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DIFFERENTIAL ROLES OF THE TWO MAJOR ENDOCANNABINOID HYDROLYZING ENZYMES IN CANNABINOID RECEPTOR TOLERANCE AND SOMATIC WITHDRAWAL

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

By

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While it was certainly not conventional for a chemical engineer to suddenly decide to become a drug abuse researcher, my experience and training during my time here at VCU was everything I hoped for…and none of the dangerous and unpleasant chemical plants I would've enjoyed otherwise.

First and foremost, I have to thank my advisor, Dr. Aron Lichtman. I honestly don't know why I somehow ended up in his laboratory over any other, if only by mixture of prodding and random choice, and yet I don't know how I could've been quite as successful as I was without him. He knew exactly when to guide my sometimes wild and random exuberance for doing *everything*, knew when to introduce new folds into my work to keep things challenging and hectic, and occasionally gave me an insanely long leash that few might, trusting me to come back with something worthwhile. He taught me not how to work hard, but work efficiently, and to always understand your outcomes on both a numerical and conceptual level.

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To my rugrat nephew Ethan, you keep me inspired to do great things and be a great success for you to look toward some day. With the wonderful people around you, you'll be ready for all you own success one day, a good start might be getting to the point you can remotely read and understand this paper in the least bit. Good luck with that! And to the future little niece to be named later (Riley), I hope to be successful enough to be around much more by your preschool years, finger crossed.

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LIST OF ABBREVIATIONS

2-AG	2-arachidonoyl glycerol
[^{3}H]CP55,940	Tritium labeled 2-[(1S,2R,5S)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol
[^{35}S]GTPγS	guanosine 5'-O-[gamma-thio]triphosphate
%MPE	maximal percent effect
AA	arachidonic acid
ABHD	α/β hydrolase
AEA	anandamide
AMYG	amygdala
ANOVA	analysis of variance
B_{max}	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
CG CTX	cingulate cortex
CPU	caudate putamen
DAGL	diacylglycerol lipase

DSE	depolarization-induced suppression of excitation
DSI	depolarization-induced suppression of inhibition
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
E_{max}	maximal effect
EC_{50}	half maximal (50%) effective concentration
eCB	endocannabinoid
FAA	fatty acid amides
FAAH	fatty acid amide hydrolase
G-protein	guanine nucleotide binding protein
G_i	cAMP inhibitory G-protein
G_s	cAMP stimulatory G-protein
GABA	γ-animobutyric acid
GDP	guanosine diphosphate
GP	globus pallidus
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GTP	guanosine triphosphate
GTPase	guanosine triphosphate hydrolase
HIPP	hippocampus
HYPO	hypothalamus
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
K_D	equilibrium dissociation constant
LC-MS-MS	liquid chromatography tandem mass spectrometry
MAPK	mitogen activated protein kinase
MAGL	monoacylglyceride lipase
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D
PAG	periaqueductal gray
PDE_{MD}	metal-dependent phosphodiesterase
PF-3845	N-(pyridin-3-yl)-4-(3-(5-(trifluoromethyl)pyridin-2-yloxy)benzyl) piperdine-1-carboxamide
PLC	phospholipase C
POA	preoptic area of the hypothalamus
Rim	rimonabant
SAMHSA	Substance Abuse and Mental Health Services Administration
s.c.	subcutaneous
SCID	severe combined immunodeficiency
SN	substantia nigra
SR1	SR141716A (rimonabant)
SR2	SR144528
SS CTX	somatosensory cortex
THC	Δ^9-tetrahydrocannabinol
TP_{N22}	tyrosine phosphatase (type N22)
Tris	tris(hydroxymethyl)aminomethane

TRPV1	transient receptor potential vanilloid 1
URB597	[3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate
WIN55,212	(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de)-1,4-benzoxazin-6-yl]-1-napthalenylmethanone

ABSTRACT

DIFFERENTIAL ROLES OF THE TWO MAJOR ENDOCANNABINOID HYDROLYZING ENZYMES IN CANNABINOID RECEPTOR TOLERANCE AND SOMATIC WITHDRAWAL

By Joel E. Schlosburg, Ph.D.

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

Virginia Commonwealth University, 2010.

Major Director: Dr. Aron Lichtman, Professor, Department of Pharmacology & Toxicology

While there is currently active debate over possible therapeutic applications of marijuana and cannabis-based compounds, consistently their primary drawbacks have been the psychoactive properties, dependence, and abuse potential. Prolonged administration of Δ^9-tetrahydrocannabinol (THC), the primary psychoactive constituent in marijuana, demonstrates both tolerance and physical withdrawal in both preclinical and clinical studies. Repeated THC administration also produces CB_1 receptor adaptations in the form of reduced activation of receptors, along with a downregulation of membrane surface receptors, in many brain regions involved in THC-associated behaviors. The increased need for drug to maintain therapeutic effects, and a withdrawal syndrome following discontinuation of use, are common risk factors in drugs of abuse. Recently, compounds have been developed that prolong the availability of the major naturally occurring endogenous cannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), through inhibition of their catabolic breakdown by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively. The overall objectives of this research are to elucidate the physiologic roles of these two endogenous ligands

and to determine if either can produce beneficial therapeutic effects without negative cannabis-like CNS effects. Therefore, we tested the impact of acute and prolonged blockade of FAAH and MAGL on a variety of cannabinoid-mediated behaviors and on precipitated cannabinoid withdrawal. Despite that acute blockade of FAAH and MAGL produce similar efficacy in reducing nociceptive responses, and both can reduce THC-induced somatic withdrawal, sustained blockade of these enzymes leads to remarkably different adaptations in CB_1 receptor functioning. Namely, prolonged elevations in brain 2-AG leads to marked antinociceptive tolerance, cross-tolerance to exogenous cannabinoid agonists, and physical dependence. In contrast, sustained elevations in brain anandamide continues to dampen pain responses without apparent signs of physical withdrawal, loss of CB_1 receptor activation as measured by $[^{35}S]GTP\gamma S$, or receptor downregulation as measured by $[^{3}H]CP,55940$. These results suggest that chronic 2-AG elicits greater compensatory changes in CB_1 receptor functions than anandamide. With similar efficacy in most therapeutic endpoints tested, and evidence of reduced impact on long-term function of the endocannabinoid system, these results distinguish FAAH as a more promising therapeutic target to treat pain and other conditions than MAGL.

INTRODUCTION

At the present time, the legalization of marijuana (*cannabis sativa*) use and growth may be closer to reality for the first time since almost 75 years ago (Mechoulam, 1986). A majority of states now allow some legal provisional use of commonly medically accepted therapeutic uses (i.e. nausea and glaucoma), with many states expanding the allowances to prescribe solely to the discretion of the doctor. The state of California is currently considering a ballot initiative that would officially decriminalize private individual use and growth of marijuana plants, regardless of intended purpose (Bogdanoski, 2010). While this may represent a new reality of drug culture in the United States, before the federal marijuana prohibition in 1937, cannabis extracts were of common use in medical tinctures and elixirs for treatment of a wide variety of psychological and physiological disorders. Recent studies have identified definitive evidence of use of cannabis materials that dates back to at least 700 B.C. (Mechoulam et al., 1991). The primary drawbacks for medical uses of cannabis-based treatments are the untoward psychoactive and cognitive effects, as well as concerns over abuse potential. In this thesis, I examine and discuss the potential for using the endogenous bioactive ligands that cannabis constituents mimic, and selectively target the regulatory system that controls their availability within the body, as a possible alternative to cannabis use. We will try and explore possible ways in which these endogenous ligands can substitute for cannabis in a number of therapeutic uses, as well as the possibility of reducing impairing effects and abuse potential. Most notably, we will examine two aspects of cannabis dependence as described in the *Diagnostic and Statistical Manual of*

Mental Disorders (DSM-IV), tolerance and physical withdrawal (American Psychiatric Association and American Psychiatric Association. Task Force on DSM-IV, 2000).

Tolerance is defined as the reduced efficacy of a chemical substance to produce bioactive effects similar to that of initial exposure, leading to the need for use increasing drug to produce the same outcomes as initial use. Withdrawal can have both psychological (i.e. craving and irritability) and physical components (i.e. chills, cramping, nausea), which results from sudden discontinuation or the application of an antagonist of the substance. Most often, the physical symptoms (combined with craving) enhance the likelihood of drug users continuing use while attempting to quit (American Psychiatric Association and American Psychiatric Association. Task Force on DSM-IV, 2000).

The discovery of cannabinoid ligands and receptors

The discoveries of what makes marijuana a biologically active compound, with the wide variety of physiological functions that it influences, are comparatively recent amongst drugs with such long histories of worldwide use. The first constituents of marijuana were isolated by Roger Adams in 1940; however these compounds were not compounds with psychoactive properties (Adams et al., 1940a; Adams et al., 1940b). In 1964, Raphael Mechoulam reported on the first studies in which chemical compounds extracted from the cannabis plant were isolated and found to have biological activity attributed to 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 discovery that the active compounds in marijuana were lipid-based led to active debate over whether activity at cellular level was targeting a specific receptor system, or merely

nonselectively altering membrane composition (Martin et al., 1988). It was finally discovered in 1988 that cannabis-based compounds require components of $G\alpha_i$ proteins to signal in cell cultures (Howlett et al., 1986), and later that cannabimimetic compounds stereoselectively bound to specific sites in the brain, suggesting a mode of action via a G-protein coupled receptor (GPCR) (Devane et al., 1988). Selective radiolabeled ligands and advances in biological tools allowed for the eventual cloning of two distinct cannabinoid receptors that THC binds to, now known as CB_1 (Matsuda et al., 1990) and CB_2 (Munro et al., 1993). Not only were CB1 receptors heavily concentrated in numerous key areas of the brain, but also it was found to be the most abundant GPCR found in brain.

Actions of cannabinoid receptors

CB_1 receptors belong to the G-protein coupled receptor superfamily and activate primarily $G\alpha_{i/o}$, resulting in inhibition of adenylyl cyclase, activation of A-type and inwardly rectifying potassium channels, inhibition of N- and P/Q-type calcium channels and stimulation of MAP kinase (Howlett et al., 2002). The functional consequences of these cellular signals are a reduction in fusion of synaptic vesicles to the outer membrane, and suppression of both excitatory and inhibitory signals in neurons, depending on the other receptor systems present in the synaptic milieu (Katona et al., 1999; Kreitzer and Regehr, 2001). CB_1 receptors are primarily distributed throughout the nervous system, both centrally and peripherally. The CB_2 receptor is commonly found on immune cells, and recent evidence had demonstrated the presence of CB_2 receptors in microglia and brainstem neurons (Cabral and Marciano-Cabral, 2005; Van Sickle et al., 2005). While their enhanced expression during induction of neuro-inflammation suggests potential neuroprotective function, the function of central CB_2 receptors is

not yet fully known. Knockout mice have been developed that lack functional CB_1, CB_2, or both receptors to further aid in studying the contributions of the activation of each subtype (Buckley et al., 2000; Zimmer et al., 1999). In addition, selective agonists and antagonists are also available for each receptor (Rinaldi-Carmona et al., 1994; Rinaldi-Carmona et al., 1998).

Using both CB_1 (-/-) mice and antagonists, several physiological changes are attributable to CB1 receptor activation, including a group of effects highly correlated to CB_1 receptor binding and activation know as the "tetrad". This battery of four tests, or subsets thereof, is often employed to screen for cannabimimetic activity, and include: spontaneous locomotor suppression, analgesia to noxious thermal stimuli, catalepsy, and hypothermia (Compton et al., 1993). Studies show that all are sensitive to CB_1 blockade or inactivation, with spontaneous activity the only response still seen at higher doses (Varvel et al., 2005). CB_1 receptor activation can also be attributed to several common features of marijuana: increased feeding (Beardsley et al., 1986; Chambers et al., 2007), reduced emesis and nausea (Darmani, 2001a; Darmani, 2001b), a wide range of analgesia or reductions in pain hypersensitivity (Lichtman and Martin, 1991; Martin et al., 1999), impairments in several aspects of memory (Lichtman and Martin, 1996; Niyuhire et al., 2007), and reduced pressure in the aqueous humor in the eye (Chien et al., 2003; Green and Pederson, 1973). Interestingly, CB1 (-/-) mice were valuable in demonstrating that cannabinoid receptor activation plays a role in the rewarding properties of other common drugs of abuse, as these mice fail to demonstrate elevated dopamine release in nucleus accumbens or substantial intake by ethanol or morphine (Hungund et al., 2003; Mascia et al., 1999).

Activation of CB2 receptors, being primarily on immune cells, has been shown to play a role in reducing inflammatory edema (Berdyshev et al., 1998; Puffenbarger et al., 2000),

inflammatory pain and pain from nerve injury (Ibrahim et al., 2005; Sanson et al., 2006), as well as reductions in hypersensitivity reactions to allergenic stimuli (Jonsson et al., 2006; Maekawa et al., 2006). While both CB_1 and CB_2 receptor subtypes share approximately 48% homology and downstream cellular signaling pathways, their respective distribution likely accounts for the majority of differential physiological response. However, it should be noted that CB_1 is heavily present throughout the body, with wide distribution outside neurons, and may also have some role in functions outside neural control such as has been shown with fat deposition (Herling et al., 2008; Ravinet Trillou et al., 2004).

The endocannabinoid system: ligands and regulatory pathways

A group of endogenous ligands, derived from phospholipid precursors and act on cannabinoid receptors, have been identified. They are collectively referred to as endocannabinoids (eCBs). Among these include nonselective agonists such as noladin ether and arachidonoyl dopamine, and the endogenous CB_1 antagonist virodhamine (Gomez-Ruiz et al., 2007). There is also *in vitro* evidence that a class of peptide derivatives of α-hemoglobin (hemopressins) may also be able to bind to CB1 receptors and alter activation, though not through G-protein activity (Gomes et al., 2009; Heimann et al., 2007). By far, the most studied and well-characterized ligands are anandamide (Devane et al., 1992) and 2-arachidonoylglycerol (Mechoulam et al., 1995; Stella et al., 1997). The available signaling pool of both ligands is tightly regulated by both a series of synthetic, as well as degradative, enzymes (summarized in Figure 1).

AEA was initially thought to be derived primarily from N-arachidonoyl-phosphatidylethanolamine (NAPE), and cleaved via a NAPE-specific phospholipase D (NAPE-

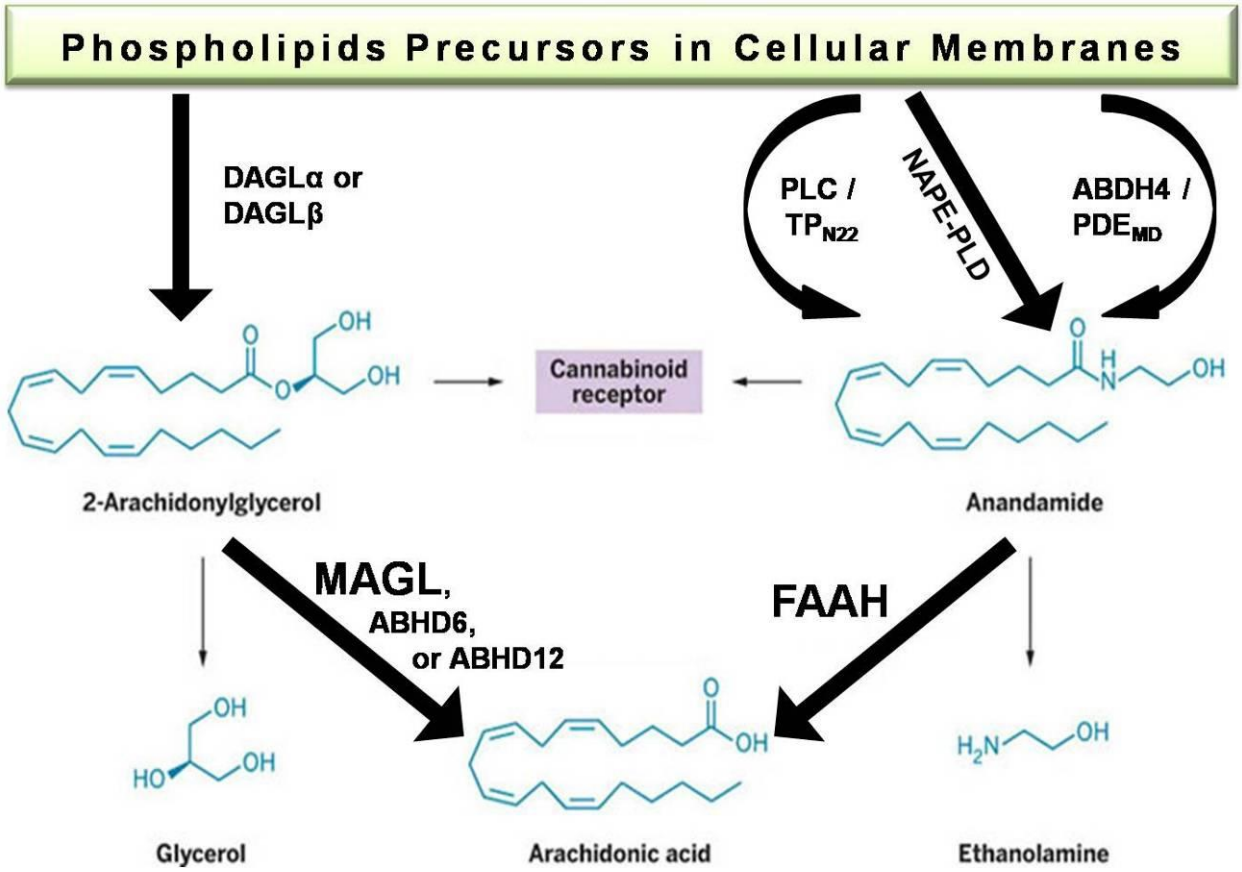

Figure 1 – Schematic of the synthetic and degradative pathways proposed for the two major endogenous cannabinoids. Both are synthesized through one of several lipases from phospholipids contained within the cell membrane to generate ligands for the CB_1 and CB_2 receptors. The respective degradative enzymes catalyze the active ligands to arachidonic acid, which is inactive at cannabinoid receptors.

PLD). However, the observation that NAPE-PLD knockout mice possess wild-type levels of AEA invalidated this theory (Leung et al., 2006). An alternative enzyme pathway proposed to be responsible for AEA biosynthesis includes α/β-hydrolase 4 (ABH4) cleavage to a lipid intermediate that is further hydrolyzed to anandamide by a mellalo-dependent phosphodiesterase (Simon and Cravatt, 2006). Subsequent studies in mice lacking the proposed phosphodiesterase, GDE1, also demonstrated similar brain AEA levels. Deleting both GDE1 and NAPE-PLD simultaneously did not significantly alter bulk tissue AEA levels, suggesting at least a third pathway is responsible for AEA synthesis (Simon and Cravatt, 2010). A third pathway was proposed in which phospholipase C (PLC)-catalyzed cleavage of NAPE generates phosphoanandamide which is subsequently dephosphorylated by a phosphatase (Liu et al., 2006). Given that all these pathways have been shown to functionally generate anandamide in succession, and the differential distribution and cellular condition in which these enzymes are activated, it is theorized that these pathways may all play a partial role and are tissue specific (Liu et al., 2008). A clearer regulatory mechanism is known for AEA degradation, which is rapidly and predominantly hydrolyzed to arachidonic acid and ethanolamine by fatty acid amide hydrolase (FAAH), and inactivation/inhibition of FAAH greatly increases levels of AEA in a variety of tissues (Cravatt et al., 2001; Fegley et al., 2005). FAAH also degrades several other fatty acid amides with known physiological functions, such as: oleamide (sleep), palmitoylethanolamide (PEA; anti-inflammatory), and oleoylethanolamide (OEA; satiety) (Cravatt et al., 2001).

2-AG is synthesized by the cleavage of diacylglycerol (DAG) by DAG lipase. Recent studies of mice with deletions of the two functional DAGL isotypes, α and β, demonstrated that a majority of the 2-AG content in brain is regulated by DAGLα, as well as all the CB_1 receptor-

mediated actions attributable to 2-AG in brain studied so far (Gao et al., 2010; Tanimura et al., 2010). 2-AG is also rapidly degraded, primarily by the enzyme monoacylglycerol lipase (MAGL). A recent proteomic analysis of the enzymes that hydrolyze 2-AG in brain showed that 3 serine hydrolases made up the majority of degradative activity. MAGL was predominantly responsible for 2-AG regulation, hydrolyzing approximately 85% of the brain's 2-AG content. Novel hydrolases discovered to be involved to lesser degrees in 2-AG hydrolysis included α/β-hydrolase 6 and 12 (ABHD6/ABHD12), which accounted for 4% and 9% of hydrolysis, respectively. Further study showed these enzymes have characteristics suggesting differential cellular localization, and new evidence points to differential distribution of these enzymes among neuronal and glial cells. FAAH, while displaying 2-AG hydrolysis activity in isolated testing, displayed negligible contribution to the overall hydrolysis of 2-AG in whole brain (Blankman et al., 2007).

While the advantages of having two distinct ligands in the brain with overlapping receptor targets are unclear, there is growing evidence that their functions and localization are as equally segregated as their regulatory mechanisms. Levels of available pools of 2-AG are almost 1000-fold higher than that of AEA, though dialysis of extracellular synaptic spaces in the nucleus accumbens reveals the differences in the pool that potentially serves to signal only about 3-fold higher (Alvarez-Jaimes et al., 2009). Cellular localization of FAAH appears to be predominantly postsynaptic, located at sites associated with calcium regulation, while MAGL is found in axon terminal localized postsynaptically (Gulyas et al., 2004).

Anandamide and fatty acid amide hydrolase inactivation

The primary methods of exploring the function of the endogenous cannabinoid system include: phenotypic changes in CB_1 receptor (-/-) mice, the use of inhibitors of FAAH that elevate AEA levels in brain and several peripheral tissues (Ahn et al., 2009; Boger et al., 2005; Fegley et al., 2005), and mice that have FAAH genetically inactivated (Cravatt et al., 2001). CB_1 receptor antagonist studies have also been used to provide evidence of endocannabinoid function; however the inverse agonist properties of available antagonists confound the potential interpretations (Landsman et al., 1997). FAAH inhibition and genetic deletion most directly examines the physiologic role and therapeutic potential of AEA activity at cannabinoid receptors, selectively elevating AEA without altering 2-AG levels. Given the other bioactive fatty acid amides regulated by FAAH, the possibility exists that mediators other than AEA may provide therapeutic benefits. Given that AEA is the only regulated fatty acid amide that binds CB_1 receptors, FAAH inhibitor effects mediated by AEA should be reversible by CB_1 inactivation.

Inhibition by URB597 produces elevations in AEA above vehicle of about 4-fold for up to 3 h, with inhibition lasting for around 12 h. Second generation inhibitors such as PF3845 are able to elevate AEA from 10- to 15- fold above vehicle, comparable to that seen in FAAH (-/-) mice, with inhibition of FAAH remaining for up to 36 h. Earlier studies performed on the actions of exogenous AEA showed immediate cannabimimetic effects using central and intravenous routes of administration, however these effects were short in duration (Smith et al., 1994). This is likely due to the rapid metabolism of exogenous AEA, often in a matter of less than 10 minutes (Willoughby et al., 1997). Administration of AEA exogenously to animals treated with URB597 or FAAH (-/-) mice demonstrate cannabinoid-mediated tetrad behavioral effects (Cravatt et al.,

2001; Fegley et al., 2005), displaying the potential for AEA to act in a manner similar to THC in the absence of rapid degradation. When examining these effects upon CB_1 antagonist, or selective deletion of non-neuronal FAAH, it is clear that all the tetrad behaviors are mediated by central CB_1 receptor activation, with some exception for hypomotility (Cravatt et al., 2001; Cravatt et al., 2004).

Inhibitors of FAAH have proven to possess therapeutic potential in a wide variety of applications (for review see Piomelli et al., 2006). The FAAH inhibitor URB597 shows anxiolytic-like activity in the elevated zero maze, as well as reducing vocalizations during isolation (Kathuria et al., 2003). URB597 also displays antidepressant activity in forced swim and tail suspension testing (Gobbi et al., 2005). While these findings have proven difficult to replicate fully, it appears that the efficacy of the anxiolytic-like and antidepressive-like activity of FAAH inhibition is enhanced during conditions of exceptional stress and aversiveness (Naidu et al., 2007). Given that endocannabinoids are produced under conditions of cellular stress "on demand" suggests that any anxiolytic actions of FAAH inhibition may only show psychoactive effects during periods of extreme distress (Haller et al., 2009).

Most applications for FAAH inhibitors have focused on the analgesic and anti-hypersensitive pain modulation properties. FAAH (-/-) mice show hypoalgesic phenotypes to a variety of painful thermal and chemical noxious stimuli (Lichtman et al., 2004). Both reversible and irreversible inhibitors of FAAH display similar analgesia in diverse pain tests (Chang et al., 2006; Lichtman et al., 2004; Suplita et al., 2005). In addition to acute pain models, FAAH inhibition is effective in reducing allergenic itch response at similar potency (Schlosburg et al., 2009). FAAH inhibition shows ever greater efficacy at reversing sensitivity and hyperalgesia due to chronic inflammation (Ahn et al., 2009; Cravatt et al., 2004; Jayamanne et al., 2006;

Jhaveri et al., 2008) and nerve injury (Chang et al., 2006; Jayamanne et al., 2006; Jhaveri et al., 2006; Kinsey et al., 2009).

In addition to reducing inflammatory pain, FAAH inhibition is able to reduce inflammatory edema (Cravatt et al., 2004; Holt et al., 2005; Wise et al., 2008), an effect that is mostly attributable to the actions of peripheral FAAH expression outside nervous tissue (Cravatt et al., 2004). FAAH inhibition also reduces inflammatory markers in visceral models of colitis and gastrointestinal inflammation (Massa et al., 2004; Storr et al., 2008). These anti-inflammatory actions are correlated with evidence of reduced cytokine release following immunological insults by inflammatory mediators such as lipopolysaccharides, which also are able to induce production of AEA (Liu et al., 2003; Maccarrone et al., 2002; Roche et al., 2008; Tham et al., 2007). However, in the case of inflammatory and anti-edema effects, there is increasing evidence that several bioactive FAAH-regulated fatty acid amides (AEA included) are targeting alternative receptor systems other than cannabinoid receptors (Chang et al., 2006; Costa et al., 2008; D'Agostino et al., 2007; Lo Verme et al., 2005; Sagar et al., 2008).

In addition to the potential beneficial therapeutic applications in which FAAH inhibition appears to demonstrate efficacy, FAAH inhibitors have been demonstrated to elicit minimal cannabinoid-mediated psychoactive effects and possess low potential for drug abuse. Initial studies of URB597 show that it does not induce a place preference with repeated associations, and does not display substitution in rats trained to discriminate THC (Gobbi et al., 2005). Combinations of inhibitors and exogenous AEA reveal that the inhibitors alone are unable to induce increase in dopamine release from the shell of the nucleus accumbens, a common hallmark of abuse potential, but can in the presence of exogenous AEA (Solinas et al., 2006). The combination of exogenous AEA with FAAH inhibition also allows for discrimination by rats

trained to identify THC-like effects, an action not found with FAAH inhibitors alone (Solinas et al., 2007). Studies of self-administration in squirrel monkeys, the only model to show self-administration of THC so far (Justinova et al., 2003), show that URB597 is not self-administered or alter drug-seeking behavior in mice trained to press for THC or cocaine, though did potentiate AEA self-administration. Also unlike THC, FAAH inhibitors do not reinstate extinguished drug use to THC, cocaine, or even AEA (Justinova et al., 2008).

2-arachidonoylglycerol and monoacylglycerol lipase inactivation

Only recently have the proper tools become available to manipulate 2-AG in the CNS, and investigate the physiological functions of this second eCB. URB602 was the first reported inhibitor of MAGL able to elevate 2-AG levels at higher doses, though only using highly localized injections in the brain. While the ability to enhance 2-AG levels was low, and not particularly selective against FAAH (Vandevoorde et al., 2007), initial work with this compound provided the capability to demonstrate that both AEA and 2-AG are responsible for the phenomena of cannabinoid stress-induced analgesia in the periaqueductal gray (Hohmann et al., 2005). Further publications have used URB602 systemically to elicit rather questionable findings attributed to enhanced 2-AG levels (Comelli et al., 2007), and at least one lead compound (URB754) was found to be completely inactive upon replication (Saario et al., 2006), later attributed to a toxic and nonselective contaminant of synthesis (Tarzia et al., 2007). Other nonselective serine hydrolase inhibitors, such as N-arachidonyl maleimide, demonstrated enhancement of 2-AG in producing CB_1 receptor-mediated behaviors and receptor activation, though not definitively via MAGL inhibition (Burston et al., 2008). The nonselective inhibition

of a variety of serine hydrolases by these drugs, especially nonselective towards FAAH, made it difficult to determine what contribution MAGL inhibition specifically played in the result found.

In 2008, our group in collaboration with the Cravatt group, reported on the first inhibitor selective and potent enough to acutely elevate 2-AG levels 8-fold when given systemically, without elevations in AEA. JZL184 was capable of cannabinoid-mediated enhancement in numerous acute pain tests, hypomotility, and hypothermia (Long et al., 2009a). JZL184 also produced anti-allodynic effects in mice with peripheral nerve injury, an effect mediated by CB_1 receptors. FAAH effects in these same models are dependent on both CB_1 and CB_2 receptors (Kinsey et al., 2009). The hypomotility and hypothermic effects seen following JZL184 represent potential differential physiological roles for MAGL, as these effects have never been reported in FAAH (-/-) mice or mice treated with FAAH inhibitors. Subsequent studies have demonstrated that JZL184 can enhance cannabinoid-mediated neuronal plasticity in the form of depolarization-induced suppression of excitation (DSE) and inhibition (DSI). Both result in cannabinoid-receptor hyperpolarization of a repetitively depolarized neuron, which depending on the nature of the neuronal cell type, suppresses subsequent vesicular release of excitatory glutamate or inhibitory GABA. These effects are not mimicked by FAAH inhibitors (Pan et al., 2009; Straiker et al., 2009). Current efforts are underway to determine comparative efficacy of JZL184 in the numerous models already established to be modulated by FAAH inhibition.

An intriguing twist to the segregated roles of MAGL and FAAH inhibition behavioral responses was a second series of studies employing simultaneous FAAH/MAGL inhibition. Using JZL184 in combination with FAAH (-/-) mice, JZL184 in combination with PF3845, or a newly described dual-endocannabinoid enzyme inhibitor JZL195, mice showed pronounced thermal analgesia and even a catalepsy-like response. These effects were absent using isolated

inhibition of either enzyme alone. Also, using mice trained to discriminate THC, JZL184 partially substituted when given as a challenge treatment alone to a wild-type mouse, but fully substituted for THC in FAAH (-/-) mice. JZL195 also demonstrated the capability to produce full substitution. This study suggests that the simultaneous elevation of both AEA and 2-AG together in brain may provide the combined CB_1 receptor activity to produce psychoactive effects similar to that of exogenous agonist such as THC (Long et al., 2009b).

Tolerance and receptor adaptations following repeated cannabinoid administration

The presence of cannabis tolerance and dependence following repeated use has long been a controversial issue, though generally accepted with greater evidence and controlled studies (Jones et al., 1976; Jones et al., 1981). Before the receptor was ever cloned, cellular adenylyl cyclase inhibition underwent tolerance during continuous exposure to THC in media, as well as cross-tolerance to other cannabinoid drugs (Dill and Howlett, 1988). Later studies have implemented the tetrad behavioral endpoints to measure levels of cross tolerance of THC towards itself, synthetic cannabinoid agonist, and exogenous anandamide. Similarly, repeated high-dose AEA and synthetic agonist can produce THC cross-tolerance (Fan et al., 1994; Fride, 1995; Pertwee et al., 1993; Welch, 1997; Wiley et al., 2005). An explanation of how exogenous AEA produces tolerance despite a very short duration of receptor activation remains unclear. Conversely, similar tolerance is noted by repeated administration of the CB_1 inverse agonist rimonabant, both behaviorally and in stimulating the cAMP/PKA signaling pathway (Rubino et al., 2000).

With these behavioral changes following repeated exposure, CB_1 receptor desensitization and downregulation are commonly reported. THC produces loss of membrane CB_1 receptor

pools (Rodriguez de Fonseca et al., 1994), and increasing reductions in receptor-mediated activation of G-protein signaling in a dose- and time- dependent manner (Breivogel et al., 1999; McKinney et al., 2008). Desensitization and downregulation is dependent on G-protein-coupled receptor kinases and beta-arrestin in a similar fashion as other GPCR proteins (Rubino et al., 2006), though the specific target site of these proteins on CB_1 receptors for desensitization or downregulation appear to be distinct (Jin et al., 1999). There are regional changes in receptor desensitization and receptor loss observed following both THC and synthetic cannabinoid treatment, with striatal regions being consistently the least sensitive to adaptation (Sim et al., 1996; Sim-Selley and Martin, 2002), which may be correlated to selective elevations in mRNA for CB1 in striatum during repeated exposure (Romero et al., 1997). Synthetic cannabinoid agonists generally produce comparable desensitization and receptor loss in most regions (Sim-Selley and Martin, 2002); however exogenous AEA produced isolated desensitization in a previous study without any receptor loss or cAMP accumulation (Rubino et al., 2000). Later studies show FAAH (-/-) mice have similar receptor number in brain compared to wild-type controls, and normal responses to acute THC administration (Cravatt et al., 2001; Lichtman et al., 2002). Our group has now recently shown exogenous AEA produces tolerance to AEA and THC tetrad behaviors in FAAH (-/-) mice, however regional measures of receptor G-protein activation were minimally affected compared to equipotent doses of THC, further indicating a reduced impact on CB_1 receptor function by FAAH inhibition and AEA elevation (Falenski et al., 2010). Currently, no studies have examined the role of acute or repeated exposure to MAGL inhibition of exogenous 2-AG.

Physical withdrawal resulting from repeated exposure to cannabinoids

Cannabis is by far the most commonly used illicit drug in the United States, representing 73% of all illicit drug use and more than half of these individuals use marijuana exclusively. Of the over 14 million people who use marijuana in the United States, almost 4 million are classified as being dependent or abusing (Substance Abuse and Mental Health Services Administration: Office of Applied Studies, 2008). While it is common public perception that marijuana poses reduced physical dependency risk compared to other drugs of abuse, repeated marijuana smoking has been demonstrated to produce a distinct abstinence syndrome in clinical settings (Budney et al., 2003; Haney et al., 1999b; Jones et al., 1976). The symptoms of this syndrome include anxiety, irritability, stomach pains, disrupted sleep, and general physical discomfort. 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., 1999a). Any abstinence syndrome may increase the desire to continue drug use and represents a complication in treating dependence.

Despite representing more than half of all classified drug abusers and an average 1 million people receiving treatment each year for marijuana dependence, there are currently no approved pharmacological treatments available for cannabis dependence. THC is also the most reliable and effective pharmacological agent identified that reduces cannabis withdrawal signs in both preclinical (Beardsley et al., 1986; Lichtman et al., 2001; Wilson et al., 2006) and clinical (Budney et al., 2007; Haney et al., 2004) studies. In fact, treatments employed for tobacco cessation and other drugs of abuse, such as bupropion and divalproex, actually worsened

marijuana withdrawal symptoms (Haney et al., 2001; Haney et al., 2004). Thus, there is a need to examine marijuana withdrawal treatment as a unique and separate area of research.

There is only one preclinical study that established clear withdrawal from THC by spontaneous cessation, measuring decreases in primate response to obtain food during abstinence from THC (Beardsley et al., 1986). However, rodent models of precipitated cannabinoid withdrawal have been well characterized since the introduction of the selective CB_1 receptor antagonist, rimonabant (Aceto et al., 1995; Tsou et al., 1995). Mice exposed to either repeated marijuana smoke or injections of THC display similar physical withdrawal symptoms (Wilson et al., 2006), with the most common signs being paw tremors and head twitches (Cook et al., 1998; Hutcheson et al., 1998). These withdrawal behaviors have been correlated with increased adenylyl cyclase activity in cerebellum (Tzavara et al., 2000), in marked contrast to acute cannabinoid actions that inhibit adenylyl cyclase activity. This effect also produces cross-tolerance to adenosine- and GABA- mediated cerebellar adenylyl cyclase inhibition (Selley et al., 2004). Previous attempts at continuous infusion of exogenous AEA were conducted prior to the availability of FAAH inhibition, with minimal results in precipitated withdrawal (Aceto et al., 1998). However, no studies have examined cannabinoid withdrawal utilizing the recent development of selective inhibitors of endocannabinoid catabolic enzymes. With cannabinoid substitution being the currently most effective treatment of cannabis withdrawal, the endogenous cannabinoid system becomes the next likely focus for therapeutic targets (Clapper et al., 2009).

Rationale and Hypothesis

Cannabinoid withdrawal

In the present series of studies, we employed FAAH (-/-) mice, MAGL (-/-) mice, FAAH inhibitors, and MAGL inhibitors to examine the role of endocannabinoid elevations in modulating established CB_1-mediated responses. The first subset of studies tests whether increasing endogenous cannabinoid levels can acutely ameliorate cannabinoid withdrawal responses. Given that cannabinoid receptor agonists administered during withdrawal can ameliorate withdrawal symptoms, we hypothesize that elevations in endocannabinoids can similarly attenuate withdrawal responses during antagonist precipitated withdrawal. First, we examined whether FAAH (-/-) mice would display a decrease in the severity of THC withdrawal responses. Next, we investigated whether acute administration of either URB597 or JZL184 would suppress the somatic signs of THC withdrawal. Finally, the liability of both FAAH and MAGL inhibition, including several combinations of simultaneous inhibition, were evaluated for potential to produce physical withdrawal themselves. We hypothesized that mice that have been subjected to prolonged FAAH inhibition will show no signs of precipitated physical withdrawal. This is based on aforementioned studies affirming minimal drug abuse potential and alterations of the cannabinoid receptor system following prolonged FAAH inhibition. MAGL inhibition may have a greater potential of physical dependence than FAAH, as 2-AG is a full agonist present in order of magnitudes greater than anandamide in brain. Simultaneous prolonged inhibition of both MAGL and FAAH is most likely to produce cannabinoid precipitated

withdrawal, 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.

Additionally, overall motor suppressant effects of both FAAH and MAGL inhibitors were examined to determine any undesirable side-effects that would have implications for therapeutic use. Again, FAAH is hypothesized to show minimal effects in this paradigm, similar to previous tests in spontaneous activity. With the enhanced THC-like signaling and locomotor suppression of 2-AG elevations, MAGL inhibition might inhibit motor performance, similar to THC.

Cannabinoid tolerance and cross-tolerance

Given the normal response to THC and receptor levels in FAAH (-/-) mice, it seems unlikely that repeated FAAH inhibition produces tolerance or cross-tolerance. JZL184 produces abundant availability of high-efficacy 2-AG for prolonged periods following repeated MAGL inhibition, which acutely produces a wider array of cannabinoid-mediated behavioral changes, especially in tetrad testing. Based on this, we hypothesize that we will see profound tolerance to the acute behavioral effects of JZL184, and subsequent cross-tolerance to other exogenous cannabinoid agonists. Given the enhanced THC-like effects, dual MAGL and FAAH inhibition should produce enhanced acute effects as previously reported, but should produce at least equivalent (if not greater) tolerance and cross-tolerance.

Endocannabinoid and receptor adaptations

Based on previous studies, we would anticipate acute FAAH and MAGL inhibition to significantly increase AEA and 2-AG in brain, respectively. With the impairment of the

degradative mechanisms in place to prevent accumulation of the upstream endocannabinoids, we would expect levels of AEA and 2-AG to further increase following prolonged inhibition, to levels equivalent to those seen in knockout animals. These elevations should be relatively similar across most brain regions, including those examined that are rich in cannabinoid receptors, as the inhibitors should distribute evenly across the brain.

Receptor adaptations should parallel the tolerance studies closely, with a loss of both G-protein stimulated activation and receptor binding sites. The magnitude of the loss for both is hypothesized to be similar for both measures, as cannabinoid receptors readily internalize and downregulate upon repeated agonist exposure. FAAH inhibition should produce minimal receptor alterations, in agreement with previous FAAH (-/-) studies. MAGL inhibition, given the predominant and established role of 2-AG in cannabinoid synaptic plasticity, is likely to produce downregulation of receptors leading to loss of overall receptor maximal efficacy. Striatal areas should be minimally affected, due to its insensitivity to THC agonist exposure. While ligand availability and receptor availability can both impact functional losses and tolerance, we hypothesize the changes in receptor activation will be the overriding correlate to cannabinoid tolerance and plasticity.

Overall, these studies should show the potential that endocannabinoids have in reducing cannabis withdrawal and treating cannabis abstinence. These studies were also designed to elucidate the ability for repeated endocannabinoid elevations to maintain their therapeutic efficacy in cannabinoid receptor mediated outcomes, without producing their own potential for physical withdrawal. While the established literature indicates FAAH inhibition produces potential therapeutic effects with minimal cannabimimetic activity and minimal functional

consequences to the endogenous cannabinoid system, we hope to explore the relative changes in activity following inhibition of both FAAH and MAGL side-by-side.

Methods

Subjects

The subjects were adult male C57BL/6J mice that were purchased from the Jackson Laboratory (Bar Harbor, ME). Also serving as subjects were adult, male and female FAAH (-/-) and (+/+) mice that were obtained from the Center Transgenic Colony at Virginia Commonwealth University (Richmond, VA) backcrossed onto a C57BL/6J (at least 13 generations) background. Mice homozygous for a gene-trap at the *Mgll* locus (MAGL -/- mice) are viable, born at the expected Mendelian frequency and display normal cage behavior compared with wild-type (MAGL +/+) and heterozygous (MAGL +/-) littermates. All MAGL mutant mice used in this study were on a mixed 129SvEv/C57Bl background, with housing and experiments performed with littermate controls at Scripps Research Institute.

Mice were kept on a 12-hour light/dark cycle, with all experiments performed during the light cycle. Mice were housed 4-6 per cage in a temperature (20–22°C) and humidity controlled environment, in a Association for Assessment and Accreditation of Laboratory Animal Care-approved facility, with food and water available *ad libitum* except during testing. All experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University, Medical College of Wisconsin, or Scripps Research Institute in accordance with the Guide for the Care and Use of Laboratory Animals. Mice were temporarily individually housed during all tolerance studies, starting 2 days prior to repeated injections, and

through all behavioral testing. After testing was complete, all mice were humanely sacrificed via CO_2 asphyxia followed by rapid cervical dislocation, unless tissue was collected as described below.

Drugs

JZL184 and JZL195 were synthesized as described previously (Long et al., 2009a; Long et al., 2009b), as was PF3845 (Ahn et al., 2009). WIN55,212 and URB597 were purchased from Cayman Chemical (Ann Arbor, MI). Rimonabant, Δ^9-THC, AEA, and CP55,940 were obtained from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). [^{35}S]GTPγS (1250 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). [^{3}H]SR141716A (44.0 Ci/mmol) was purchased from Amersham Pharmacia (Piscataway, NJ). Scintillation fluid (ScinitSafe Econo 1) was purchased from Thermo Fisher Scientific (Waltham, MA) and Whatman GF/B glass fiber filters (Whatman, Clifton, NJ) were obtained through Fisher Scientific (Pittsburgh, PA). GDP, GTPγS, adenosine deaminase, bovine serum albumin (BSA), and all other chemicals unless stated otherwise were purchased from Sigma-Aldrich (St. Louis, MO).

URB597 was dissolved in a vehicle containing Tween 80/DMSO/saline in a ratio of 1:2:7. In initial experiments, JZL184 was dissolved in a vehicle of PEG 200/Tween 80 in a ratio of 4:1 for THC withdrawal studies, and was injected at a volume of 4 µL/g body mass to limit vehicle effects. All other drugs, including all subsequent JZL184 experiments, were dissolved in a vehicle consisting of ethanol, Alkamuls-620 (Sanofi-Aventis, Bridgewater, NJ), and saline in a ratio of 1:1:18, sonicated as necessary, and injected intraperitoneally in a volume of 10 µl/g body mass. URB597 was administered 1 h before testing to coincide with previous findings of peak

anandamide elevations at this time point (Fegley et al., 2005). Similarly, JZL184 and PF3845 were administered 2 h before testing to coincide with peak levels of 2-AG elevations following systemic administration (Long et al., 2009a).

Rimonabant-Precipitated Withdrawal

In THC withdrawal, mice were given subcutaneous injections of THC to induce dependence under either a high or low dosing regimen. In the high-dose regimen, mice were given two daily injections of THC (50 mg/kg, s.c.) for five and a half days, with each injection separated by approximately 10-12 h. This paradigm was also used to compare high-dose AEA administered exogenously (50 mg/kg, s.c.), as other testing showed THC and AEA roughly equipotent in acute tetrad responses (Falenski et al., 2010). In the low-dose regimen, each mouse was given a single, daily injection of THC (10 mg/kg, s.c.) for six days. In both conditions, the mice were given an i.p. injection of rimonabant 30 min after THC. All mice were then monitored and scored as described below for one hour following rimonabant injection.

For acute treatments, drugs were given coinciding with peak endocannabinoid elevations at the time of rimonabant injection, at times described above. For evaluation of withdrawal potential of the respective enzyme inhibitors themselves, URB597 was given twice-daily (10 mg/kg, i.p.), while JZL184 (40 mg/kg, i.p.) and PF3845 (10 mg/kg, i.p.) were given once-daily for 6 days, the last injections given at times as described above.

Behavioral Evaluation of Somatic Withdrawal Signs

Animals were pretreated with test drugs at times described above. All animals were placed into white (for contrast) acrylic chambers (20 cm x 20 cm x 20 cm), with a clear acrylic front

panel and a mirrored back panel, for a 30 min period for acclimation to the test chamber. The chambers were enclosed in sound-attenuating cabinets, designed and custom built at Virginia Commonwealth University, that contained an indirect filtered LED light source and fans for air circulation and white noise. At the 30 min time point, the animals were briefly removed from the chambers, were given an i.p. injection of rimonabant, and were immediately returned to the chambers for a 1 h observation period. The chambers were wiped clean with water just before the mice were returned for the observation period. Behavior was recorded through the clear front panel using a series of Fire-i™ digital cameras (Unibrain, San Ramon, CA) and the videos were processed and saved using ANY-maze™ video tracking software (Stoelting Co., Wood Dale, IL). Chambers were fully sanitized at the end of each testing day using ammonia-based cleansers and soap, then left to air dry at least two days to dissipate any odors.

The videos were subsequently placed in randomized order in a separate ANY-maze™ protocol for a trained observer to score using a keyboard-based behavioral tracking system, blinded to treatment group. ANY-maze™ software was used to track key presses assigned to somatic withdrawal behaviors for both time pressed and/or number of occurrences. Videos were scored using 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 tremors that included a range of behavior from single-paw twitches to full fluttering/shaking of both paw 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 rats. The third behavior that was quantified was hind leg scratching that involved any repetitive scratching motion of the head or torso by either hind leg. All behaviors were counted as new incidences if either separated by at least 1 s, and/or interceded by another distinct behavior (i.e. crawling, climbing, grooming).

Rotarod Motor Coordination Testing

Mice were trained for at least three days before testing to remain on a rotating 1¼" rotarod (IITC Life Sciences, Woodland Hills, CA) until able to stay on a rotarod maintained at 16 RPM. On drug test days, the rotarod was set to accelerate from 1 RPM to 16 RPM over the course of 60 s. The data shown reflect the RPM speed at which the animal fell off, 16 RPM representing animals that remained on the rotarod during testing.

On test days, a baseline test was given prior to drug administration. THC (40 mg/kg) was administered at a dose that demonstrated significant motor impairment in preliminary testing, and then was tested for CB_1 receptor specificity by treating animals with rimonabant (3 mg/kg) 10 min before THC administration. For the enzyme inhibitor tests, URB597 (10 mg/kg) and JZL184 (16 mg/kg) were given at the same doses as those used in withdrawal experiments. All drugs were tested at time points before, during, and after times observed during the withdrawal tests.

Cumulative dose-responses

In order to reduce the number of animals used and reduce individual animal variability, behavioral dose-responses were evaluated using a cumulative dosing regimen. Previous study shows slow elimination of the drugs, so that cumulative dosing closely parallels brain levels seen after bolus dosing (Falenski et al., 2010). For evaluation of responses to exogenous cannabinoid agonists, baseline behavioral endpoints were measured, and then each animal administered the first dose intraperitoneally. After 30 minutes, the mouse was evaluated for drug effects, then immediately injected with a dose necessary to achieve the next cumulative dose (i.e. to go from 1 mg/kg to 3 mg/kg, the mouse received a 2 mg/kg injection). This process was repeated through the entire dose-response, with the entire procedure taking less than 4 h from start to finish.

Behavioral assessment of cannabinoid activity

During tolerance studies, mice were injected with either vehicle daily for six days, vehicle for five days and drug (JZL184 40 mg/kg or PF3845 10 mg/kg, i.p.) on the sixth day (single groups), or drug daily (JZL184 40 mg/kg or PF3845 10 mg/kg, i.p.) for six days (repeated groups). These doses have been previously established to show complete inhibition of the enzyme, maximal behavioral efficacy acutely, and are active for at least 24 h (Ahn et al., 2009; Long et al., 2009a). The six-day dosing is meant to parallel previous studies demonstrating precipitated withdrawal and tolerance in both THC and AEA (Falenski et al., 2010).

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. Hyperreflexive popping and jumping away from the bar was scored, along with attempts to bite and chew on the bar upon presentation. 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 either 52.0° (MAGL tolerance and cross-tolerance) or 56.0° C (MAGL/FAAH timeline). The latency for the animal to withdraw its tail from the water within a 10 s cutoff time was scored. In the hot plate test, each mouse was placed within an open-topped polycarbonate cylinder (7.5 cm inner diameter) on a hot plate (IITC Inc., Woodland Hills, CA) that was maintained at 56.0° C, and the latency to jump or lick/shake a hind paw within a 60 s observation period 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. Mice were then evaluated either at times described in figures, or every 30 minutes according to procedures described for cumulative dose-responses above.

Brain preparation during THC withdrawal

To quantify AEA and 2-AG levels in brain during THC withdrawal, both FAAH (+/+) and (-/-) mice were administered twice-daily injections of either vehicle or THC (50 mg/kg, s.c.) for five and a half days, and withdrawal was precipitated by rimonabant (10 mg/kg, i.p.) as described above. Thirty minutes into the withdrawal period, mice were decapitated and brains were extracted. Brains were removed, not including brainstem or olfactory bulb, and the brain

was further dissected by separation of the cerebellum from the forebrain/midbrain. Activity of cAMP-dependent pathways in cerebellum has been identified in previous studies as a potential direct contributor to precipitated cannabinoid somatic withdrawal in mice (Tzavara et al., 2000), while many midbrain and cortical regions have shown greatest plasticity in response to chronic THC administration (Gonzalez et al., 2004; Sim-Selley and Martin, 2002). Both sections were snap frozen in liquid nitrogen, and then stored at -80°C until the time of processing.

Brain preparation following enzyme inhibition/inactivation

Whole brains were removed from decapitated mice after 2 h or 26 h following final injections of either JZL184 or PF3845. Brain sections were snap frozen in liquid nitrogen and stored at -80°C until extraction. For the regional dissections for eCB quantification, the sections were landmarked and removed as follows:

The cingulate cortex was dissected from the dorsal surface of the brain from approximately the genu of the corpus callosum (~Bregma +1.15) to the midpoint of the hippocampus (~ Bregma -2 mm), both of which are visible after removal of the cortical tissue. Forceps were used to dissect the sample by aligning one jaw of the dissecting forceps with the longitudinal fissure and the other approximately 1-1.5 mm laterally, then pinching. The sample includes both anterior and posterior (retrosplenial) cingulate cortices, and probably a small portion of adjacent motor/parietal cortex.

The striatum was removed after the cingulate cortex by carefully resecting the remaining cortex from the surface of the brain. This procedure exposes the striata bilaterally. The striatum is visible as an almond shaped, striated structure on each exposed surface. The forceps are aligned on each side of the structure, and tissue is gently pinched to remove the striatum while

leaving the underlying cortex intact. The sample includes dorsal (caudate-putamen) and ventral (nucleus accumbens) striatum, as well as the adjacent rostral extent of the globus pallidus.

The hippocampus is visible on the dorsal surface of the brain after resection of the cortex. The rostral extent is identified visually, and then pinched with forceps to free from the brain. Forceps are used to gently hold the free end of the hippocampus and the tissue is gently peeled from the underlying brain structures. The ventral aspect of the tissue is then pinched to free the hippocampus from the brain. The sample contains the isolated hippocampal complex throughout its rostral-caudal extent.

The PAG was dissected from an approximately 2 mm section collected using the superior and inferior colliculi as anterior and posterior landmarks, respectively (Bregma -3 to Bregma -5). Cortex and hippocampus were discarded, and then the colliculi were trimmed. Tissue ventral to the PAG was removed at the midpoint of the section, and the lateralmost aspect of the sample was removed. The sample included the PAG throughout its rostral-caudal extent, as well as surrounding reticulum and adjacent colliculi.

The cerebellum was removed by gently pulling the structure away from the brainstem and severing the cerebellar peduncles. The sample includes the entire cerebellar cortex, as well the deep nuclei. Spinal cord was taken from the point of decapitation to the hip joint, and forced out of the spinal column via syringe using forced saline pressure.

Extraction & quantification of endocannabinoids by LC/MS

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 4000 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 3000 rpm and 4°C to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml 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 micron (Supelco, USA). 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 AEA-d8; (379>287) and (279>269) for 2-AG; and (387>96) for 2AG-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 pmol to 40 pmol for AEA and from 0.05nmol to 64 nmol for 2-AG.

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 allowed to incubate 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.

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

Mice were sacrificed by decapitation, and brains were removed and frozen in isopentane at -30°C and stored at -80°C. Assays were conducted as previously published (Sim et al., 1995). Briefly, coronal sections (20 µm) were cut on a cryostat at -20°C, thaw-mounted onto gelatin-subbed slides, and stored desiccated at 4°C overnight. Slides were then stored desiccated at -80°C until use. For assay, slides were brought to room temperature, then equilibrated in 50 mM Tris-HCl buffer (pH 7.4) with 3 mM $MgCl_2$, 0.2 mM EGTA, and 100 mM NaCl (Assay Buffer) for 10 min at 25°C. Next, slides were transferred to Assay Buffer + 0.5% BSA, with 2 mM GDP and 10 mU/ml adenosine deaminase for 15 min at 25°C. Slides were then incubated in Assay Buffer + 0.5% BSA containing 0.04 nM [^{35}S]GTPγS in the presence or absence (basal) of maximally effective concentrations of appropriate drug (3 µM CP55,940) and/or vehicle for 2 hrs at 25°C. After final incubation, slides were rinsed twice in 50 mM Tris buffer (pH 7.4) at 4°C, then in deionized water. Slides were then dried, and exposed to Kodak BioMax MR film with [^{14}C] standards for 24-36 hrs. Films were digitized at 8-bits per pixel with a Sony XC-77 video camera. Regions of interest were selected using anatomical landmarks and measured using NIH ImageJ software.

Data presentation & analysis

All data are reported as mean ± SEM. The somatic withdrawal behaviors were the scored observations of a 30 min sample observation period from the 1 h recording. Noncontinuous behaviors, such as head twitches and paw tremors, are presented as number of incidences observed. The continuous behavior of hind leg scratching is presented as total time observed scratching. Endocannabinoid levels are reported as mole per gram tissue. With 2-AG being far

more abundant in bulk tissue, AEA reported as pM/g and 2-AG as nM/g. Rotarod data are expressed as the average RPM value at which the animal fell off the apparatus. Experiments with only two treatment groups were analyzed for statistical significance using the Student's *t* test. Experiments with more than two groups were analyzed using one- and two- way analysis of variance (ANOVA; treatment or genotype factors). Time courses and cumulative dose-responses, such as rotarod testing and cross-tolerance experiments, were analyzed using repeated measures ANOVA. Significant ANOVAs were followed by either Tukey's post-hoc tests for multiple comparisons, or Dunnett's post hoc test was used for dose-responses and comparisons to controls. Resulting *p* values of less than 0.05 were considered significant.

[^{35}S]GTPγS binding experiments were performed in triplicate, and all data points are reported as mean ± SEM of four experiments. Nonspecific binding was first subtracted from all binding data. Stimulated binding was determined as agonist-stimulated binding minus basal binding, and values are reported as percentage stimulation above basal. All receptor binding experiments were performed in duplicate and reported as mean ± SEM of four experiments. Nonspecific binding was first subtracted from total binding, yielding specific binding data. Nonlinear regression analyses of agonist concentration-effect curves were performed with Prism 5.0 using a sigmoidal dose-response model or specific binding of single site model (GraphPad Software Inc., San Diego, CA). Values reported from regressions as mean ± SEM for interpolated results. ANOVA was run using Prism for comparing multiple regression values, with confidence intervals used to determine post-hoc significance following significant ANOVA F-value. Regional G-protein stimulation autoradiography data are reported as mean ± SEM of triplicate sections from 7-8 brains/group. Net [^{35}S]GTPγS binding is defined as (agonist-stimulated

[^{35}S]GTPγS binding – basal [^{35}S]GTPγS binding). Analysis was performed in GraphPad Prism Version 5 using Student's *t*-test between the two treatments for each individual region analyzed.

Chapter 1: Endocannabinoid elevations in attenuation of THC physical withdrawal and potential for endocannabinoid induction of withdrawal symptoms

1.1 Rimonabant precipitates similar somatic withdrawal signs in FAAH (-/-) and (+/+) mice given repeated injections of THC.

Toward determining the potential for increased AEA availability substituting for THC during withdrawal, the purpose the first series of experiments was to determine whether THC dependence would be reduced in FAAH (-/-) mice compared to FAAH (+/+) mice. In the first experiment, FAAH (-/-) and (+/+) mice were treated in the high THC dosing regimen or given vehicle for 5.5 days. On the sixth day, the vehicle-treated mice were given an acute injection of vehicle or rimonabant (10 mg/kg), while all the THC-treated mice were given an acute injection of rimonabant (10 mg/kg). Previous research from our laboratory indicated that mice treated repeatedly with THC and challenged with vehicle do not exhibit any withdrawal symptoms (Lichtman et al., 2001; Wilson et al., 2006). Front paw tremors/fluttering were the primary somatic sign observed. As seen in Figure 2a, a significant main effect of treatment on the number of paw tremors was observed [$F(2,30) = 54.0$, $p < 0.001$] in which rimonabant precipitated increases in paw flutters only in groups that received repeated THC compared to the other groups ($p < 0.001$). However, there was no significant effect for either genotype ($p = 0.44$) or interaction between genotype and treatment ($p = 0.55$). Figure 2b shows similar results for head twitches, with a significant effect of treatment [$F(2,30) = 47.0$, $p < 0.001$], but no significant differences for the main effect of genotype or the genotype by treatment interaction.

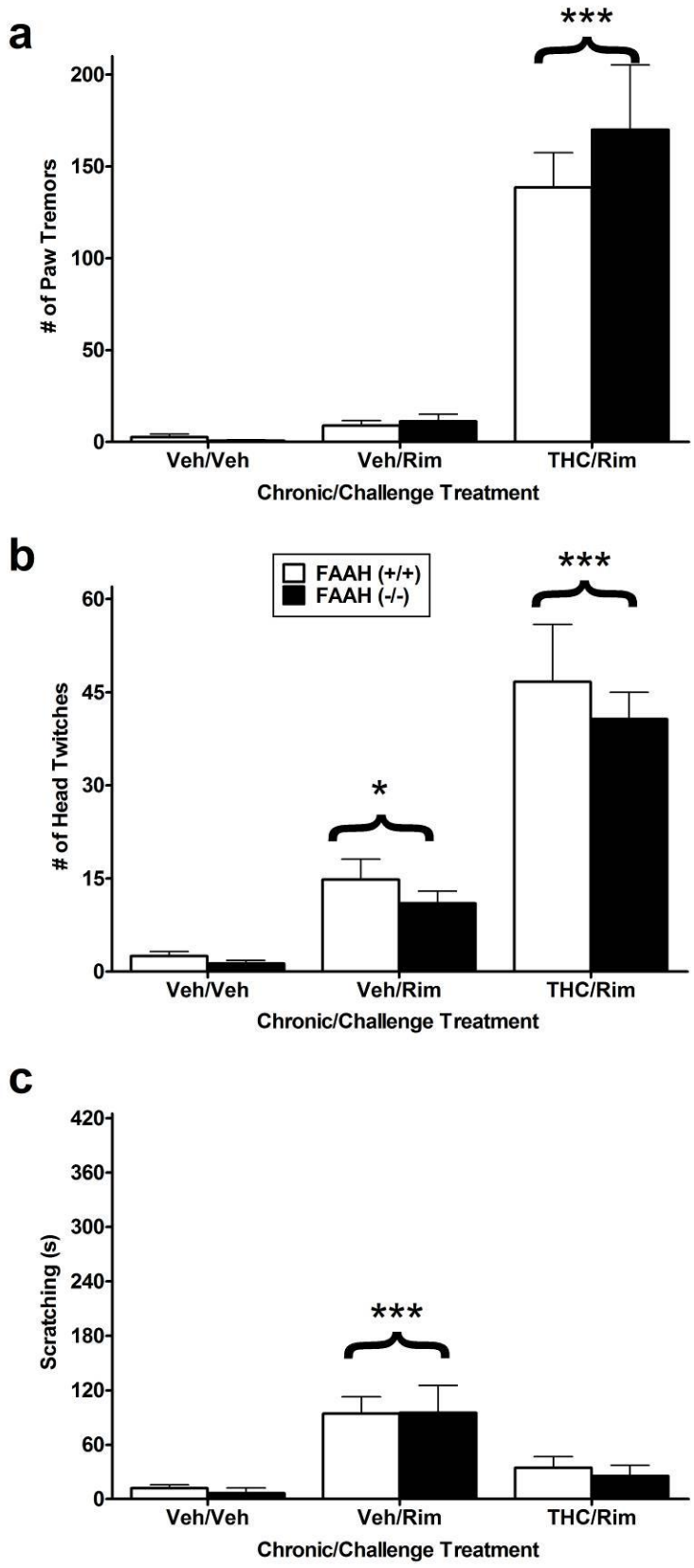

Figure 2 – FAAH (-/-) and (+/+) mice show similar somatic withdrawal signs following a high THC (50 mg/kg twice daily for 5.5. days) dosing regimen. Rimonabant precipitated significant increases in paw tremors (**a**) and head twitches (**b**) in mice treated repeatedly with THC, regardless of genotype. No genotype differences were found. (**c**) Rimonabant elicited a increase in scratching behavior in mice treated repeatedly with vehicle, regardless of genotype. $n = 6$ mice per group. Comparisons collapsed across genotype: $*p < 0.05$ versus vehicle controls, $***p < 0.001$ versus all other treatments.

Rimonabant precipitated significantly more head twitches in THC-dependent mice than in each of the other groups ($p < 0.001$). However, mice treated repeatedly with vehicle and challenged with rimonabant showed a small, but significant, increase in head twitching compared to vehicle control mice ($p < 0.05$). A significant treatment effect was found for hind leg scratching behavior [$F(2,30) = 14.9$, $p < 0.001$; Figure 2c], and again there was no influence of genotype. Rimonabant increased scratching in mice given repeated vehicle injections compared to the other two groups ($p < 0.001$).

Although FAAH (-/-) mice did not display significant decreases in withdrawal behavior, it is possible that ceiling effects caused by the high THC dosing regimen obscured subtle genotype differences influenced by elevated AEA. Thus, a follow-up experiment was conducted using a mild THC dosing regimen to examine whether severity of rimonabant precipitated withdrawal is altered in FAAH (-/-) mice. Rimonabant precipitated paw tremors [$F(2, 26) = 97.5$, $p < 0.001$; Figure 3a] and head twitching [$F(2, 26) = 5.9$, $p < 0.05$; Figure 3b] in mice treated repeatedly with low-dose THC compared to the other two groups of mice, though at a lower magnitude for both endpoints than seen in high-dose THC treated mice. However, there was no effect of genotype on either of these withdrawal responses. As seen in Figure 3c, vehicle-treated mice receiving rimonabant alone, regardless of genotype, spent significantly more time scratching than the other two treatment groups [$F(2, 26) = 34.9$, $p < 0.001$]. Because we and others have found that rimonabant induces scratching behavior in drug naïve mice (Darmani and Pandya, 2000; Wilson et al., 2006), this behavior is not considered a withdrawal response and is not reported in subsequent experiments.

In the next experiment, we examined whether rimonabant would be more potent in precipitating withdrawal in FAAH (-/-) mice than in FAAH (+/+) mice. Both genotypes were

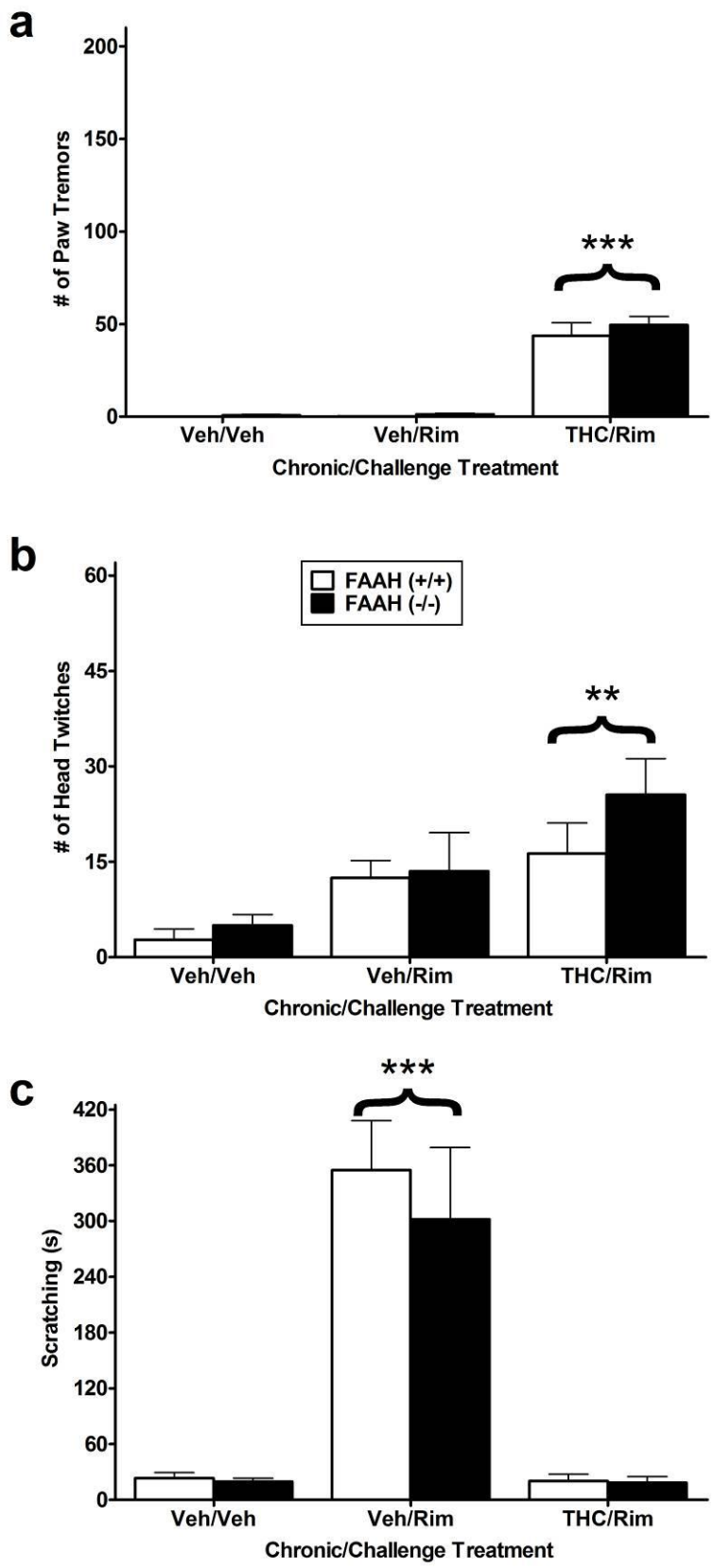

Figure 3 - FAAH (-/-) and (+/+) mice show similar somatic withdrawal signs following a low THC (10 mg/kg, once daily for 6 days) dosing regimen. Rimonabant precipitated a significant increase in paw tremors (**a**) and head twitches (**b**) in mice treated repeatedly with THC, regardless of genotype. No significant genotype differences were found. (**c**) Rimonabant only elevated scratching in mice receiving repeated injections of vehicle. $n = 6$ mice per group. Comparisons collapsed across genotype: $^{**}p < 0.01$ versus vehicle control, $^{***}p < 0.001$ versus all other treatment groups.

subjected to the high THC dosing regimen (i.e., 50 mg/kg twice a day for 5.5 days) and the dose-response relationship of rimonabant in precipitating paw tremors and head shakes was determined. Rimonabant elicited a significant dose-responsive effect on paw tremors [$F(3, 37) = 38.4$, $p < 0.001$; Figure 4a], with both 3 and 10 mg/kg of rimonabant precipitating tremors significantly above those of mice given an acute injection of vehicle ($p < 0.001$). The ED_{50} values in FAAH (+/+) and (-/-) mice were 2.9 mg/kg (95% C.I. 2.1 to 4.0 mg/kg) and 2.9 mg/kg (95% C.I. 1.7 to 4.7 mg/kg), respectively. The observation that rimonabant was equipotent in precipitating withdrawal in both genotypes further demonstrates that deletion of FAAH does not affect withdrawal responses in THC-dependent mice. Rimonabant challenge also precipitated a significant increase in head twitching [$F(3, 37) = 12.4$, $p < 0.001$; Figure 4b]. Each dose of rimonabant increased this effect compared to vehicle ($p < 0.01$).

1.2 Comparison of rimonabant precipitated withdrawal in high-dose THC versus high-dose AEA

Rimonabant (Rim) precipitated a similar magnitude of withdrawal responses once again in FAAH (-/-) and (+/+) mice treated subchronically with high-dose THC. A two-way ANOVA, with genotype and treatment (subchronic vehicle-challenge vehicle, subchronic vehicle-challenge rimonabant, subchronic THC- challenge rimonabant) as between subject factors, revealed main effects of treatment for both head twitches [$F(2, 29) = 48$, $p < 0.001$; Figure 5b] and paw flutters [$F(2, 29) = 65$, $p < 0.001$; Figure 5a]. Post hoc analyses revealed that rimonabant challenge elicited significant increases in both head shakes and paw tremors in mice treated with subchronic THC, but not subchronic vehicle. Interestingly rimonabant challenge precipitated paw flutters in FAAH (-/-) mice treated subchronically with AEA (Figure 5a; $t_{10} = 5.3$, $p = 0.001$), but produced no changes in the number of head twitches (Figure 5b).

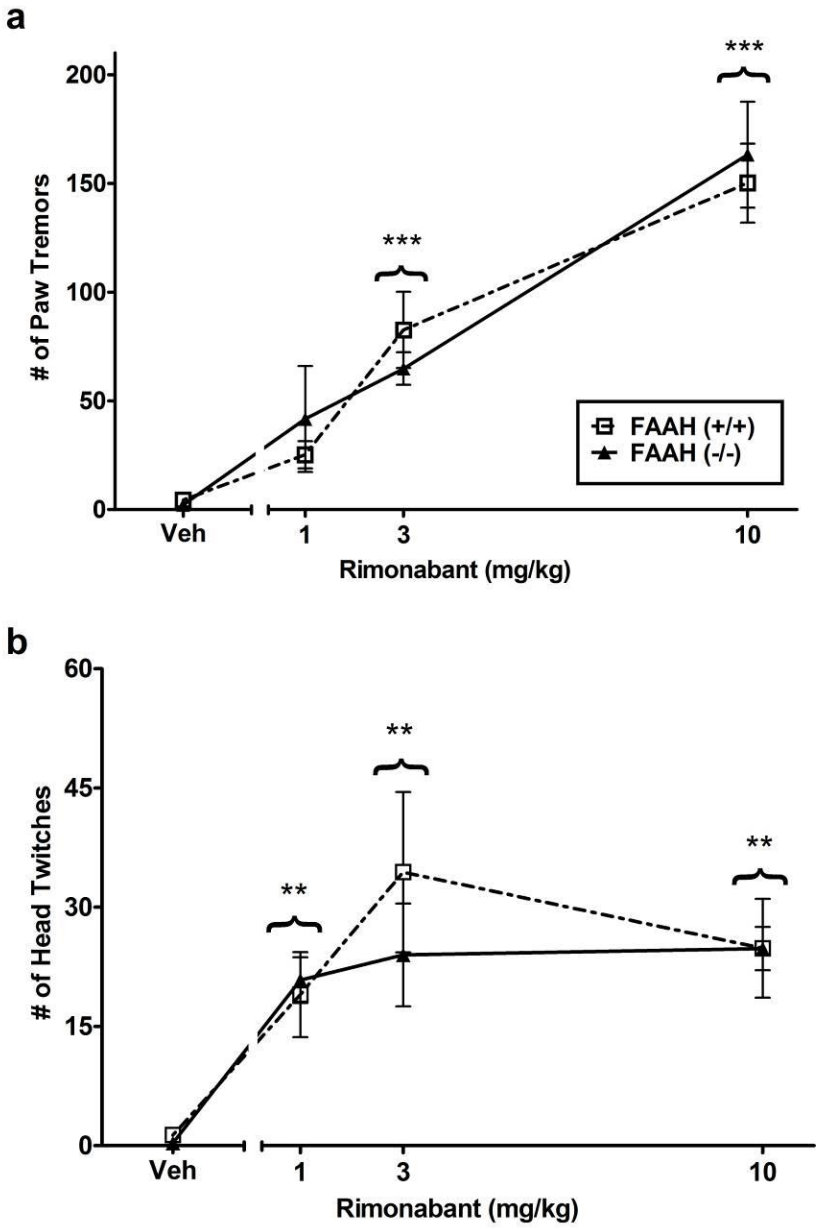

Figure 4 - Rimonabant (1, 3, and 10 mg/kg) dose-dependently increased the incidence of paw tremors (**a**) in mice treated with a high THC dosing regimen. Rimonabant was equipotent in eliciting paw tremors between FAAH (+/+) mice and FAAH (-/-) mice. (**b**) Rimonabant also precipitated a significant increase in head twitching compared to vehicle in THC-dependent mice. $n = 6$ mice per group. Comparisons collapsed across genotype: $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle control.

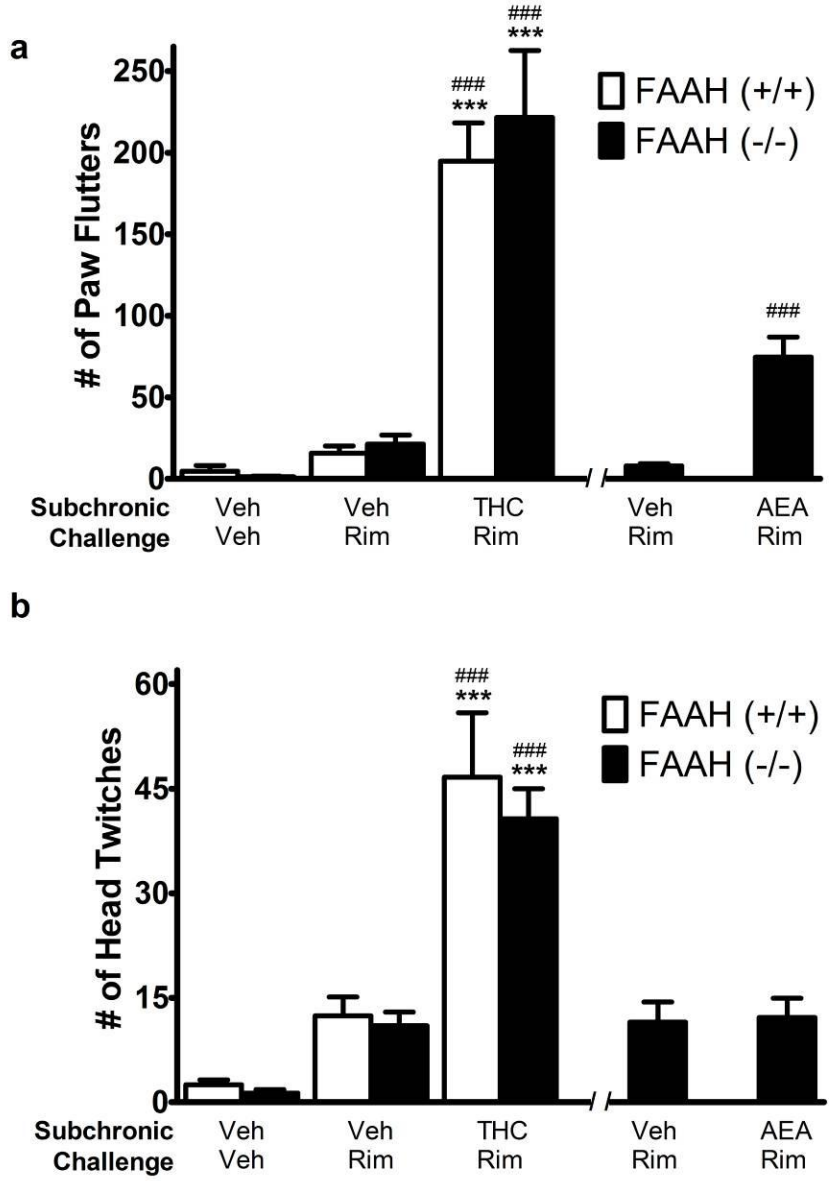

Figure 5 - Evaluation of rimonabant precipitated withdrawal in FAAH (-/-) mice treated subchronically with THC (50 mg/kg twice daily for 6 days) or AEA (50 mg/kg twice daily for 6 days), and FAAH (+/+) mice treated subchronically with THC. (**a**) Rimonabant precipitated significant increases in paw tremors in mice treated repeatedly with THC, regardless of genotype. The magnitude of this effect was considerably less for AEA treated mice than in mice treated repeatedly with THC. (**b)** Rimonabant precipitated significant increases in head twitches in mice treated repeatedly with THC, regardless of genotype, but did not elicit increases in AEA-treated mice. ***$p < 0.001$ versus corresponding subchronic vehicle-vehicle challenge group of the same genotype. $^{###}p < 0.001$ versus corresponding subchronic vehicle-rimonabant challenge group of the same genotype. $n = 6$ mice/condition.

Additionally, the magnitude of the rimonabant precipitated paw flutters following subchronic AEA administration was smaller than following subchronic THC (AEA mean $\pm$ SEM = 74 ± 13; THC mean $\pm$ SEM = 222 ± 41).

1.3 Acute administration of the FAAH inhibitor, URB597, reduces the severity of rimonabant-precipitated withdrawal in THC-dependent mice

FAAH (-/-) mice possess constitutively elevated levels of AEA that would have occurred presumably across the development of dependence as well as during rimonabant challenge. Thus, in the next experiment we investigated whether acute blockade of FAAH using the irreversible FAAH inhibitor, URB597, would reduce the severity of rimonabant-precipitated withdrawal responses in THC treated mice. URB597 was given at a high dose (10 mg/kg) prior to rimonabant precipitation, so as to allow FAAH inhibition to be complete, and AEA to accumulate, over the time of withdrawal. A comparative study was done looking at URB597's potential effectiveness in both high and low dosing regimens.

URB597 altered the overall expression of fluttering behavior, as denoted by a significant main effect of acute treatment [$F(1, 36) = 10.9$, $p < 0.01$], as well as a significant interaction between URB597 and THC treatments [$F(2, 36) = 4.1$, $p < 0.05$]. Split post-hoc analysis of URB597 treatment for each THC regimen shows that URB597 significantly reduced paw fluttering in both the high and low THC dosing paradigms ($p < .05$; Figure 6a). While there was a main effect of head twitching based on THC dosing [$F(2, 36) = 7.9$, $p < 0.01$], signifying that high-dose THC elicited greater head shaking than the other groups ($p < .05$; Figure 6b), there appeared no effect of URB597 treatment on head shaking intensity.

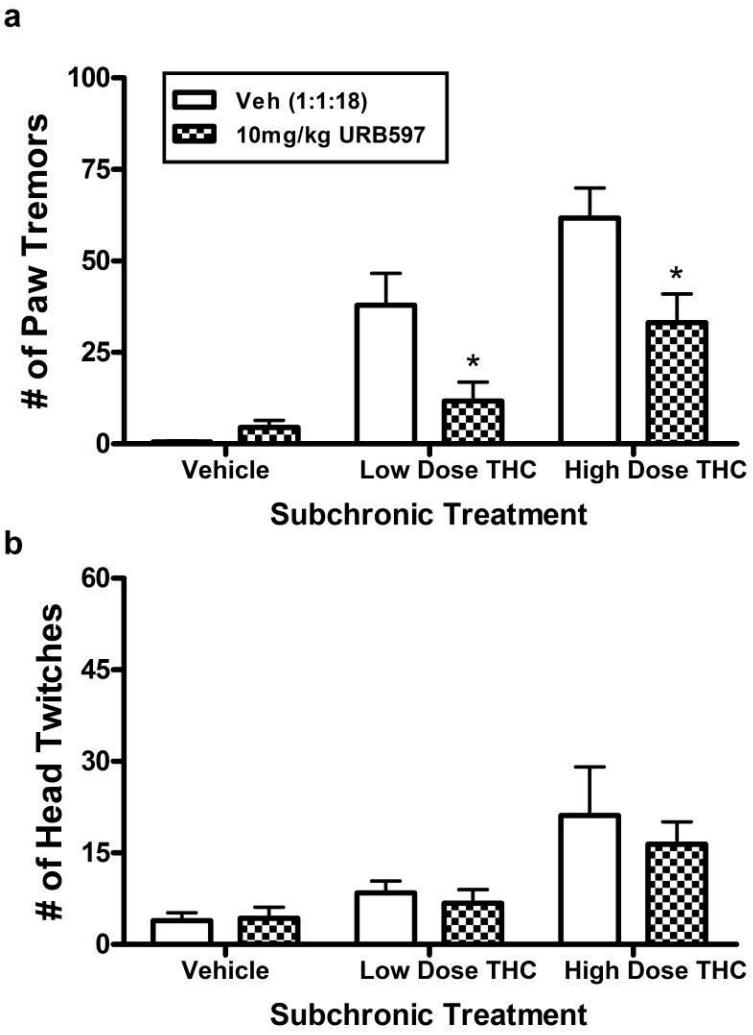

Figure 6 - Assessment of pretreatment by the irreversible FAAH inhibitor, URB597 (10 mg/kg), on the incidence of rimonabant-precipitated withdrawal behavior in mice that were treated subchronically with either low (10 mg/kg daily) or high THC (50 mg/kg twice-daily) dosing regimens. (**a**) URB597 reduced the incidence of rimonabant-precipitated paw tremors in both low and high THC dosing conditions. (**b**) URB597 did not appear to alter the expression of head twitches from THC withdrawal. $n = 8$ mice per group. $^{*}p < 0.05$ versus respective vehicle control group.

The experiment was repeated, and conducted in both FAAH (+/+) and (-/-) mice to determine the specificity of any URB597 effects to its actions on FAAH activity. As seen in Figure 7a, URB597 reduced paw tremors during THC withdrawal by approximately 40% in FAAH (+/+) mice, but had no effect in FAAH (-/-) animals, as reflected by an interaction between URB597 treatment and FAAH genotype [$F(1, 27) = 4.4$, $p < 0.05$]. ANOVA revealed a main effect of URB597 on head twitches during THC withdrawal [$F(1, 27) = 4.5$, $p < 0.05$]. However, there was no main effect of FAAH genotype and no interaction between FAAH genotype and URB597 treatment. Planned comparisons did not reveal significant differences between URB597 and vehicle for each respective genotype (Figure 7b).

1.4 Rimonabant-precipitated withdrawal potential following repeated FAAH inhibition

The purpose of this experiment was to examine whether repeated administration of FAAH inhibitors produces a cannabimimetic 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. No irregular behaviors were observed or noted during the recording or scoring of the videos, and all the same somatic signs tracked during THC withdrawal were quantified (Figure 8). Rimonabant produced no significant differences between mice that were given repeated injections of URB597 and vehicle on paw tremors [$t(14) = 0.8$, $p = 0.44$] as well as head twitching [$t(14) = 0.4$, $p = 0.68$].

Upon availability, we also tested the longer-acting second generation FAAH inhibitor PF3845. Its inhibition of FAAH persists for 24 h, is far more selective amongst serine hydrolases, and the elevations in AEA are far more comparable to those of a FAAH (-/-) mouse. Once-daily PF3845 (10 mg/kg) elicited minimal signs following rimonabant precipitation, exhibiting even fewer

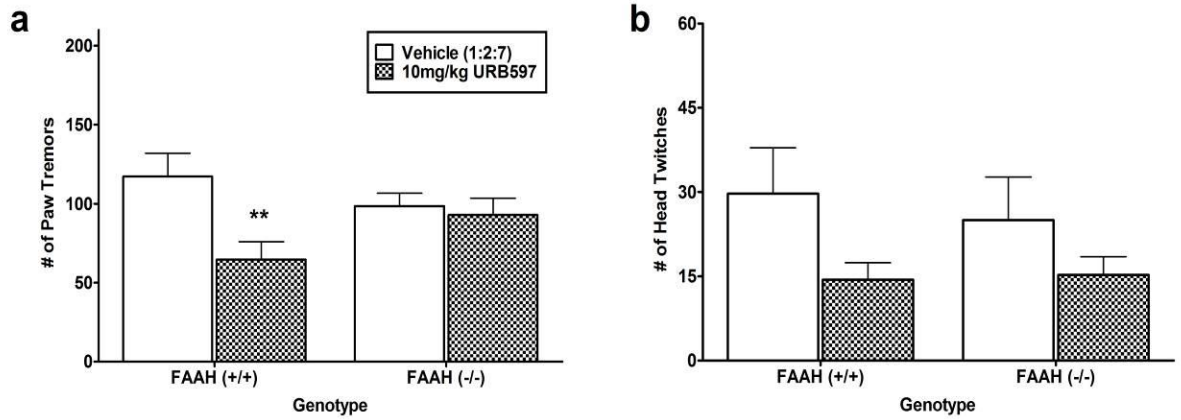

Figure 7 - Assessment of the irreversible FAAH inhibitor, URB597 (10 mg/kg), on the incidence of rimonabant-precipitated withdrawal behavior in FAAH (+/+) and (-/-) mice that were treated subchronically with a high THC dosing regimen. (**a**) URB597 reduced the incidence of rimonabant-precipitated paw tremors in FAAH (+/+) THC-dependent mice, but was without effect in FAAH (-/-) mice. (**b**) URB597 reduced the incidence of rimonabant-precipitated head twitches, regardless of genotype. $n = 8$ mice per group. $^{**}p < 0.01$ versus FAAH (+/+) vehicle control group.

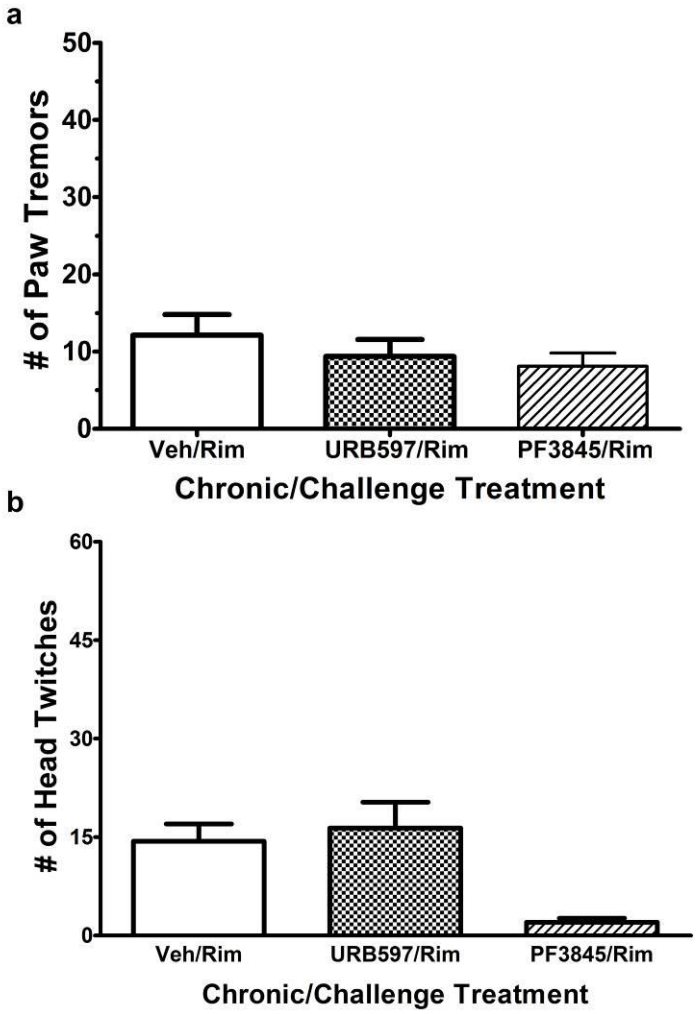

Figure 8 – Evaluation of rimonabant-precipitated withdrawal signs following repeated high-dosing of FAAH inhibitors. The first generation, short-acting inhibitor URB597 was given at 10 mg/kg twice daily, while the more potent and longer-acting inhibitor PF3845 was given daily at a dose of 10 mg/kg. Neither FAAH inhibitor given subchronically elicited paw fluttering (**a**) or head twitching (**b**) symptoms compared to vehicle. The vehicle group shown is representative of collapsed data across separate URB597/PF3845 experiments, while statistical comparisons were made across specific control groups to particular experiment. n = 8-16 mice per condition.

incidences than URB597, and comparable to vehicle treatment for fluttering [$t(14) = 1.1$, $p = 0.30$] and head twitching [$t(14) = 0.9$, $p = 0.39$].

1.5 Acute administration of the MAGL inhibitor, JZL184, reduces the severity of rimonabant-precipitated withdrawal in THC-dependent mice

The first selective MAGL inhibitor reported, JZL184, shows a partial set of CB_1 receptor mediated behavioral effects in the cannabimimetic tetrad test (hypomotility, hypothermia, and analgesia) (Long et al., 2009a). To examine if acute elevation of 2-AG levels can reduce somatic signs of rimonabant precipitated-withdrawal in THC-dependent mice, vehicle or JZL184 (16 mg/kg in PEG vehicle) was administered 2 h before rimonabant injection. The high THC dosing regimen was used. Testing was performed in both FAAH (+/+) and (-/-) mice to examine the specificity of drug effects to FAAH, and to ascertain whether simultaneous elevation of AEA and 2-AG levels causes differential responses.

As seen in Figure 9a, JZL184 reduced the incidence of paw tremor activity during THC withdrawal by approximately 50% [$F(1, 21) = 33.3$, $p < 0.001$]. Unlike the reduction seen in URB597, JZL184 was equally effective in reducing tremors in both FAAH (+/+) and (-/-) mice. Also in contrast to URB597, there were no effects of JZL184 treatment on the occurrence of head twitching during withdrawal (Figure 9b). However, it should be noted that the signal of rimonabant precipitated head shakes in THC-dependent mice was considerably lower than the signal in the previous experiments (see Figures 2b, 4b, and 6b). This reduced signal of head twitches may have been the consequence of the PEG-based vehicle used for JZL184.

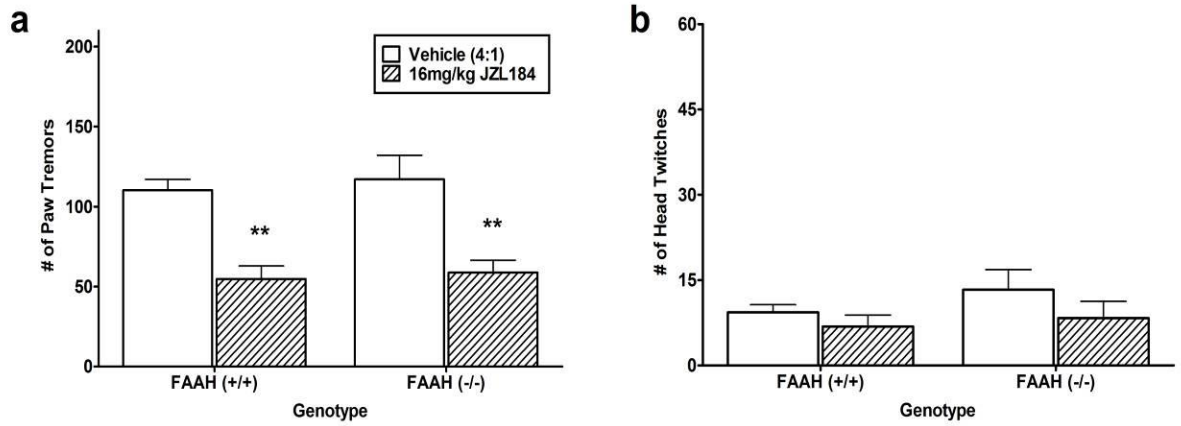

Figure 9 - Assessment of the irreversible MAGL inhibitor JZL184 (16 mg/kg in PEG vehicle), on the incidence of rimonabant-precipitated withdrawal behavior in FAAH (+/+) and (-/-) mice that were treated subchronically with a high THC dosing regimen. (**a**) JZL184 reduced the incidence of rimonabant-precipitated paw tremors in both FAAH (+/+) and (-/-) mice. (**b**) No significant effect was found on head twitches. n = 6-7 mice per group. $^{**}p < 0.01$ versus respective genotype vehicle control.

1.6 Precipitated withdrawal potential during repeated MAGL inhibition alone, and in combination with FAAH inhibition

Since acute MAGL inhibition was equally effective in attenuating THC withdrawal as seen with FAAH inhibition, repeated MAGL inhibition was tested to determine if it has any potential for physical dependence. As the PEG-based vehicle seemed to produce bioactivity on its own, such as suppressing open field behaviors independent of drug effects, the traditional 1:1:18 vehicle was used to test repeated daily JZL184 (40 mg/kg). In order to examine additional withdrawal potential due to simultaneous elevations in AEA and 2-AG, both FAAH (+/+) and (-/-) mice were given repeated JZL184. In contrast to the absent withdrawal response in FAAH (-/-) mice and repeated FAAH inhibitors, rimonabant precipitated significant paw fluttering [$F(4, 74) = 12.8$, $p < 0.001$] in mice treated subchronically with JZL184, with fluttering significantly increased compared to vehicle in both FAAH genotypes ($p < 0.001$; Figure 10a). The magnitude of withdrawal was comparable to the magnitude seen in mice treated with the low-dose THC regimen (10 mg/kg). There was also a main effect of treatment for head twitching [$F(4, 74) = 3.5$, $p < 0.05$], however only THC elicited significant increases compared to vehicle treatment ($p < 0.05$; Figure 10b).

To control for the possibility of developmental adaptations in FAAH (-/-) mice, follow-up experiments were performed using pharmacological means of dual MAGL-FAAH inhibition. The first experiment tested combined administration of JZL184 (40 mg/kg) and PF3845 (10 mg/kg). The precipitated withdrawal magnitude of PF3845 and JZL184 were both similar to previous tests described above, however the combination of JZL184 and PF3845 together [$F(3, 28) = 10.0$, $p < 0.001$; Figure 11a] did not alter the magnitude of paw fluttering compared to

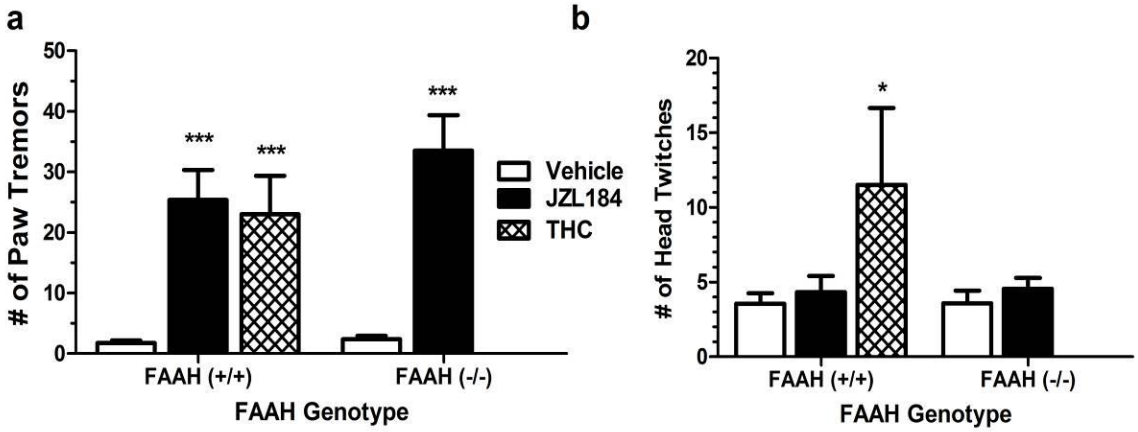

Figure 10 - Prolonged elevation of 2-AG, by prolonged MAGL inhibition, leads to signs of cannabinoid physical dependence. (**a**) Mice treated with 6-day JZL184 (40 mg/kg) show significant paw fluttering withdrawal behavior upon precipitation with the cannabinoid antagonist rimonabant (10 mg/kg) equally 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 that THC does. n = 8-15 per group, ***$p < 0.001$ versus respective vehicle controls.

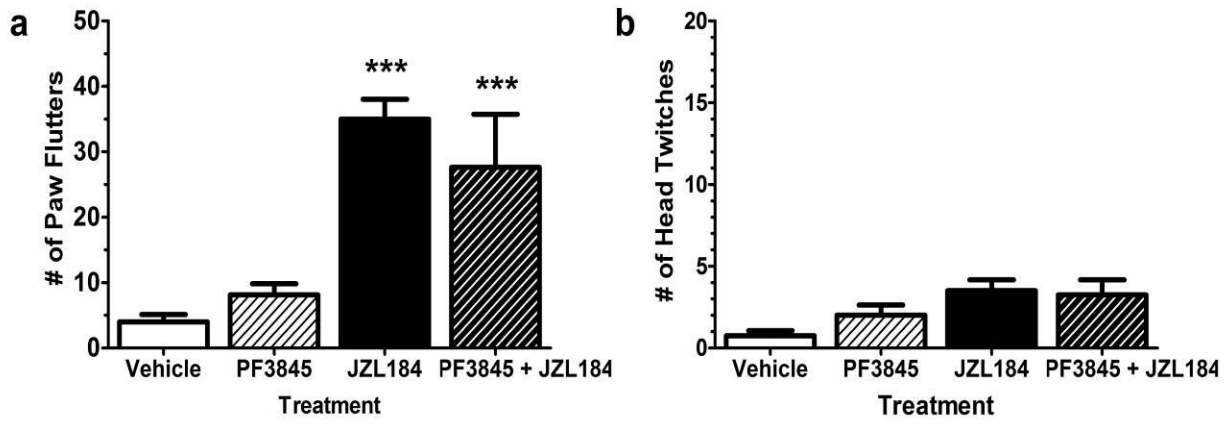

Figure 11 – Cannabinoid precipitated withdrawal elicited by prolonged MAGL inhibition is not altered by simultaneous inhibition of FAAH. (a) Mice treated with 6-day JZL184 (40 mg/kg) show significant paw fluttering withdrawal behavior upon precipitation with the cannabinoid antagonist rimonabant (10 mg/kg), which is absent following 6-day FAAH inhibition via PF3845 (10 mg/kg), nor enhanced by co-administration of both enzyme inhibitors simultaneously. (b) None of the combination of enzyme inhibitors elicited head twitching behavior. $n = 8$ per group, ***$p < 0.001$ versus respective vehicle controls.

JZL184 alone. While there was an overall treatment effect on head twitches [$F(3, 28) = 3.5$, $p < 0.05$; Figure 11b], no individual group was significantly elevated from vehicle.

The next experiment examined the withdrawal potential of the dual enzyme inhibitor JZL195, which has been shown to elevate 2-AG and AEA simultaneously to the equivalent of JZL184 and PF3845, respectively. Due to the minimal experience with repeated treatment and time course data of JZL195, two dosing regimens were tested. The first was a high-dose daily regimen (40 mg/kg), and another that gave an equipotent dose to that of JZL184 twice-daily (20 mg/kg). As seen in Figure 12, the daily high dose of JZL195 failed to elicit paw fluttering significantly elevated from vehicle, while the twice-daily JZL195 produced paw fluttering equivalent to that JZL184 [$F(3, 27) = 14.5$, $p < 0.001$]. No treatment effect was observed for head twitches ($p > 0.05$).

In our final evaluation of withdrawal behaviors elicited by prolonged elevations of endogenous cannabinoids, we tested the recently developed MAGL (-/-) mice for rimonabant-precipitated withdrawal. Due to limited availability, and their SV129 background strain, MAGL heterozygous mice treated with repeated JZL184 were used as positive controls and to test for strain difference. As seen in Figure 13, MAGL (-/-) treated with rimonabant failed to produce enhanced fluttering behavior compared to (+/+) controls. However, multiple observers noted many common signs of cannabinoid withdrawal in the MAGL (-/-) mice that are not typically quantifiable, such as hunching and ptosis. In contrast, JZL184 treated mice produced almost identical fluttering results in the SV129 mice as seen in previous tests with C57 mice [$F(3, 18) = 6.2$, $p < 0.01$]. Similarly, no head twitching was found to be increased amongst any group ($p > 0.05$).

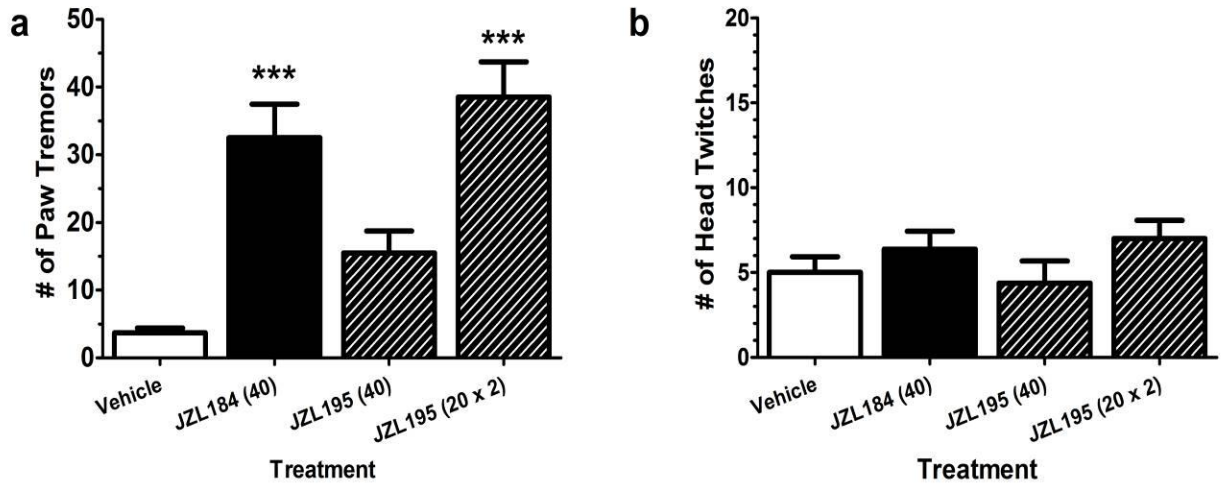

Figure 12 – The dual MAGL/FAAH inhibitor JZL195, though apparently shorter-acting *in vivo* than JZL184, produces similar withdrawal effects to that of MAGL inhibition alone. (**a**) While a high once-daily dose of JZL195 (40 mg/kg) fails to elicit precipitated paw tremoring, twice-daily lower doses (20 mg/kg) elicited similar flutters to that of once-daily JZL184. (**b**) No combination of enzyme inhibition elicited head twitch behavior above that of vehicle. $n = 6$ per treatment group. ***$p < 0.001$ versus vehicle control group.

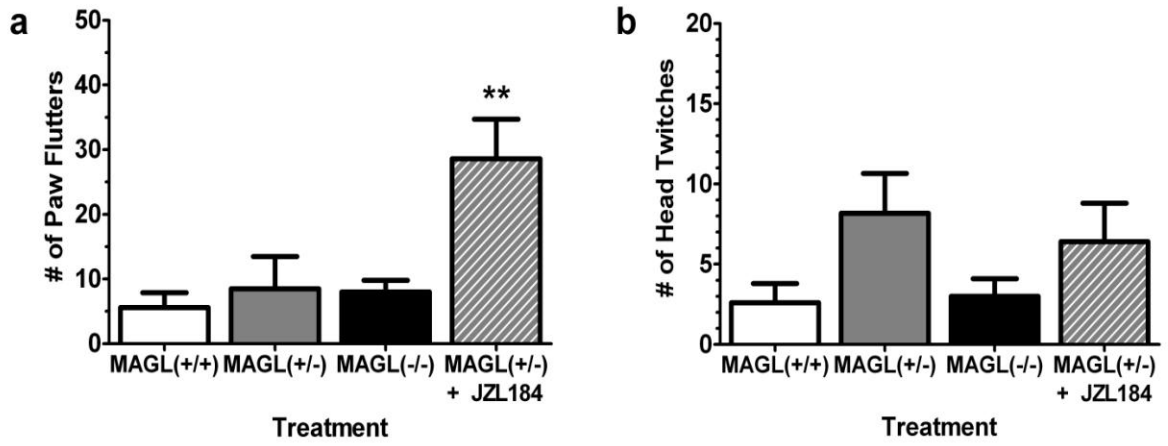

Figure 13 – Cannabinoid precipitated withdrawal elicited by prolonged pharmacological MAGL inhibition, but not exhibited in mice with MAGL genetically inactivated. (a) Mice treated with 6-day JZL184 (40 mg/kg) show significant paw fluttering withdrawal behavior upon precipitation with the cannabinoid antagonist rimonabant (10 mg/kg), which is absent in naïve mice regardless of MAGL genotype. (b) Neither JZL184 treatment nor MAGL genotype influenced head twitching behavior in rimonabant-treated mice. $n = 6$ per group, $^{**}p < 0.01$ versus all other groups.

1.7 Rotarod motor coordination tests

While URB597 does not appear to affect locomotor activity (Piomelli et al., 2006), JZL184 has been reported to suppress spontaneous activity (Long et al., 2009a); however, neither compound has been examined in the rotarod test, an assay used to assess motor coordination. In order to evaluate whether URB597 or JZL184 elicits motor deficits that may interfere with the expression of somatic withdrawal signs, both endocannabinoid modulators were evaluated in this assay. In an initial experiment, we examined the effects of THC (40 mg/kg) vs. rimonabant (3 mg/kg) on performance in the rotarod test. As shown in Figure 14a, THC significantly impaired performance in the rotarod test, with a significant interaction between THC treatment and time [$F(6, 60) = 2.4$, $p < 0.05$]. THC reduced performance from baseline beginning at 30 min post-injection and continued to impair performance up to 6 h ($p < 0.05$). Rimonabant significantly blocked THC-induced rotarod impairment for up to 2 hours ($p < 0.05$).

The final experiment examined whether URB597 (10 mg/kg) or JZL184 (16 mg/kg) would impair performance in the rotarod test (Figure 14b). URB597 showed no significant impairment of rotarod performance compared to baseline or vehicle-treated control mice for up to 2 h post-treatment, which includes the time period that was used for observation in the withdrawal tests, as well as the peak of AEA enhancement (Fegley et al., 2005). JZL184, which elevates 2-AG brain levels up to 8 hours post-injection (Long et al., 2009a), also showed no evidence of motor impairment compared to baseline or vehicle controls for a 24 h period following treatment.

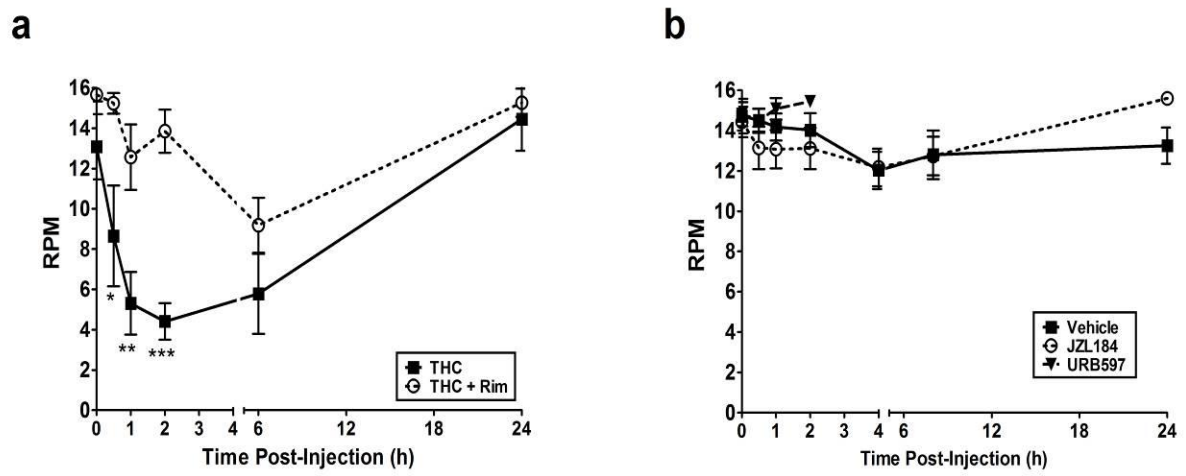

Figure 14 - THC, but neither URB597 nor JZL184, impaired motor performance in the rotarod task. (**A**) THC (40 mg/kg) impairs rotarod performance, expressed as RPM at which point mice fell off the rod. Rimonabant (3 mg/kg) pretreatment blocked THC-induced rotarod impairment. (**B**) Neither URB597 (10 mg/kg) nor JZL184 (16 mg/kg in PEG vehicle) adversely affected motor coordination in the rotarod test. Doses of each inhibitor used in rotarod test were found effective in suppressing somatic withdrawal signs. n = 6-12 mice per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus both baseline time point and rimonabant control group.

1.8 Discussion: Substitution during cannabinoid withdrawal

In the present studies, we investigated the role of endocannabinoid degradative enzymes in THC dependence. Specifically, we examined the impact of increasing AEA or 2-AG levels on somatic withdrawal signs precipitated by the CB_1 receptor antagonist rimonabant in THC dependent mice. Curiously, FAAH (-/-) mice showed no alterations in withdrawal responses across a variety of conditions. Despite constant elevation of AEA above that of wild-type mice, the FAAH (-/-) responses suggest that constitutive absence of this enzyme, including across the development of dependence and rimonabant challenge, does not affect withdrawal responses. Strikingly, the FAAH inhibitor URB597 and the MAGL inhibitor JZL184 ameliorated withdrawal responses in THC-dependent mice when administered acutely. In FAAH (-/-) mice, URB597 no longer reduced precipitated paw tremors, while JZL184 maintained its efficacy. This pattern of findings is consistent with the notion that these drugs produce their effects through the inhibition their distinct ascribed enzymes. Unlike direct-acting cannabinoid agonists that possess dependence liability, repeated administration of URB597 or the longer-acting second generation inhibitor PF3845, alone did not lead to signs of precipitated cannabinoid withdrawal after repeated administration. These findings indicate that increasing endogenous cannabinoid signaling may represent a novel strategy to treat cannabis dependence.

The characteristic pattern of behavior associated with rimonabant-precipitated somatic withdrawal signs in THC-dependent mice reported here is similar to that previously characterized in the literature (Cook et al., 1998; Lichtman et al., 2001). Tremors in the front paws continue to be the most consistent, quantifiable, and consistently dose-responsive. Paw tremors was dose-responsive to both the dose of THC that was subchronically administered and dose of rimonabant used to precipitate withdrawal, making it the most principal behavior in

defining THC dependence in mice. Rimonabant elicited head twitches in non-dependent mice, but this effect was augmented in THC dependent mice. On the other hand, scratching behavior appears to be an intrinsic effect of rimonabant. Previous research has demonstrated that rimonabant induces scratching in a dose-responsive manner, and is blocked by cannabinoid agonists (Janoyan et al., 2002). In fact, our research has shown that the endocannabinoid system may play a modulatory role in scratching behavior (Schlosburg et al., 2009).

Given the observation that URB597 reduced rimonabant precipitated withdrawal signs in THC dependent mice, the lack of a FAAH (-/-) phenotype in this withdrawal model was somewhat surprising. Though one might expect that enhanced endocannabinoid signaling might provide a protective mechanism against cannabinoid withdrawal, especially as AEA is discretely produced on-demand under conditions of stress (Hohmann et al., 2005), it is also possible that elevated endocannabinoids during the development of dependence may have enhanced the severity of precipitated withdrawal. Despite having consistently elevated AEA levels nearly 10-fold above that of the wild-type animals, FAAH-deficient mice have previously been demonstrated to display similar responses to acute THC in a battery of cannabinoid sensitive behaviors as wild type animals (Cravatt et al., 2001). FAAH (-/-) and (+/+) mice also have identical levels of CB_1 receptors in brain and possess similar binding affinities to [^{3}H]CP-55,940, suggesting no abnormalities in receptor number or function (Lichtman et al., 2002). However, the possibility exists that other compensatory actions occurred due to genetic deletion of this gene across ontogeny. At any rate, genetic deletion of FAAH does not appear to influence rimonabant-precipitated withdrawal responses in THC dependent mice across a variety of different conditions.

The pharmacological inhibition of FAAH via URB597 was timed to elevate AEA levels during the period withdrawal is precipitated. The short-term elevation of AEA during this period significantly attenuated the severity of the withdrawal behavior, as seen primarily in the reduced amount of paw tremors (Figure 6A & 7A). This reduction in paw tremors by URB597 was completely absent in FAAH (-/-) mice, showing that the effect seen was specific to the actions of URB597 on FAAH activity. Conversely, it appears that the mechanism by which URB597 altered expression of head twitching was FAAH independent. Although FAAH blockade leads to increased levels of AEA, this enzyme also regulates the catabolism of noncannabinoid fatty acid amides, including *N*-palmitoyl ethanolamine (PEA), *N*-oleoyl ethanolamine (OEA), oleamide, and the N-acyl taurines (Cravatt et al., 2001; Saghatelian et al., 2006). Thus, it is unclear whether the beneficial effects of URB597 in reducing THC withdrawal responses is related to elevated levels of AEA and/or the other substrates of FAAH. Regardless, the present results suggest that URB597 or other FAAH inhibitors may be a promising pharmacotherapeutic approach to alleviate THC withdrawal responses, both mild and severe.

With recent advances allowing systemic examination of MAGL inhibition and consequential 2-AG elevations, we examined whether elevating 2-AG could similarly reduce somatic THC withdrawal symptoms. While less is known about the behavioral consequences of 2-AG inhibition, the concentration of this endocannabinoid is more than one hundred-fold greater than that of AEA in the brain. However, it is possible much of the 2-AG in the body does not play a role in cannabinoid signaling (Bisogno et al., 1999). With the use of JZL184 to inhibit MAGL function, and subsequent elevations of 2-AG, we found that there was a clear attenuation of paw tremor incidence after acute inhibition. One might predict an increased efficacy of JZL184 in reducing withdrawal symptoms in FAAH (-/-) mice, since both major endogenous cannabinoids,

AEA & 2-AG, are elevated above that of wild-type mice simultaneously. However, this was not the case. The observation that JZL184 was equally efficacious in FAAH (+/+) and (-/-) mice indicates that the mechanism of JZL184 was independent of FAAH activity. However, given the limits with knockout animals, full characterization of dual inhibition of both enzymes on cannabinoid withdrawal is warranted in future studies.

The lack of rimonabant-precipitated cannabinoid withdrawal signs by repeated URB597 or PF3845 injection is an important observation for the clinical development of these drugs and has important implications for the development of other FAAH inhibitors. The present findings examining endocannabinoid attenuation of withdrawal adds to a growing body of literature demonstrating that URB597 lacks the rewarding properties that are typical of exogenous cannabinoids. In FAAH (-/-) mice, AEA is equipotent at eliciting full tetrad behavioral effects as THC (Falenski et al., 2010). However even under a worst case scenario, hitting FAAH (-/-) with repeated high doses of AEA (ED_{84} = 50 mg/kg twice daily) that produce effective cross-tolerance to THC, AEA was only capable of eliciting a fraction of the withdrawal response as that of THC. This could be due to a faster elimination, faster disassociation from receptors in the brain, or perhaps differential receptor activation of downstream pathways leading to physical dependence. While there are no indications of direct correlation, previous studies of AEA activation of CB_1 receptors indicates maximal efficacy *in vitro* equal to or higher than that of THC.

The ability of prolonged MAGL inhibition to elicit precipitated cannabinoid withdrawal represents a potential drawback of using these inhibitors in treatment of cannabis abuse disorders. Cannabis withdrawal is not recognized in the current *Diagnostic and Statistical Manual of Mental Disorders* (*DSM-IV*), but is currently being debated for inclusion in the next edition (Crowley, 2006). Without widespread medical consensus as to the severity (or even

existence) of such a condition, possible treatment options must present minimal risk in contributing to any further dependence problems. It should be noted that the severity of withdrawal is comparable in magnitude to that of the lowest level of consistent withdrawal quantifiable using THC. This may indicate that while the risk of physical dependence may exist, it is minimal compared to exogenous cannabinoid agonists, and which is the only current effective treatment proven in preclinical and clinical settings. Also promising is the observation that though simultaneous inhibition of FAAH and MAGL produces enhanced acute cannabinoid-mediated activity (Long et al., 2009b), there appears to be little enhanced magnitude of precipitated withdrawal compared to MAGL inhibition alone. This observation was replicated using a wide variety of genetic and pharmacological tools.

The absence of precipitated withdrawal in MAGL (-/-) further underscores the potential for compensatory mechanisms in mice with genetic deletions, as well as adaptive behaviors in mice with developmental and chronic losses in biological functions. An important control was the eliciting of precipitated withdrawal in JZL184-treated heterozygous mice. The magnitude of withdrawal demonstrates that strain differences between the SV129 and C57 mice are unlikely to account for the absent MAGL phenotype. However, further study once mice are backcrossed onto the C57 background will be necessary to properly compare between studies. In addition, MAGL (-/-) mice treated with repeated JZL184 should be planned for future studies in order to confirm the withdrawal signs elicited by JZL184 are a consequence of MAGL inhibition.

The lack of effects of URB597 in the rotarod test complement the results of other studies showing that genetic deletion or pharmacological inhibition of FAAH does not elicit any apparent untoward motor effects. In contrast, THC elicited motor incoordination that persisted for up to six hours. This impairment was reversed by rimonabant, demonstrating a CB_1 receptor

mechanism of action. While mice receiving JZL184 display a decrease in spontaneous activity, and exhibit a flattened posture reminiscent of mice receiving THC, these mice were able to perform normally in the rotarod test throughout the full time course of demonstrated 2-AG elevations (Long et al., 2009a). These findings suggest that endocannabinoid elevation, through blockade of enzymatic degradation, is not sufficient to cause dependence or loss of motor coordination typical of high doses of THC and other exogenous cannabinoid receptor agonists.

In summary, acute administration of the selective FAAH inhibitor URB597, or the selective MAGL inhibitor JZL184, significantly reduced somatic withdrawal symptoms precipitated by the CB_1 receptor antagonist rimonabant in THC-dependent mice. These findings suggest that inhibitors of endocannabinoid metabolizing enzymes may offer an effective pharmacotherapy to treat cannabis withdrawal. Neither FAAH nor MAGL inhibition impaired gross motor function, and repeated FAAH inhibition did not lead to cannabinoid physical dependence. MAGL elicited a moderate precipitated withdrawal profile, though minimal compared to the intense THC withdrawal it was able to attenuate. Collectively, these data suggest that endocannabinoid modulation represents a promising avenue of treatment for a challenging, yet still controversial syndrome.

Chapter 2: Prolonged endocannabinoid elevations in the induction of cannabinoid behavioral tolerance and cross-tolerance

2.1 Repeated JZL184 produces tolerance to cannabinoid-mediated effects

The first step in examining the potential for tolerance to develop from prolonged elevations in 2-AG levels in brain, was to examine the pharmacological effects of JZL184 when given acutely (Long et al., 2009). We ran a subset of the cannabinoid tetrad tests that show sensitivity to JZL184, and given the acute synergistic effects in combination with FAAH (Long et al, 2009), were run simultaneously in FAAH (+/+) and (-/-) mice. With all the behavioral measures, we saw JZL184 acute effects following the general time course of the drug activity and returning to values similar to vehicle by 24 h.

As shown in Figure 15a, latency for tail withdrawal from a heated water bath increased for mice acutely treated with JZL184 in both genotypes. The effect of dual FAAH-MAGL inhibition synergy was replicated as signified by a significant interaction between treatment and genotype [$F(2, 39) = 3.3$, $p < 0.05$] based on the increased level of analgesia produced compared to MAGL inhibition in FAAH (+/+) mice. There were also significant interaction terms for the timeline based on both treatment and genotype [$F(8, 156) = 4.0$, $p < 0.001$]. When performing post-hoc analysis split by both genotype and time, the acute JZL184 groups were elevated in both genotypes above vehicle starting at 2 h post-injection, and remained so for at least 8 h, with FAAH (-/-) showing significant increases out to 24 h. The repeated JZL184 treatment groups,

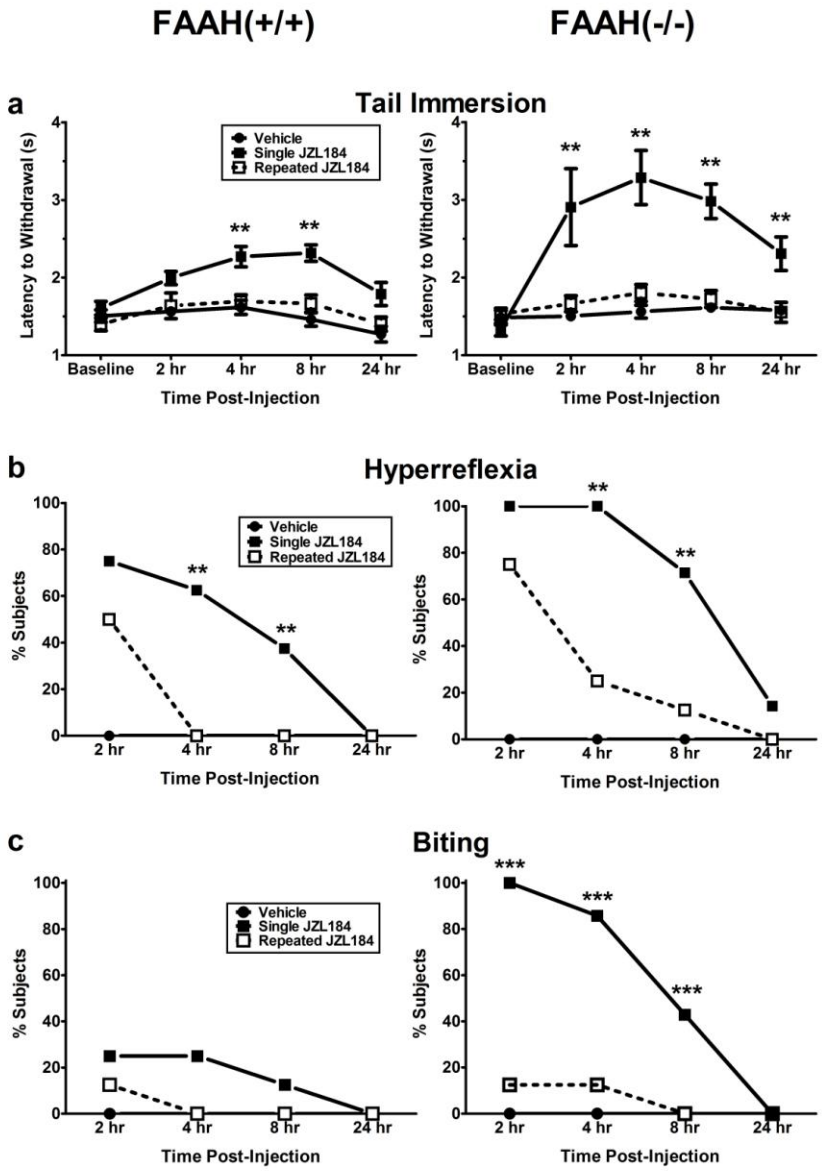

Figure 15 – While FAAH inhibition enhances acute behavioral effects of MAGL inhibition, prolonged MAGL inhibition induces tolerance regardless of FAAH inhibition. Time-course comparison of tail immersion antinociception (**a**), hyperreflexive "popcorning" behavior (**b**), and biting behavior upon presentation of a bar (**c**). Mice were treated with either vehicle (circles), 1-day JZL184 (40 mg/kg; closed squares), or 6-day JZL184 (open squares and dashed line) administration. While the acute effects of JZL184 are enhanced in FAAH -/- animals compared to wild-type littermates similar to Long et al (2009), these behaviors undergo complete tolerance following repeated JZL184 administration. $n = 8$ per group. $^{**}p < 0.01$, $^{***}p < 0.001$ denote significant differences between single and repeated JZL184 groups.

regardless of genotype, showed complete tolerance to these effects, and at no point were tail immersion latencies increased above those of vehicle-treated mice.

Figure 15b shows the percent of subjects that were scored as hyperreflexive or "popcorning" when presented with the catalepsy bar. This behavior was originally reported as a common effect of JZL184 treatment, and is also common amongst mice treated with moderate to high dose cannabinoid agonists. As with the tail immersion data, we see an interaction between time and treatment [$F(6, 117) = 10.2$, $p < 0.001$], and a main effect of genotype [$F(1, 39) = 6.9$, $p < 0.05$], but there was no interaction of treatment and genotype. Upon post-hoc analysis of hyperreflexia split by time, the acute JZL184 groups showed an increased percentage of incidences, as initially does the repeated JZL184 groups. However, by 4 h the repeated JZL184 groups returned to near vehicle levels, and the acute groups remain actively hyperreflexive for 8 h. Again, the diminished magnitude and time-course of these effects indicate some form of tolerance regardless of FAAH genotype.

While it is unclear the exact nature of the biting behavior upon presentation of the catalepsy bar, it has been noted as a behavior selectively enhanced by dual FAAH-MAGL inhibition. As seen in Figure 15c, only FAAH (-/-) mice showed an enhanced likelihood of biting behavior, as indicate by the three-way interaction term of genotype on time and treatment [$F(6, 117) = 2.5$, $p < 0.05$]. Upon split post-hoc analysis, at no time did any JZL184 treatment differ from vehicle in FAAH (+/+) mice, while FAAH (-/-) mice showed enhanced biting in acutely treated JZL184 mice compared to both vehicle and repeated JZL184 mice. This is a behavior only enhanced under dual inhibition conditions, and yet still undergoes complete tolerance following 6 days of prolonged inhibition.

While JZL184 in saline-based vehicle does not produce hypothermia, we have found that challenging JZL184-treated mice with hypothermic drugs or environments can elicit a thermal dysregulation, mediated by the CB_1 receptor (see Fig. A1). Upon placing animals in a 4°C environment, vehicle treated animals show minimal loss of body temperature over time (Figure 16). However, JZL184 treated mice lose several degrees of body heat, that returns to normal upon returning to ambient room temperatures, as indicated by a significant interaction term between time and treatment [$F(18, 120) = 5.1$, $p < 0.001$]. Examining treatment effects over time, acutely exposed JZL184 mice show greater loss in body temperature starting at 3 h, and maintain this temperature loss until an hour following removal from the cold environment. Meanwhile, rimonabant pretreatment prevents JZL184 hypothermia, and repeated JZL184 treated mice also fail to produce significant hypothermia, again demonstrating tolerance following 6-day treatment.

2.2 Analgesic tolerance following prolonged MAGL inhibition versus FAAH inhibition

FAAH inhibition does not produce nearly the range of cannabinoid receptor-mediated effects reported in JZL184-treated mice (i.e. hypothermia, hyperreflexia, locomotor suppression, etc.). However, either FAAH or MAGL inhibition elicits hypoalgesic phenotypes. Thus, a direct comparison was made by examining the time-course of analgesic responses to 56°C tail immersion. Both JZL184 (40 mg/kg) and PF3845 (10 mg/kg) were daily for six days, with testing of analgesic responses tested at the 2 h post-injection point on days 1, 3, and 6. Both vehicle groups, and acute drug groups that received vehicle until day 6, were used as controls and for comparisons for tolerance. The time courses for the change in latency from baseline are shown in Figure 17a-b, with the values of all groups on day 6 highlighted in Figure 17c.

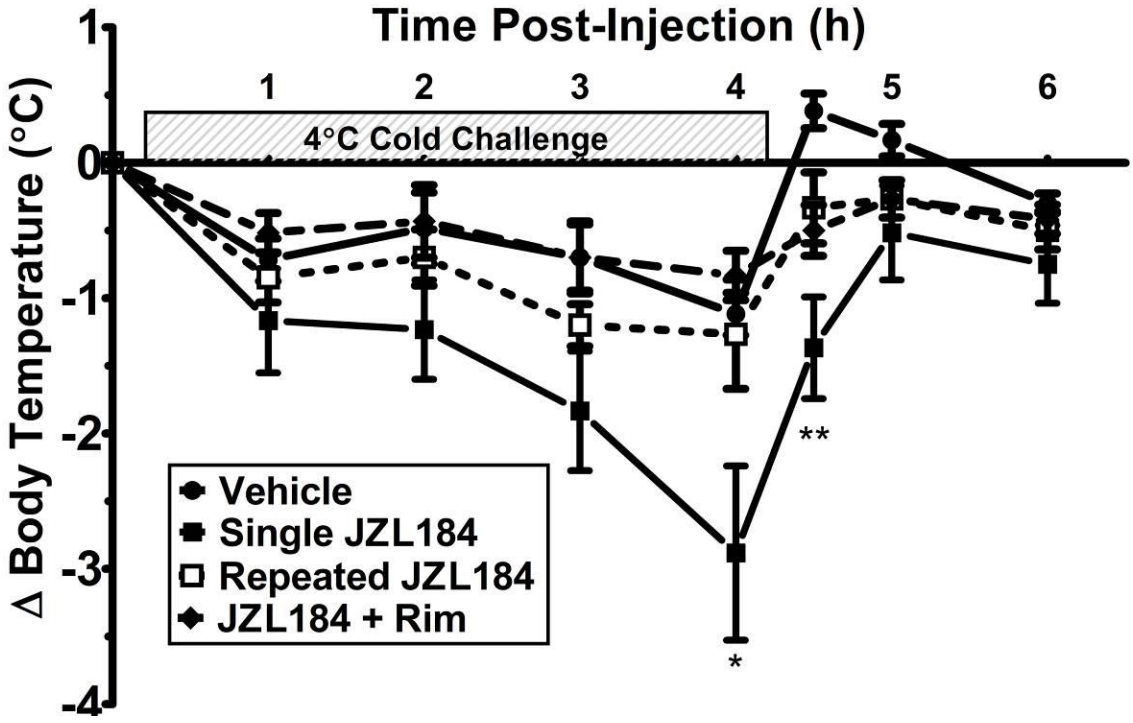

Figure 16 – Hypothermic tolerance following prolonged MAGL inhibition, as demonstrated under cold challenge conditions for 4 hours (4°C). Vehicle treated mice (circles) show minimal decreases in body temperature in cold conditions, however JZL184 (40 mg/kg; closed squares) induced a nearly 3°C drop. This effect is CB1 receptor mediated, as evidenced by reversal with rimonabant (3 mg/kg) co-treatment (diamonds with dashed line). Upon repeated administration, JZL184 hypothermic effects undergo near complete tolerance (open squares with dotted line). *n* = 6 per group. $*p < 0.05$, $**p < 0.01$ denote significant differences between single and repeated JZL184 groups.

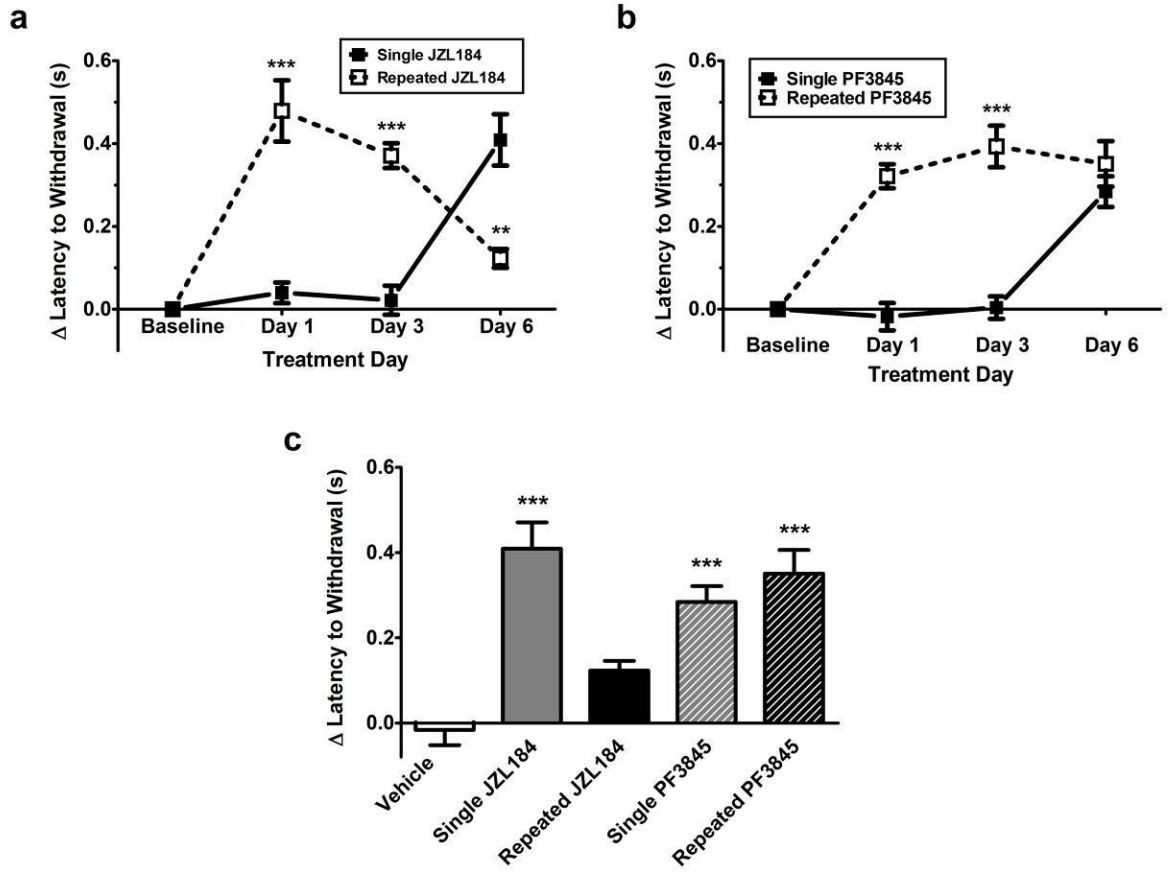

Figure 17 – In contrast to the tolerance exhibited following prolonged MAGL inhibition, prolonged FAAH inhibition maintains efficacy in tail immersion antinociception. Time line comparison of changes in tail immersion latency from baseline over 1, 3, and 6 days following either JZL184 (**a**) or PF3845 (**b**) treatment. (**c**) Direct comparison of change in latency on day 6. $n = 6$ mice per treatment. $^{***}p < 0.001$ denotes significant difference from vehicle, or between single and repeated drug treatment groups.

Latencies remained consistent through repeated testing and vehicle injections, while both drugs showed similar immediate effects, as indicated by day 1 in the repeated groups and day 6 of the single-exposure groups. There was a significant interaction term between treatment and testing day [$F(8, 70) = 19.5$, $p < 0.001$], and split analysis by testing day shows a diminishing effect of JZL184 over repeated treatment, which returns to vehicle latencies by day 6. Meanwhile, PF3845 maintains similar analgesia across repeated treatment days to the same levels as initial exposure, with both repeated and single PF3845 groups having significantly greater latencies than vehicle ($p < 0.001$).

It was at this time that the newly-generated MAGL (-/-) mice became available for evaluation. We tested the responses of MAGL (+/+), (+/-), and (-/-) mice while still on the SV129 background on which they were created. As shown in Figure 18, MAGL (-/-) mice show a hyperalgesic phenotype compared to (+/+) controls in the 52°C tail immersion test ($p < 0.05$; Figure 18a), a temperature at which our previous studies were conducted, and a temperature at which FAAH (-/-) latencies match those of wild-type controls. Hyperalgesic responses were also noted in the 56°C hot plate test ($p < 0.05$; Figure 18b), a temperature at which FAAH (-/-) show a hypoalgesic phenotype. These results contrast to those found in FAAH (-/-) mice, which display a normal or hypoalgesic phenotype in similar tests, though those tests were run on mice with a C57 background strain.

2.3 Chronic MAGL blockade leads to behavioral cross-tolerance to exogenous cannabinoids

To further explore potential alterations in the endocannabinoid system, we compared the behavioral effects of cannabinoid receptor agonists in animals with chronic disruptions in MAGL

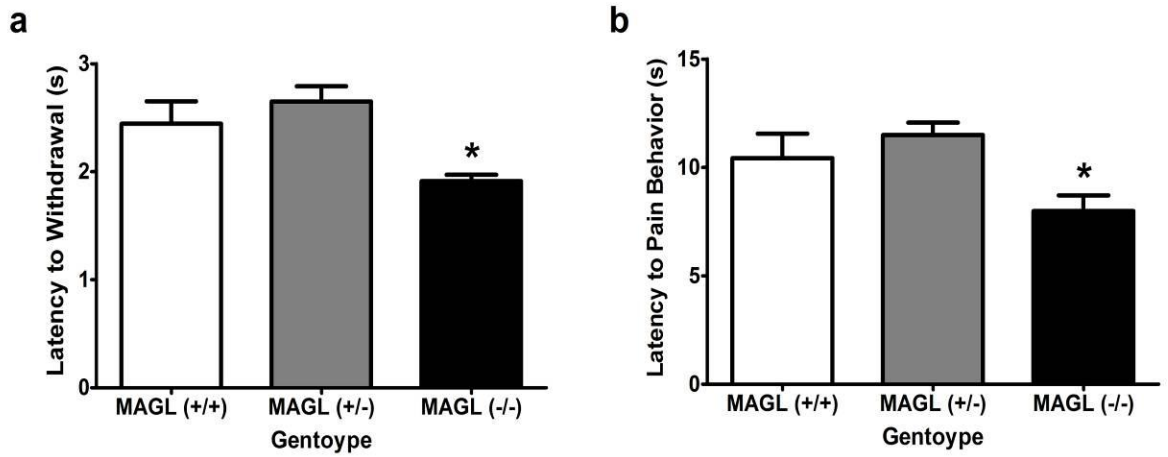

Figure 18 – Baseline pain sensitivity of mice with genetically inactivated MAGL activity. In both the nociceptive 52°C water bath tail immersion (**a**) and 56°C hot plate (**b**) assays, latencies were lower in MAGL(-/-) mice compared to wild-type controls. n = 6-8 per group. *$p < 0.05$ versus MAGL (+/+) group.

versus controls. It has previously been shown that FAAH (-/-) mice exhibit wild-type responses in antinociceptive, hypothermic, and cataleptic assays when treated with THC, indicating normal CB_1 function in these animals. However, MAGL (-/-) mice and repeated enzyme inhibitor administration has not been tested under similar conditions. JZL184 (40 mg/kg), PF3845 (10 mg/kg), MAGL (-/-), and FAAH (-/-) mice were all employed to profile the potential for prolonged elevations in endocannabinoids to produce cross-tolerance to exogenous cannabinoid activation.

The first experiment looked at the effect of repeated JZL184 in FAAH (+/+) and (-/-) mice in response to cumulative dose-responses of THC. All tests were run 26 h following the final JZL184 injection, based on the previous above studies indicating that the majority of acute behavioral alterations are returned to baseline levels by 24 h. In all measures, THC increased the magnitude of effect with increasing dose, with the responses between FAAH genotype nearly identical. While by the highest dose tested (100 mg/kg), almost all measures were at or approaching maximal effect in vehicle treated animals, interaction terms for treatment by THC dose indicate repeated JZL184 treated mice showed significantly reduced response to the antinociceptive [$F(6, 84) = 35.9$, $p < 0.001$; Figure 19a] and hypothermic [$F(6, 84) = 19.5$, $p < 0.001$; Figure 19b] characteristics of THC. The potency of THC was not only shifted, but the maximal efficacy of THC was severely depressed in these endpoints to a point of minimal response even at extraordinary doses. Interestingly, the cataleptic properties of THC were not significantly altered by repeated JZL184 pretreatment [ANOVA(THC dose x JZL184), $p > 0.05$; Figure 19c]. The extent of cross-tolerance observed was not altered in any way by FAAH genotype.

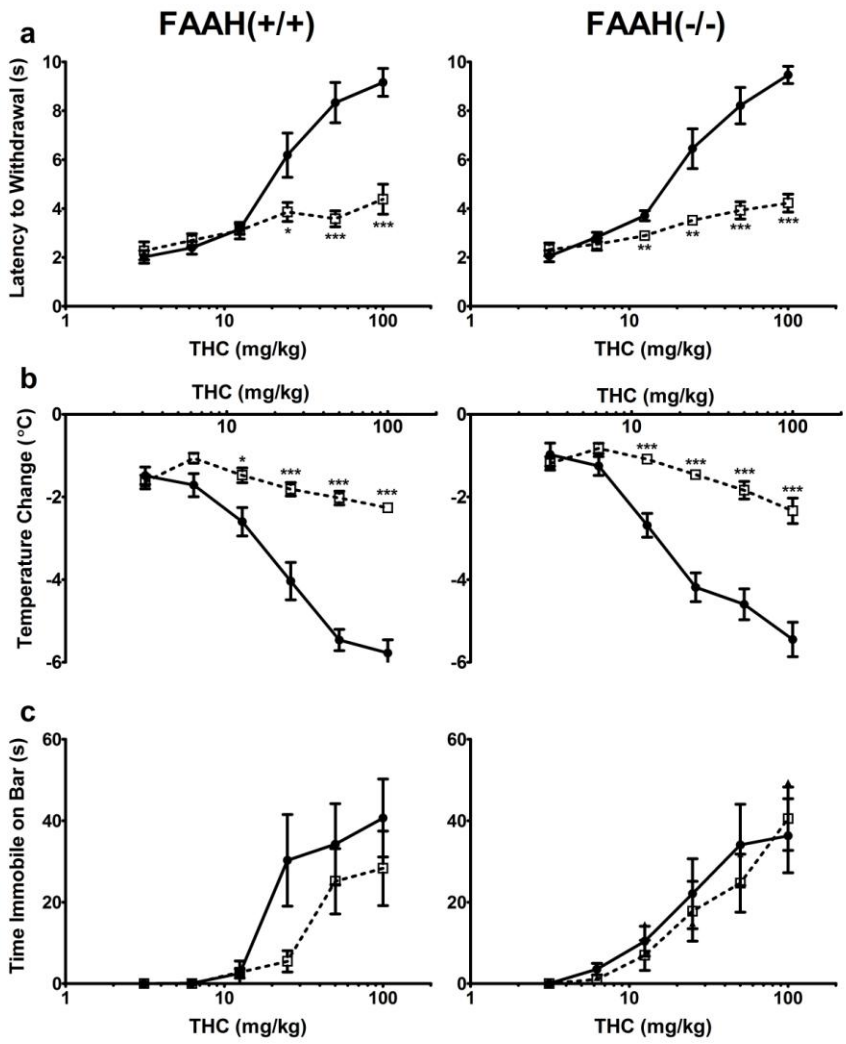

Figure 19 - Prolonged JZL184 administration produces behavioral cross-tolerance to the partial cannabinoid receptor agonist THC. Cumulative dose-response comparison of the behavioral effects of THC in mice pretreated with either repeated vehicle (circles with solid line) or 6-day JZL184 (40 mg/kg; open squares with dashed line). All tests were run in both FAAH (+/+) mice (left column) and FAAH (-/-) mice (right column). Mice were tested 26 h after their final pretreatment for tail immersion antinociception (**a**), hypothermia (**b**), and catalepsy (**c**) in response to escalating doses of THC. Repeated JZL184 produced significant decreases in response to the antinociceptive and hypothermic effect of THC, but not catalepsy. FAAH genotype did not alter the response to THC in vehicle treated mice, and did not alter the degree of cross-tolerance to THC following repeated administration. $n = 8$ per group, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ denote significant differences from vehicle controls.

The extent of cross-tolerance to THC was nearly complete, possibly due to its partial agonist properties at CB_1 receptors, and 2-AG being a full agonist demonstrated by *in vitro* testing. Further tests were conducted against the synthetic full agonist WIN55,212-2 (WIN), which is the most commonly used and best characterized synthetic agonist in comparison to THC, including comparable regional CB_1 receptor downregulation (Sim-Selley and Martin, 2002). MAGL (-/-) mice were tested first for comparison of dose-responses to WIN versus MAGL (+/+) and (+/-) controls. As seen with the hyperalgesia tests shown earlier, the MAGL (+/+) and (+/-) groups showed similar response to WIN, which showed maximal responses in all measures in a manner about 10-fold more potent than seen with THC. There were significant statistical interaction terms for genotype and WIN dose observed for the antinociceptive [$F(12, 120) = 3.6$, $p < 0.001$; Figure 20a] and hypothermic [$F(12, 120) = 4.6$, $p < 0.001$; Figure 20b] measures. Upon examining individual doses, the MAGL (-/-) mice showed significantly reduced responses to WIN at 3 to 10 mg/kg for tail immersion, and 10 to 30 mg/kg for hypothermia. The tail immersion data converges again at the highest dose, though any differences in response are obscured by the test cutoff for maximal responses, something that was unable to be achieved by THC. However, as seen with JZL184 in THC, the cataleptic response to WIN was not altered by MAGL genotype (Figure 20c).

Due to the differences in background strain that could represent a potential confound to the MAGL (-/-) results, tests were replicated with repeated JZL184 treated mice. Response and potency of WIN was roughly equal to that seen in the MAGL (-/-) test, and the results confirmed those seen in both previous experiments. Repeated JZL184 mice showed reduced response to WIN-induce antinociception [$F(6, 84) = 25.1$, $p < 0.001$; Figure 20d] and hypothermia [$F(12, 120) = 40.8$, $p < 0.001$; Figure 20e]. As seen with THC, there was a shift in potency as well as

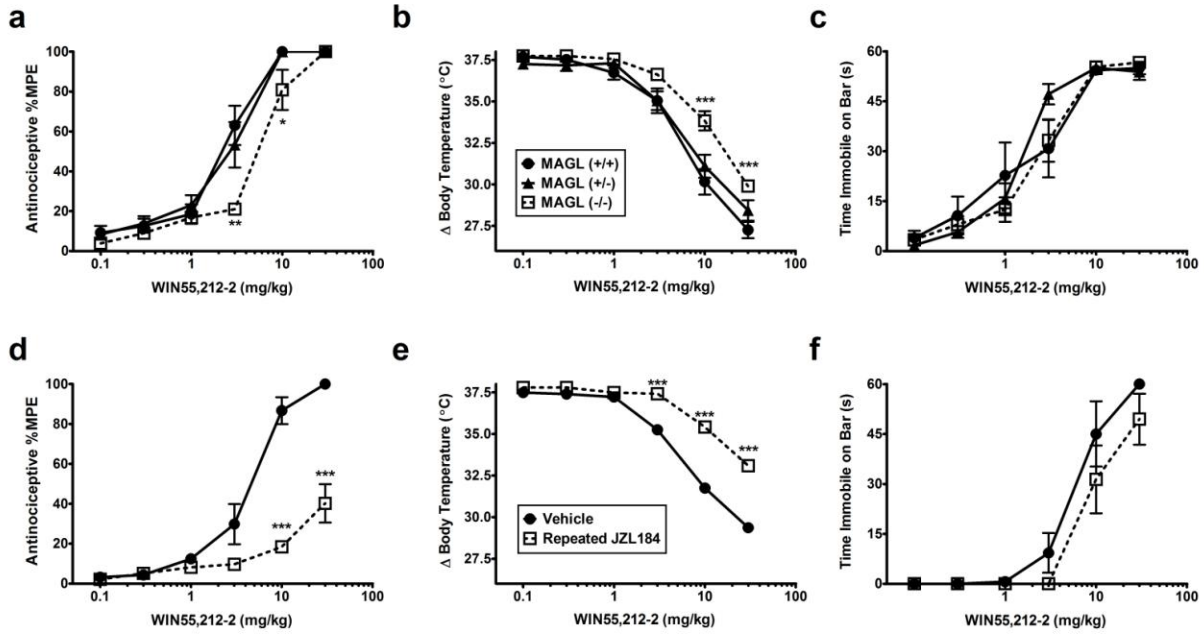

Figure 20 - Repeated JZL184 administration, or genetic deletion of MAGL, produces behavioral cross-tolerance to a cannabinoid receptor agonist. (**a**, **b**, **c**) Cumulative dose-response comparison of WIN55,212-2 behavioral effects in MAGL (+/+; circles), (+/-; triangles), and (-/-; open squares) mice. Mice were tested for tail antinociception (**a**), hypothermia (**b**), and catalepsy (**c**) in response to increasing doses of WIN. (**c**, **d**, **e**) Cumulative dose-response comparison of the behavioral effects of WIN55,212-2 in mice pretreated with either repeated vehicle (circles with solid line) or 6-day JZL184 (40 mg/kg; open squares with dashed line). Mice were tested 26 h after their final pretreatment for tail antinociception (**c**), hypothermia (**d**), and catalepsy (**e**) in response to WIN. Repeated JZL184 and MAGL (-/-) mice exhibited significant decreases in response to the antinociceptive and hypothermic effect of WIN, but not catalepsy. $n = 8$ per group in the JZL184 study, and $n = 6$ per group in the MAGL genotypic study. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle pretreatment or MAGL (+/+) controls.

an overall depression of maximal effect, though not of the same magnitude. Catalepsy continued to be unresponsive to change by prolonged MAGL inhibition.

As a final cross-tolerance comparison in response to elevated endocannabinoids, repeated PF3845 was tested against increasing doses of WIN. As shown in Figure 21, dose-responses of antinociception, hypothermia, and catalepsy are all overlapping between PF3845 and vehicle pretreated groups. These tests indicate that prolonged elevation of 2-AG, but not AEA, produce cross-tolerance to exogenous cannabinoid receptor activation.

2.4 Discussion: Tolerance following prolonged endocannabinoid elevation

The MAGL inhibitor JZL184 produced a near full profile of behavioral effects in cannabimimetic tetrad studies reported in the original article (Long et al., 2009a). However, since then, we have learned that many of these effects are the result of interactions between the drug and the polyethylene glycol-based vehicle in which it was tested. In accordance with these findings, we transitioned to using traditional ethanol:emulphor:saline vehicle. While JZL184 is less soluble and the behavioral profile is less pronounced, similar MAGL inhibition and 2-AG elevations can be achieved with slightly higher concentrations. Upon testing JZL184 tetrad behavior in the new vehicle, tail flick antinociception latencies remained enhanced, and hyperreflexive popcorning maintained its prevalence.

In our effort to replicate the findings of enhanced behavioral effects by simultaneous inhibition of FAAH and MAGL, JZL184 was also tested in FAAH (-/-) mice. What we observed was a replication of the original findings of the Cravatt lab (Long et al., 2009b), with FAAH (-/-) mice showing longer latencies to noxious thermal heat, and a longer timeline of significant

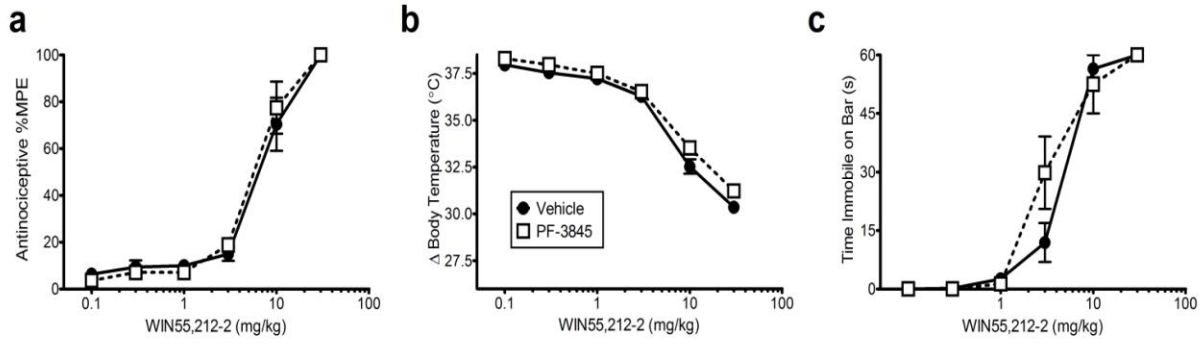

Figure 21 – Prolonged inhibition of FAAH via repeated PF3845 administration fails to produce behavioral cross-tolerance to a cannabinoid receptor agonist. (a, b, c) Cumulative dose-response comparison of WIN55,212-2 behavioral effects in mice treated with either vehicle (circles) or PF3845 (10 mg/kg daily; open squares). Mice were tested for tail antinociception (a), hypothermia (b), and catalepsy (c) in response to increasing doses of WIN. n = 8 per group.

efficacy compared to wild-type controls. We also observed a greater percentage of mice presenting as hyperreflexive for a longer period, and a new behavior in the form of mice gnawing and biting at the catalepsy bar upon presentation. This was observed almost exclusively in mice with both FAAH and MAGL inhibited. This behavior is unlikely a measure of aggression, as mice did not display aggressive behavior toward caged littermates upon return to home cages, though the biting seems to be generalized to most objects placed in front of them, which may indicate increase anxiety-like behavior. Follow-up with studies of feeding behavior, and anxiety tests such as plus maze and light-dark box, might further clarify potential causes for such behavior.

Regardless of FAAH phenotype, all JZL184-elicited behaviors showed reduced magnitude and duration following six days of treatment. The analgesic tolerance resulted in essentially complete absence of effect. When examining the hypothermic cold challenge paradigm, which is not responsive at all to FAAH inhibition (see Fig. A1), repeatedly-treated JZL184 mice showed no significant drop in body temperature compared to vehicle, again demonstrating complete tolerance. Since our original test conditions for tail immersion were designed to be mild enough as to not elicit FAAH analgesia at baseline (52°C; Lichtman et al., 2004), we repeated our tests comparing MAGL and FAAH inhibitors head-to-head over repeated treatment to see which target shows higher efficacy, and whether either maintain their analgesic activity. The FAAH inhibitor PF3845 showed slightly lower increases in withdrawal latency upon initial exposure compared to JZL184. However, JZL184 lost the entirety of its analgesic efficacy by six days, while PF3845 maintained its original effectiveness throughout. FAAH inhibitors also maintained their efficacy to reduce mechanical and cold allodynia in a nerve injury model of pain (Kinsey et al., 2009), as well as increased anti-inflammatory properties with repeated

treatment. In both these models, repeated MAGL inhibition also underwent complete tolerance (see Fig. A2). This maintenance of beneficial outcomes in pain and inflammation testing is a very promising trait for FAAH inhibitors as potential clinical analgesics, as even the most widely effective analgesics in use today (i.e. acetaminophen, opioids) undergo some level of tolerance.

Upon examining the baseline pain sensitivity of MAGL (-/-) mice, we see a hypersensitivity to noxious heat in the spinally-dominant tail immersion assay, and the supraspinally-dominant hot plate assay. This is in stark contrast to previous study of FAAH (-/-) mice, which show phenotypic hypoalgesia (Lichtman et al., 2004). This further underscores the adaptations occurring following prolonged MAGL inhibition, and apparent lack of adaption in the presence of chronic FAAH inhibition.

Given the complete tolerance to a wide variety of cannabinoid-like behaviors following repeated JZL184, we expected to see effective cross-tolerance to exogenous cannabinoid agonists under the same conditions. Mice showed identical baseline behavior given a 26 h washout period following the last injection of JZL184, suggesting JZL184 behavioral activity has subsided. We were surprised to see not only profound shifts in THC's antinociceptive and hypothermic potency in these mice, but also a substantial depression of THC efficacy as well, almost to the point of inactivity. As seen with the tolerance data, this effect appears to be entirely driven by prolonged MAGL inhibition, as no differences were noted in FAAH (-/-) mice. The absence of catalepsy tolerance represents a noticeable difference between tolerance by an endogenous ligand and an exogenous agonist. THC would typically produce tolerance to all tetrad effects, including catalepsy, though when given systemically is expected to hit all receptors in the brain equally. These catalepsy data may suggest that expression levels of MAGL in various brain regions may dictate the level of tolerance seen for various behaviors.

Since THC cross-tolerance to JZL184 was approaching full insensitivity, we chose to examine the efficacy of the test drug as a factor in the level of tolerance observed. Typically, it is harder to suppress activity of a full receptor agonist compared to a partial agonist. Follow-up testing of WIN in JZL184-treated and MAGL (-/-) mice provided measures of the maximal tolerance possible by MAGL in the face of maximal possible cannabinoid receptor activation. While the level of cross-tolerance was still clear following prolonged MAGL inhibition, in both tests it appeared that the behavioral effects were surmountable with increasing doses of WIN. This fits with expected models of receptor activation from *in vitro* studies, and suggesting 2-AG produces complete cross-tolerance to partial agonists, and shifts in potency of other full agonists. Also fitting with this model, elevated partial agonism by AEA during prolonged FAAH inhibition was incapable of producing any signs of cross-tolerance to WIN challenge.

Finally, to demonstrate the functional consequences the phenomena of tolerance and cross-tolerance have on neuronal communication and plasticity, electrophysiological studies were conducted in mice treated with repeated JZL184 and PF3845 (see Fig. A4). While previous studies have shown JZL184 acutely enhances cannabinoid-mediated DSI, we've demonstrated that in a pair of brain regions studied (hippocampus and cingulate cortex), repeated JZL184 treated mice show diminished DSI, a functional corollary to tolerance. Furthermore, exogenous application of cannabinoid agonist normally suppresses neuronal activity, which is also diminished by repeated JZL184 administration, a functional consequence of reduced CB_1 receptor activity. Neither of these effects was seen in repeated PF3845 treated mice.

These data are potential complications for MAGL inhibition as a therapeutic target, as not only does the analgesic activity of MAGL inhibition diminish with repeated dosing, but so does the behavioral potency and neuronal function of at least one entire class of compounds. Given

the extensive cross-talk of cannabinoid and opioid systems, especially with pain-related endpoints, testing for extrinsic cross-tolerance to morphine analgesia would need to be evaluated to determine the extent of functional losses. Yet to be examined as alternative therapeutic options are either intermittent long-term dosing that takes into account the extraordinary length of JZL184 activity in brain, or loser dosing of JZL184 that might not produce the level of elevations in 2-AG or as prolonged an increase as seen with the high-dose regimen used here. Meanwhile, FAAH inhibitors continue to present no signs of significant consequences on the normal functioning of cannabinoid receptor action.

Chapter 3: Enhanced endocannabinoid availability and functional receptor adaptation corresponding with behavioral responses

3.1 Endocannabinoid quantification during THC withdrawal

To examine any possible alterations in endogenous cannabinoid levels during THC withdrawal, AEA and 2-AG were quantified from brains of FAAH (+/+) and (-/-) mice, treated with either repeated vehicle or THC (50mg/kg), and precipitated with Rimonabant (10 mg/kg). The two brain tissue sections examined were cerebellum and combined forebrain/midbrain, the results summarized in Table 1. The cerebellum displayed no significant differences in AEA with regard to treatment, however FAAH (-/-) mice showed approximate 10-fold AEA elevations [$F(1, 28) = 242.4$, $p < 0.001$] regardless of treatment. FAAH (-/-) mice also showed significant decreases in cerebellum 2-AG content regardless of treatment [$F(1, 28) = 5.5$, $p < 0.05$]. In the forebrain/midbrain, in addition to the elevations in AEA in FAAH (-/-) mice [$F(1, 29) = 313.9$, $p < 0.001$], there was an interaction between genotype and treatment [$F(1, 29) = 5.6$, $p < 0.05$]. FAAH (-/-) mice showed reduced AEA levels in midbrain/forebrain tissue during THC withdrawal when compared to FAAH (-/-) mice receiving rimonabant alone ($p < .05$). There were no differences in 2-AG levels in forebrain/midbrain tissues between any groups.

Table 1. Endocannabinoid Quantification Following Rimonabant Precipitation

	AEA (pM/g tissue)	**2-AG (nM/g tissue)**
Cerebellum		
FAAH (+/+) , Veh/Rim	2.7 ± 0.4	14.1 ± 0.7
FAAH (+/+) , THC/Rim	3.0 ± 0.6	12.4 ± 1.1
FAAH (-/-) , Veh/Rim	29.7 ± 2.0	11.8 ± 0.7
FAAH (-/-) , THC/Rim	32.2 ± 2.9	10.3 ± 1.1
Forebrain/Midbrain		
FAAH (+/+) , Veh/Rim	3.4 ± 0.5	12.6 ± 1.0
FAAH (+/+) , THC/Rim	2.7 ± 0.4	11.3 ± 0.6
FAAH (-/-) , Veh/Rim	27.6 ± 1.6	10.1 ± 0.8
FAAH (-/-) , THC/Rim	21.1 ± 1.8*	12.1 ± 1.5

*$p < .05$ vs. FAAH (-/-) Veh/Rim group

3.2 Prolonged MAGL, but not FAAH, inhibitors lead to accumulation of 2-AG in brain

The elevation of whole-brain 2-AG levels following pharmacological MAGL inhibition by JZL184 has been previously established (Long et al., 2009). We further compared the AEA and 2-AG levels following 6 days of JZL184 (40 mg/kg daily), and found a similar accumulation of eCBs following prolonged inhibition. At the peak 2 h time point (Figure 22a), we see a modest increase in AEA level only in the repeated JZL184 group, which is gone by 26 h (Figure 22c). This is likely a nonselective effect of JZL184, which has been shown previously to inhibit FAAH transiently at higher doses by upward of 60% when given acutely. At the 2 h peak, JZL184 given repeatedly produces 2-AG levels around 15-fold above vehicle-treated levels, and represent a 79% increase above that observed with JZL184 given just once. This increase diminishes post-injection, as repeated JZL84 enhancement over single exposure is down to 39% by 26 h. When testing the peak 2 h effects in FAAH -/- mice, we find JZL184 does not alter the already elevated AEA levels, and shows similar patterns of 2-AG elevation to FAAH (+/+) mice (Figure 22b).

When examining the endocannabinoid accumulation in brain following daily dosing of PF3845 (10 mg/kg), we see AEA elevations acutely 2 h post-injection of more than 15-fold above that of vehicle (Figure 23a). There does not appear to be any substantial difference in the level of AEA accumulation following repeated dosing compared to acute exposure. Also, PF3845 shows clear selectivity, as 2-AG levels are unaltered by either single or repeated dosing (Figure 23b).

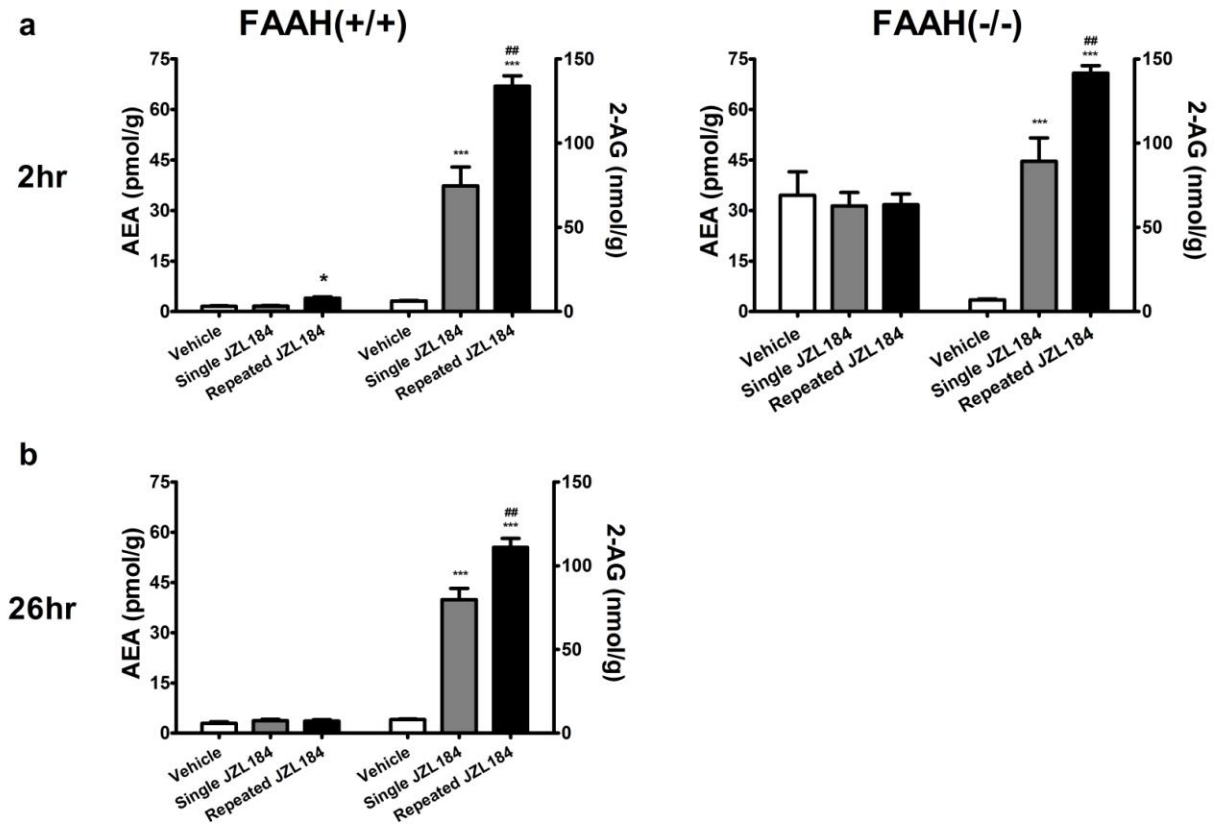

Figure 22 - Whole-brain AEA & 2-AG levels following either single or repeated JZL184 treatment. (**a**) 2 h after treatment JZL184 (40 mg/kg) selectively increases 2-AG about 10-fold after acute administration, and demonstrates an accumulation of 2-AG with repeated JZL184 administration (left column). FAAH (-/-) mice show similar 2-AG elevations, with typical 10-fold AEA elevations regardless of treatment. (**b**) 26 h following final administration, 2-AG levels are still elevated in JZL184 treated mice, with diminished accumulation effects. (c) PF3845 elevates AEA levels almost 20-fold, with similar elevations following either single or repeated administration, and without any elevations in 2-AG levels. $n = 6$ per group. $^{*}p < 0.05$, $^{***}p < 0.001$ versus vehicle controls, $^{\#\#}p < .01$ versus single JZL184 treatment.

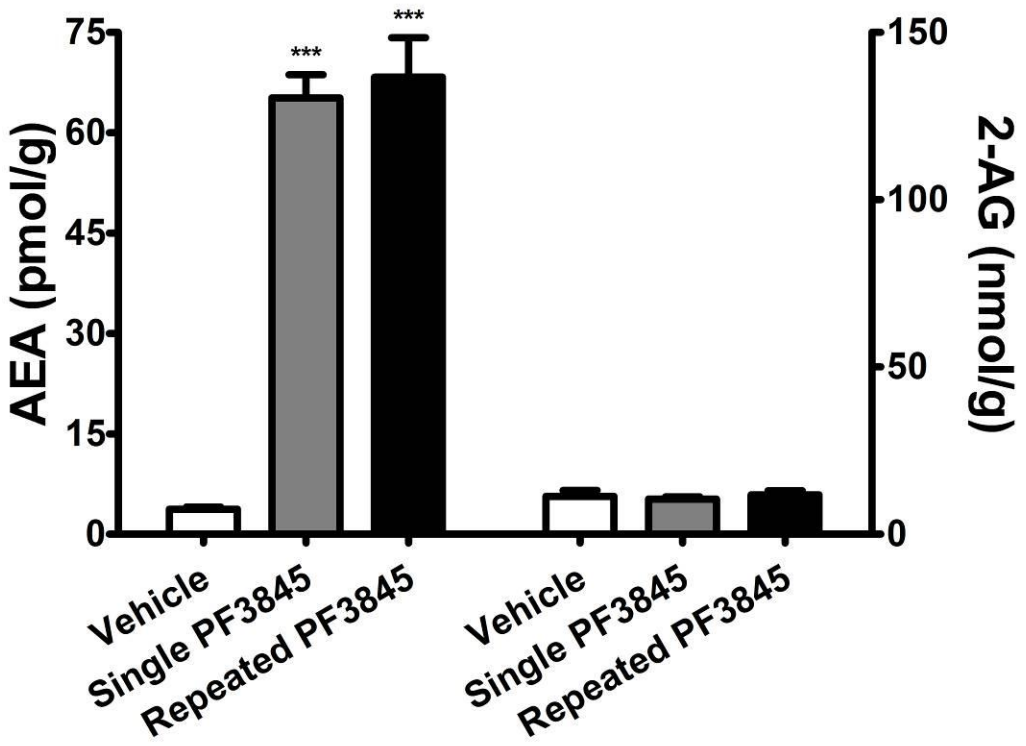

Figure 23 - Whole-brain AEA & 2-AG levels following either single or repeated PF3845 treatment. PF3845 elevates AEA levels almost 20-fold 2 h post-injection, with similar elevations following either single or repeated administration, and without any elevations in 2-AG levels. *n* = 6 per group. ***$p < 0.001$ versus vehicle controls.

3.3 Genetic inactivation of MAGL enhances 2-AG in brain

The created MAGL (-/-) mice show reduced protein expression and almost 88% impairment in hydrolysis rates of 2-AG (see Fig. A3). The resulting decrease rate of hydrolysis allows for the accumulation of several glycerol-containing lipids, though 2-AG is by far the most prominent, with nearly 15-fold increases observed in MAGL (-/-) mice, a level comparable to that seen in repeated JZL184-treated mice (Figure 24b). The resulting accumulation is also apparent when examining the free fatty acid degradation product, whose levels decrease in MAGL (-/-) animals by nearly 70% compared to (+/+) littermates, a decrease not seen in MAGL (+/-) mice (Fig. 24c). Importantly, NAE levels, including that of the other major endocannabinoid AEA, are not significantly altered based on MAGL genotype (Figure 24a).

3.4 Brain CB_1 receptors are impaired by chronic MAGL, but not FAAH, blockade

The loss of most all acute behavioral responses to JZL184 with repeated treatment, and occurrence of cannabinoid cross-tolerance in mice with genetic or prolonged pharmacological disruption of MAGL suggested that CB_1 receptors might be downregulated and/or desensitized in these animals. To test this possibility, we examined CB_1 receptor expression and function through specific binding of [^{3}H]-SR141716A and CB_1 agonist CP55,940-stimulated [^{35}S]-GTPγS binding, respectively, in whole-brain homogenates from mice repeatedly treated with JZL184. Prolonged MAGL disruption led to decreases in CB_1 receptor binding curves compared to vehicle, as well as a suppressed magnitude of [^{35}S]-GTPγS binding (Fig. 25a and 25b). Nonlinear sigmoidal regression on the activation curves revealed a significantly lower maximal efficacy of agonist stimulation (E_{max}), with no apparent change in the potency as measured by EC_{50}. The non-linear regression of the binding data revealed a significantly reduced maximal

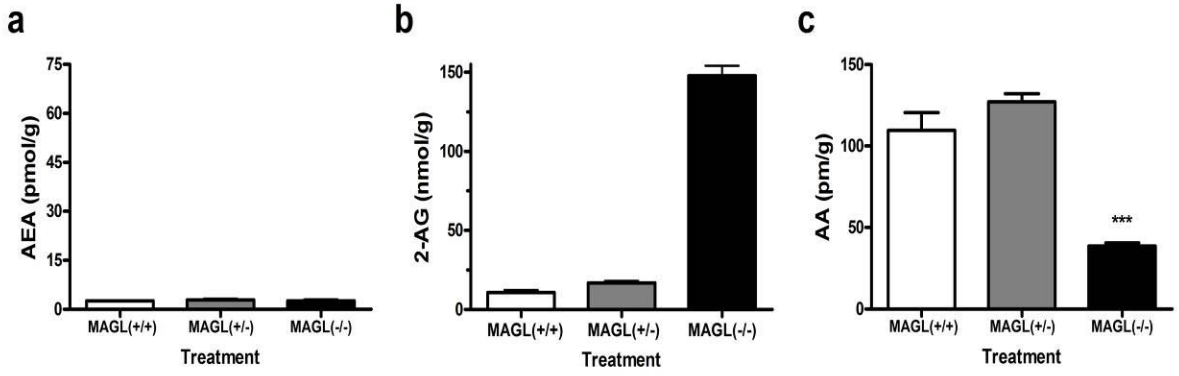

Figure 24 – Lipid profile of mice with genetically inactivated MAGL activity. Without any alteration in AEA levels in whole brain (**a**), MAGL (-/-) mice have 15-fold elevations in 2-AG whole brain content (**b**). MAGL (+/-) mice, despite only 50% 2-AG hydrolysis activity, show normal wild-type levels of 2-AG. (**c**) With the increase in 2-AG, a decrease in 2-AG's metabolite, arachidonic acid, is also seen in MAGL (-/-) mice. ***$p < 0.001$ versus vehicle MAGL (+/+) controls.

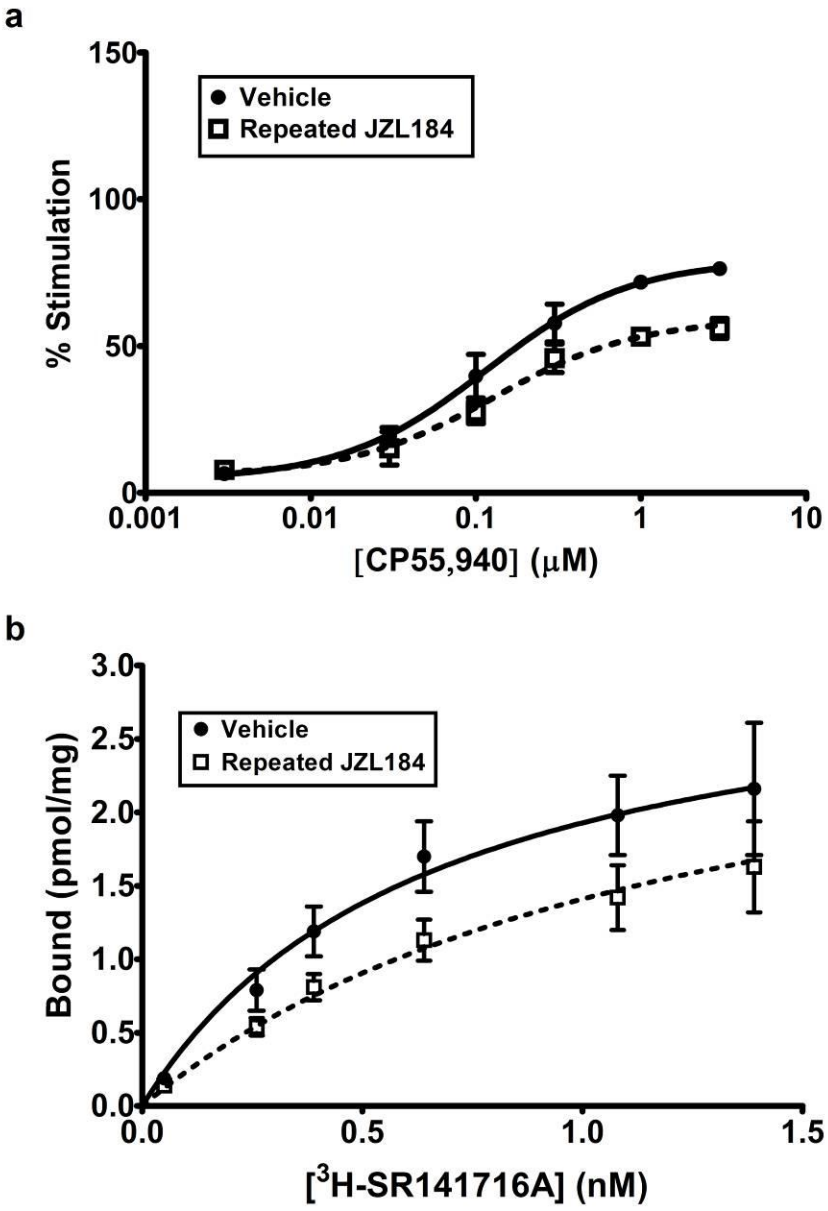

Figure 25 - Loss of receptor activation and membrane receptor levels following 6-day MAGL inhibition by JZL184. Comparison of the membrane receptor GTPγS binding (**a**) stimulated by the cannabinoid agonist CP55,940, and the specific CB_1 receptor binding (**b**) by the tritiated antagonist ^{3}H-SR141716A, from homogenate whole brains following pretreatment with either vehicle (circles), or 6-day JZL184 (open squares with dashed line). Repeated JZL184 decreases both the overall level of receptor signaling as well as the number of surface receptors available. n = 4 tissue samples per group, run in separate experiments with each point run in triplicate for GTPγS and duplicate for receptor binding.

binding (B_{max}) without alteration of the K_D (Table 2). The magnitude of overall loss in receptor efficacy closely matched that of loss in receptor binding. These results are typical of receptor loss without fundamental changes to the receptor function that may alter ligand affinity.

As a generous donation by the Cravatt lab at the Scripps Research Institute, we were given access to whole-brain samples from control mice that were part of a cancer study. The mice were severely compromised immune deficient (SCID) mice that were littermates of mice implanted with tumorigenic cells, and were treated with 30 days of either vehicle or JZL184 (40 mg/kg) by oral gavage. The [^{35}S]-GTPγS curves are shown in Figure 26a, displaying a substantial reduction in E_{max} of 53% [Vehicle: 117.3 ± 6.3, JZL184: 55.8 ± 6.2] without any significant shift in EC_{50} [Vehicle: 0.06 ± 0.03, JZL184: 0.12 ± 0.06]. In contrast, the loss of receptor binding in 30-day JZL184 mice was comparable in magnitude to that seen by 6-day JZL184 treatment (Figure 26b), representing a 33% loss of maximal binding [Vehicle: 3.87 ± 0.30, JZL184: 2.88 ± 0.38], and no shifts in the K_D [Vehicle: 0.69 ± 0.12, JZL184: 0.71 ± 0.23]. These decreases in receptor activation in the absence of further receptor loss are typically indicative of desensitization; however there could also be strain differences.

The questions arising from the previous data were confirmed against MAGL (-/-) mice, which have genetic inactivation of this enzyme and 2-AG elevations from development, and represent yet a third different strain of mice against which these receptor adaptations were tested. The MAGL (-/-) mice showed far greater depressions in maximal receptor activation of 44%, with MAGL (+/-) mice showing a slight lower and nonsignificant loss of 21% (Figure 27a). A similar pattern was seen in the receptor binding, with MAGL (-/-) showing 49% decrease in total binding sites compared to (+/+) controls, and (+/-) mice did not significantly differ from wild-

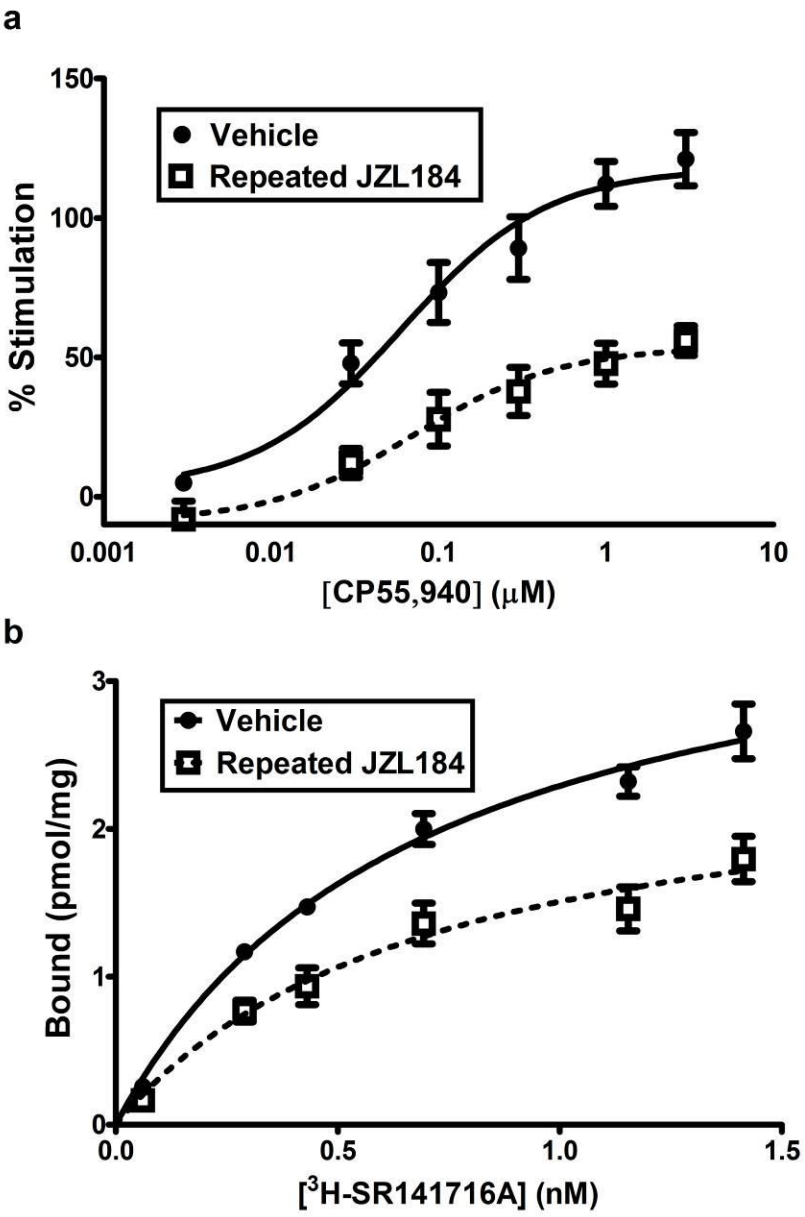

Figure 26 - Loss of receptor activation and membrane receptor levels following 30-day oral JZL184 administration. Comparison of the membrane receptor GTPγS binding (**a**) stimulated by the cannabinoid agonist CP55,940, and the specific CB_1 receptor binding (**b**) by the tritiated antagonist ^{3}H-SR141716A, from homogenate whole brains following pretreatment with either vehicle (circles), or 30-day oral gavage or JZL184 (open squares with dashed line) in SCID mice. 30-day JZL184 decreased receptor activation and the number of surface receptors available, the magnitude of the reduction greater than seen with 6-day JZL184 treatment. n = 4 tissue samples per group, run in separate experiments with each point run in triplicate for GTPγS and duplicate for receptor binding.

type controls (Figure 27b). As with prior tests, the EC_{50} and K_D values indicate no alterations in receptor affinity and function. The pattern of loss of both activation capacity and binding sites parallels that seen in the 6-day JZL184 brains, but to a greater magnitude (Table 2).

In contrast, prolonged blockade of FAAH by PF-3845 did not impact CB_1 receptor expression or function (Figure 28 & Table 2). These findings are consistent with previous work showing no loss of CB_1 receptor number or function in $FAAH^{-/-}$ mice.

3.5 Regional analysis of brain CB_1 receptor adaptation and eCB accumulation

The observation that prolonged MAGL blockade caused profound cross-tolerance to the antinociceptive and hypothermic, but not cataleptic effects of cannabinoid receptor agonists could indicate that CB_1 receptors were differentially affected in specific brain regions. To test this hypothesis, we performed an extensive regional analysis of CP55,940-stimulated [^{35}S]GTPγS binding in mice treated using the 6-day either vehicle or JZL184 (40 mg/kg) dosing regimen. Representative autoradiograms illustrate CP55,940-stimulated [^{35}S]GTPγS binding in both groups at several coronal levels, including caudate putamen, hippocampus, periaqueductal gray (PAG), and cerebellum (Figure 29a). Visual inspection shows chronic JZL184 treatment led to a heterogeneous reduction in CP55,940-stimulated [^{35}S]GTPγS binding. Notable brain regions showing significant CB_1 desensitization include the cingulate cortex, hippocampus, somatosensory cortex, and PAG (Fig. 29b). In contrast, chronic JZL184 treatment did not elicit desensitization in the striatum or globus pallidus, two brain regions associated with catalepsy (Pertwee and Wickens, 1991; Wickens and Pertwee, 1993).

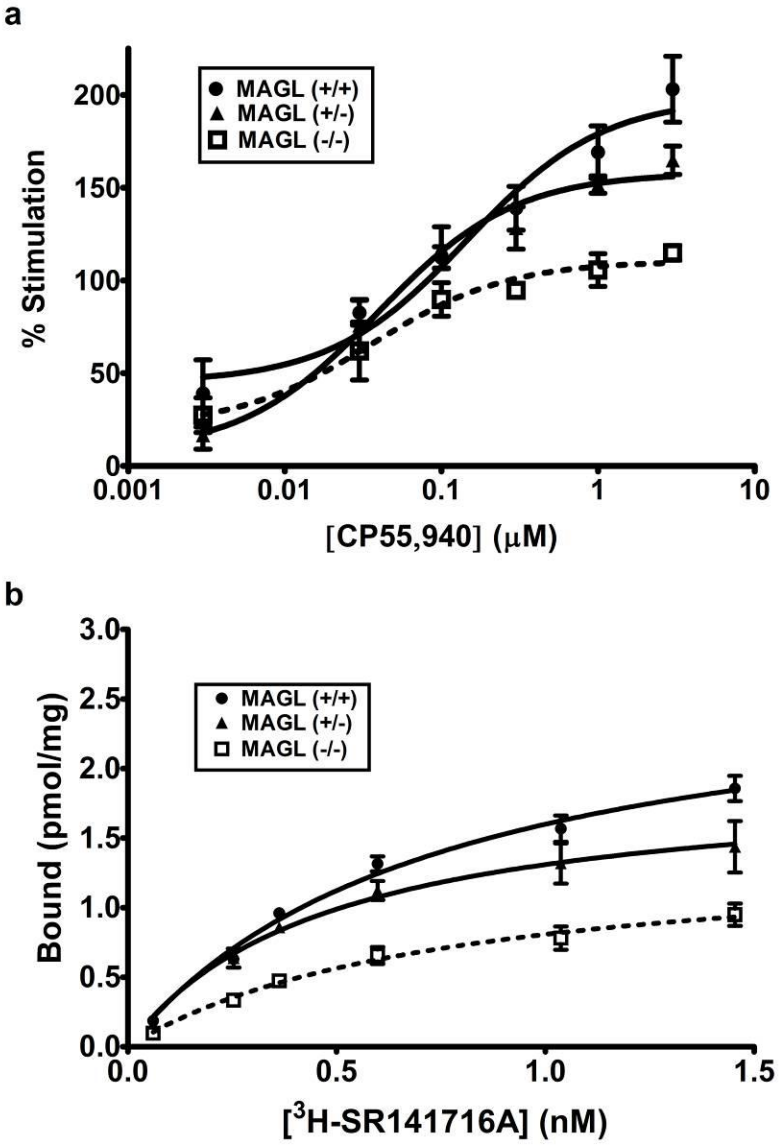

Figure 27 - Loss of receptor activation and membrane receptor levels in mice with genetically inactivated MAGL activity. (**a**, **b**) Comparison of the GTPγS binding (**a**) and specific CB_1 receptor binding (**b**) from homogenate whole brains of MAGL (+/+; circles), MAGL (+/-; triangles), or MAGL (-/-; open squares with dashed line) mice. MAGL (-/-) mice show significantly reduced overall receptor activation and membrane receptor numbers compared to wild-type controls, with MAGL (+/-) showing partial, but still significant reductions. n = 4 tissue samples per group, run in separate experiments with each point run in triplicate for GTPγS and duplicate for receptor binding.

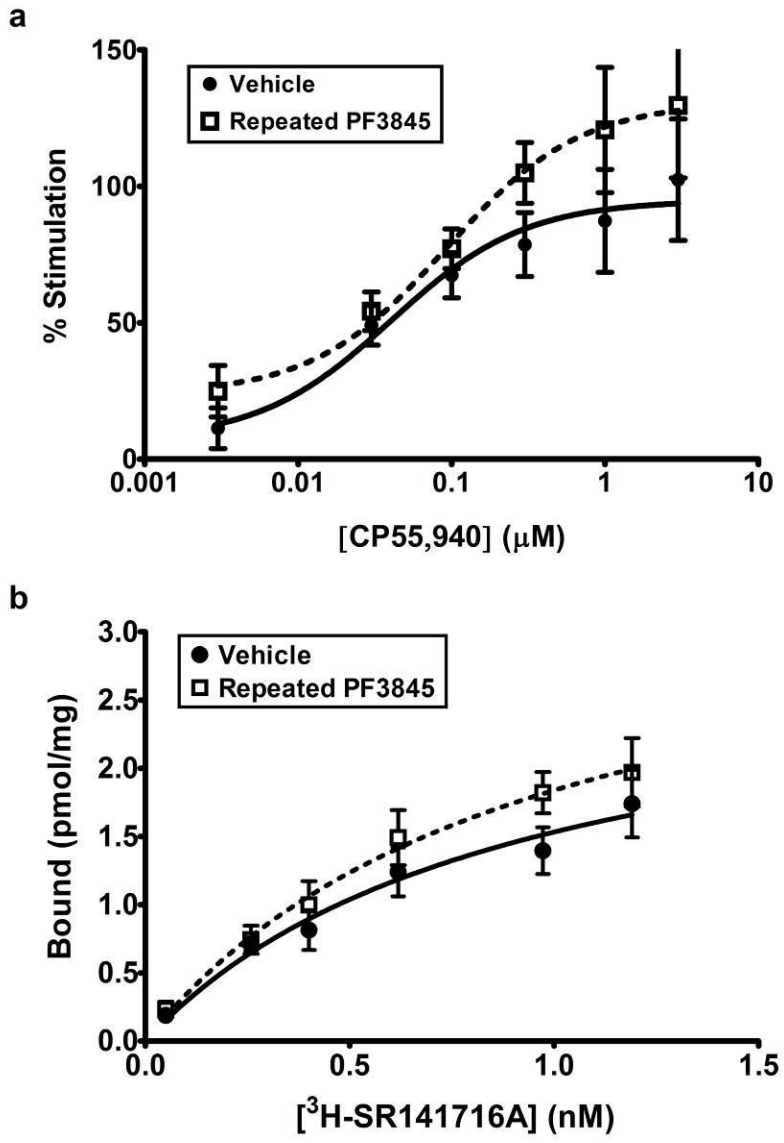

Figure 28 – Prolonged FAAH inhibition by repeated PF3845 does not alter CB_1 receptor function. GTPγS binding (**a**) stimulated by the cannabinoid agonist CP55,940, and specific CB_1 receptor binding (**b**) by the tritiated antagonist ^{3}H-SR141716A, was assayed from homogenate whole brains following pretreatment with either vehicle (circles), or 6-day PF3845 (open squares with dashed line). No significant changes in either measure were noted based on PF3845 treatment. n = 4 tissue samples per group, run in separate experiments with each point run in triplicate for GTPγS and duplicate for receptor binding.

Table 2. CB1 receptor affinity, activation, and binding following prolonged eCB elevations. Best-fit values (mean ± SEM) for the G-protein activation and receptor binding curves. $^{**}p < 0.01$, $^{***}p < 0.001$ decrease versus respective control group.

Treatment	EC_{50} (μM)	E_{max} (% stimulation)	% Loss (vs. control)
MAGL Genotype			
MAGL +/+	0.05 ± .02	183.3 ± 11.1	
MAGL +/-	0.03 ± .01	157.0 ± 6.5	21%
MAGL -/-	0.02 ± .01	107.5 ± 5.7	44%***
JZL184 Treatment			
Vehicle	0.10 ± 0.02	78.3 ± 3.4	8%
Repeated JZL184	0.09 ± 0.02	58.1 ± 3.1	25%**
PF3845 Treatment			
Vehicle	0.03 ± 0.02	93.8 ± 8.7	
Repeated PF3845	0.05 ± 0.02	125.8 ± 10.9	N/A
Treatment	**K_D (nM)**	**B_{max} (pmol/mg)**	**% Loss (vs. control)**
MAGL Genotype			
MAGL +/+	0.74 ± 0.12	2.79 ± 0.22	
MAGL +/-	0.48 ± 0.06	1.94 ± 0.09	30%
MAGL -/-	0.76 ± 0.11	1.42 ± 0.10	49%***
JZL184 Treatment			
Vehicle	0.74 ± 0.33	3.41 ± 0.28	3%
Repeated JZL184	0.96 ± 0.49	2.74 ± 0.16	26%**
PF3845 Treatment			
Vehicle	0.91 ± 0.29	2.93 ± 0.50	
Repeated PF3845	0.96 ± 0.16	3.60 ± 0.33	N/A

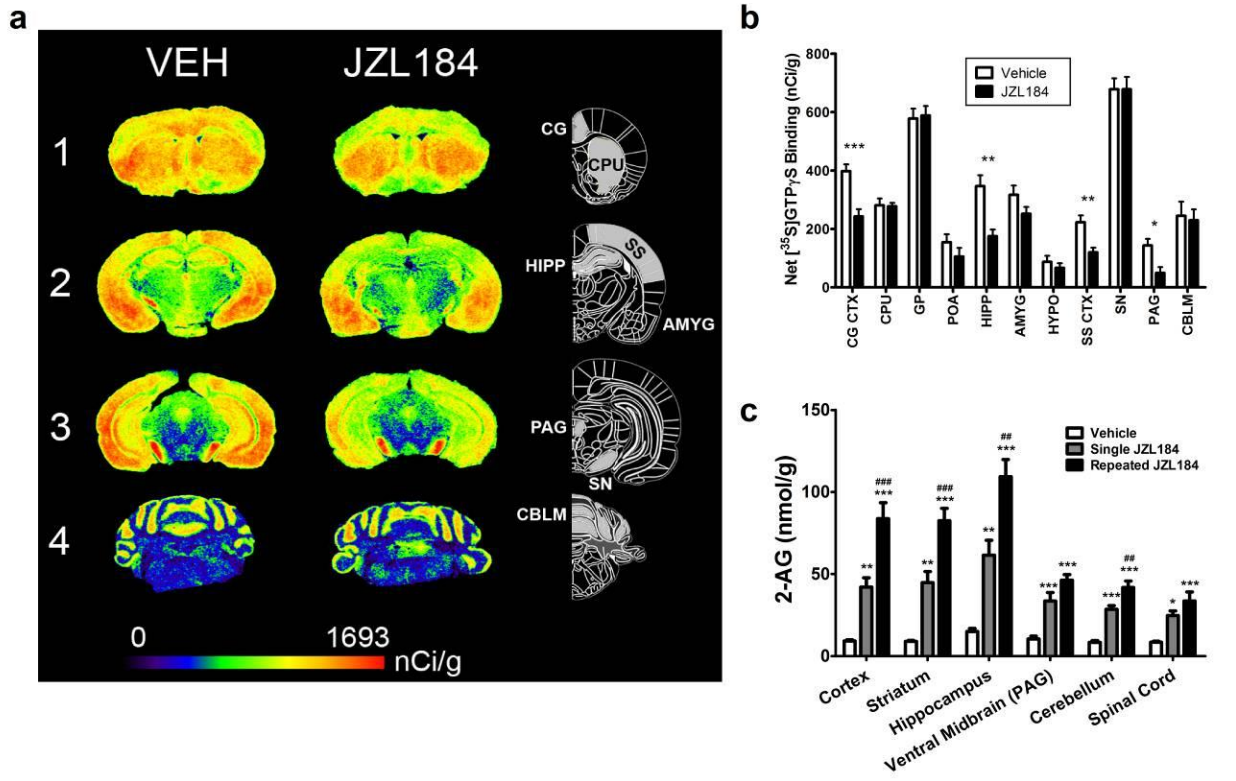

Figure 29 - Regional changes in cannabinoid agonist-stimulated [^{35}S]GTPγS binding and 2-AG levels following prolonged inhibition of MAGL. (**a**) Representative autoradiograms showing CP55,940-stimulated [^{35}S] GTPγS binding in coronal brain sections following either repeated vehicle (left column) or JZL184 (right column) treatment. Pseudocolor images indicate levels of receptor-mediated G-protein activity and highlight significant decreases in CB1 receptor activation in the cingulate cortex (row 1), hippocampus (row 2) and periaqueductal gray (row 3), while no differences are apparent in the caudate-putamen (row 1) or cerebellum (row 4). (**b**) Densitometric analysis of CP55,940-stimulated [^{35}S]GTPγS binding in selected regions, including: cingulate cortex (CG CTX), caudate putamen (CPU), globus pallidus (GP), preoptic area of the hypothalamus (POA), hippocampus (HIPP), amygdala (AMYG), hypothalamus (HYPO), somatosensory cortex (SS CTX), substantia nigra (SN), periaqueductal gray (PAG), & cerebellum (CBLM). (**c**) Regional 2-AG levels following JZL184 treatment, many

corresponding to regions quantified for CB_1 receptor activation. Increases, while varying somewhat in intensity, were less regionally dependent than CB_1 receptor activation. n = 8 brains per group, run in triplicate slices for each targeted region. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus vehicle treatment for specific region. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus single JZL184 treatment for specific region.

To confirm that the differential behavioral adaptations are due to CB_1 receptor activation, and not regional differences in eCB signaling pools, we examined gross regional changes in endocannabinoids to match key regions examined in [^{35}S]GTPγS binding. Vehicle, single JZL184, and repeated JZL1284 groups were sacrificed 26 h after their final injection to match the timeline of the autoradiographic study. Levels of AEA were not substantially altered across regions by any treatment, and directional changes varied by region (Figure 30). While the elevations in 2-AG vary between rostral and caudal regions, similar relative accumulation effects as those in whole-brain are seen following repeated JZL184 in each region. Each region showed significant elevation in 2-AG following acute JZL184 (Figure 29c), which was further enhanced by repeated administration. 2-AG content is not predictive of CB_1 receptor dysfunction, as areas such as neocortex and striatum have identical 2-AG profiles, but divergent levels of receptor inactivation. Given that many of the areas associated with motor coordination and function (i.e. globus palladus, caudate putamen, and cerebellum) do not show desensitization following repeated JZL184, evidence points to CB_1 receptor deficits as the overriding correlate to behavioral tolerance rather than potential regional differences in 2-AG elevation.

3.6 Discussion: Endocannabinoid accumulation and cannabinoid receptor adaptations

Given that all the behaviors observed involved either direct alteration of the regulatory function of endocannabinoid ligands, or repeated administration of exogenous cannabinoid agonists that may alter normal endocannabinoid activity, it is important to understand the changing endogenous ligand availability in order to predict possible changes in endocannabinoid function. We found that inhibitors of endocannabinoid degradation reduced antagonist-precipitated behaviors in mice undergoing THC withdrawal; however no phenotypic differences

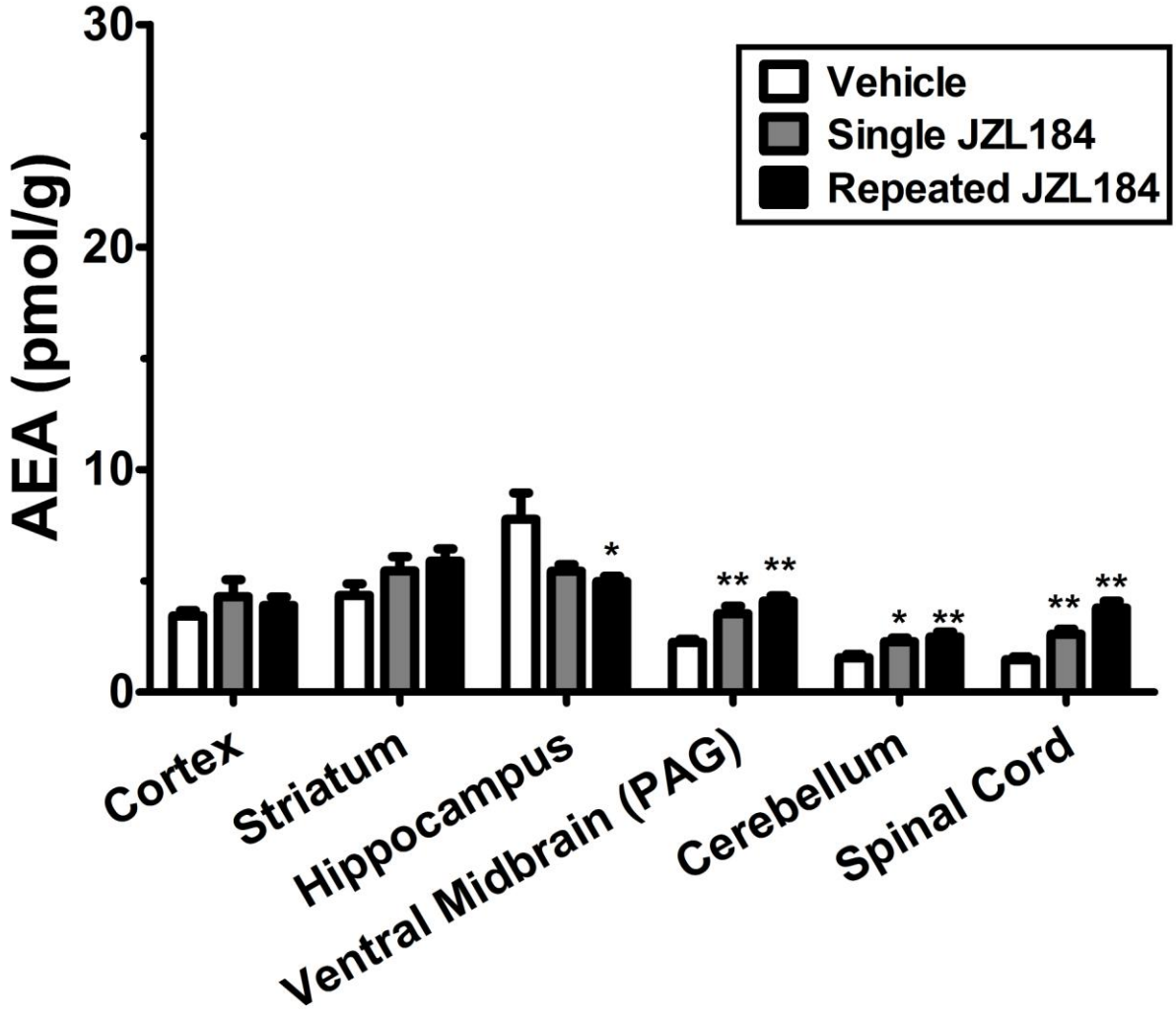

Figure 30 - Regional AEA levels following JZL184 treatment, many corresponding to regions quantified for CB_1 receptor activation. Changes in AEA content were bidirectional, with direction of changes regionally-dependent. No effects of repeated JZL184 were observed when compared to acute JZL184 effects. n = 8 brains per group, run in triplicate slices for each targeted region. $*p < 0.05$, $**p < 0.01$ versus vehicle treatment for specific region.

were observed in FAAH (-/-) mice. Follow-up studies shown into AEA and 2-AG content in the brains of FAAH (-/-) mice showed lower 2-AG content in the cerebellum compared to wild-type mice, which may be a compensatory mechanism for consistent AEA elevations. There was also a downregulation of AEA content in midbrain/forebrain regions following chronic THC, which may be a feedback response on AEA synthesis due to repeated CB_1 receptor activation. Reductions in AEA content of midbrain/forebrain regions have been previously reported in rats during THC withdrawal, and similarly no alterations were seen in cerebellum (Gonzalez et al., 2004). Cerebellum also presents less CB_1 receptor desensitization and downregulation than midbrain areas, such as thalamus and hippocampus, following chronic THC (Sim-Selley and Martin, 2002). Either of the reduced endocannabinoid responses observed in FAAH (-/-) mice could account for normalized sensitivity to THC withdrawal. Given the possibility that AEA is not increased under cannabinoid withdrawal conditions, but rather subject to specific decreases, may provide justification for why FAAH inhibition may be valuable for the stabilization of endocannabinoid function during THC withdrawal.

When testing the endogenous cannabinoid content in whole brain following enzyme inhibitor administration, we were addressing two main questions of repeated inhibition: 1) does FAAH inhibition or MAGL inhibition maintain selectivity for their respective endocannabinoid target when continuously inactivated over a period of several days, and 2) can repeated inhibition enhance ligand availability above that of just a single exposure, or are the levels seen following initial inhibition representative of a stable plateau for endocannabinoids levels? While PF3845 represents a highly selective inhibitor, even compared to URB597, JZL184 always been reported to possess an ability to inhibit FAAH acutely (Long et al., 2009a; Long et al., 2009b). However, the ability to inhibit FAAH is far less potent than its MAGL activity, and levels of enzyme

inhibition never reached an extent capable of producing AEA elevations in brain when given acutely. Meanwhile, though approximately 10-fold increases in AEA and 2-AG via treatment of their respective enzyme inhibitors seems substantial, no studies to date have determined if these levels continue to stay enhanced, much less elevate further with repeated exposure. Further elevations would be the result of continued enhancement of ligand availability, likely a consequence of additional synthesis under conditions of diminished degradative mechanisms. Conversely, newly established stable levels would be the result of cellular adaptations that allow for negative feedback mechanisms that stabilize extraphysiologic elevations in endogenous cannabinoids. These feedback mechanisms could include reduced synthetic mechanisms of endocannabinoids, induction of increased synthesis of the degradative enzymes, or even degradation by alternative lower-affinity hydrolases as levels reach higher concentrations.

What we observed when examining repeated JZL184 treated mice was a cumulative effect of inhibition. Transient enhancements of AEA levels were observed a few hours following injection only in those receiving repeated JZL184, indicating a slight impact of partial FAAH inhibition over a period of several days. While this increase is comparatively minimal and brief compared to elevations seen by FAAH inhibitors and FAAH knockout mice, it does raise questions as to any contribution AEA is playing in any observed effects in mice treated with repeated JZL184. Given its relatively minor elevation and absence by 26 h post-injection, it likely plays minimal role in the long-term adaptations observed. Since MAGL (-/-) show no elevations in AEA, this confirms that these observations are likely nonselective actions of JZL184, and studies of repeated JZL184 in MAGL (-/-) mice will confirm the contribution of simultaneous AEA and 2-AG enhancement on drug action. Importantly, most behavioral and

receptor studies performed in MAGL (-/-) so far follow the pattern of findings seen in mice treated with repeated JZL184.

FAAH (-/-) mice show typical stable 10-fold increases in AEA, and repeated FAAH inhibition by PF3845 produces similar AEA elevations when comparing initial exposure to prolonged inhibition. Interestingly, these chronic elevations do not appear to impact the available pool of precursors for 2-AG, as 2-AG levels in FAAH (-/-) mice and repeated PF3845 treated animals are unaltered compared to respective controls.

It appears that with prolonged inhibition of MAGL, a continual enhancement and further accumulation occurs for 2-AG above that of a single exposure. With the high abundance and rapid kinetics of 2-AG formation, it is not surprising that increased time with MAGL inhibited produces even greater elevations. After six days, 2-AG levels reach up to 70% higher than that of a single exposure to JZL184, with significant elevations remaining above those of acute exposure for over 24 h. This may suggest we may not have obtained the peak physiological 2-AG concentrations possible using pharmacological inhibition. However, this theory is not confirmed by the results from the MAGL transgenic mice. The 2-AG levels following repeated JZL184 inhibition closely match those of the MAGL knockouts, suggesting that the approximately 15-fold increases are about the maximal attainable, which would fit with the fact that at least two alternate enzymes that are still actively degrading 2-AG in brain tissue in these mice. More interesting is the fact that heterozygous mice show now major differences compared to wild-type controls. The initial findings using JZL184 showed that doses that could inhibit ~50% of MAGL brain hydrolysis were capable of modest increases in 2-AG on the order of 3-fold. However, the same inhibition of hydrolysis fails to produce similar magnitudes of 2-AG

content in heterozygous MAGL mice, potentially suggesting some forms of compensatory developmental compensation of reduced 2-AG hydrolysis.

With the enhancement of 2-AG availability in brain tissue with repeated JZL184 administration, this would provide evidence for enhanced drug activity after six days of injections. However, the profound tolerance seen points to evidence of receptor level adaptations in these neuronal circuits. Upon examining whole-brain CB_1 receptors, we see that MAGL inhibition produces profound losses in the maximal stimulation of CB_1 receptor pools by an exogenous agonist. The stable measure of potency across all tests suggests that the membrane preparations properly removed any 2-AG from the samples, and its presence did not alter the measurement of CB_1 function or binding. Loss of maximal activation is typically indicative of desensitization or 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 promotes substantial receptor internalization and degradation. Microscopy studies of GFP-tagged CB_1 receptors in drug-treated cells could confirm this hypothesis, and is of current focus within the Cravatt lab.

Regional analysis of the receptor activation following repeated JZL184 shows that regions typically associated with high levels of cannabinoid receptor plasticity to exogenous agonist, such as hippocampus and cortical regions, show extensive loss of stimulated function. In addition, a region with established function in cannabinoid-mediated analgesia, the periaqueductal gray, showed the largest relative decrease in receptor activation. Meanwhile areas associated with catalepsy, such as globus pallidus and caudate putamen, showed no apparent changes in receptor activity. It should be noted that these regions also show

comparative resistance to functional losses following treatment with exogenous cannabinoid agonist, though significant functional loss is typically noted (Sim-Selley and Martin, 2002; Sim-Selley et al., 2006). When examining the 2-AG content available in these respective regions, we see the regional variations in intrinsic activity and susceptibility to internalization of the cannabinoid receptors is independent of 2-AG availability. Similar elevations are seen across all brain regions examined, and nearly identical 2-AG levels are quantified in regions that show functional losses compared to those which do not show any loss in activity. This suggests that while increased 2-AG likely the catalyst for inducing the loss of receptor activity, it is other factors such as receptor density, cellular regulatory mechanisms, and turnover rate of receptor pools themselves that dictate the level of plasticity in each region. These changes in receptor pools appear to, in turn, alter the resultant behavioral outcomes.

Chapter 4: General Discussion and Conclusions

The purpose of the preceding studies was to further elucidate the potential of elevating endogenous cannabinoids, via inhibition of the degradative enzymes FAAH and MAGL, to elicit cannabimimetic activity. We hypothesized that elevating endogenous cannabinoids would demonstrate potential therapeutic benefits, such as reducing cannabinoid withdrawal or as an analgesic, through actions at cannabinoid receptors. Both endpoints showed responses following inhibition of either FAAH or MAGL, often with similar efficacy. In addition, we aimed to evaluate the comparative consequences of acute and prolonged inhibition of either major degradative enzyme on potential negative effects associated with exogenous cannabinoids and marijuana. These consequences included impairment of motor coordination, tolerance to drug actions, and potential for physical dependence as evidenced by precipitated somatic withdrawal. Our hypothesis that FAAH inhibitors would show minimal impact on cannabinoid function, similar to previously reported in FAAH (-/-) mice, held for all the endpoints we tested. No withdrawal, tolerance, or receptor adaptations were observed following FAAH inhibitor treatment.

We also confirmed our hypothesis about the potential for endocannabinoid system adaptations observed by MAGL inhibition. However, it is important to place these adaptations in perspective, as we found no impairment to motor coordination or alterations in catalepsy response. We also found that withdrawal intensity was equivalent to that of a THC regimen that

is minimally required to see reliable withdrawal behaviors. Receptor adaptations and tolerance were actually greater than expected following prolonged MAGL inhibition, but was more regionally and behavior specific than that seen with exogenous cannabinoid agonists.

We further investigated the cannabimimetic activity of enzymatic inhibition of FAAH and MAGL by examining the selectivity of ligand elevations, as well as enhanced accumulation of endogenous cannabinoid content in brain by prolonged inhibition. With repeated inhibitor administration, there is equal possibility of either enhanced ligand availability or a new stable elevated plateau being established. Equally important was evaluation of adaptations in the function and number of cannabinoid receptors following prolonged elevations in endogenous cannabinoids. In addition to tolerance to any beneficial effects of elevated endocannabinoids, desensitization and downregulation of receptors can also impact normal signaling functions under physiologic levels of endocannabinoids.

Most importantly, these studies allowed the first direct comparisons of elevated AEA and 2-AG to elicit the same cannabimimetic endpoints, and compare their functional changes to the endogenous cannabinoid system following prolonged elevation. Collectively, our findings show that elevations in either AEA or 2-AG can exhibit therapeutic cannabimimetic activity. However, 2-AG plays a dominant role in the plasticity of cannabinoid receptor function, and also elicits undesirable adaptations within the endogenous cannabinoid system. Remarkably, AEA activates cannabinoid receptors following FAAH inhibition in a manner seemingly devoid of functional loss of cannabinoid activity or function.

In perfect congruence with previous studies of FAAH (-/-) mice, repeated treatment with PF3845 does not alter CB_1 receptor function or number compared to vehicle control. This adds to a growing literature with examples of how FAAH inhibition exerts cannabimimetic activity

with inexplicably little consequence to long-term function of the cannabinoid system. Unlike exogenous agonists, URB597 fails to elicit conditioned place preferences or aversions (Gobbi et al., 2005), and also fails to generalize in rats trained to discriminate the drug effects of THC (Gobbi et al., 2005; Solinas et al., 2007). In addition, URB597 does not increase dopamine release in the shell of the nucleus accumbens (Solinas et al., 2006), a common hallmark of almost all substances of abuse. Moreover, it has recently been shown that monkeys previously trained to administer other drugs of abuse, including THC, will not self-administer URB597. Finally, URB597 also lacks the ability to prime reinstatement, and fails to increase self-administration, in monkeys receiving either THC or cocaine (Justinova et al., 2008). The aforementioned study suggests that not only do FAAH inhibitors lack rewarding properties, but they also do not enhance the dependence liability of common drugs of abuse. It is important to follow up on these reports by testing the second-generation FAAH inhibitors such as PF3845, which not only elevate AEA to higher levels than that of URB597, but also for a far longer period of time. While our data and findings from FAAH (-/-) mice (Falenski et al., 2010) suggest these differences may not result in discrepancies from current findings, with improved duration and selectivity it is important to further underscore the minimal consequences of FAAH inhibition under any conditions.

The pattern of findings presented here, and in previous studies, suggests MAGL inhibition may show much of the potential for rewarding effects and hallmarks of drugs of abuse that FAAH inhibition does not. Already, we've established that MAGL inhibitors do produce some subjective generalization to mice trained to determine THC discriminative stimuli. We've also shown cannabinoid precipitated withdrawal, behavioral and functional tolerance, and cannabinoid receptor cross-tolerance following prolonged MAGL inhibition. While many of

these effects are comparatively low versus moderate to high doses of THC, MAGL inhibitors showed little advantage in efficacy above FAAH inhibitors in those measures tested in these studies included within, and therefore provides little evidence for MAGL as the more advantageous target.

Prolonged MAGL inhibition by JZL184 also induced nearly complete tolerance to all of its currently known acute measures of cannabinoid activity. Most importantly, this included loss of analgesic and anti-allodynic (see Fig. A2) efficacy. Simultaneous inhibition of FAAH and MAGL together does produce significant elevations in both AEA and 2-AG, producing enhanced acute cannabinoid behavioral effects. However, similar tolerance was seen following prolonged inhibition of both enzymes as was observed during prolonged MAGL inhibition alone. In addition to JZL184 tolerance, prolonged MAGL inhibition also produced cross-tolerance to exogenous cannabinoid agonists and reductions in cannabinoid membrane receptors. This inactivation of the cannabinoid system following repeated MAGL inhibitor administration presents a potential limitation in the therapeutic advantages of these compounds over exogenous cannabinoid agonists. In contrast, FAAH inhibitors maintained their analgesic and anti-inflammatory efficacy, with no apparent impact or long-term hindrance on cannabinoid receptor system function. Taken together, these results suggest that FAAH inhibition represents a promising target for enhancing cannabinoid signaling without the many negative side-effects associated with cannabinoid agonists and marijuana.

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List of References

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Appendix

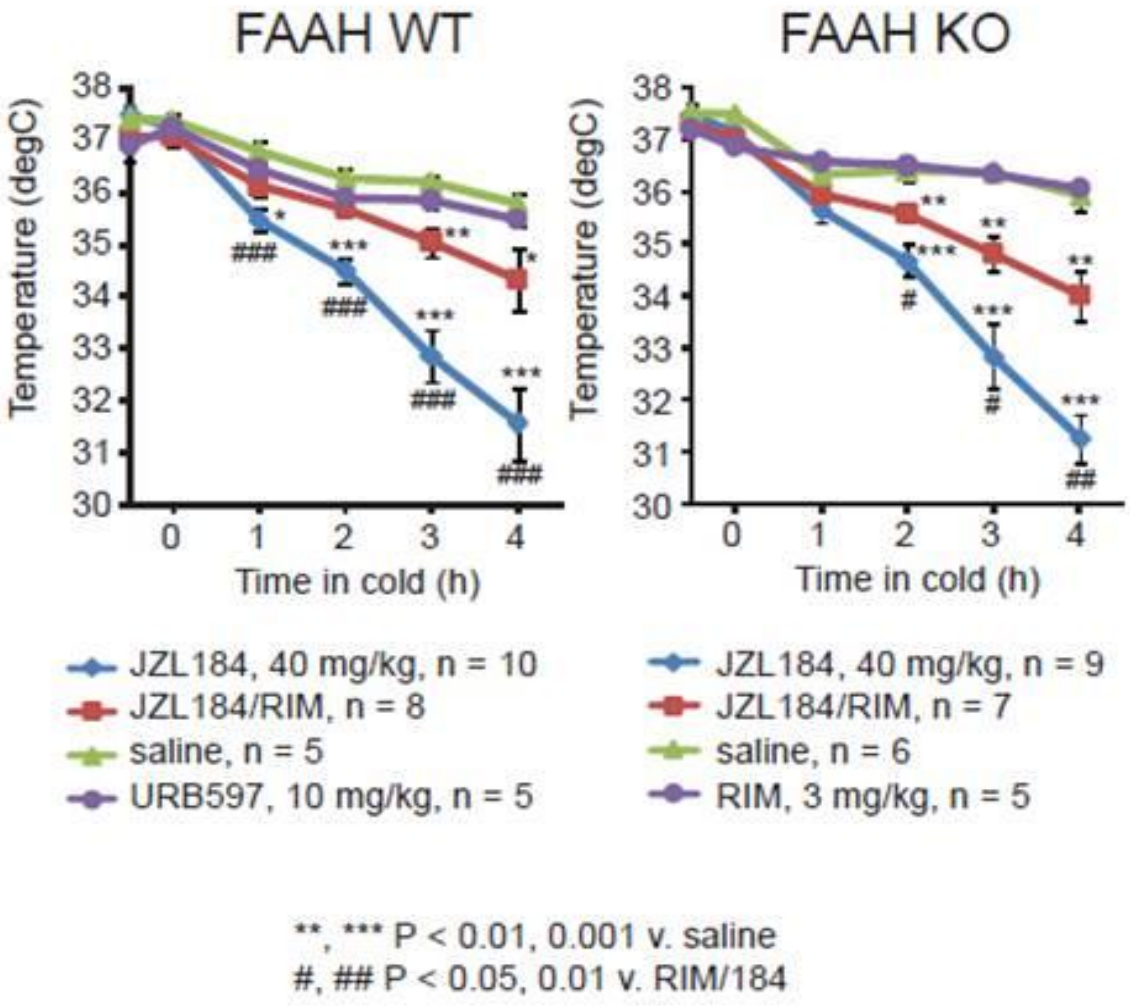

Figure A1. MAGL inhibitors produce hypothermia, as demonstrated under cold challenge conditions for 4 hours (4°C). Vehicle treated mice (triangles) show minimal decreases in body temperature in cold conditions, however JZL184 (40 mg/kg; diamonds) induced profound drops in body temperature. This effect is CB_1 receptor mediated, as evidenced by reversal with rimonabant (3 mg/kg) co-treatment (squares). This effect is not apparent in vehicle-treated FAAH (-/-) mice (right panel), nor the FAAH inhibitor URB597 (circles). Data collected by Jon Long as a part of the Cravatt group at Scripps Research Institute.

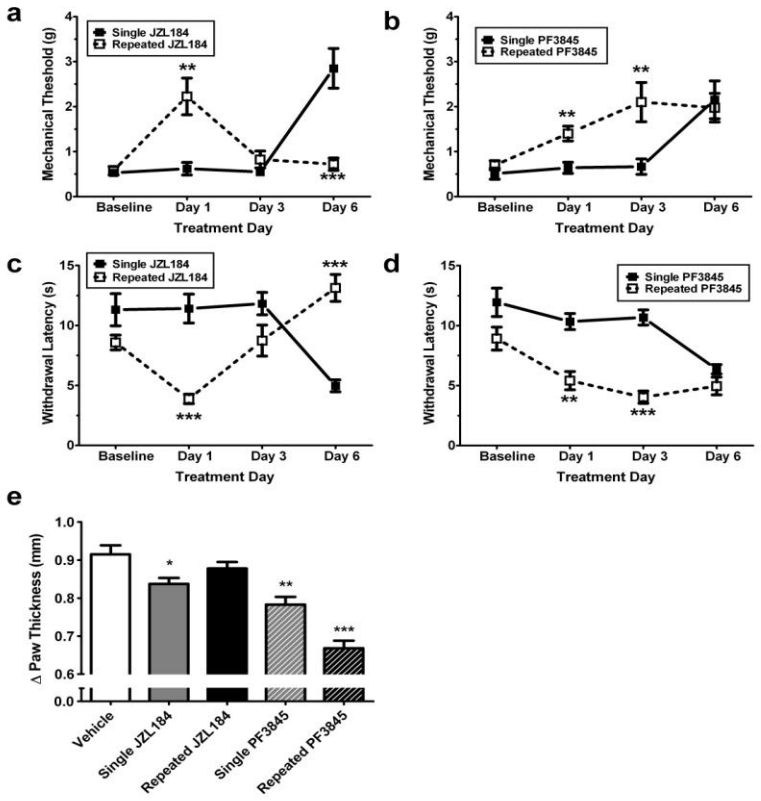

Figure A2. Tolerance to anti-allodynic and anti-edema effects of JZL184 versus PF3845 following repeated administration. Comparison of pain-related endpoints following 1-day drug treatment (JZL184 40 mg/kg or PF3845 10 mg/kg; closed squares) or 6-day drug treatment (open squares and dashed line) administration. (a-d) Tolerance to either JZL184's (a, c) or PF3845's (b, d) anti-allodynic effects over time on mice that received chronic constriction nerve injury, which manifests allodynic effects to von Frey mechanical stimulation (a-b) and cold allodynia (c-d) when challenged with acetone. Single treatment groups (closed squares) received vehicle treatment until day 6, while repeated JZL184 group (open squares and dashed line) received drug every day tested. (e) Measurement of increased paw thickness 6 h after local paw injection of carageenan. Mice treated with single injections of JZL184 and PF3845 both show decreases in edema, with repeated JZL184 showing tolerance, while PF3845 effects are enhanced with repeated treatement. n = 8 per group in all studies, with the same mice tested in (a, c), as well as (b, d). *$p < .05$, **$p < .01$, ***$p < .001$ denote significant differences between single and repeated drug (JZL184 or PF3845) groups, or difference from vehicle controls in panel (e). Data collected by Dr. Steve Kinsey and Sudeshna Ghosh in the Lichtman lab.

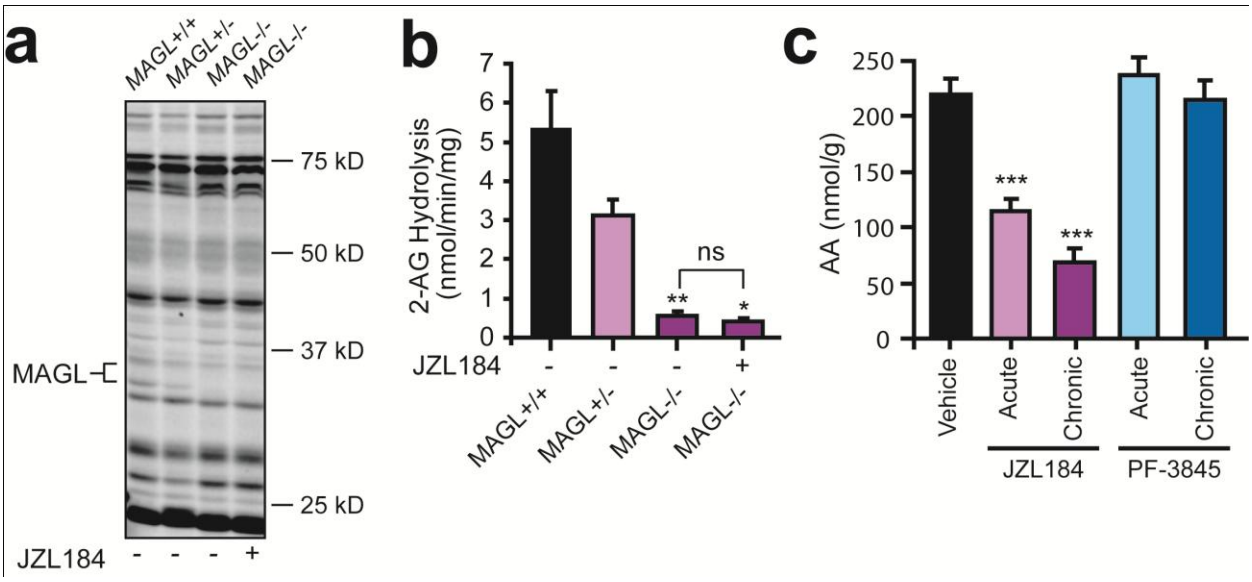

Figure A3. Further characterization of endocannabinoid metabolism in mice with chronic disruptions of MAGL or FAAH**.** (**a**) Activity-based protein profiling of MAGL$^{+/+}$, $^{+/-}$, and $^{-/-}$ soluble brain proteomes with or without JZL184 (5 µM) pre-treatment. (**b**) 2-AG hydrolytic activities of MAGL$^{+/+}$, $^{+/-}$, and $^{-/-}$ soluble brain homogenates with or without JZL184 pre-treatment (1 µM); n = 4 mice per group. (**c**) Brain levels of arachidonic acid (AA) in mice treated acutely or chronically with JZL184 (acute dosing regime: 40 mg/kg, i.p.; chronic dosing regime: six days, one dose per day, evaluated 2 hr after final dose) or PF-3845 (acute dosing regime: 10 mg/kg, i.p.; chronic dosing regime: six days, one dose per day, evaluated 2 hr after final dose); n = 5-6 mice per group. Data are presented as means ± s.e.m. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus vehicle-treated or wild-type littermate control mice (Dunnett's test). Data collected by Jacqueline Blankman as part of the Cravatt group at Scripps Research Institute.

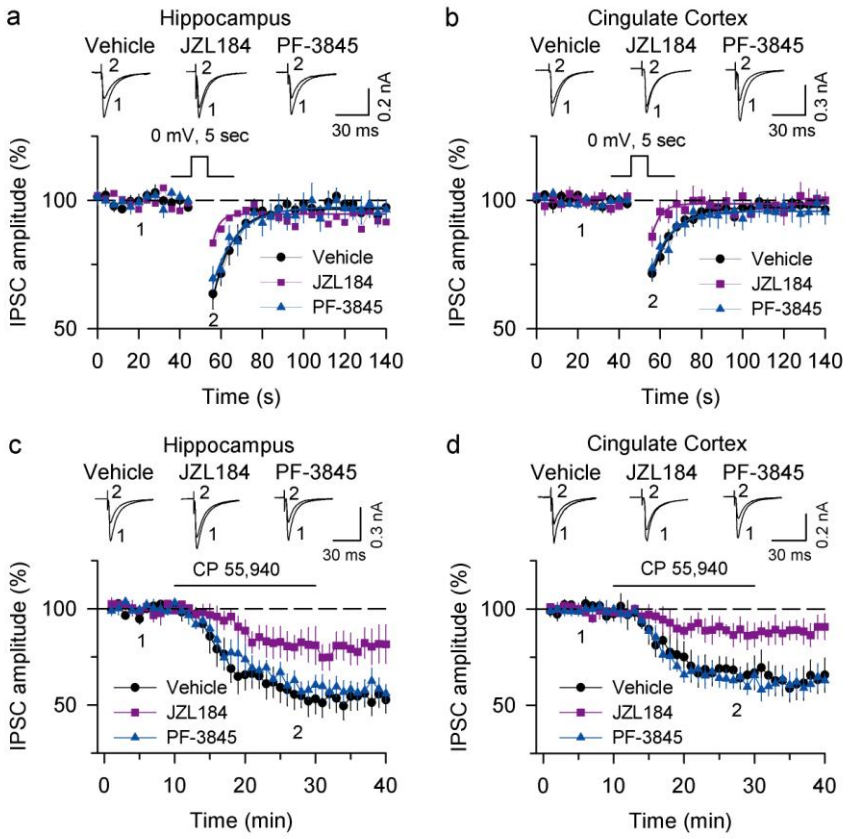

Figure A4. Effects of repeated *in vivo* administration of JZL184 and PF-3845 on DSI and CP55,940-induced depression of IPSCs in the hippocampus and cingulate cortex. (A, B) Repeated JZL184 treatment attenuated DSI in hippocampal CA1 pyramidal neurons (A) and layer V pyramidal neurons of the cingulate cortex (B), whereas repeated PF-3845 treatment did not have significant effect (n = 11-15). The lines superimposed are the single exponential fitting curves of the decay of DSI. (C, D) Bath application of CP55,940 (3 μM) induced significantly less depression of IPSCs in the hippocampus (C) and cingulate cortex (D) in JZL184-treated mice than that in vehicle-treated mice (n = 6 for each group), whereas CP55,940-induced depression of IPSCs in both brain regions in PF-3845-treated mice were not significantly different from that vehicle-treated mice (n = 6-7). Data collected by Bin Pan as a part of the Liu group at Medical College of Wisconson.

Vita

Joel Schlosburg was born on January 7th, 1983 in Washington, D.C. and currently resides in Damascus, MD. He is a U.S. citizen, and began his studies at VCU in the Fall of 2005, graduating in Spring 2010.

EDUCATION

Virginia Commonwealth University, Richmond, VA

Ph.D. in Pharmacology & Toxicology (in progress) **2005-2010**

P.I. Dr. Aron Lichtman

Research Concentrations: Cannabinoids and somatic signs (withdrawal, pruritus) / FAAH mechanisms in pain modulation

Rensselaer Polytechnic Institute, Troy, NY

B.S. in Psychology (cum laude) **2001-2005**

P.I. Dr. Larry Reid

Thesis: "Estradiol Injections Enhance Female Rats' Intake of Fat and Sugar and Induce Weight Gain"

Rensselaer Polytechnic Institute, Troy, NY

B.S. in Chemical Engineering (cum laude) **2001-2005**

Areas of Concentration: Process Design, Controller Design

AWARDS

Lauren Woods Award (1st Place - Best Graduating Researcher), Department of Pharmacology & Toxicology, VCU **2010**

Research Retreat Poster Competition (T-1st), Department of Pharmacology & Toxicology, VCU **2009**

Ruth L. Kirschstein National Research Service Award for Individual Predoctoral Fellows (F31), NIDA, **"**Endocannabinoid Modulation of Pruritus" **2009-Present**

Anthony Ambrose Award (Best 3rd-Year Student), Department of Pharmacology & Toxicology, VCU **2008**

NIDA Institutional Training Grant Trainee, Department of Pharmacology & Toxicology, VCU **2008-2009**

International Cannabinoid Research Society Student Travel Award **2007, 2008, 2009**

Graduate Fellowship, Department of Pharmacology & Toxicology, VCU **2005-2010**

Lewis S. Coonley Prize (Excellence in Process Design), Department of Chemical Engineering, RPI **2005**

MEMBERSHIPS AND TITLES

Virginia Academy of Sciences	**2009-2010**
The American Association for the Advancement of Science	**2008-Present**
American Society for Pharmacology and Experimental Therapeutics	**2008-Present**
International Cannabinoid Research Society	**2007-Present**
Omega Chi Epsilon (Chem. Eng. National Honor Society), RPI Charter President	**2004-2005**
American Institute of Chemical Engineers	**2002-Present**

TEACHING EXPERIENCE / SERVICE

2009

Virginia Commonwealth University, Richmond, VA

Student Evaluator & Judge, VCU Brain Day / Virginia Academy of Sciences

As a part of the outreach to school-age children, both VCU's Department of Psychology & the Virginia Junior Academy of Sciences hosted events where young students investigate an issue concerning a particular area of interest, or results of a small experiment of their design. I was a volunteer judge to evaluate posters or provide feedback and questions for presenters in the areas of pharmacology or behavioral sciences.

Pharmacology & Toxicology Research Retreat Entertainment Director **2008-2009**

As a part of the annual departmental research retreat, the students present entertainment to promote cooperation and enhance camaraderie amongst the faculty and new students. I was charged with planning the games played, collecting information and implementing the presentation of the events, and hosting the evening's activities.

Student Coordinator, Questers Program **2007**

Questers is a high school weekend educational program for students interested in various fields of advanced science. Along with an appointed faculty representative, it was my duty to organize the pharmacology program and experiments performed. I also organized the other student volunteers, and ran the lecture / experiment on "Pain and Analgesia".

Rensselaer Polytechnic University, Troy, NY **2005**

Teaching Assistant/Project Developer – for Dr. B Wayne Bequette in "Chemical Process Dynamics and Control"

Met with students for supplemental instruction, proctored tests, and managed various open-session classes for during final projects. Also worked to develop a potential new final project for course based on a real-life chemical process (food extruder).

Junior Museum, Troy, NY **2003-2004**

Educational Aid

I worked at this scientific museum for young children as an aid to the educational planner, doing any tasks required to be completed. Educational duties included activity planning, exhibit research, and interactive teaching activities and demonstrations with visitors.

2002-2005

Walter Reed Army Medical Center, Washington, DC

Educational and Training Technician

Performed ordinary duties as a member of the School-Age Services staff, while also responsible for planning summer educational programs for both the youth & teen divisions and all major scientific experiments/demonstrations.

PUBLICATIONS

- Schlosburg JE, Blankman JL, Pan B, Nguyen PT, Ramesh D, Kinsey SG, Booker L, Burston JJ, Abdullah RA, Long JZ, Nomura DK, Ghosh S, Wise LE, Selley DE, Sim-Selley LJ, Liu QS, Cravatt BF, & Lichtman AH. (2010) Sustained inactivation of monoacylglycerol lipase produces functional antagonism of the brain endocannabinoid system. Submitted to *Nature Neuroscience* March 2010.
- Falenski KW, Thorpe AJ, Schlosburg JE, Cravatt BF, Abdullah RA, Smith TH, Selley DE, Lichtman AH, Sim-Selley LJ. (2010) FAAH -/- Mice Display Differential Tolerance, Dependence and Cannabinoid Receptor Adaptation Following Δ^9-Tetrahydrocannabinol and Anandamide Administration. *Neuropsychopharm* (Epub March 31, 2010).
- Schlosburg JE, Carlson BLA, Ramesh D, Abdullah RA, Long JZ, Cravatt BF, Lichtman AH. (2009) Inhibitors of endocannabinoid metabolizing enzymes reduce precipitated withdrawal responses in THC dependent mice. *AAPS J* **11**(2):342-52
- Schlosburg JE, Kinsey SG, Lichtman AH. (2009) Targeting fatty acid amide hydrolase (FAAH) to treat pain and inflammation. *AAPS J* **11**(1):39-44
- Schlosburg JE, Boger DL, Cravatt BF, Lichtman AH. (2009) Endocannabinoid modulation of scratching response in an acute allergenic model: A new prospective neural therapeutic target for pruritus. *J Pharmacol Exp Ther* **329**(1):314-23
- Long JZ, Li W, Booker L, Burston JJ, Kinsey SG, Schlosburg JE, Pavon FJ, Serrano AM, Selley DE, Parsons LH, Lichtman AH, Cravatt BF. (2009) Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. *Nat Chem Biol* **5**:37-44.

MANUSCRIPTS IN PREPARATION

- Schlosburg JE, Abdullah RA, Conrad DH, Lichtman AH. (2010) The cannabinoid receptor antagonist/inverse agonist rimonabant produces scratching via action on central CB_1 receptor activity.

- Schlosburg JE, Ramesh D, Kinsey SG, Booker L, Abdullah RA, Blankman JL, Long JZ, Selley DE, Sim-Selley LJ, Cravatt BF, & Lichtman AH. (2010) Enhanced cannabimimetic behaviors resulting from simultaneous inhibition of both FAAH & MAGL undergo tolerance following prolonged inhibition.

- Rogosch T, Sinning C, Podlewski A, Watzer B, Schlosburg JE, Lichtman AH, Cascio MG, Bisogno T, Di Marzo V, Nüsing R, Imming P. (2010) A novel possible mechanism of action of dipyrone (metamizol).

ABSTRACTS

- Schlosburg JE, Burston JJ, Ramesh D, Kinsey SG, Booker L, Abdullah RA, Long JZ, Selley DE, Cravatt BF & Lichtman AH. *CB1 Agonist-like CNS Effects Following Prolonged Inhibition of Monoacylglycerol Lipase (MAGL) Undergo Tolerance*. 2009 Gordon Research Conference: Cannabinoid Function in the CNS, Biddeford, ME.
- Schlosburg JE, Burston JJ, Kinsey SG, Booker L, Abdullah RA, Long JZ, Selley DE, Cravatt BF & Lichtman AH. *Behavioral and Functional Adaptation of the Endocannabinoid System Following Repeated Monoacylglycerol Lipase (MAGL) Inhibition*. International Cannabinoid Research Society 2009: 19th Annual Symposium of the Cannabinoids, St. Charles, IL.
- Schlosburg JE & Lichtman AH. *Endocannabinoid Modulation of Scratching Response in an Acute Allergenic Model*. Carolina Cannabinoid Collaborative 2008, Williamsburg, VA.
- Schlosburg JE & Lichtman AH. *Endocannabinoid Modulation of Pruritus: Further Investigations Into Itch*. International Cannabinoid Research Society 2008: 18th Annual Symposium of the Cannabinoids, Aviemore, Scotland.
- Schlosburg JE, Carlson BLA, & Lichtman AH. *The FAAH inhibitor URB597 ameliorates cannabinoid withdrawal in mice*. Experimental Biology 2008, San Diego, CA.
  - Also presented at VCU Watts Research Day
- Schlosburg JE. *Attenuation of Somatic Precipitated THC Withdrawal Symptoms by FAAH Inhibition*.

  Carolina Cannabinoid Collaborative 2007, Wrightsville Beach, NC.
- Schlosburg JE & Lichtman AH. *Cannabinoid Modulation of Pruritus: Potential Agents Against Itch*. International Cannabinoid Research Society 2007: 17th Annual Symposium of the Cannabinoids, St. Sauveur, PQ.
  - Also presented at VCU Watts Research Day
- Thorpe AJ, Schlosburg JE, Cravatt BF, Martin BR, Sim-Selley LJ, & Lichtman AH. *FAAH (-/-) Mice Exhibit Normal CB1 Receptor Function Following Acute or Repeated Administration of Cannabinoids*, International Cannabinoid Research Society 2006: 16th Annual Symposium of the Cannabinoids, Budapest.
- Reid, LD, Boswell, KJ, Klein, LA, Caffalette, CA, Schlosburg, JE, Stitt, KT, Reid, ML *Further Studies of Estradiol and Intake of Palatable Ingesta*. Abstracts of the International Behavioral Neuroscience Society, Volume 14, June 2005, Santa Fe.

INVITED SEMINARS

- "Elevating Endogenous Cannabinoids: Insights into the Endocannabinoid System, and Novel Therapeutic Applications". Scripps Research Institute, Committee on the Neurobiology of Addictive Disorders, October 23rd, 2009.
- "Inhibition of Endocannabinoid Degradation: Camparing Two Enzymatic Targets for THC-like Therapeutics". NIDA, Intramural Research Program, October 8th, 2009.
- "Tale of Two Enzymes: The Potential and Pitfalls of Endocannabinoids as Therapeutics". Medical University of South Carolina, Department of Neurosciences, September 17th, 2009.