mice with either vehicle or JZL184 (4, 16, or 40 mg/kg, i.p.). Both 40 and 16 mg/kg JZL184 significantly reduced jumping at 4, 6 and 8 h post-pellet removal (Fig. 16A). JZL184 dose-responsively reduced the intensity of total paw flutters [$F(3,28) = 26.4$; $p < 0.001$; Fig. 16B], total head shakes [$F(3,28) = 12.1$; $p < 0.001$; Fig. 16C], and weight loss across all time points [$F(12, 112) = 4.0$; $p < 0.001$; Fig. 16D]. All doses of JZL184 completely blocked the occurrence of diarrhea in morphine-dependent mice undergoing withdrawal (Fig. 16E).

We next evaluated whether the anti-withdrawal effects of JZL184 were mediated by the CB_1 receptor. An additional group of mice was co-administered JZL184 (40 mg/kg) and rimonabant (3mg/kg, i.p). Rimonabant reversed the anti-withdrawal effects of JZL184, as indicated by the increased percentage of spontaneous withdrawal jumps (Fig. 16A), number of total paw flutters [$F(2,22) = 24.8$; $p < 0.001$; Fig. 16B], number of total head shakes [$F(2,22) =$

9.7; $p < 0.001$; Fig. 16C] and intensity of weight loss [$F(8,84) = 5.3$; $p < 0.001$; Fig. 16D]. In addition, rimonabant also reversed the anti-diarrheal effects of JZL184 (Fig 16E).

4.3. The FAAH Inhibitor PF-3845 and THC also reduce the intensity of abrupt withdrawal signs

To elucidate the role of FAAH in morphine withdrawal, we evaluated the irreversible FAAH inhibitor, PF-3845 (10 mg/kg, i.p.) on spontaneous morphine withdrawal. PF-3845 significantly attenuated the frequency of paw flutters [$p < 0.001$; Fig 17B] and head shakes [$p < 0.001$; Fig 17D], but did not affect jumping behavior, weight loss or the expression of diarrhea (Fig 17A, 17D and 17E). The phytocannabinoid THC blocked withdrawal induced jumping (Fig 17A), reduced the intensity of total paw flutters [$p < 0.001$; Fig 17B], head shakes [$p < 0.01$; Fig 17C] and weight loss [$F(4,52) = 3.966$; $p < 0.01$; Fig. 17D], and completely prevented the occurrence of diarrhea (Fig. 18E). This experiment shows that acute FAAH inhibition attenuates a subset of withdrawal signs (i.e., paw flutters and head shakes), while the cannabinoid receptor agonist THC attenuates all the measured behavioral signs.

4.4. Combined partial MAGL inhibition and complete FAAH inhibition attenuates spontaneous withdrawal signs through the activation of CB_1 receptors

To evaluate the role of dual inhibition of MAGL and FAAH on spontaneous opioid withdrawal signs a set of mice was co-administered a low dose of JZL184 (4 mg/kg, i.p.) or vehicle and a high dose of PF-3845 (10 mg/kg, i.p.) or vehicle. These doses were selected to optimize the anti-opioid withdrawal effects of endocannabinoid catabolic inhibitors and avoid the dependence potential of high doses of JZL184. Combination of JZL184 and PF-3845, but not either drug alone, significantly reduced jumping at 4, 6, and 8 h after morphine pellet removal (Fig. 18A). In addition, the combination as well as each drug by itself significantly reduced the

intensity of total paw flutters [$F(3,26) = 12.3$; $p < 0.001$; Fig. 18B] and head shakes [$F(3,26) = 4.4$; $p < 0.01$; Fig. 18C]. However, only the combination reduced the intensity of weight loss [$F(12,104) = 1.7$; $p < 0.01$; Fig. 18D], as indicated by significant interactions between group and time. In addition, the combination treatment significantly blocked the occurrence of diarrhea while the groups treated with either drug alone presented with diarrhea (Fig. 18E). Either individual treatment reduced only the intensity of paw flutters and head shakes, whereas the combination reduced the full spectrum of abrupt withdrawal signs.

Rimonabant completely blocked the anti-withdrawal effects of combined administration of JZL184 and PF-3845, indicating a CB_1 receptor mechanism of action. Rimonabant co-treatment completely reversed the effects of the combination as indicated by a significant interaction for paw flutters [$F(1,37)$; $p<0.01$, Fig 19B], head shakes [$F(1,37)$; $p<0.05$; Fig 19C] and weight loss [$F(12,108)=2.752$; $p<0.01$; Fig 19D]. In addition, rimonabant also reversed the effects of the combination in blocking jumps and the occurrence of diarrhea (Fig 19A & E).

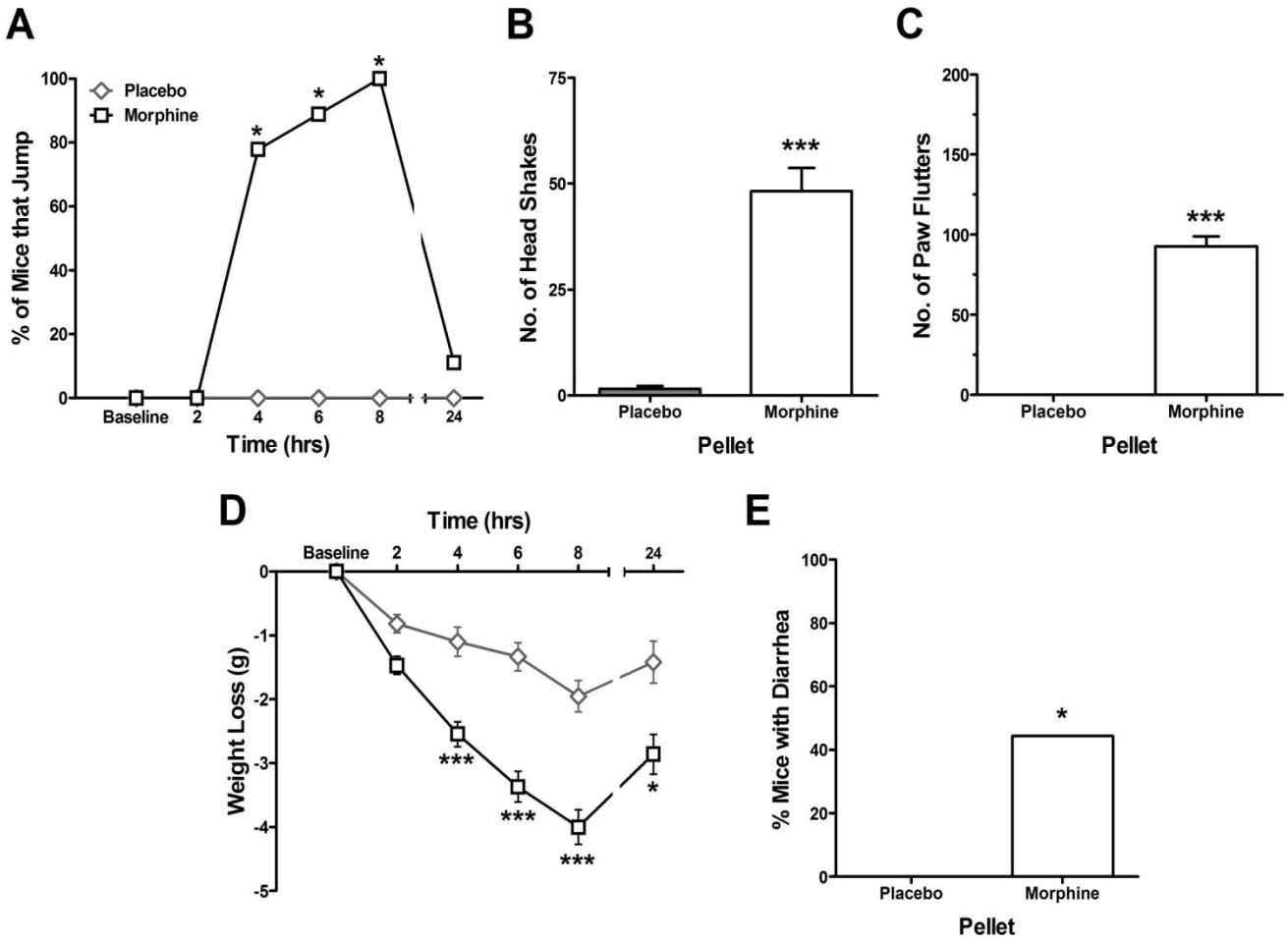


Figure 15. Evaluation of spontaneous withdrawal signs in mice.

Removal of morphine pellets leads to increased spontaneous withdrawal signs compared to mice implanted with placebo pellets. (A) Percentage of mice that displayed platform jumping, (B) number of paw flutters, (C) number of head shakes, (D) weight loss and (E) expression of diarrhea. Data expressed as mean ± SEM for panels B-D. *$p<0.05$,**$p<0.01$,***$p<0.001$ vs. placebo; n = 6-9 mice/group.

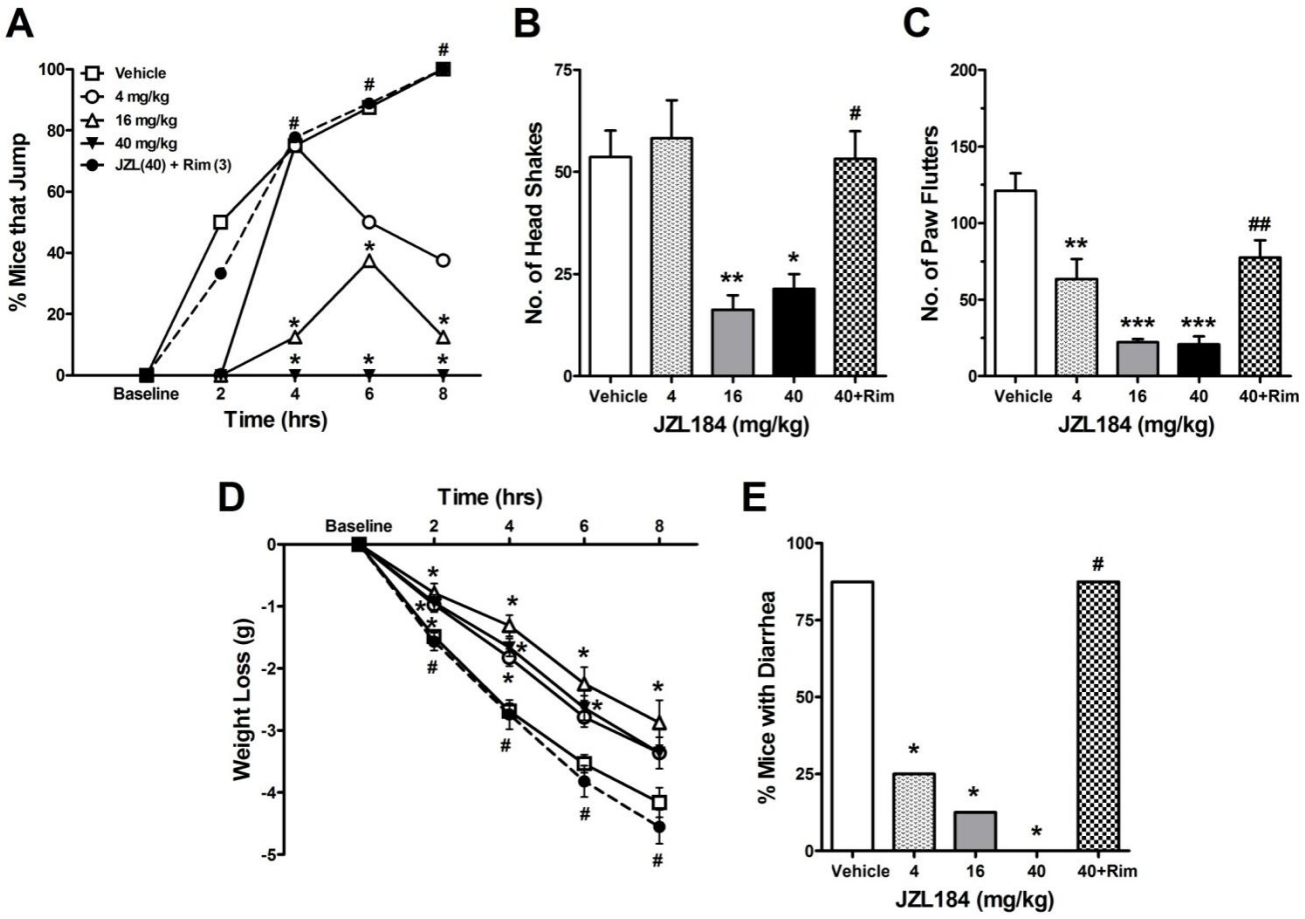


Figure 16. JZL184 reduces spontaneous withdrawal signs in a CB_1-dependent manner

The MAGL inhibitor JZL184 dose-dependently attenuates the intensity of the following spontaneous withdrawal signs in morphine-dependent mice through the activation of CB_1 receptors. Rimonabant (3 mg/kg) when co-administered with JZL184 (40mg/kg) blocked it anti-withdrawal effects. The withdrawal signs measured include (A) jumps, (B) paw flutters, (C) head shakes, (E) weight loss and (E) diarrhea. Data expressed as mean ± SEM for panels B-D. *$p<0.05$ vs. morphine control; # $p<0.05$ v/s JZL184 treated group; n = 7-9mice/group.

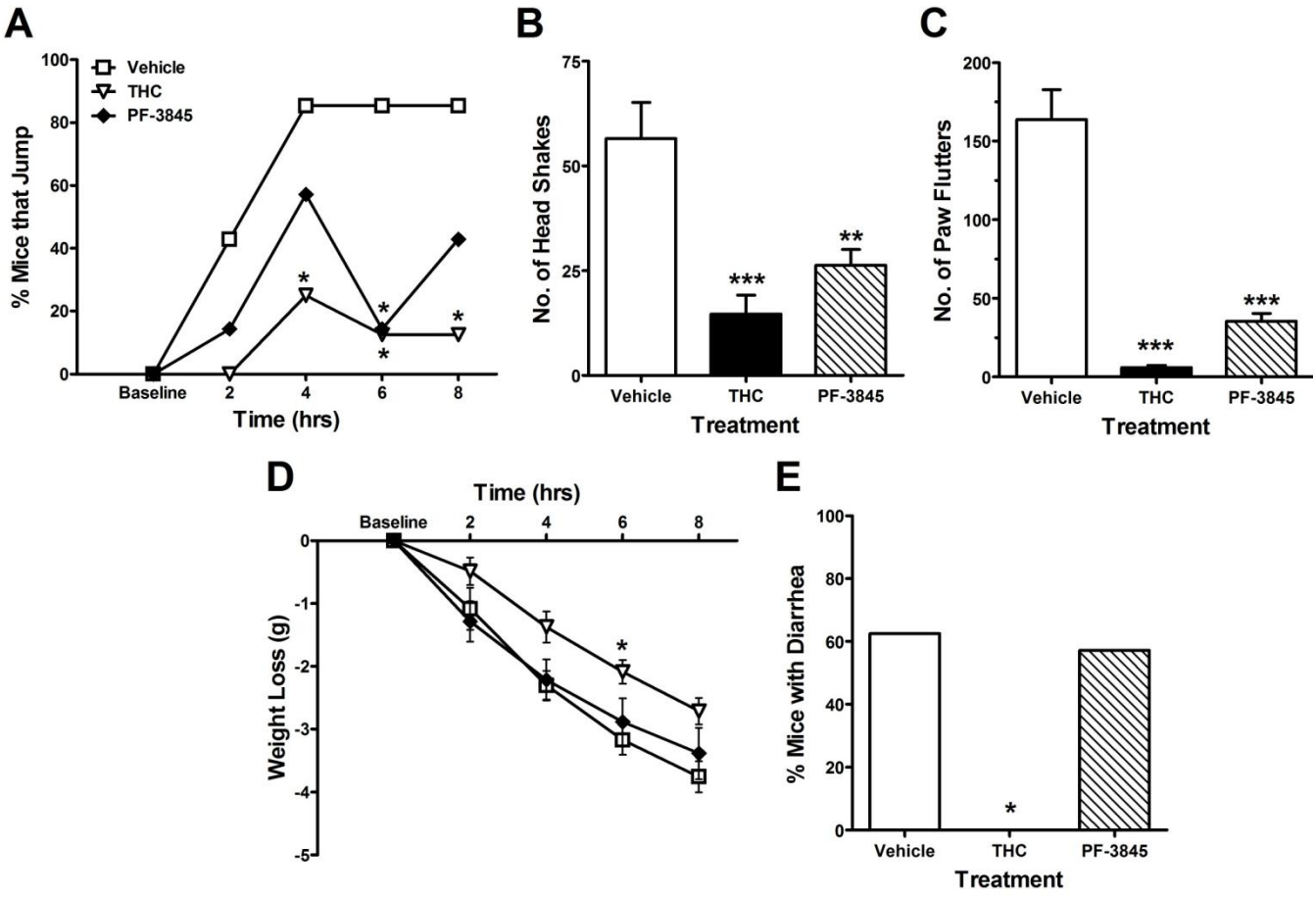


Figure 17. THC and PF-3845 reduce spontaneous withdrawal signs

The phytocannabinoid THC attenuates the intensity of the spontaneous withdrawal signs in morphine-dependent mice while the FAAH inhibitor PF-3845 reduces the intensity of some withdrawal signs. The withdrawal signs measured include (A) jumps, (B) paw flutters, (C) head shakes, (E) weight loss and (E) diarrhea. Data expressed as mean ± SEM for panels B-D. *$p<0.05$ vs. morphine control; n = 7-8 mice/group.

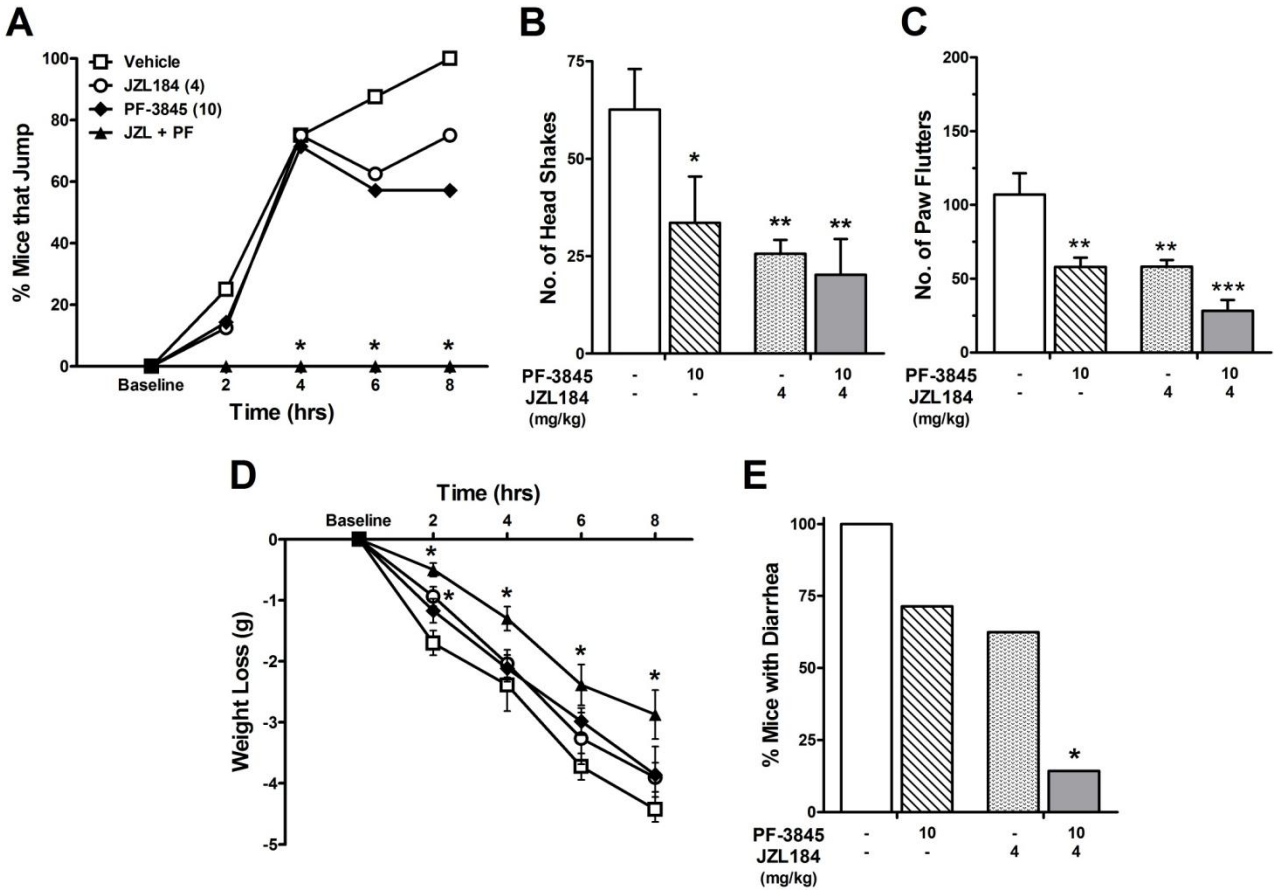


Figure 18. Combination of low-dose JZL184 and high-dose PF-3845 reduces abrupt withdrawal signs

The combination of low dose of the MAGL inhibitor JZL184 (4 mg/kg) and high dose of the FAAH inhibitor PF-3845 (10 mg/kg) reduces the intensity of the spontaneous withdrawal signs in morphine dependent mice to a greater extent than either inhibitor by itself. The withdrawal signs measured include (A) jumps, (B) paw flutters, (C) head shakes, (E) weight loss and (E) diarrhea. Data expressed as mean ± SEM for panels B-D. *$p<0.05$ vs. morphine control; n = 7-8mice/group.

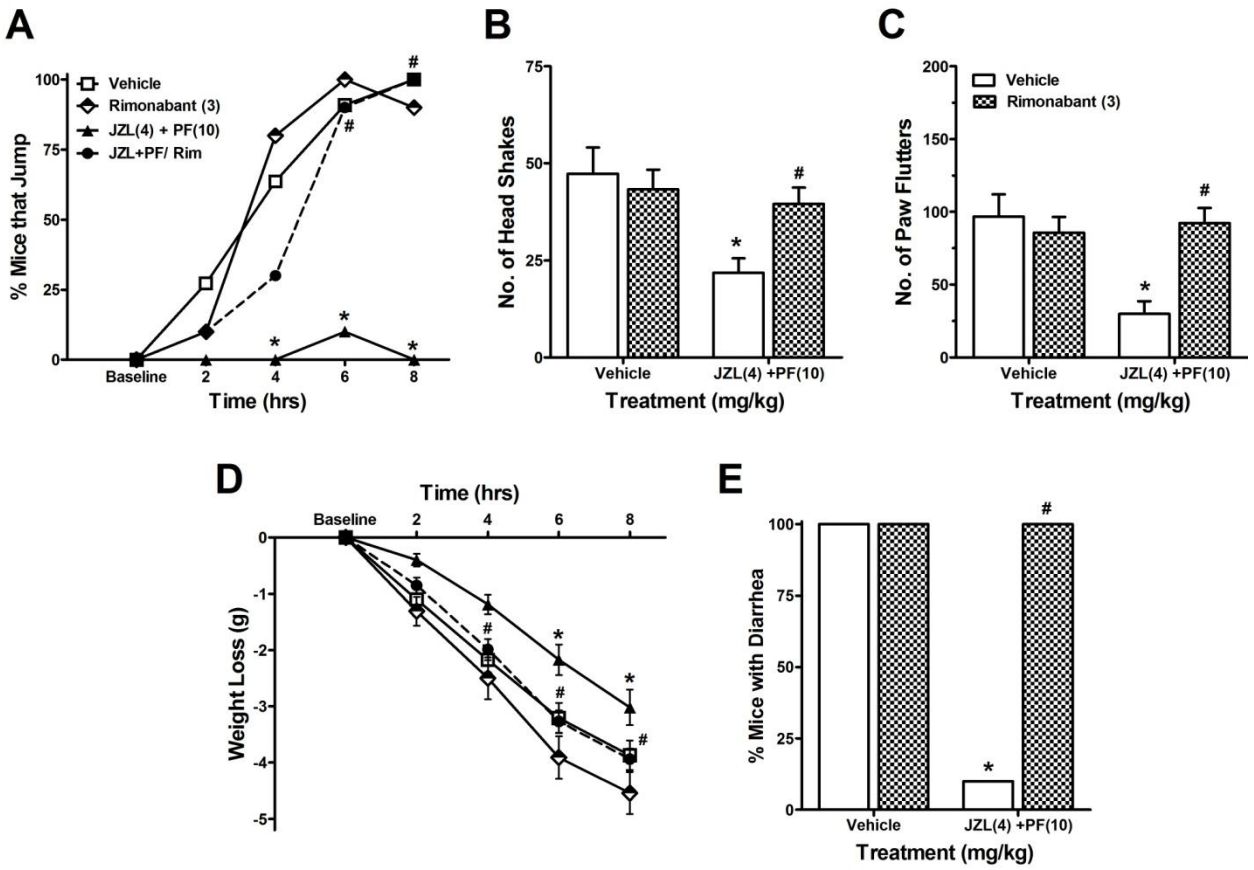


Figure 19: Effects of the combination of partial MAGL and complete FAAH inhibition are CB_1 mediated

Rimonabant blocks the effects of the combination of low dose of the MAGL inhibitor JZL184 (4 mg/kg) and high dose of the FAAH inhibitor PF-3845 (10 mg/kg) in reducing the intensity of the spontaneous withdrawal signs in morphine dependent mice. The withdrawal signs measured include (A) jumps, (B) paw flutters, (C) head shakes, (E) weight loss and (E) diarrhea. Data expressed as mean ± SEM for panels B-D. *$p<0.05$ vs. morphine control; n = 10-11 mice/group.

4.5. Evaluation of tetrad effects and measurement of endocannabinoid levels in brain following acute MAGL and FAAH inhibition

Mice treated acutely with either vehicle, THC (10 mg/kg), low-dose JZL184 (4 mg/kg), PF-3845 (10 mg/kg), combination of low-dose JZL184 + PF-3845 and high-dose JZL184 (40 mg/kg) were assessed in the tetrad assay (Table 4). After assessment of tetrad effects, brains were harvested at 2h post-injection. Neither treatment produced any change in the open field locomotor behavior or cataleptic effects in ICR mice. Additionally, only THC produced significant hypothermia ($\Delta^{o}C$= -2.02 ± 0.7; $p<0.05$) and antinociception (Δ sec= 2.21 ± 0.9; $p<0.05$) in the tail withdrawal test, while the other treatments had no effect.

The elevation of whole-brain endocannabinoid levels following acute and prolonged MAGL and FAAH inhibition by JZL184 and PF-3845 has been previously established in C57BL/6J mice (Long et al., 2009a; Schlosburg et al., 2010). Additionally, MAGL inhibition reduces AA levels in the brain which is a hydrolytic product of 2-AG catabolism. A recent report suggests that 2-AG hydrolysis by MAGL is responsible for up to 80% of AA generated in the brain (Ahn et al., 2009; Long et al., 2009a; Nomura et al., 2011). To determine whether acute treatment with MAGL and FAAH inhibitors produces altered levels of endocannabinoids and arachidonic acid in whole brains from ICR mice we measured the levels of AEA, 2-AG,OEA, PEA and AA. Acute treatment with low-dose JZL184 (4 mg/kg), alone and in combination with FAAH inhibition, significantly elevated 2-AG levels 2-4 fold [$F(3,28) = 25.7$; $p<0.001$; Fig.20A]. Notably, mice treated with the combination showed a higher elevation in 2-AG than mice treated with JZL184 (4mg/kg) alone. High-dose JZL184 elevated 2-AG levels approximately 10 fold ($p<0.001$ v/s vehicle control. Fig. 19A), comparable to levels observed in C57BL/6J mice (Long et al., 2009b). Acute treatment with high-dose JZL184 led to

approximately 40% decrease in AA levels ($p<0.05$) and the combination moderately lowered AA levels [$F(3,28) = 4.6$; $p<0.01$, Fig. 20B] compared to vehicle controls. When examining endocannabinoid accumulation in brain 2 h following acute treatment with PF-3845 or combination of PF-3845+JZL184, AEA was elevated more than 8-fold above that of vehicle [$F(3,28)=309.3$; $p<0.001$; Fig 20C)].

4.6. Brain CB_1 receptors are impaired by prolonged MAGL, but not FAAH blockade

Genetic or prolonged pharmacological disruption of MAGL, but not FAAH, leads to down-regulation and desensitization of CB_1 receptors in C57BL/6J mice (Falenski et al., 2010; Schlosburg et al., 2010). To determine whether these CB_1 adaptations also occurred in outbred ICR mice, we measured endocannabinoid and AA levels, examined CB_1 receptor expression and function through specific binding of [^{3}H]-SR141716A and CP55,940-stimulated [^{35}S]-GTPγS binding, respectively, in whole-brain homogenates from mice treated with either vehicle, low-dose JZL184 (4 mg/kg), PF-3845 (10 mg/kg), combination of JZL184 (4mg/kg) + PF-3845 (10mg/kg), or high-dose JZL184 (40 mg/kg). Mice were treated with inhibitors/vehicle once a day for 5 days and brains were harvested 24 h later or once a day for 6 days and brains extracted 2 h later.

Repeated low dose JZL184 did not lead to increased levels of 2-AG at 24 h, though high dose JZL184 increased brain 2-AG levels at this time point approximately 8 fold compared to vehicle ($p<0.001$; Fig. 21A). Repeated treatment with high-dose JZL184 led to about 40% decrease in AA levels ($p<0.05$) and the combination moderately lowered AA levels [repeated treatment, $F(3,20)= 3.7$; $p<0.05$, Fig. 21B] compared to vehicle controls. On the contrary, low dose JZL184 elevated 2-AG levels, at 2 h following repeated dosing, approximately 10 fold while the combination elevated 2-AG 8-fold [$F(4,24)=62.445$; $p<0.001$; Fig. 20D]. These

treatments also significantly attenuated AA levels by approximately 40%. The high dose of JZL184 elevated 2-AG levels almost 20-fold and produced about 70% decrease in AA levels [$F(4,24) = 19.5$; $p<0.05$, Fig. 21E] compared to vehicle controls. Neither low dose nor high dose of repeated JZL184 injections produced significant increases in whole brain AEA levels at 2 h or 24 h (Fig. 21 C & F). Prolonged FAAH blockade did not induce changes in AEA or 2-AG levels 24 h post treatment. Prolonged FAAH inhibition with or without concomitant MAGL inhibition led to elevated AEA levels at 2 h similar to acute treatment [$F(4,24)=46$; $p<0.001$; Fig 21F].

Consequently, the CP55,940-stimulated [^{35}S]-GTPγS concentration-effect curves (Fig. 22 A and B) display a substantial reduction in E_{max} of 44% from mice receiving repeated high dose-JZL184 (40 mg/kg). Two-way ANOVA analysis shows that there is a significant attenuation of E_{max} by JZL184 (4 mg/kg) treatment irrespective of simultaneous FAAH inhibition [$F(1,20)=11.9$; $p<0.01$; Fig.22A]. Nonlinear sigmoidal regression on the activation curves revealed a significantly lower maximal efficacy of agonist stimulation (E_{max}). Repeated treatment with high dose-JZL184 (40 mg/kg) also resulted in decreases in whole brain CB_1 receptor binding curves compared to vehicle. The non-linear regression of the binding data revealed a significantly reduced maximal binding (B_{max}) by 38% compared to vehicle (Fig 22C and D). None of the other treatments significantly altered whole brain CB_1 receptor binding levels. Thus, prolonged complete MAGL inhibition leads to both down-regulation and desensitization of CB_1 receptors in whole brain. Low dose JZL184 alone or in combination with FAAH inhibition resulted in receptor desensitization in the absence of apparent receptor loss.

Table 4. Tetrad effects of MAGL and FAAH inhibitors in ICR mice

Treatment (mg/kg)	Hypothermia ($\Delta^{o}C$)	Tail flick latency (Δ sec)	Catalepsy (sec)	Time Immobile (sec)
Vehicle	0.46 ± 0.3	-0.23 ± 0.2	0.00	11.2 ± 1.5
JZL184 (4)	0.19 ± 0.3	0.01 ± 0.1	0.00	9.72 ± 2.9
PF-3845 (10)	0.18 ± 0.2	0.10 ± 0.2	0.00	10.7 ± 1.8
JZL(4) + PF(10)	0.31 ± 0.2	-0.26 ± 0.2	0.00	19.1 ± 7.0
JZL184 (40)	-0.70 ± 0.4	1.19 ± 0.8	0.00	14.6 ± 3.2
THC (10)	**-2.02 ± 0.7***	**2.21 ± 0.9***	9.05 ± 6	23.6 ± 16

Data represented as mean ± SEM; *p<0.05 v/s vehicle control; n=9/group

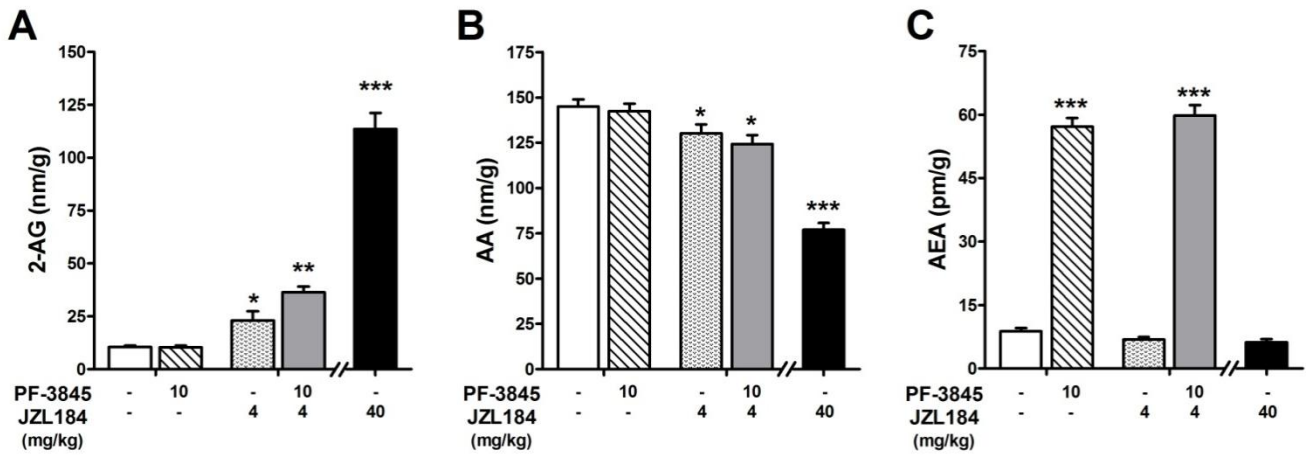


Figure 20: Whole brain levels of endocannabinoids and arachidonic acid in mice with acutely disrupted MAGL and/or FAAH.

Levels of (A) 2-AG, (B) arachidonic acid, and (C) AEA in the whole brain from mice treated acutely (single dose i.p. evaluated 2 h later) treated with either vehicle, low-dose JZL184(4 mg/kg), PF-3845 (10 mg/kg), combination of JZL184(4mg/kg) + PF-3845(10mg/kg) and high-dose JZL184(40 mg/kg). Data expressed as mean ± SEM. *$p<0.05$; **$p<0.01$, ***$p<0.001$ vs. vehicle control; n=8 mice/group.

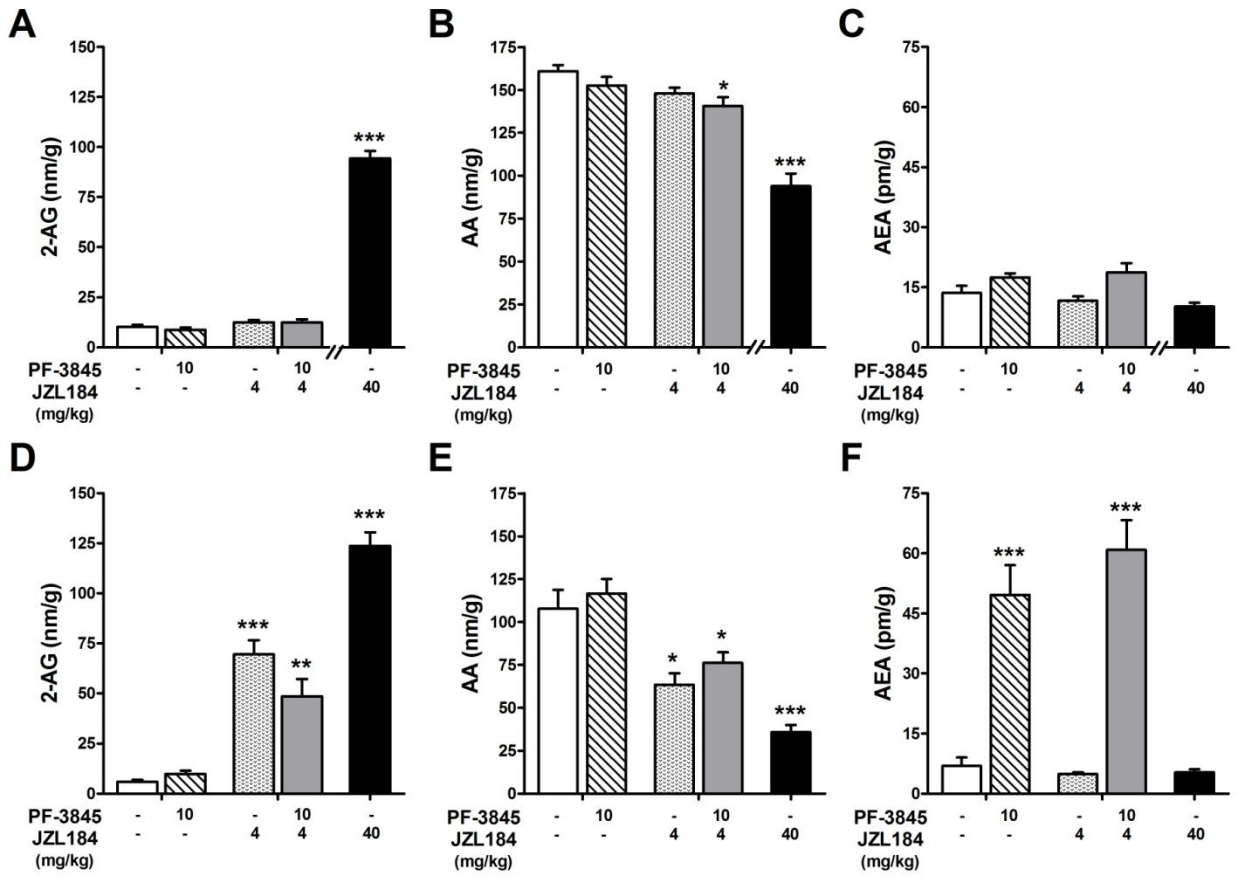


Figure 21. Whole brain levels endocannabinoids and arachidonic acid from mice repeatedly treated with MAGL and/or FAAH inhibitors.

Levels of AEA, 2-AG and AA (treatment: 5 d, one dose per day (i.p.), and evaluated 24 h after final dose; Panel A-C) and (treatment: 6 d, one dose per day (i.p.), and evaluated 2 h after final dose; Panel D-F). Data expressed as mean ± SEM. *p<0.05; **p<0.01, ***p<0.001 vs. vehicle control; n=6 mice/group.

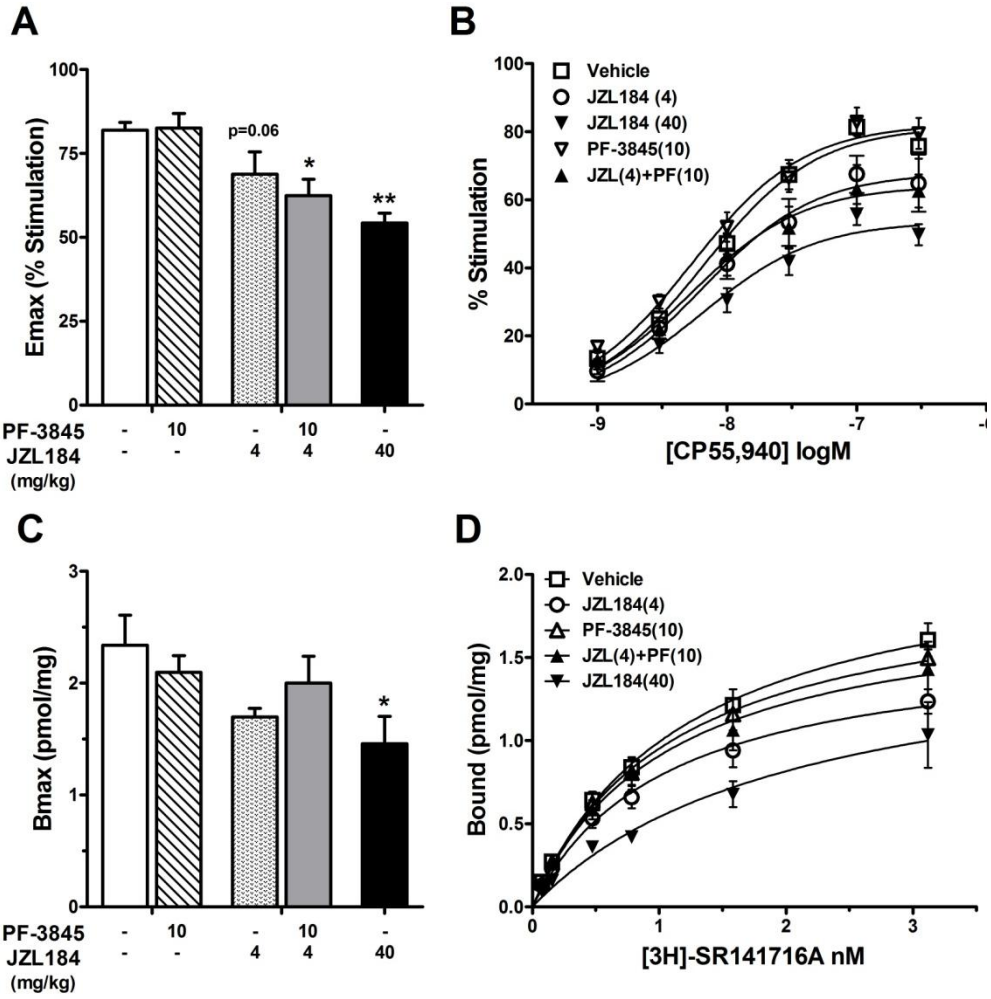


Figure 22. CB_1 receptor function in mice following prolonged disruption of MAGL and/or FAAH.

CP55,940-stimulated [^{35}S]-GTPγS binding as indicated by (A) best fit E_{max} and (B) mean % stimulation of CP55,940 concentration curve; Membrane-specific CB_1 receptor binding by the antagonist [^{3}H]-SR141716A, as evaluated by the (C) best-fit B_{max} of binding curves and (D) specific binding curves, from whole brain homogenates from mice repeatedly treated with MAGL and/or FAAH inhibitors **(**treatment: 5 d, one dose per day (i.p.), and evaluated 24 h after final dose**.** Data represented as mean ± SEM. *p<0.05; **p<0.01, ***p<0.001 vs. vehicle control; n=6 mice/group.

4.7. Discussion

Acutely administered JZL184, which 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 spontaneous withdrawal, including paw flutters, head shakes, jumps, diarrhea, and weight loss. The FAAH inhibitor PF-3845 reduced only spontaneous withdrawal-induced paw flutters and head shakes, but did not attenuate intensity of jumps, diarrhea or body weight loss. Furthermore, combination of low-dose JZL184 and high dose of the FAAH inhibitor PF-3845 significantly attenuated all measured signs of spontaneous withdrawal. Rimonabant blocked these effects, indicating a necessary role of CB_1 receptors. The combination elevated appropriate levels of AEA and 2-AG and did not produce any cannabinoid tetrad effects. However, repeated treatment of the combination led to CB_1 receptor desensitization.

The observation that complete MAGL inhibition significantly blocked all opioid withdrawal symptoms, while FAAH inhibition only reduced a subset of these effects corroborates with our previous findings in the precipitated withdrawal model (Ramesh et al., 2011a) (see Chapter 3). However, the ability of prolonged MAGL inhibition at high doses of JZL184 to elicit cannabinoid dependence represents a drawback of using these inhibitors in treatment of abuse disorders (Schlosburg et al., 2010). Alternatively, a combination of low-dose JZL184 (4 mg/kg) and PF-3845 (10 mg/kg) possessed nearly full efficacy in attenuating physical opiate withdrawal signs. The magnitude of these effects was comparable to that of complete MAGL inhibition (40 mg/kg) and augmented in comparison to low-dose JZL184 (4 mg/kg) or PF-3845 (10 mg/kg) alone. The doses of PF-3845 and JZL184 selected for combination were intended to maximize the therapeutic efficacy of inhibiting both enzymes while avoiding the undesirable side-effects of complete MAGL inhibition. This combination also produces augmented anti-

allodynic effects in the carrageenan pain assay (Wise, Kinsey et al, unpublished data) and repeated treatment does not lead to functional CB_1 receptor adaptations in C57BL/6J mice.

In these experiments, we stimulated CB_1 receptors by elevating endogenous cannabinoid through the use of inhibitors of their hydrolytic enzymes. We quantified brain levels of endocannabinoids following acute repeated treatment of JZL184, PF-3845, and combined JZL184+PF-3845. Notably, the combination of partial MAGL/complete FAAH inhibition acutely resulted in approximately 2-fold higher levels of 2-AG compared to partial MAGL inhibition by itself. FAAH blockade by itself did not alter 2-AG levels compared to control levels.The enhanced elevation produced by the combination may be due to the ability of FAAH to degrade 2-AG (Blankman et al., 2007). To test this idea, we could examine whether PF-3845 further inhibits 2-AG hydrolysis activity in brain tissue from ICR mice treated with JZL184. Additionally, it would be necessary to determine if the enhanced elevation is observed in all relevant brain regions (ex. cerebelleum, LC, nucleus accumbens, striatum, PAG) or restricted to a specific area.

In a clinical setting, therapeutic agents used to manage of opiate withdrawal would likely be given repeatedly; therefore, it is important to compare the impact of repeated administration of compounds on endogenous cannabinoid levels as well as to assess CB_1 receptor function. Repeated administration of 40 mg/kg JZL184 elevated 2-AG whole brain levels 24 h after the last injection. These results are in agreement with studies in C57BL/6J mice in which repeated MAGL blockade resulted in up to 12-fold elevations in 2-AG up to 26 h after the last exposure of JZL184 (Schlosburg et al., 2010). Repeated JZL184 treatment results in more prolonged inhibition of MAGL and greater 2-AG levels than that produced by an acute injection. The further increase in 2-AG levels following repeated treatment is probably due to delayed

recovery of MAGL due to the long half-life of JZL184. Accordingly, repeated administration of high dose JZL184 also produced significant losses in the maximal stimulation of CB_1 receptors in whole brain by the cannabinoid receptor agonist CP55,940. Loss of maximal activation is usually indicative of desensitization of receptors, in which GRK phosphorylation and β-arrestin binding prevents typical activation in the presence of ligand. However, given that the magnitude of loss in receptor activation almost exactly equals the loss of receptor binding sites in almost all cases, it appears that prolonged 2-AG elevation with high-dose JZL184 promotes significant receptor internalization and degradation. However, repeated treatment with low-dose JZL184 treatment alone or in combination with PF-3845 did not lead to changes in 2-AG levels 24 h post-final injection, suggesting that the levels of 2-AG are normalized over the course of repeated treatment. We confirmed this observation this observation by also examining an earlier time point (i.e. 2h) following the final injection of the inhibitors. Accordingly, these groups showed CB_1 receptor desensitization, but no changes in CB_1 receptor number in whole brain. Repeated FAAH inhibition did not lead to accumulation of AEA levels 24h after the last dose. Thus, FAAH inhibition produces a transient elevation of AEA that does not accumulate in the brain following repeated treatment. These results confirm previous findings that prolonged complete MAGL inhibition leads to acculmulation of brain 2-AG and reduction of AA levels (Schlosburg et al., 2010). Taken together, these findings indicate that the moderate elevations in 2-AG produced by low-dose JZL184, as well as the combination (2-4 fold), induced desensitization of CB_1 receptors, but was insufficient to produce receptor down-regulation. However, given that cannabinoids induce region-dependent changes in CB_1 receptor function (Breivogel et al., 1999; Schlosburg et al., 2010), regional differences may not be reflected in whole brain homogenate binding.

Chapter 5: Anti-diarrheal actions of MAGL inhibition: Effects on naloxone-precipitated hypermotility and hypersecretion in morphine-dependent ilea

In both the naloxone-precipitated morphine withdrawal and spontaneous morphine withdrawal models, MAGL inhibition, but not FAAH inhibition, blocked the occurrence of diarrhea in morphine-dependent mice. The antecedents for diarrhea include increased muscle contractility and hypersecretion of fluids and electrolytes in the intestinal tract. In this Chapter, we employed the isolated ileum preparation to investigate the mechanism by which JZL184 blocked diarrhea. Ileum was exposed to morphine for 1 h at which time naloxone was added to the tissue bath to precipitate contractions and hypersecretion. The ileum offers a useful *in vitro* model to investigate opioid withdrawal (Paton, 1957), since it shows tolerance and withdrawal to prolonged application of morphine. The cannabinoid receptor agonists THC and WIN-55,212 have been shown to reduce naloxone-precipitated contractions in ilea subjected to prolonged morphine exposure (Frederickson et al., 1976; Basilico et al., 1999).

Intestinal motility is mediated predominantly by longitudinal muscle, which is innervated by the myenteric plexus, while secretion is controlled by the submucosal plexus innervating the mucosa and the submucosa (for cross-section of the ileum see Fig. 23). CB_1 receptors are believed to be located enteric neurons and extrinsic primary afferent nerves in the submucosa of ileum. Furthermore, primary afferent nerves that express the CB_1 receptor predominantly innervate regions of the submucosal ganglia where cholinergic secretomotoneurons are located (MacNaughton et al., 2004). In addition, MAGL mRNA and protein is expressed abundantly in the submucosal plexus of the rat ileum (Duncan et al., 2008). MORs are also abundantly expressed in the myenteric and submucosal plexus of the ileum and

control muscle contractility and secretion/absorption of electrolytes by modulating Ach release (Paton, 1957; Sheldon et al., 1990; Bagnol et al., 1997). In these experiments, we investigated whether JZL184 and PF-3845 would ameliorate naloxone-precipitated contractions and secretion in morphine-exposed ilea.

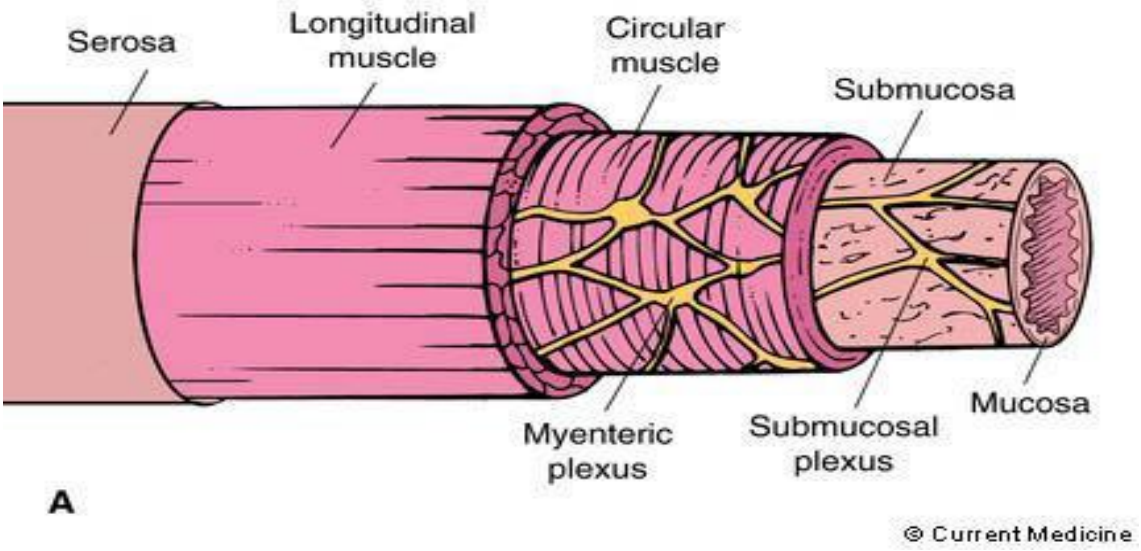


Figure 23: Cross-section of ileum

Outer-inner layers; Serosa, longitudinal muscle, circular muscle, submucosa amd mucosa. Muscular layers are innervated by myenteric plexus while submucosa and mucosa receive innervations from the submucosal plexus. Cannabinoid and opioid receptors are predominantly located in the neuronal tissue.

5.1. Methods

Subjects

For EFS studies and studies measuring levels of endogenous ligands, male ICR mice were used as subjects (Harlan laboratories; Indianapolis). The mice weighed between 26 and 30 g and were housed 4 per cage in a temperature controlled (20-22^{o}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.

For Ussing Chamber studies female C57BL/6J as well as female CB_1 (-/-) and (+/+) mice backcrossed onto a C57BL/6J background for at least 13 generations were used as subjects. The mice weighed between 18-22g and were housed 4 per cage in a temperature controlled (20-22^{o}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 the University of Maryland at Baltimore in accordance with the Guide for the Care and Use of Laboratory Animals.

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 were 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.

Measurement of electrogenic ion transport in vitro in Ussing's chambers.

Small intestinal tissue (including neuromuscular layer; whole SI except the proximal jejunum) was mounted in Ussing chambers 0.126 cm^2 of tissue to 5 ml Krebs buffer containing 10 mM glucose (serosal buffer) and 11.2 mM mannitol (mucosal buffer). The buffer was maintained at 37^oC and continuously aerated 95% oxygen, 5% carbon dioxide. Agar-salt

bridges and electrodes were used to measure potential difference. Every 50 s, the tissues were short circuited at 1 V (DVC 1000 voltage clamp; World Precision Instruments, Sarasota, FL), and the short circuit current (I_{sc}) was continuously monitored.

To test the reactivity/viability of the tissue a high concentration of glucose is applied to the mucosal level to activate the co-transporter which results in an increased short-circuit current. Next, the tissue is washed and the glucose and mannitol buffers are applied at the serosal and mucosal side respectively. Once the short-circuit current (I_{sc}) reaches a stable baseline, morphine (30µM) is applied to the serosal side. Tissues that do not show an acute decrease in I_{sc} in response to morphine treatment are not included in the study. After 20 minutes, the buffer is replaced and morphine is applied again. Ten minutes later JZL184 (10 µM) or vehicle (DMSO) is applied to the serosal side. Ten minutes later, the tissue is washed again and morphine and JZL184/DMSO is immediately re-applied to the tissue. Twenty minutes later naloxone is applied to the serosal side of the tissue (30µM) to precipitate withdrawal.

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^{o} 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. 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. EFS contraction height was compared by paired t-test. Differences of $p<0.05$ were considered significant.

Results

5.1. 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) was used to precipitate contractions. The height of naloxone-precipitated contractions was been 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. 24A]. 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. 24B. 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. 24C; see Fig. 24D for representative traces of contractions in the three conditions].

5.2. JZL184 but not PF-3845 attenuates neurogenic field-stimulated contractions in naïve ileal tissue

To investigate further, 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 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. 25C; Fig 23A for a representative trace]. In contrast, PF-3845 did not inhibit EFS contractions in the ileum ($p = 0.36$, Fig. 25C; Fig. 25B for a representative trace).

5.3. Both JZL184 and PF-3845 inhibit naloxone-precipitated hypersecretion in morphine-treated ilea

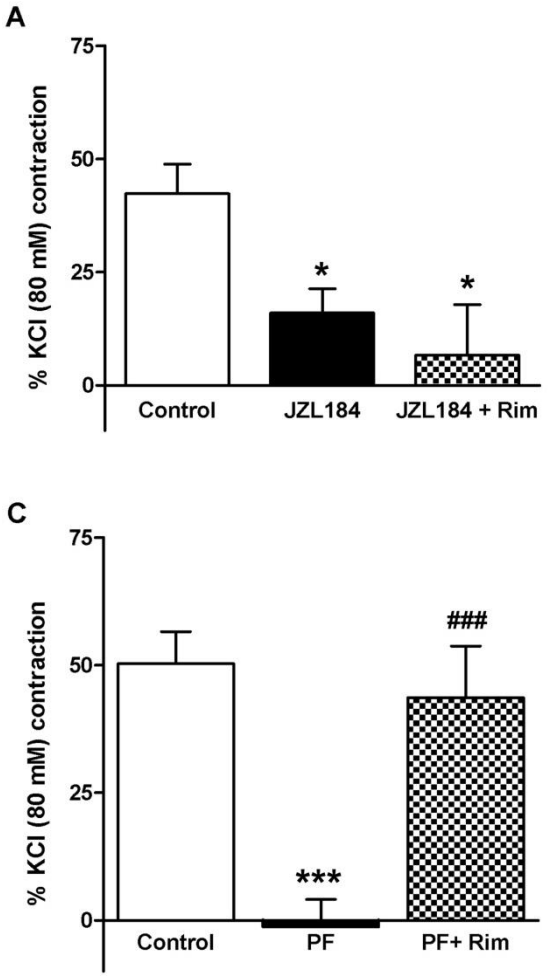
Since both MAGL and FAAH inhibition reduces naloxone-precipitated hypercontractility, the effects of MAGL and FAAH inhibition was evaluated in an *in vitro* model of secretion in ileal tissue as measured by changes in short-circuit current (I_{sc}). The measured current was normalized to the surface area of the tissue. Acute morphine treatment by itself caused a mild decrease in the I_{sc} current. Naloxone exposure (30μM) to both CB_1(+/+) and CB_1(-/-) ileal tissue treated with morphine (30μM) for 60 min, resulted in a profound increase in I_{sc} current ($p<0.05$ v/s morphine control; Fig. 23A). The MAGL inhibitor JZL184 (10μM) and the FAAH inhibitor PF-3845 blocked naloxone-induced increase in I_{sc} current in wild-type ileum tissue ($p<0.05$ v/s morphine-naloxone control; Fig. 26A and 26C). The anti-secretion effects of JZL184 were not observed in CB_1(-/-) tissue (Fig. 26B). Taken together, these data suggest that both MAGL and FAAH inhibition attenuate naloxone-induced hypersecretion in morphine-dependent ileum tissue.

5.4. Evaluation of effects of JZL184 on endocannabinoid, arachidonic acid and prostaglandin levels in ileum

The data depicted in Fig. 24 and Fig. 25 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. 24A), 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 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, AEA (table 5) 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). PF-3845 on the other hand elevated levels of AEA (table 5) but did not alter levels of 2-AG, AA or prostaglandins (data not shown).

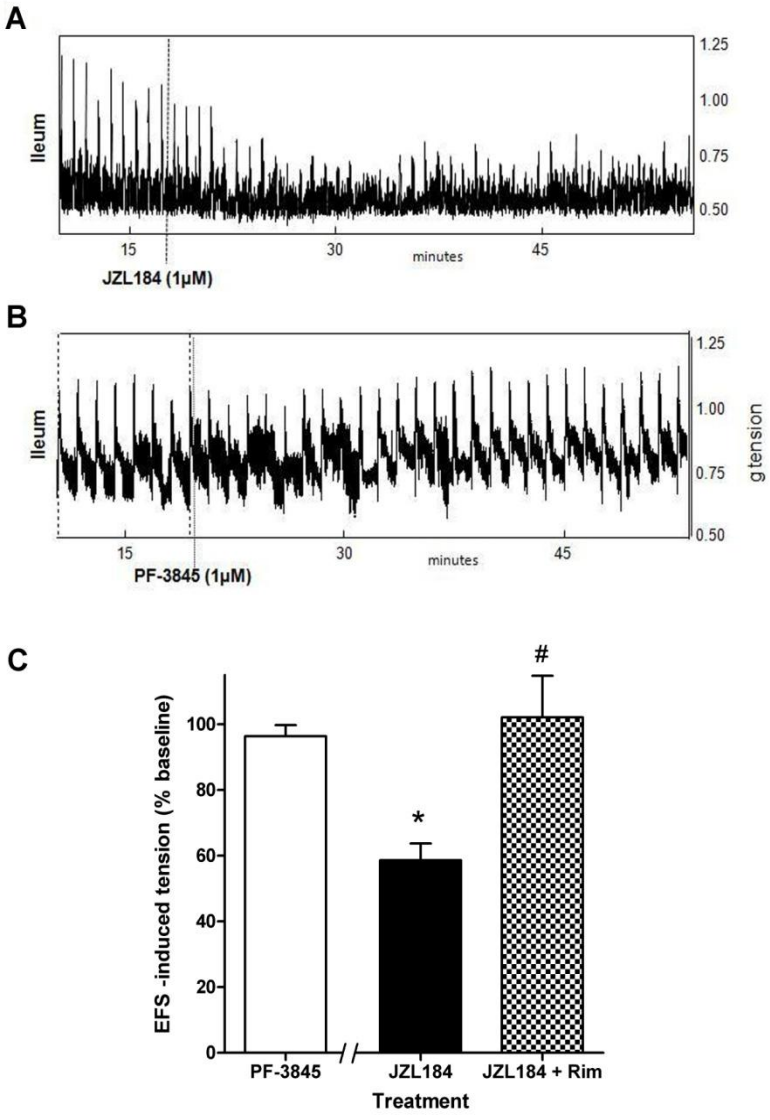
Figure 24. Inhibitors of endocannabinoid catabolic enzymes reduced naloxone-precipitated contractions of morphine-treated ilea, as measured by isometric tension recording.



(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.

Figure 25: 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.

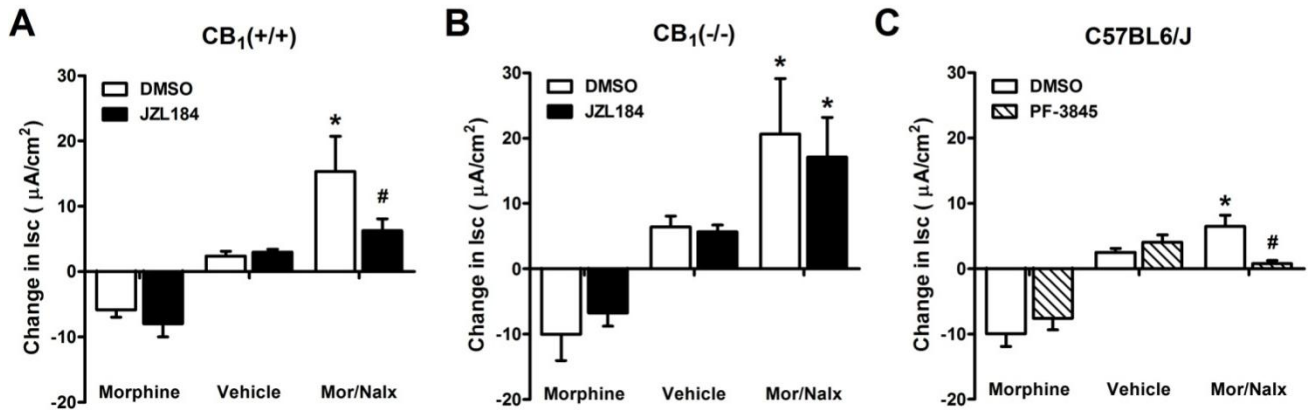


Figure 26. Effects of MAGL and FAAH inhibition on naloxone-precipitated hypersecretion in CB_1(+/+) and CB_1(-/-) mice.

(A) JZL184 blocks naloxone-precipitated hypersecretion in morphine-treated CB_1(+/+) ileum tissue as measured by change in I_{sc} current normalized to surface area. (B) JZL184 treatment does not alter naloxone-precipted hypersecretion in CB_1(-/-) tissue (C) PF-3845 blocks naloxone-precipitated hypersecretion in morphine-treated C57BL6/J ileum. Data expressed as mean ± SEM. *$p<0.05$ v/s appropriate morphine control; #$p<0.05$ v/s corresponding morphine-naloxone treated tissue. n=4-5 mice/ group; Data collected by Dr. Terez Shea-Donohue and Dr. Tim Vanuytsel at the University of Maryland, Baltimore

Table 5. Effects of FAAH and MAGL inhibitors on AEA and 2-AG levels 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

5.5. Discussion

Based on the differential anti-diarrheal effects between JZL184 and PF-3845, we predicted a similar association on intestinal motility and secretion in *in vitro* models of naloxone-precipitated withdrawal in morphine-treated ilea. To test this hypothesis, we investigated the effects of endocannabinoid catabolic enzyme inhibitors in *in vitro* models of electrogenic mucosal ion transport and neurogenic contractions to account for the anti-diarrheal effects of MAGL inhibition. JZL184 attenuated the intensity of naloxone-precipitated ileal contractions, but rimonabant failed to reverse these effects, suggesting that these effects are non-CB_1 mediated. Rimonabant blocked the effects of PF-3845 on naloxone-precipitated contractions in morphine-dependent ilea. Next, we demonstrated that naloxone can precipitate hypersecretion of electrolytes in morphine-dependent ileal tissue. In this model, effects of JZL184 in attenuating naloxone-precipitated hypersecretion in morphine-dependent ilea were abolished in CB_1(-/-) mice suggesting that CB_1 receptors modulate the anti-secretory actions of JZL184.

Cannabinoid receptor agonists have been shown to inhibit the electric field stimulated (EFS) contractions of myenteric plexus-longitudinal muscle preparations through prejunctional CB_1 receptors. This results in inhibition of contractile activity by blocking the release of acetylcholine from neurons of the myenteric plexus (Coutts and Pertwee, 1997). Acute exposure of ileal tissues to morphine also results in inhibition of EFS contractions via inhibition of Ach release (Paton, 1957). However, prolonged exposure leads to adaptations and naloxone can precipitate hypercontactility by inducing excess Ach release in these tissues (Tsou et al., 1982). Given the co-localization of MAGL, FAAH, CB_1 receptors and opioid receptors in myenteric plexus (Bagnol et al., 1997; Coutts et al., 2002; MacNaughton et al., 2004; Bashashati et al.,

2011), it is possible that both MAGL and FAAH inhibitors indirectly block hypercontractility during morphine withdrawal by attenuating the release of Ach from myenteric cholinergic neurons innervating the longitudinal muscle of the ileum.

Acutely, opiates have anti-diarrheal effects by inducing constipation via inhibition of intestinal motility as well as electrolyte and fluid transport. Opiate withdrawal results in gastro-intestinal distress including vomiting and diarrhea. Opiate agonists have been shown to have anti-secretory activity by affecting ion transport in the small intestine of mice (Sheldon et al., 1990). Both MOR and DORs have been implicated in the anti-secretory actions of opiates. In our studies, we have shown that *in vitro*, these actions of opiates undergo adaptations on prolonged treatment. This study is first to show that naloxone precipitates a hypersecretory response in tissues treated with morphine for a prolonged period of time in the Ussing's chamber. Cannabinoids also have been shown to have anti-secretory effects. The synthetic cannabinoid WIN blocks hypersecretion evoked by capsaicin or EFS in *in vitro* guinea pig and rat ileum preparations in the Ussing's chamber in a CB_1 dependent manner (Tyler et al., 2000; MacNaughton et al., 2004). These actions of cannabinoids are mediated by CB_1 receptors in the submucosal plexus of the guinea pig ileum. JZL184 possibly exerts its effects indirectly via CB_1 receptors that are co-localized with MORs in the submucosal neurons (Maguma et al., 2010).

The antecedents for diarrhea include enhanced contractility and increased secretion of fluids and electrolytes. Two observations from the present study indicate that the *in vitro* contraction 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 in vitro, but only JZL184 blocked the occurrence of diarrhea in vivo. Second, rimonabant blocked the anti-diarrheal effects of JZL184 in vivo, but did not reduce

naloxone-precipitated contractions in ileum in vitro. 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*. Additionally, both JZL184 and PF-3845 reversed naloxone-precipitated secretion in ileal tissue from CB_1(+/+) and C57BL6/J mice, respectively. JZL184's anti-secretion effects were not observed in CB_1(-/-) mice suggesting that CB_1 receptors modulate the actions of MAGL inhibition in this model. The lack of agreement between the effects of these inhibitors in the in vitro model v/s the differential anti-diarrheal effects observed in vivo may be due to different antecedents in each of the studies. First, the length of exposure of morphine is only 1 h compared to 3 days in the behavioral assays. For secretion studies, female C57 mice served as subjects while in the in vivo studies ICR male mice were used. Finally, in the in vitro studies, exposure to various drugs was ex vivo in organ baths, while in vivo they were given systematically.

Curiously, JZL184 did not increase 2-AG levels in ileum. However, the effects of JZL184 in elevating 2-AG levels are tissue specific. Likewise, JZL184 does not elevate 2-AG in other peripheral tissues, including testes, white adipose tissue, and lung, though it increases this endocannabinoid in other tissues, including liver, kidney, brown adipose tissue, and spleen (Long et al., 2009b). 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 PGs and other eicosanoids. Additionally, JZL184's anti-withdrawal effects *in vivo* as well as in the *in vitro* secretion model were reversed by rimonabant and in CB_1 (-/-) mice respectively, implicating the involvement of CB_1 receptors. JZL184 did not reduce levels of PGs in the ileum which corroborates with recent evidence that MAGL inhibition does not alter

AA and PG levels in gut (Nomura et al., 2011), which further argues against this alternative mechanism of action. Therefore, 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).

Chapter 6: Discussion

Summary

Previous studies have examined the ability of THC or exogenous cannabinoid agonists to reduce somatic opioid withdrawal signs in rodents. The purpose of the studies performed in this dissertation was to elucidate the potential of elevation of endocannabinoids, via inhibition of their degradative enzymes, to inhibit opioid withdrawal in pre-clinical rodent models of dependence. We hypothesized that elevating endogenous cannabinoids following inhibition of their appropriate catabolic enzymes would inhibit opioid withdrawal, through actions at cannabinoid receptors. Both MAGL and FAAH inhibition reduced both naloxone-precipitated as well as spontaneous somatic withdrawal signs with differential efficacy. Given the undesirable effects of prolonged complete MAGL inhibition and the reduced efficacy of FAAH inhibition to reduce somatic withdrawal signs compared to MAGL inhibition, we investigated a combination of partial MAGL and complete FAAH inhibition to maximize efficacy. This combination also reduced withdrawal signs without any associated dependence liability and mild receptor desensitization on repeated treatment. Finally, to elucidate the mechanism of anti-diarrheal effects, we hypothesized that MAGL but not FAAH inhibition would block hypercontractility and hypersecretion in the ileum. Both inhibitors blocked hypercontractility indicating that this model alone does not account for diarrhea. However MAGL inhibition significantly reduced withdrawal induced hypersecretion suggesting that MAGL inhibitors block diarrhea via anti-secretory actions.

Differential effects of MAGL v/s FAAH inhibition

Notably, high doses of 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.

Anti-withdrawal mechanism of action of MAGL and FAAH inhibition in vitro

In addition to attenuating somatic withdrawal signs with a higher efficacy, MAGL inhibition also completely blocked occurrence of diarrhea in vivo, while FAAH inhibition lacked anti-diarrheal efficacy. In order to understand the underlying mechanism, we examined the effects of both MAGL and FAAH inhibitors in *in vitro* assays of diarrhea. The precursors of diarrhea include enhanced contractility of the intestinal muscle (i.ie enhanced motility) as well as hypersecretion of water and electrolytes. Both JZL184 and PF-3845 reduced naloxone-precipitated contractions and reduced hypersecretion in the ileum in a CB_1-dependent manner. There are several mechanisms by which CB_1 activation may modulate anti-secretory effects and block contractility. One possibility is that activation of CB_1 receptors innervating cholinergic

secretomotoneurons (MacNaughton et al., 2004) may block Ach induced activation of transport proteins in the mucosa and submucosa (Fig 27). Additionally, CB_1 activation in myenteric neurons may block acetylcholine mediated hypercontraction of longitudinal muscle. Thus, inhibitors of endocannabinoid catabolic enzymes attenuate opiate withdrawal effects in two distinct *in vitro* models in the small intestine.

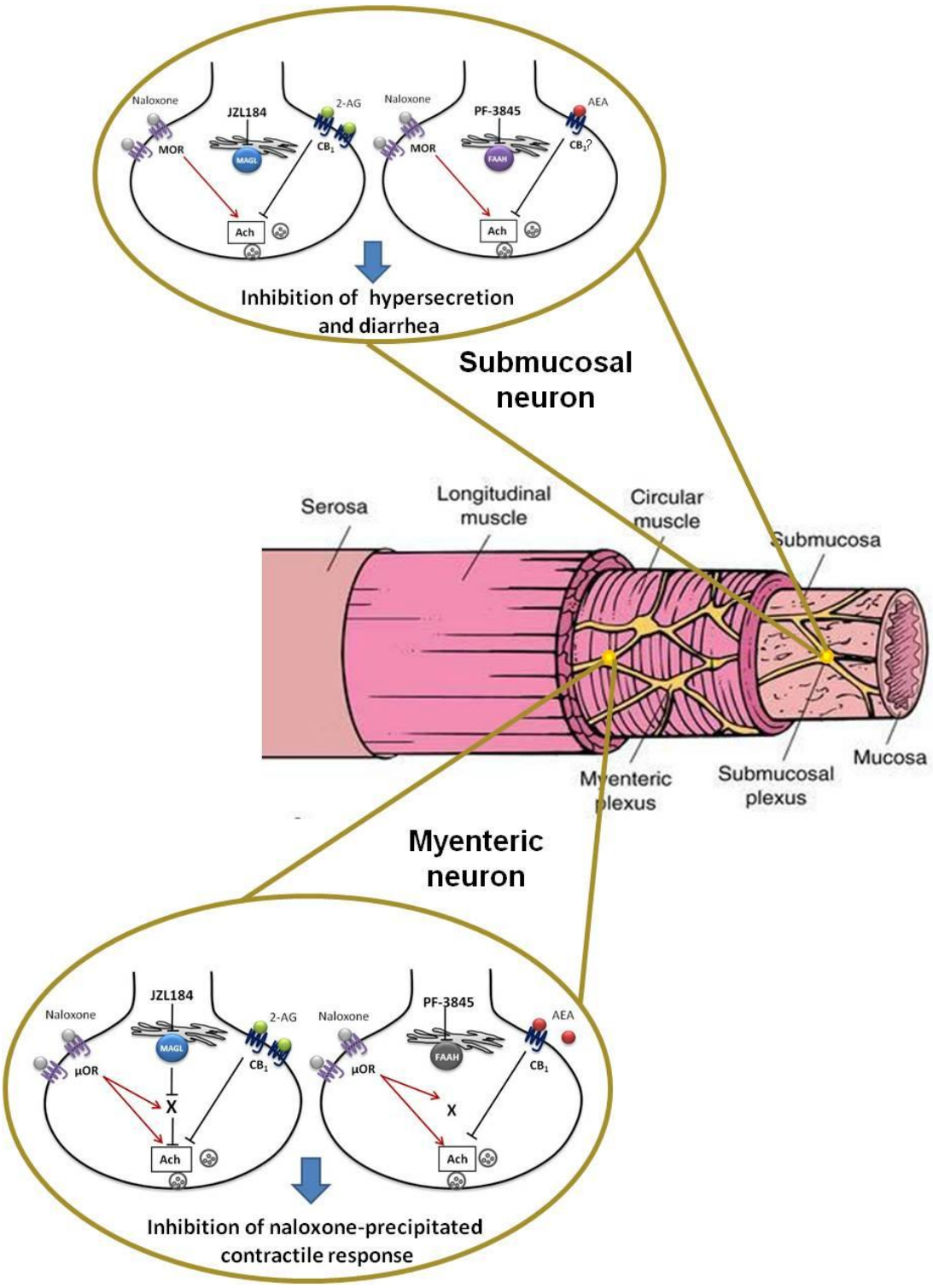


Figure 27: Ant-diarrheal mechanism of action of MAGL and FAAH inhibitors in the ileum (Top) JZL184 and PF-3845 reduces mucosal hypersecretion in the ileum diarrhea through activation of CB_1 receptors in the submucosal neurons (Bottom) Both JZL184 (non-CB_1) and PF-3845 (via CB_1) reduce naloxone-precipitated neurogenic contraction of the longitudinal muscle by affecting Ach release from myenteric neurons.

Advantages of MAGL and FAAH inhibitors over traditional cannabinoids

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, hypothermia and hyper-reflexia, than FAAH inhibitors, 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; 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.

Advantages of combining partial MAGL and complete FAAH inhibition

On the other hand, other studies indicate that complete MAGL inhibition may possess potential for rewarding effects and share other hallmarks in common with drugs of abuse. High dose JZL184 partially substitutes for THC in the drug discrimination paradigm (Long et al.,

2009c). In addition, repeated JZL184 elicits cannabinoid dependence, behavioral and functional tolerance, and cannabinoid receptor cross-tolerance (Long et al., 2009c; Schlosburg et al., 2010). It should still, however, be noted that many of the cannabimimetic side-effects of JZL184 are comparatively low in intensity versus moderate to high doses of THC or marijuana. However, while MAGL inhibitors possess far more side-effects than FAAH inhibitors, they showed much higher efficacy than FAAH inhibitors in reducing opioid withdrawal symptoms. Taken together, these results suggest that while both FAAH and MAGL inhibitors individually represent promising targets for reducing opioid dependence, they also have limitations. In order to circumvent some of these drawbacks, we investigated whether a combination of partial MAGL inhibition and complete FAAH inhibition would achieve enhanced efficacy in attenuating opioid withdrawal signs, without the negative side-effects associated with complete MAGL inhibition. Partial MAGL inhibition is achieved with lower dose of JZL184 (4 mg/kg) that produces a physiologically relevant response by elevating 2-AG levels (2-4 fold). The combination of partial MAGL and complete FAAH inhibition elevates AEA levels similar to FAAH inhibition alone, but elevated 2-AG levels higher than that observed with MAGL inhibition alone in ICR mice. Thus, it is possible that the combination exerts beneficial anti-withdrawal effects due to enhanced levels of 2-AG. Although this combination did not produce CB_1 receptor down-regulation, it induced moderate CB_1 desensitization following repeated treatment. In C57BL/6J mice, prolonged exposure to the combination did not induce any CB_1 receptor functional tolerance, analgesic tolerance in pain assays (Wise, Kinsey et al., unpublished data) or physical cannabinoid withdrawal. Taken together, the anti-withdrawal and analgesic effects of this combination coupled with its lack of dependence potential are important findings for the clinical development of a combined drug that is a partial MAGL and

a complete FAAH inhibitor. To this effect, a novel inhibitor SA-57 which has differential potency at MAGL and FAAH has been synthesized (Niphakis et al., 2011). The in vivo pharmacological actions of this inhibitor remains to be characterized.

Anti-withdrawal mechanism of action: Brain

Although determining the molecular mechanisms for endocannabinoid dampening of opioid withdrawal is beyond the scope of this dissertation, findings from previously published reports provide insight. A section of studies has suggested that opioid and cannabinoid receptors may interact due to their close proximal location within the neuron via signaling with a common G-protein pool, by forming functional heterodimers, or by allosterically modulating reciprocal functions (Rios et al., 2006; Hojo et al., 2008; Scavone et al., 2010). It is well established that acute morphine inhibits adenylyl cyclase at the cellular level, while opiate withdrawal leads to increased production of adenylyl cyclase and downstream signaling molecules in the LC and PAG (Nestler and Tallman, 1988; Guitart et al., 1992; Punch et al., 1997). These signaling events are associated with the expression of somatic and autonomic behavioral withdrawal signs (Shaw-Lutchman et al., 2002). Thus, increasing endocannabinoid activity may counteract the consequences of cAMP overshoot during opioid withdrawal (Fig. 28) 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). The other less explored hypothesis is that CB_1 may modulate signaling of MOR at the extra receptor level by altering signaling at the second messenger level (cAMP cascade, MAPK cascade or

Ca^{2+} signaling); affecting transcription of dependence induced genes or directly modulates release of neurotransmitters such as dopamine at the interneuronal level. Alternatively, CB_1 receptor activation is well known to reduce the release of GABA, glutamate, norepinephrine, and other neurotransmitters believed to be mediated through the inhibition of the adenylyl cyclase and the downstream mediators (Piomelli, 2003). Thus, dampening of other transmitters systems caused by CB_1 receptor activation represents an alternative mechanism by which endogenous cannabinoids could reduce opioid withdrawal.

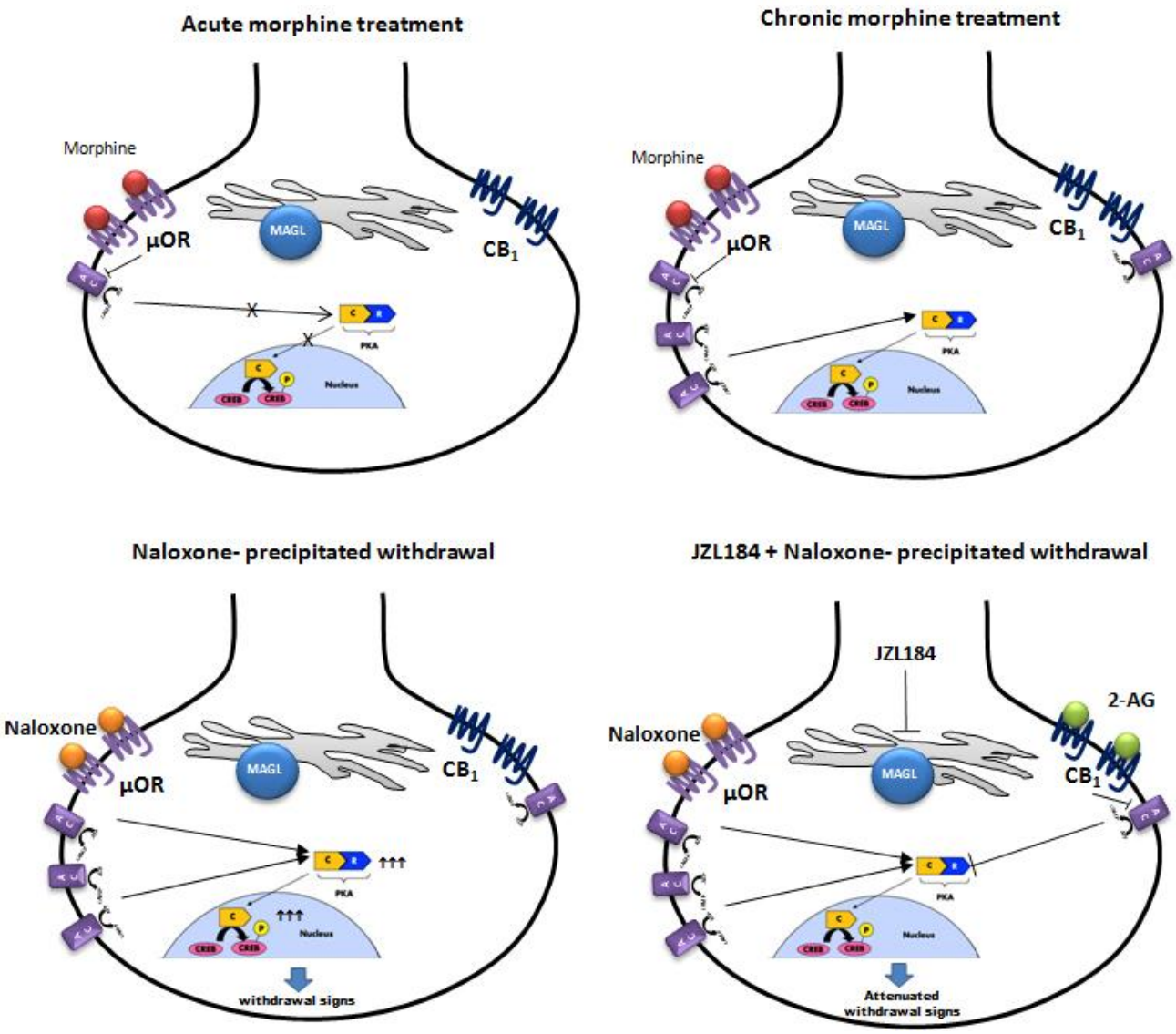


Figure 28: Proposed mechanism of action of anti-withdrawal effects of JZL184 in the brain

(Clock-wise) Acute morphine treatment causes suppression of AC activity and its downstream effects; Chronic morphine exposure leads to upregulation of AC and normalization of downstream signaling; Induction of withdrawal following chronic morphine exposure causes cAMP "overshoot" and increasing signaling of cAMP cascade resulting in expression of withdrawal signs; Acute, indirect CB1 activation by JZL184 inhibits AC and its downstream cascade, thereby attenuating withdrawal.

Another important consideration is whether endocannabinoid catabolic enzymes inhibitors will affect other components of opioid addiction, such as drug reward, extinction, relapse, and craving. Nonetheless, the results from the present study are the first to show that elevation of endocannabinoid levels ameliorates the expression of opioid withdrawal. The preliminary *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. These inhibitors offer advantages over current treatment options such as benzodiazepines, methadone, buprenorphine or naltrexone. These inhibitors produce selective elevation of eCBs, do not substitute for opioids as they do not have the same dependence liability and lack side-effects of benzodiazepines and opiate substitutes. Additionally, cannabinoids and morphine act at similar brain regions and affect similar signal transduction pathways. Rather than management or symptomatic treatment of withdrawal signs, elevating endocannabinoids may mechanistically alleviate abstinence signs by directly acting on signaling machinery in brain areas associated with opiate dependence. Thus, the enzymes responsible for endocannabinoid degradation offer promising targets for possible new treatments for opioid dependence.

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Awards and Honors

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Publications

Joel E. Schlosburg, Brittany L.A. Carlson, **Divya Ramesh**, Jonathan Z. Long, Benjamin F. Cravatt and Aron H. Lichtman. "Inhibitors of endocannabinoid metabolizing enzymes reduce precipitated withdrawal responses in THC dependent mice". *AAPS J,* 2009 Jun; 11(2):342-52.

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(In preparation) **Divya Ramesh**, Scott T. O'Neal, Tim Vanuytsel, Robert A. Owens, Rehab A. Abdullah, Anu K. Mahadevan, Benjamin F. Cravatt, Terez Shea-Donohue and Aron H. Lichtman. "MAGL inhibition attenuates opioid withdrawal induced somatic signs and diarrhea through activation of CB_1 receptors"

(In preparation) Steven G. Kinsey, Laura E. Wise, Sudeshna Ghosh, **Divya Ramesh**, Rehab A. Abdullah, Anu K. Mahadevan, Benjamin F. Cravatt, and Aron H. Lichtman. "Prolonged partial inhibition of monoacylglycerol lipase maintains CB_1 receptor-mediated antinociceptive and gastroprotective effects in mice".

Presentations

Paper Presentation: **Divya Ramesh**, Scott T. O'Neal, Robert A. Owens, Anu K. Mahadevan, Benjamin F. Cravatt, and Aron H. Lichtman. "MAGL and FAAH inhibition reduces spontaneous withdrawal signs in morphine-dependent mice". *Carolina Cannabinoid Collaborative meeting*, Oct 2011, Raleigh-Durham, NC.

Poster presentation: **Divya Ramesh**, Robert A. Owens, Steven G. Kinsey, Benjamin F. Cravatt, and Aron H. Lichtman. "Effects of MAGL and FAAH inhibition on morphine-dependent mice undergoing spontaneous withdrawal". *International Cannabinoid Research Society meeting*, July 2011, St. Charles, IL.

Paper Presentation: **Divya Ramesh**, Joel E. Schlosburg, Gracious R. Ross, Rehab A. Abdullah, Steven G. Kinsey, Jonathan Z. Long, Benjamin F. Cravatt, Hamid I. Akbarali, Laura J. Sim-Selley and Aron H. Lichtman**. "**Targeting endocannabinoid catabolic enzymes for the treatment of opioid withdrawal"**.** *International Cannabinoid Research Society meeting*, July 2010, Lund, Sweden.

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