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Targeting the Endocannabinoid System to Reduce Nociception

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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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
AA	arachidonic acid
ACEA	arachidonyl-2-chlorethylamide
AEA	anandamide
ANOVA	analysis of variance
B _{max}	maximal specific binding sites
cAMP	cyclic adenosine monophosphate
CB ₁	cannabinoid receptor, subtype 1
CB ₂	cannabinoid receptor, subtype 2
CBC	cannabichromene
CBD	cannabidiol
CBN	cannabinol
DAGL	diacylglycerol lipase
ED ₅₀	half maximal (50%) effective dose
eCB	endocannabinoid
FAA	fatty acid amides
FAAH	fatty acid amide hydrolase

G-protein	guanine nucleotide binding protein
GABA	γ -aminobutyric acid
GPCR	G-protein coupled receptor
i.p.	intraperitoneal
IRTX	5'-Iodoresiniferatoxin
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
K _i	dissociation constant for an inhibitor
LC/MS/MS	liquid chromatography tandem mass spectrometry
LPS	lipopolysaccharide
MAGL	monoacylglycerol lipase
MK886	[(1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]- α,α -dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid sodium salt]
NAL	naltrexone
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D
O-3223	Selective CB ₂ agonist
O-4395	THCV; Δ^9 -tetrahydrocannabivarin
O-4950	CBC; Cannabichromene

PAG	periaqueductal gray
PF-3845	N-(pyridin-3-yl)-4-(3-(5-(trifluoromethyl)pyridin-2-yloxy)benzyl) piperdine-1-carboxamide
PWT	paw withdrawal threshold
Rim	Rimonabant
s.c.	Subcutaneous
SR ₁	SR141716A (rimonabant)
SR ₂	SR144528
THC	Δ^9 -tetrahydrocannabinol
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

TARGETING THE ENDOCANNABINOID SYSTEM TO REDUCE NOCICEPTION

By Lamont Booker, 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, 2011

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

Pain of various etiologies (e.g., visceral, inflammatory) can be a debilitating disorder that presents a problem of clinical relevance. While it is known that Δ^9 -tetrahydrocannabinol (THC) the primary psychoactive constituent found in marijuana produces analgesia in various rodent models of pain, its pharmacological properties are overshadowed by its psychomimetic effects. THC is the primary phytocannabinoid found in marijuana though other prevalent constituents such as the phytocannabinoids (e.g., cannabidiol (CBD), cannabinol (CBN), cannabichromene (CBC), tetrahydrocannabivarin (THCV)) may possess antinociceptive actions without the psychomimetic effects associated with THC. Indeed, these phytocannabinoids act upon the endocannabinoid system (ECS) that is comprised of the CB1 and CB2 cannabinoid receptors, endogenous ligands (anandamide (AEA), 2-arachidonoylglycerol (2-AG)), and endocannabinoid biosynthetic and catabolic enzymes. We hypothesize that phytocannabinoids as well as endocannabinoid catabolic enzyme inhibitors reduce nociception preclinical models of pain. In the first series of studies, the antinociceptive effects of prevalent phytocannabinoids

were evaluated in the acetic acid stretching test, a rodent visceral pain model. While CBN and THC both produced antinociceptive effects via a CB1 mechanism of action, CBC, and CBD had no effect on nociception. Conversely, THCV antagonized the antinociceptive effects of THC. These results suggest that various constituents of marijuana may interact in a complex manner to modulate pain.

Since the THC and CBN displayed their effects via specific endogenous cannabinoid receptors, we investigated whether increasing endocannabinoids block nociceptive behavior. Blockade of the catabolic enzyme fatty acid amide hydrolase (FAAH) elevates AEA levels and elicits antinociceptive effects, without psychomimetic issues associated with THC. Similarly, blockade of another endocannabinoid catabolic enzyme monoacylglycerol lipase (MAGL) elevates (2-AG) and elicits antinociceptive effects. Therefore, we tested the hypothesis that FAAH and/or MAGL inhibition blocks nociception in the acetic acid abdominal stretching model, and the LPS-induced allodynia (i.e. painful response to a non-noxious stimuli) model of inflammation.

Genetic deletion or pharmacological blockade of FAAH or pharmacological blockade of MAGL significantly reduced the total number of abdominal stretches in the visceral pain model. Additionally, blockade of both enzymes simultaneously produced an enhanced antinociceptive effect versus blocking the enzymes individually. These effects were mediated through CB1 receptors. However, in the LPS-induced allodynia model, FAAH inhibited anti-allodynic effects through a CB1 and CB2 receptor mechanism. In both assays other potential targets of FAA

substrates (i.e., mu-opioid, TRPV1, and PPAR-alpha receptors) did not play an apparent role in FAAH inhibited antinociceptive responses. Taken together, these results illustrate that targeting the endocannabinoid system via direct acting agonists such as the phytocannabinoids, or indirect methods (i.e. inhibiting degradative enzymes of the endogenous cannabinoids), represents a promising strategy to treat pain.

Chapter 1. Introduction

Pain (e.g. visceral, inflammatory) can be a debilitating disorder that greatly affects the quality of life. It accounts for one of the top reasons for emergency department visits according to the National Health Statistics Report (CDC, 2008). In addition, analgesic compounds tend to be the leading type of therapeutic agents mentioned during these visits. While these analgesic compounds range in degree of effectiveness, they present greater issues when taken chronically. For example, repeated opioid use can lead to tolerance, opiate-induced hyperalgesia, constipation, and the potential of addiction (Mitra, 2008; Ossipov et al., 2003). Another example is the chronic use of non-steroidal anti-inflammatory drugs (NSAIDs). Chronic use of these drugs can cause gastric ulcers (Chan and Leung, 2002), and ultimately potentiate visceral pain. As a part of this dissertation, we investigate alternative targets for alleviating visceral and inflammatory pain. More specifically, we explore the effects of plant-derived cannabinoids as well as the endogenous cannabinoid system (ECS) and their impact on modulating nociceptive behavior. To date, marinol, which is synthetic THC, and cesamet a synthetic cannabinoid are the only FDA-approved class of cannabinoids and can be used to treat nausea and emesis elicited by cancer chemotherapy and as an appetite stimulant in patients suffering from AIDS-related cachaxia.

Cannabis sativa

Marijuana has been used and cultivated for a variety of purposes as archaeological evidence dates back to the 2350 B.C (cannabinoids in nature and medicine 2009). Such examples are the use of cannabis for its cooking oil from cultivated seeds, fiber for making rope and paper, and medicinally (headaches, parasites, antibiotic, analgesic, hypnotic) (Russo, 2007). In 1937, the US Congress implemented and adopted the Marijuana Tax Act. The tax act posed a tax on buyers, sellers, importers, growers, physicians, veterinarians, and any other persons who deal in marijuana commercially, prescribe it professionally, or possess it. Ultimately the tax act eliminated further medicinal use and nearly halted all research in the field for several years.

Although marijuana was illustrated to have medicinal value, its psychoactive properties sparked an exponential increase in its recreational use. According to a recent 2009 survey, marijuana is the most commonly used and abused illicit drug in the United States of America with an estimated 16.7 million people reporting past-month use (NIDA- SAMHSA, 2010). Today marijuana is defined as a schedule I controlled substance considered not to be legitimate for medicinal use. *Cannabis sativa* is comprised of 489 identified constituents of various terpenoids, cannabinoids, flavonoids, amino acids, carbohydrates, fatty acids, and hydrocarbons (Elsohly and Slade, 2005). Delta-9-Tetrahydrocannabinol (THC) is the most prevalent and well characterized constituent of the approximately 70 cannabinoids identified in cannabis (Elsohly and Slade, 2005), and largely accounts for the psychoactive properties of this plant. Other

prevalent phytocannabinoids that are structurally similar to THC include cannabitol (CBN), cannabidiol (CBD), cannabichromene (CBC), and tetrahydrocannabivarin (THCV) (see Table 1). Each of these compounds has been found to possess pharmacological properties of their own. The extent to which these phytocannabinoids and other constituents found in marijuana interact with each other, may contribute to marijuana's overall pharmacological effects.

In 1899 the first attempt was made to identify a cannabis constituent which was discovered to be that of CBN from the resin of Indian hemp (Wood et al., 1899). Almost half a century later other cannabinoid constituents were isolated and identified. Cannabidiol (CBD) was the second phytocannabinoid isolated (Adams et al., 1940) however its structure identification would come years later (Mechoulam & Shvo, 1963). CBD was tested in volunteer studies and reported as not being the active ingredient resulting in the narcotic activity reported with the use of marijuana. In 1964, the main psychoactive ingredient found in marijuana, tetrahydrocannabinol (THC) was isolated from hashish by column chromatography and its structure was determined by correlations with known terpenoids (Gaoni & Mechoulam, 1964). The development of the mass spectrometry, infrared spectroscopy, and nuclear magnetic resonance allowed for ease in the future identification of other prevalent phytocannabinoids found in marijuana. Cannabichromene (CBC) was identified as a new active principle in hashish in 1966 (Gaoni & Mechoulam, 1966) followed by cannabivarin (CBV) and tetrahydrocannabivarin (THCV) in 1971 (Merkus, 1971). Soon after the elucidation of the structure of THC there was a growing interest in the production of synthetic THC analogs. To

date, there are numerous synthetic cannabinoids that have been synthesized that share pharmacological properties similar to THC, although more potent. An example of a synthetic cannabinoid is CP-55,940 synthesized by Pfizer (Koe et al., 1985). CP-55,940 along with several other synthetic compounds such as HU-210 (Howlett et al., 1990), and WIN55-212 (D'Ambra et al., 1992) were synthesized soon after which helped advance the study about cannabinoids and their pain-relieving effects. These synthetic cannabinoids as well as THC show different binding affinities for the CB1 and CB2 receptors which results in differences in potency. Additionally, unlike THC which is a partial efficacy agonist in vitro, synthetic cannabinoids produced full agonist like properties (Matsuda et al., 1990). The range of published affinities (nM) in reverse order of potency of these compounds for the CB1 receptor are THC (35.3-80.3), WIN 55,212-2 (1.89-123), CP-55,940 (0.5-5.16), HU-210 (0.061-0.82), and CB2 receptor are THC (3.9-75.3), CP-55,940 (0.69-19.8), WIN 55,212-2 (0.28-16.2), HU-210 (0.17-0.52) (Howlett et al., 2002; Ibrahim et al., 2003) (see Table 2). Due to the fact that these new synthetic compounds were very potent, and assuming they bound to a specific receptor, they were used as labeled ligands. In 1988 tritium-labeled CP-55,940 was used to identify specific binding sites in the brain for cannabinoids, which provided evidence and prompted a search for an endogenous receptor system (Devane et al., 1988). The selective binding of this compound helped in identifying the cannabinoid receptors and the evolution/expansion of knowledge of the endocannabinoid system.

Table 1. Discovery and isolation of prevalent cannabinoid constituents found in marijuana.

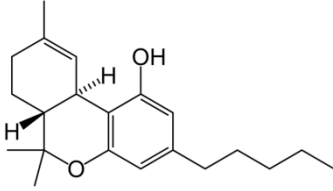
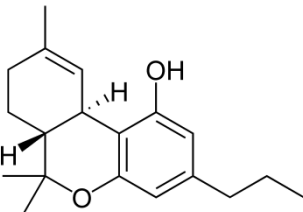
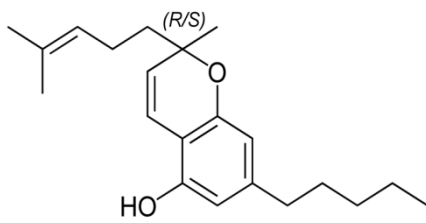
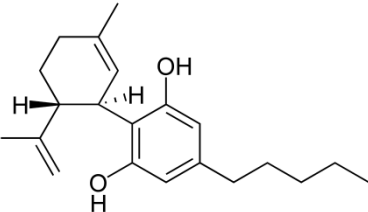
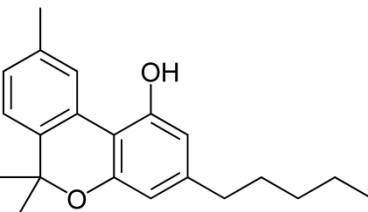
<i>Phytocannabinoids</i>	<i>Structure</i>	<i>Year Isolated</i>
Tetrahydrocannabinol (THC)		1964 (Gaoni & Mechoulam, 1964)
Tetrahydrocannabivarin (THCV)		1971 (Merkus, 1971)
Cannabichromene (CBC)		1966 (Gaoni & Mechoulam, 1966)
Cannabidiol (CBD)		1940 (Adams et al., 1940)
Cannabinol (CBN)		1899 (Wood et al., 1899)

Table 2. Synthetic cannabinoids and their relative potencies at the CB1 receptor. The potency range is depicted as the concentration of unlabeled drug which displaced tritiated compounds from CB1 receptor see references for review (Howlett et al., 2002; Pertwee et al., 2010).

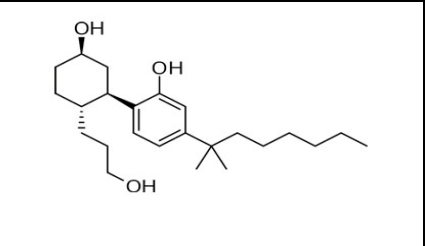
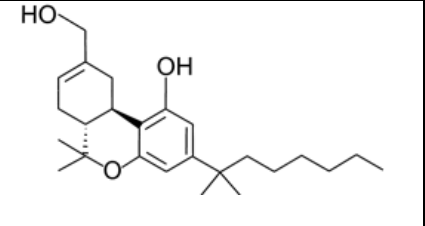
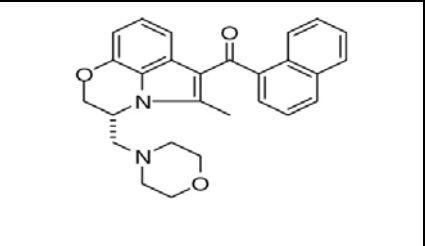
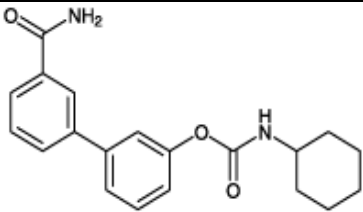
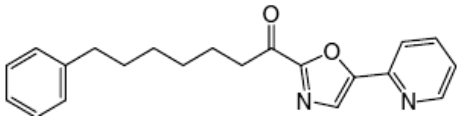
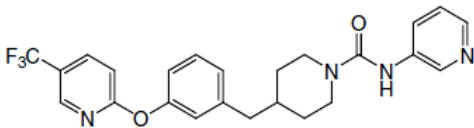
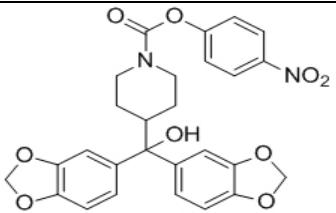
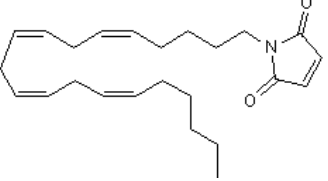
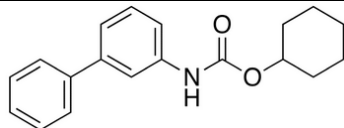
<i>Synthetic Cannabinoids</i>	<i>Structure</i>	<i>Potency at CB₁ receptor</i>
CP-55,940		0.5-5.0 nM
HU-210		0.06-0.73 nM
WIN55-212		1.89-123

Table 3. Endocannabinoid catabolic enzyme inhibitors.

<i>Inhibitor</i>	<i>Structure</i>	<i>Target</i>
URB597		Irreversible FAAH (Boger et al., 2005, Piomelli et al., 2006)
OL-135		Reversible FAAH (Boger et al., 2005)
PF-3845		Irreversible FAAH (Ahn et al., 2009)
JZL184		MAGL (Labar et al., 2010)
<i>N</i> -arachidonylmaleimide		MAGL (Labar et al., 2010)
URB602		MAGL and FAAH (Labar et al., 2010)

Endocannabinoid system

The endocannabinoid system consists of G-protein coupled receptors which are associated with Gi/o G-proteins (for review see (Howlett et al., 2002). Activation of these receptors decreases cAMP production via blockade of adenylyl cyclase (Howlett et al., 1990), activation of inwardly rectifying potassium (GIRK) channels via G $\beta\gamma$ subunits (Mackie et al., 1995; McAllister et al., 1999). Furthermore activation of the cannabinoid receptor inhibits N- and P/Q-type calcium channels, which reduces synaptic vesicle fusion to the nerve terminal thereby inhibiting the release of excitatory and inhibitory neurotransmitters. The consequence of this effect leads to a decrease in post-synaptic depolarization. To date, two primary cannabinoid receptors have been cloned. The first receptor is the cannabinoid receptor type 1 (CB1) and is located heterogeneously throughout the central nervous system (CNS) (Matsuda et al., 1990; Munro et al., 1993; Zimmer et al., 1999), and is believed to mediate marijuana's psychomimetic effects. In support of this notion, cannabinoids induce tetrad effects (decrease in locomotor activity, hypothermia, catalepsy, and analgesia) are reversed with a CB1 antagonist and in CB1 (-/-) mice (Compton et al., 1996; Rinaldi-Carmona et al., 1994). The cannabinoid receptor type 2 (CB2) was identified in a human promyelocytic leukaemia cell line soon after the CB1 receptor was discovered (Munro et al., 1993). CB2 receptors are expressed predominately in cells of the immune and hematopoietic systems (Cabral and Marciano-Cabral, 2005), though CB2 receptor messenger RNA and protein are expressed in microglia (Carlisle et al., 2002; Nunez et al., 2004) and brainstem neurons (Onaivi et al., 2006; Van Sickle et al., 2005). Activation of CB2

receptors also modulates cytokine secretion (Klein et al., 2003), reduces proliferation (Lombard et al., 2007), and suppresses monocyte chemotaxis through PI3K/Akt and ERK1/2 signaling (Montecucco et al., 2008). CB1 and CB2 receptors share approximately 44% homology with each other (Munro et al., 1993). CB1 receptors are located primarily in neuronal tissue and therefore may play a major role in analgesia. Conversely, CB2 receptors are located primarily on immune cells and may be more involved in reducing inflammatory mediated effects. The distribution of both cannabinoid receptors provides an anatomical basis for the analgesic effects of cannabinoids. Cannabinoid receptors have been localized on presynaptic terminals of both GABAergic (Katona et al., 1999) and glutamatergic neurons (Huang et al., 2001; Szabo and Schlicker, 2005). Transient suppression of the inhibitory transmission (i.e. GABA) is termed depolarization-induced suppression of inhibition (DSI). Conversely transient suppression of the stimulatory neurotransmitter (e.g. glutamate) is called depolarized-induced suppression of excitation (DSE). Both result in cannabinoid receptor hyperpolarization of a repetitively depolarized neuron, which suppresses subsequent vesicular fusion and release of glutamate or GABA. This is the case with activation of cannabinoid receptors found on glutamatergic neurons (Maejima et al., 2001).

Endogenous ligands which bind to and activate the cannabinoid receptors were discovered and termed endocannabinoids (eCBs) (Di Marzo and Fontana, 1995)). These endocannabinoids are derived from phospholipid precursors in the postsynaptic neuron. Unlike classical neurotransmitters or neuromodulators, endocannabinoids are not stored in vesicles, but

are released on demand and travel in a retrograde manner from post-synaptic terminals to the pre-synaptic terminals to act on cannabinoid receptors. The first endocannabinoid isolated from the brain was anandamide (AEA) (Devane et al., 1992), followed by 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). Additionally, three other endocannabinoids have been discovered which are derivatives of arachidonic acid. These putative endocannabinoids which have non selective activity are noladin ether (Hanus et al., 2001), virodhamine (Porter et al., 2002), and N-arachidonoyldopamine (NADA) (Huang et al., 2002). However, the most studied and well characterized ligands are AEA and 2-AG.

Different enzymes are responsible for the synthesis of AEA and 2-AG. AEA synthesis is regulated by multiple pathways, but the most widely accepted pathway is via the cleavage of N-arachidonoyl-phosphatidylethanolamine (NAPE). NAPE is then hydrolyzed by NAPE-phospholipase D forming AEA. However, a previous study (Leung et al., 2006) showed that NAPE-PLD knockout mice still possess wild-type levels of AEA. Alternatively at least two other hypotheses arose such as AEA synthesis via phosphodiesterase (PDE), or synthesis via cleavage of phospholipase C (PLC) and a phosphatase (Liu et al., 2006). On the other hand, 2-AG is synthesized by the cleavage of diacylglycerol (DAG) by DAG lipase-alpha (DAGL α) (Gao et al., 2010; Tanimura et al., 2010). In these previous studies they demonstrated that DAGL α knockout mice when compared to DAGL β knockout mice showed a significantly lesser production of 2-AG levels.

As with most signaling messengers, AEA and 2-AG are rapidly inactivated soon after they are released. AEA is taken back into the post-synaptic terminal and degraded by the enzyme fatty acid

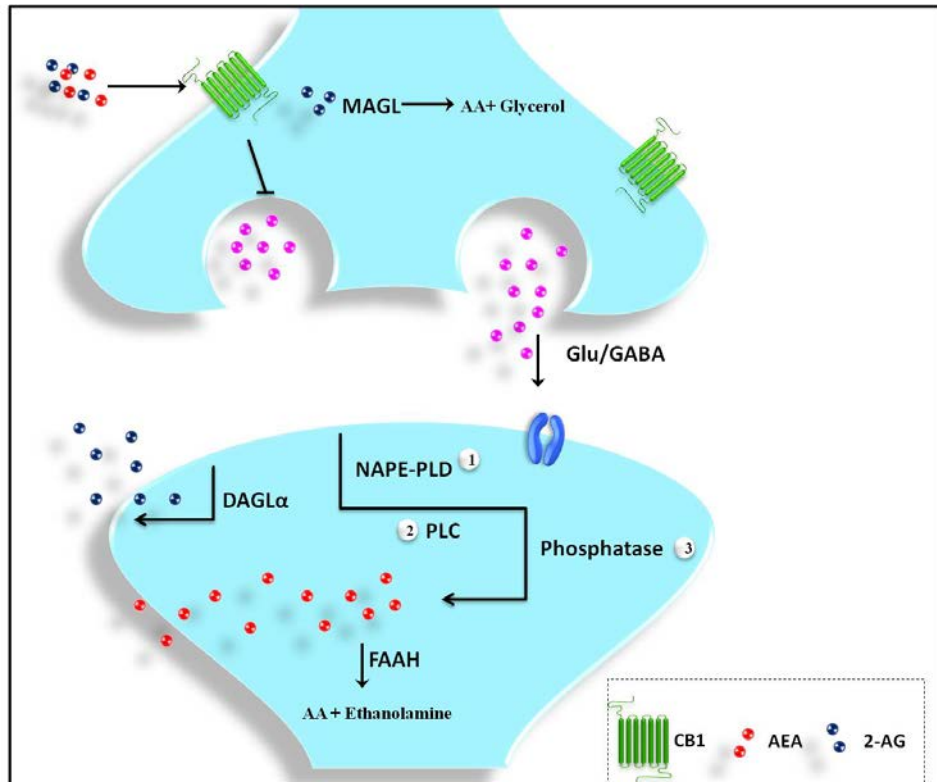
amide hydrolase (FAAH) (Cravatt et al., 1996)(Giang and Cravatt, 1997).

FAAH is located within the post-synaptic terminal and is responsible for the degradation of other amides such as oleamide,

the sleep agent, palmitoylethanolamide (PEA), the anti-inflammatory agent, and

oleoylethanolamide (OEA) the satiety lipid (Cravatt et al., 1995). Conversely, approximately 85% of 2-AG is degraded within the presynaptic terminal by the enzyme monoacylglycerol lipase (MAGL). The remaining 2-AG is degraded by enzymes alpha/beta hydrolase 6 and 12 (ABHD) (Blankman et al., 2007). Although 2-AG is present at levels 170-1000 (Stella et al., 1997; Sugiura et al., 2002) fold greater than AEA, both endocannabinoids produce some

Figure 1. Overview of the Endocannabinoid Signaling System



cannabinoid effects as evaluated by the classical “Tetrad”. This battery of four tests, is used to assess for cannabimimetic activity, and includes: spontaneous locomotor suppression, analgesia to noxious thermal stimuli, catalepsy, and hypothermia (Compton et al., 1993; Martin et al., 1991b). Externally administered THC or other synthetic cannabinoids do not mimic the physiological effects of locally released on demand endocannabinoids because their overall effect is to cause a persistent inhibition of neurotransmitter release, and not the localized and transient effects seen with endocannabinoids (Vaughan and Christie, 2005). Thus exploitation of the endocannabinoid system may have a more applicable implication in the clinical setting.

Several genetic and pharmacological tools have been developed to help better understand the role of the endocannabinoid system such as FAAH knockout mice, FAAH neuronal specific knock-in mice, selective pharmacological agents against FAAH, and pharmacological agents selective for inhibiting MAGL. FAAH (-/-) mice display 15 fold elevated AEA levels in the brain and show an antinociceptive phenotype ((Cravatt et al., 2001; Lichtman et al., 2004)). In addition to genetic blockade of FAAH, pharmacological effects have also been demonstrated. Irreversible (PF-3845, URB597) and reversible (OL-135) inhibitors of FAAH (see Table 3) have been demonstrated to elevate AEA levels in the brain ((Ahn et al., 2009; Boger et al., 2005; Fegley et al., 2005)) and produce analgesia in a variety of animal models of pain (see review (Schlosburg et al., 2009b)). Additionally, complementary approaches to investigate CB1 and CB2 receptor involvement includes genetically modified mice lacking either the CB1 or CB2 receptor. In addition, selective CB1 (rimonabant, AM251) and CB2 receptor antagonists

(SR144528, AM630) have also been generated. These complementary genetic and pharmacological approaches are used in this dissertation to determine cannabinoid receptor mechanism of action.

Rationale and Hypothesis

The overall goals for this dissertation were to investigate the effect of prevalent phytocannabinoids in commonly used mouse models of pain, and determine whether elevating endocannabinoids by blocking their hydrolysis is a viable approach to treat pain. We hypothesize that direct activation of the endocannabinoid system by phytocannabinoids or indirectly activating the endocannabinoid system by blocking hydrolyzing enzymes reduce pain.

Phytocannabinoids and abdominal stretching

Although THC has been well established to produce antinociceptive effects in the several models of nociception such as the tail-flick (Martin et al., 1984), inflammatory pain (Smith et al., 1998), and neuropathic pain (De Vry et al., 2004), other prevalent phytocannabinoids that are structurally similar to THC have not been assessed. These compounds include CBN, CBD, CBC, and THCV. CBD has been demonstrated to have anti-edema effects (Costa et al., 2004; Lodzki et al., 2003) and potentiate the antinociceptive effects of THC (Hayakawa et al., 2008; Varvel et al., 2006). However, orally administered CBD was inactive in the acetic acid stretching model and CBN was only effective at high concentrations (Sanders et al., 1979; Sofia et al., 1975; Welburn et al., 1976). In addition, neither CBC nor THCV has been characterized in visceral pain models.

Interestingly, THCv has been shown to act as a competitive cannabinoid receptor agonist and antagonist (Pertwee, 2008; Thomas et al., 2005). We hypothesize that prevalent phytocannabinoids that are structurally similar to THC produce analgesia via a cannabinoid receptor mechanism of action. To test this hypothesis, we compared the antinociceptive effects of THC to other prevalent phytocannabinoids, including CBC, CBD, CBN, and THCv, in the acetic acid stretching model. Furthermore, we tested the ability of THCv to antagonize the effects of THC in our model since it was shown previously to act as an antagonist in vitro (Thomas et al., 2005). Previously, it was discovered that CBD did not show affinity for the CB1 receptor and CBN was reported to have moderate to low affinity for the CB1 receptor (Devane et al., 1988). THCv and CBC binding affinities have not been reported to date. Therefore, we assessed the binding affinities of prevalent marijuana constituents. A secondary goal of this chapter is to determine whether phytocannabinoids produce their antinociceptive effects through a specific cannabinoid receptor mechanism of action. Accordingly, we examined the involvement of CB1 and CB2 receptors using rimonabant and SR144528, selective antagonists for these respective receptors. Because cannabinoids elicit antinociceptive effects as well as motor suppressive effects, in the final set of experiments, we evaluated each active drug for hypomotility.

Endocannabinoid degradative enzyme inhibitors and abdominal stretching

To test the idea that the endogenous cannabinoid system may represent a target for treating visceral pain we employed the acetic acid abdominal stretching model. The acetic acid stretching model offers great benefits in that it can rapidly depict compounds that have analgesic properties. A second major goal of this project was to determine whether elevating endocannabinoids, anandamide or 2-arachidonoylglycerol by blocking their hydrolysis attenuates visceral pain. Initially, we examined the role of the endocannabinoid system and the possible mechanisms through which it can reduce abdominal stretching. Previous studies have shown that blocking FAAH produces antinociception without producing cannabinomimetic effects such as hypothermia, catalepsy, and hypomotility (Cravatt et al., 2001; Gobbi et al., 2005; Kathuria et al., 2003; Lichtman et al., 2004). Additionally, no study to date has assessed the effects of MAGL inhibition on abdominal stretching. Hence, we hypothesize that inhibiting FAAH and/or MAGL produces antinociceptive effects similar to that of the phytocannabinoid, THC, through a CB1 mediated mechanism of action in the acetic acid abdominal stretching model. To test the effects of FAAH inhibition, we examined whether FAAH (-/-) mice or FAAH wild-type mice treated with FAAH inhibitors (URB597, OL-135, PF-3845) would display reduced nociceptive behavior in the acetic acid induced abdominal stretching test. Second, we determined the receptor mechanism of action underlying the antinociceptive phenotype of FAAH-knockout mice. In order to determine the receptor mechanism of action, mice were evaluated with the respective CB1 and CB2 receptor antagonists, rimonabant and SR144528. Because the

antinociceptive effects of FAAH inhibitors have been suggested to include an opioid receptor mechanism of action (Chang et al., 2006), we also evaluated whether naltrexone would block the antinociceptive effects of URB597 and OL-135.

Previous studies illustrated that coadministration of an NSAID and a synthetic cannabinoid agonist, WIN55-212 elicited additive analgesic effects in the acetic acid abdominal stretching model (Ulugol et al., 2006). However, WIN55-212 produces THC-like cannabimimetic properties such as hypomotility, catalepsy, and hypothermia. Conversely, FAAH inhibition does not produce these side effects, therefore we tested the hypothesis that dual FAAH and COX inhibition reduce acetic acid abdominal stretching. Furthermore, since FAAH and COX regulate different signaling pathways we conducted isobolographic analysis to determine if there is a synergistic interaction by blocking both enzymes.

To test the hypothesis that blockade of MAGL, another degradative enzyme in the endocannabinoid system, produces antinociception in an acute model of visceral pain, we employed the use of the selective MAGL inhibitor JZL184 (Long et al., 2009). Furthermore, we determined if the observable antinociceptive effects of MAGL inhibition are mediated through CB1 receptors. Given the fact that FAAH and MAGL are localized in different regions of the neuron (e.g., FAAH postsynaptically located, MAGL presynaptically), and regulate different endocannabinoids, the possibility exists that they may have different roles in regulating physiological functions including nociception. Thus, simultaneous inhibition of FAAH and MAGL might offer an attractive therapeutic approach that maintains analgesic efficacy while

minimizing untoward side effects associated with direct acting cannabinoid agonists. For that reason, we determined if dual inhibition of FAAH and MAGL would enhance the antinociceptive effects in the acetic acid abdominal stretching model compared to inhibiting one enzyme.

FAAH and LPS-induced allodynia

Another preclinical model of pain is the LPS-induced allodynia model. Unlike the acetic acid model of visceral nociception which affects the internal organs of the viscera, the LPS model of allodynia models clinical diseases such as fibromyalgia, postherpetic neuralgia and various skin disorders. In the final series of experiments, we examined the role of FAAH and MAGL inhibition on reversing allodynia. Lipopolysaccharide (LPS) is a bacterial endotoxin derived from the outer cell wall of gram (-) bacteria. When LPS is injected into the plantar surface of the mouse paw, it elicits a mild innate or non-specific inflammatory response. The innate response is characterized by an infiltration of immune cells, the release of cytokines and chemokines, and activation of complement cascades to remove the bacteria. The resulting response is the induction of tactile allodynia (painful response to a non-noxious stimuli) as seen in patients with fibromyalgia, post herpetic neuralgia, and mild skin injuries (Rowbotham and Fields, 1989; Staud and Domingo, 2001). Hence, we tested the hypothesis that genetic deletion or pharmacological inhibition of the endocannabinoid catabolic enzyme FAAH, blocks tactile allodynia associated with inflammation.

Although an LPS model of inflammatory pain was recently characterized, administration of these high concentrations of LPS resulted in overt paw edema, weight loss, and malaise in mice (Naidu et al., 2010). Therefore our first aim was to modify the previously characterized LPS model of inflammation to induce tactile allodynia without producing overt edema of the paw or eliciting a general malaise. To ensure that our model can detect compounds that produce analgesia, we tested the GABA analog gabapentin, which possesses efficacy in treating various types of pain (Staahl et al., 2009) and serve as our positive control. In addition, we examined the effects of global activation of cannabinoid receptors, using THC. Since FAAH (-/-) mice show elevated levels of anandamide and hypoalgesia in acute models of pain (Lichtman et al., 2004) we determined if genetic deletion of FAAH reduces LPS-induced allodynia. FAAH-NS mice were previously generated to distinguish the function of endogenous fatty acid amides in the nervous system and peripheral tissues (Cravatt et al., 2004). These mice were developed by our collaborators by excising FAAH cDNA from the pcDNA3 vector and subcloned into the pNSE-Ex4 vector by blunt end cloning for expression under the neural-specific enolase promoter. This construct was injected into embryos and transgenic FAAH-NS offsprings were identified. The transgenic mice were intercrossed with FAAH (+/-) mice and FAAH (-/-) mice and backcrossed onto the C57BL/6 strain. With the use of southern blot analysis, it was determined that FAAH-NS mice expressed FAAH in the nervous system (brain and spinal cord) and not peripheral tissues (e.g. liver, spleen, kidney, testis, neutrophils) (Cravatt et al., 2004). We used FAAH-NS mice to distinguish whether inhibiting FAAH expressed in the peripheral and/or nervous

system(s) mediates the observed anti-allodynic effects. In a similar manner, we examined whether pharmacological blockade of FAAH would reverse LPS-induced allodynia by comparing the anti-allodynic effects of the reversible FAAH inhibitor OL-135, two irreversible FAAH inhibitors, URB597, and PF-3845. In addition, we used LC/MS/MS analysis to quantify endocannabinoid levels and compare them to mice treated with vehicle after systemic or local administration of PF-3845. Several studies have implicated a role for CB₁ receptors in reducing hyperalgesia and CB₂ receptors in ameliorating edema (see review (Anand et al., 2009)). Thus, we sought to determine whether these two cannabinoid receptors play a role in the anti-allodynic effects of FAAH (-/-) mice or wild type mice treated with FAAH inhibitors. Due to the fact that FAAH regulates other fatty acid amides other than anandamide, and since anandamide has affinities for other receptors besides cannabinoid receptors, we examined the effect of inhibiting various non-cannabinoid receptors. These receptors include the μ -opioid receptor, which was previously shown to mediate the anti-hyperalgesic response in the rat spinal nerve ligation and mild thermal injury models (Chang et al., 2006), the TRPV1 receptor, which was reported to play a vital role in the antinociceptive effects of AEA in the thermal hyperalgesia model of inflammation (Horvath et al., 2008), and the PPAR- α receptor, which was shown to mediate the antihyperalgesic effects of URB597 in an acute model of inflammation (Jhaveri et al., 2008).

Chapter 2: General Methods

Subjects

The subjects consisted of male ICR mice (Harlan Laboratories, Indianapolis, Indiana), adult male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME), adult male and female FAAH (-/-) mice backcrossed for at least 13 generations onto a C57BL/6J background, and male and female FAAH (+/+) mice derived from the same line of FAAH (+/-) breeders used to derive FAAH (-/-) mice. Additionally, male and female nervous system FAAH-restricted (FAAH-NS) (Cravatt et al., 2004) mice backcrossed onto a C57BL/6J background for at least 13 generations were used. FAAH (+/-) littermates were used as controls, because they express wild type levels of AEA and non-cannabinoid fatty acid amides (FAAs) (Cravatt et al., 2001). Lastly, male and female CB₁ (-/-) and CB₂ (-/-) mice, along with respective matched CB₁ (+/+) and CB₂ (+/+) littermates were used to determine receptor mechanisms of action. All genetically modified mice were bred in the Center Transgenic Colony at Virginia Commonwealth University. The genotype of each genetically altered mouse was confirmed via rt-PCR. Subjects weighed between 20-30 g and were housed 4-6 per cage in a temperature-controlled (20-22°C) environment. Mice were randomly assigned to treatment conditions, although a block design was used to evenly distribute transgenic and knockout mice, by sex, across treatments. Mice were kept on a 12-h light/dark cycle with food and water available ad libitum. All animal protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and were in concordance with the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory

Animal Resources, 1996). After testing was completed, mice were humanely sacrificed by CO₂ asphyxiation, followed by cervical dislocation.

Drugs

Phytocannabinoids and acetic acid writhing. THC, CBD, and CBN were obtained from the National Institute on Drug Abuse (Bethesda, MD, USA). SR141716 (rimonabant) and SR144528, respective antagonists for CB₁ and CB₂ receptors, were obtained from National Institute on Drug Abuse (Bethesda, MD), and Δ⁹-tetrahydrocannabivarin (O-4395, THCv), cannabichromene (O-4950, CBC) were supplied by Drs. Raj Razdan and Anu Mahadevan (Organix Inc, MA, USA).

FAAH and COX inhibition in acetic acid writhing. Diclofenac sodium and naltrexone HCl were purchased from Sigma-Aldrich (St. Louis, MO). URB597 was purchased from Cayman Chemical (Ann Arbor, MI). WIN55,212 was purchased from Tocris Bioscience (Ellisville, MO). Rimonabant (CB₁ receptor antagonist) and SR144528 (CB₂ receptor antagonist) were obtained from the National Institutes of Health National Institute on Drug Abuse (Rockville, MD). Diclofenac, URB597, or OL-135 was given via the subcutaneous route of administration 60 min before acetic acid administration. In the antagonism studies, rimonabant (3 mg/kg) and SR144528 (3 mg/kg) were given 70 min before acetic acid, whereas naltrexone (1 mg/kg) was administered 30 min before acetic acid. Each of these doses and pretreatment times was based on previous reports from the literature (Compton et al., 1996;

Lichtman et al., 1996; Rinaldi-Carmona et al., 1996) and from previous studies from our laboratory . Dose-response curves for URB597 and diclofenac were obtained using at least six animals at each dose. Mice were given subcutaneous injections of vehicle, diclofenac (3, 10, or 30 mg/kg), or URB597 (1, 3, or 10 mg/kg) and 60 min later were given an intraperitoneal injection of acetic acid.

FAAH and MAGL inhibition in acetic acid writhing. The MAGL inhibitor JZL184 was synthesized by our collaborators at TSRI (The Scripps Research Institute) as previously described (Long et al., 2009a). Male C57Bl/6J mice (6–8 weeks old, 20–26 g) were intraperitoneally administered JZL184 or vehicle (4:1 v/v PEG300/Tween80) at a volume of 4 ml/g weight (16, 40 mg/kg). In studies assessing dual inhibition of both FAAH and MAGL, JZL184, PF-3845, and JZL195 were dissolved in 1:1:18 (ethanol:emulphor:saline) vehicle mixture and the drug was sonicated for up to 3 min. Mice were given a subcutaneous injection of JZL184 (16 mg/kg), JZL195 (20 mg/kg), PF-3845 (10 mg/kg), or vehicle 120 min before acetic acid administration. Rimobant (3 mg/kg) or vehicle was given subcutaneous 10 min before either drug or vehicle.

FAAH inhibition and LPS-induced allodynia. URB597 (1-10 mg/kg i.p.), gabapentin (3-30 mg/kg i.p.), and MK886 [(1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]- α,α -dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid sodium salt] were purchased from Cayman Chemical (Ann Arbor, MI). The dose of MK886 used was shown to antagonize the

PPAR- α receptor in a previous study (Kehrer et al., 2001). This compound is also known to antagonize lipoxygenase, which can decrease leukotriene production, an action implicated in modulating inflammatory pain (Masferrer et al., 2010). OL-135 (1-30 mg/kg i.p.) (Boger et al., 2005), and PF-3845 (1-10 mg/kg i.p.; 0.1-10 μ g i.pl.) (Ahn et al., 2009) were synthesized as described previously. THC, rimonabant (CB₁ receptor antagonist) and SR144528 (CB₂ receptor antagonist) were obtained from the National Institute on Drug Abuse (Bethesda, MD). 5'-Iodoresiniferatoxin (IRTX; TRPV1 receptor antagonist) was purchased from LC Laboratories (Woburn, MA) and used at a concentration previously described to antagonize TRPV1 receptors (Wahl et al., 2001). The aforementioned drugs were dissolved in a vehicle consisting of ethanol, alkamuls-620, and 0.9% saline in a ratio of 1:1:18. Naltrexone HCl was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in 0.9% saline. For all systemic injections, the intraperitoneal (i.p.) route of administration was employed, using an injection volume of 10 μ l/g body weight. Gabapentin and OL-135 were administered 1 h before testing. THC, PF-3845, and URB597 were administered 2 h before testing. For the receptor antagonist experiments, rimonabant (3 mg/kg i.p.), SR144528 (3 mg/kg i.p.), MK886 (3 mg/kg i.p.), IRTX (0.5 mg/kg i.p.), or naltrexone (1 mg/kg i.p.) was administered 10 min prior to the administration of the FAAH inhibitors/analgesic compounds. For experiments evaluating local effect of FAAH inhibition, PF-3845 was administered via the intraplantar (i.pl.) route of administration into either the LPS-treated paw or the saline-treated control paw 2 h prior to testing, using a total

volume of 5 μ l. The nomenclature of receptors and ligands follows the Guide to Receptors and Channels (GRAC) (Alexander et al., 2008).

Acetic Acid Stretching Model

The acetic acid stretching test (Koster et al. 1959) was employed to evaluate visceral nociception. A total of 6-10 naive mice was used per condition in each experiment. For each desired concentration analyzed, subjects were given a subcutaneous (s.c.) injection of drug or vehicle 60 min (phytocannabinoid study) and 120 min (FAAH and MAGL inhibition studies) before an intraperitoneal (i.p.) injection of 0.6% acetic acid. In studies examining the cannabinoid receptor mechanism of action, rimonabant (3 mg/kg), SR144528 (3 mg/kg), or vehicle was administered through the i.p. route of administration 10 min before the agonist or vehicle. All injections were given in a volume of 10 μ l/g body weight. After administration of acetic acid, the subjects were placed in clear cages (11 x 7 x 5 in) and allowed time to acclimate for 3 min prior to being scored for abdominal stretches during a 20 min observation period. Stretching was defined as body contortions, belly pressing, and extension of the hind limbs from which visceral nociception was inferred.

Inflammatory pain model

Inflammatory pain was induced by injecting lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 Sigma (St. Louis, MO) (Naidu et al., 2010) in 20 μ l of physiological saline into the

plantar surface of one hind paw of each mouse (Kanaan et al., 1996). Saline was administered into the opposite hind paw. Thus, each mouse served as its own control, thereby reducing the total number of mice required.

Animals were tested for mechanical allodynia 24 h post LPS administration using calibrated von Frey filaments, ranging from 0.16 g - 6.0 g (Stoelting, Wood Dale, IL), as described previously (Kinsey et al., 2010). At 23 h after LPS administration, mice were placed into Plexiglas cylinders on an elevated wire mesh screen, allowing access to each hind paw by the filaments. Mice were allowed to acclimate to the test apparatus for 60 min prior to testing. Paw withdrawal threshold was determined by using the “up-down” method (Chaplan et al., 1994). The plantar surface of each paw was stimulated 5 times each and a positive response was scored if each mouse clutched, lifted, or fluttered the paw upon 3 of 5 stimulations. The thickness of the LPS-treated and saline-treated paws was measured both before and 24 h after LPS injection, using digital calipers (Traceable Calipers, Friendswood, TX) and expressed to the nearest ± 0.01 mm (Naidu et al., 2010). The 24 h paw thickness values were measured immediately after allodynia assessment.

Tail withdrawal assay

Each mouse was placed into a small pouch created from absorbent under pads with the tail extending from the end of the pouch. Mice were loosely held in the pouch while their tails

were immersed into the water bath maintained at 52°C. The latency for each mouse to withdraw its tail from the water (tail-flick) within a 10 s cutoff time was tabulated.

Intrathecal injections

Intrathecal injections were conducted the day of the study. Briefly, mice were held firmly under an absorbent cloth with their lower lumbar region exposed. The lumbar region (L4-L6) was located in reference to the hip placement. Mice were then injected with drug or vehicle in a total volume of 5 µl using 50 µl hamilton syringes. Correct injections were noted after needle penetration if the subject tail flared.

Stereotaxic i.c.v. surgeries

ICV surgeries were conducted the day before allodynia testing to allow for wound healing and recovery from anesthesia. Briefly, mice were anesthetized under isoflurane or given pentobarbitol (45mg/kg; i.p.). Mice were then placed on the digital stereotaxic instrument (Stoelting) and immobilized using earbars and a muzzle with their teeth inserted into the muzzle stabilizer (Franklin & Paxinos, 1997). An incision was made in the scalp to expose bregma. A peroxide soaked swab was used to remove the surrounding layer of brain film so that bregma is clearly exposed. Next, a hole (2 mm depth) was punctured into the lateral ventricle using coordinates (-1.0, -0.6) of bregma targeting the lateral ventricles (Allen mouse brain atlas). Mice were then allowed to recover overnight in their home cages. The day after surgery mice were injected with drug or vehicle in a total volume of 5 µl using 50 µl hamilton syringes.

Extraction and Quantification of Endocannabinoids by LC/MS/MS

AEA and 2-AG levels were quantified in the whole brain, whole spinal cord, and paw tissue of male C57BL/6J mice treated with a systemic dose of PF-3845 (10 mg/kg, i.p.) or local dose (1.0 µg, i.pl.) as described above. Two hours after drug or vehicle administration, the mice were decapitated and tissues were harvested. All tissue samples were snap frozen in liquid nitrogen and stored at -80°C until the endocannabinoids were extracted.

On the day of extraction, 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-AGd8) (Kinsey et al., 2009a). Homogenates were then mixed with 0.3 ml of 0.73% w/v NaCl, vortexed, and then centrifuged for 10 min at 4,000 rpm (4°C). The aqueous phase plus debris were collected and extracted two more times with 0.8 ml chloroform. The organic phases from the three extractions were pooled, and the organic solvents were evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml chloroform and mixed with 1 ml ice-cold acetone. The mixtures were then centrifuged for 5 min at 3,000 rpm and 4°C to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml methanol and placed in autosampler vials for analysis. LC/MS/MS was used to quantify AEA and 2-AG. The mobile phase consisted of (10:90) water/methanol with 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery HS C18, 4.6×15 cm, 3 µm

(Supelco, PA). The mass spectrometer was run in Electrospray Ionization, in positive mode. Ions were analyzed in multiple-reaction monitoring mode, and the following transitions were monitored: (348>62) and (348>91) for AEA; (356>62) for AEAd8; (379>287) and (279>269) for 2-AG; and (387>96) for 2AG-d8 as described previously (Kinsey et al., 2009a). A calibration curve was constructed for each assay based on linear regression using the peak area ratios of the calibrators. The extracted standard curves ranged from 0.03 to 40 pmol for AEA and from 0.05 to 64 nmol for 2-AG.

Motor Impairment

In an effort to assess motor impairment, subjects were pretreated 60 min (6-8 mice per group) with a subcutaneous (s.c.) injection of THC (1-50 mg/kg). Each mouse was then placed in a clear Plexiglas box (17.5 x 8.5 in) situated in a sound attenuating chamber that contained an indirect filtered light source and fans for air circulation. Locomotor activity was recorded using Fire-*i* digital camera software (Unibrain Inc, San Ramon, CA) web camera that was located above the activity box and behavior was analyzed using the AnyMaze Software (Stoelting, Wood Dale, IL) as described previously (Schlosburg et al., 2009a).

Binding assay

Radioligand binding was performed following the method of (Devane et al., 1988) and modified by (Compton et al., 1993). In brief, binding was initiated by the addition of 75 µg whole rat brain protein to silanized tubes containing [³H]-CP-55,940, a potent synthetic cannabinoid analog, (139.6 Ci/mM NEN, DuPont, Boston, MA) and sufficient volume of buffer A (50mM Tris-HCl, 1 mM Tris-EDTA, 3mM MgCl₂, and 5 mg/ml fatty acid-free BSA, pH 7.4) to bring the total volume up to 0.5 ml. Unlabelled (cold) CP-55,940 (1 µM) was used to assess non-specific binding. CP-55,940 was suspended without evaporation, in buffer A from 1 mg/ml ethanolic stock, as were all cannabinoid constituents. After adding tissue, the reaction mixture was incubated at 30°C for 60 min. Saturation experiments were conducted with 8 concentrations of [³H]-CP-55,940 ranging from 30 nM to 10 µM. Binding was terminated by the addition of 2 ml ice-cold buffer B (50 mM Tris-HCl, and 1 mg/ml BSA, pH 7.4), and vacuum filtration (Millipore, Bedford, MA) through pretreated (>4 hr, 0.1% solution of PEI, pH 7.4) GF/C glass-fiber filters (2.4 cm, Baxter, McGaw Park, IL). The reaction tubes were then rinsed once with 2 ml and twice with 4 ml of ice-cold buffer B. Before radioactivity was quantified by liquid scintillation spectrometry, the filters were incubated in 4 ml Budget-Solve (RPI Corp., Mount Prospect, IL) scintillation fluid, and shaken for 60 min. All assay conditions were conducted in triplicate, and the results reflect three independent experiments.

Data analyses

Abdominal stretching. The total number of abdominal stretches was tabulated for each subject and ED50 values were calculated using least squares linear regression. Data were analyzed using one-way and two-way ANOVA. Post hoc analyses were conducted with the Tukey test or Dunnett's test in dose-response experiments. All differences were considered significant at $p < 0.05$. The K_i values for the binding assay were generated from the Radlig Ligand program from the Kell software package version 6 for Windows, (Biosoft, Milltown, NJ).

Isobolographic analysis for dual inhibition. Isobolographic analysis was used to determine the nature of the drug interactions, as described previously (Tallarida, 2000). The dose of diclofenac required to elicit a 50% effect was plotted on the abscissa, and the isoeffective dose of URB597 was plotted on the ordinate. The theoretical additive effect of the two drugs was represented by the straight line connecting the two points. If the experimentally determined data points and their confidence interval lie on this line, the drug effects are considered additive. If the points lie below this line, the interaction is considered to be superadditive (synergistic); however, if they lie above the line of additivity, the interaction is defined as subadditive (antagonistic). To determine whether the interaction between two drugs was synergistic, additive, or antagonistic, the theoretical additive ED50 value of the two drugs combined (referred to as Z_{add}) was calculated from the dose-response curves of each drug administered individually, in which the combination is assumed to equal the sum of the individual effects of each drug. The experiment ED50 value of the two drugs in combination (referred to as Z_{mix}) in which the two drugs were

summed at each concentration was then determined by linear regression. The statistical difference between Z_{add} (the theoretical ED50 value) and Z_{mix} (the experimental ED50 value) were analyzed using Fisher's test. These calculations were performed using the program of Pharm Tools Pro (version 1.20; The McCary Group Inc., Elkins Park, PA), based on Tallarida (2000). $p < 0.05$ were considered significant.

LPS-induced allodynia. The dependent measures included changes in paw edema (24 h - baseline paw thickness values) and mechanical paw withdrawal thresholds 24 h after LPS. All data are reported as mean \pm SEM and were analyzed using one-way analysis of variance (ANOVA). Dunnett's test was used for post hoc analysis in the dose-response experiments to compare the effects of each drug dose to those of vehicle. Tukey-Kramer post hoc analysis was used for all tests comparing different treatment groups, as well as genotype distinctions. Differences were considered significant at the $p < 0.05$ level.

Chapter 3: Phytocannabinoids assessment in the blockade of acetic acid induced abdominal stretching

The first goal of this dissertation was to investigate the effect of prevalent phytocannabinoids in commonly used mouse models of pain. To date over 70 phytocannabinoid constituents have been identified, however the pharmacological effect of several remain unknown. THC is well established to produce analgesia in various models of pain. However, orally administered CBD was inactive in the acetic acid stretching model and CBN was only effective at high concentrations (Sanders et al., 1979; Sofia et al., 1975; Welburn et al., 1976). Neither CBC nor THCV has been characterized in the acetic acid abdominal pain model.

Briefly, male ICR mice were given a subcutaneous pretreatment (60 min) of THC, CBC, CBN, CBD, or THCV then placed back in their homecages. After the 60 min pretreatment, mice were injected intraperitoneally with acetic acid and placed in observation chambers for a 3 min acclimation period. Abdominal stretching was then recorded for 20 min beginning at the end of the acclimation period. The concentration of acetic acid was chosen based on our studies that 0.6% acetic acid resulted in robust abdominal stretching in mice (Figure 2A). Additionally, the observation and scoring window was chosen based on our studies showing that the peak abdominal stretching response rate takes place between 3 and 23 minutes (Figure 2B).

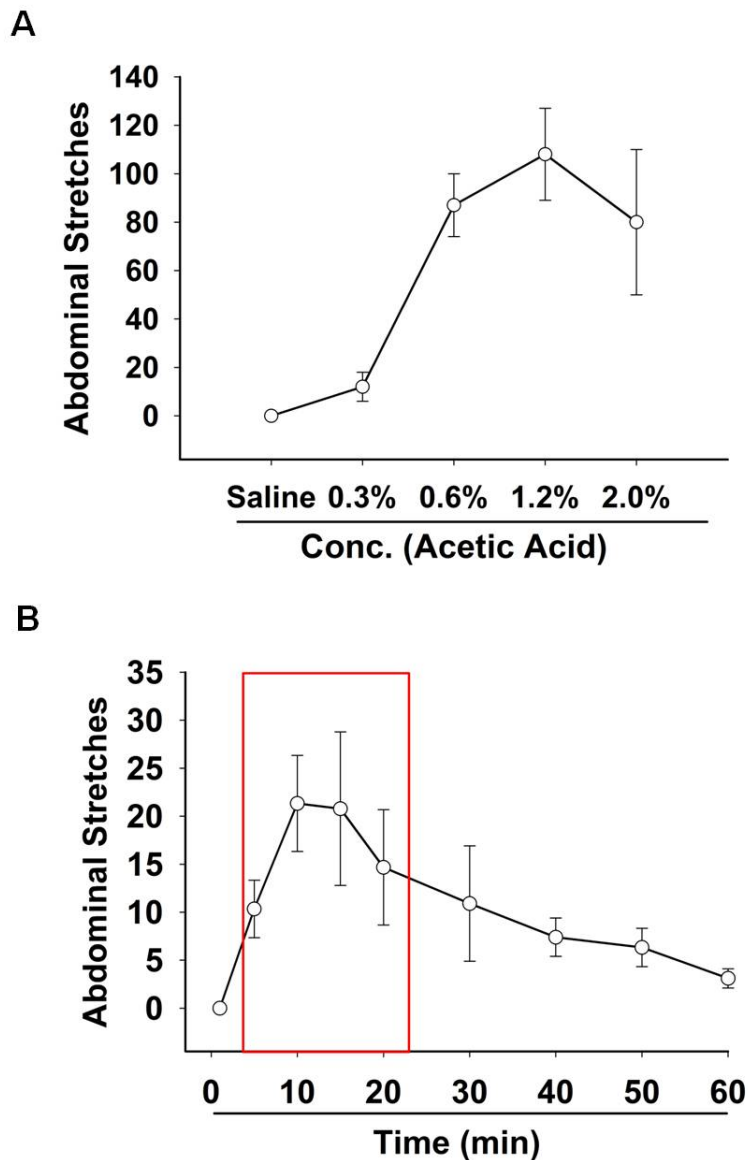


Figure 2. Increasing concentrations of acetic acid significantly increases abdominal stretching in mice (**A**). 0.6% acetic acid produces peak abdominal stretching effects between 3 min and 23 min after intraperitoneal administration (**B**). Data represents the mean \pm SEM abdominal stretching. n=6 mice per group.

3.1 THC dose-dependently blocks acetic acid induced stretching through a CB1 receptor mechanism of action

To determine the effect of phytocannabinoids on abdominal stretching, mice were pretreated in this first study with Δ^9 -THC (0.03-5.0 mg/kg; s.c.) then placed in observation boxes and observed for a total of 23 minutes. The total number of stretches was plotted on the Y-axis (ordinate). In a follow up study mice were also tested for locomotor activity to assess whether hypomotility may have contributed to decreases in abdominal stretches. As shown in Figure 3, Δ^9 -THC dose-dependently suppressed abdominal stretching, with an ED50 value of 1.1 mg/kg (95% confidence interval 0.8–1.6 mg/kg). This drug was considerably less potent in decreasing locomotor activity than in producing antinociception. Its ED50 value in suppressing locomotor activity was 7.7mg/kg (95% confidence interval 4.2–14.3 mg/kg) (see Table 4). Δ^9 -THC was 8.5 (95% confidence interval: 3.4–20.6) fold more potent in eliciting antinociception than in decreasing locomotor activity. Based on these results, we employed 3 mg/kg Δ^9 -THC to evaluate the underlying receptor mechanism of action, as this dose did not significantly interfere with locomotor activity after a 60 min pretreatment time compared to vehicle (Table 4). Rimonabant, but not SR144528, significantly blocked THC's antinocicpetive effects [$F(3, 22) = 37.1, p < 0.0001$], indicating a CB1 receptor mechanism involvement (Figure 4A). Administration of either rimonabant or SR144528 alone did not significantly affect abdominal stretching behavior (Figure 4B).

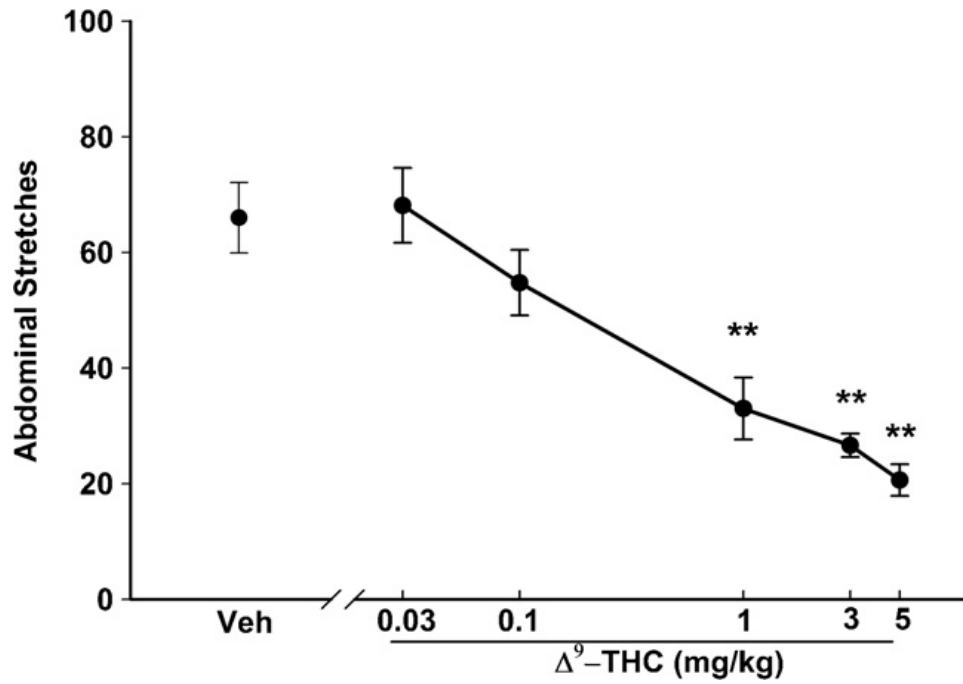


Figure 3. Subcutaneous administration of Δ^9 -THC reduced abdominal stretching in a dose-dependent manner; ED50 (95% confidence interval) value = 1.1 mg/kg (0.8–1.6). Each data point represents 6–8 mice. ** $p < 0.01$ compared with vehicle. Data reflect the mean \pm SEM number of abdominal stretches during the 20 min observation period.

Table 4. Evaluation of Δ^9 -THC in spontaneous locomotor activity behavior. Mice were given a subcutaneous injection of various concentrations of Δ^9 -THC and evaluated 60 min later for locomotor activity for a total of 20 min. Data are represented as the mean \pm SEM Percentage of time spent immobile or total distance traveled, $n = 6$ mice per group. % Time Immobile Total Distance Traveled (m).

	%Time Immobile	Total Distance Traveled (m)
Δ^9-THC (mg/kg)		
Vehicle	21.1 \pm 4.2	46.54 \pm 5.66
1.0	11.2 \pm 2.2*	41.79 \pm 2.63
3.0	21.5 \pm 5.0	43.59 \pm 4.70
10.0	37.6 \pm 9.2	45.86 \pm 7.76
50.0	49.0 \pm 6.8*	35.34 \pm 3.61*
CBN (mg/kg)		
50.0	17.6 \pm 2.9	39.02 \pm 7.75
* $p < 0.05$ vs vehicle treated mice		

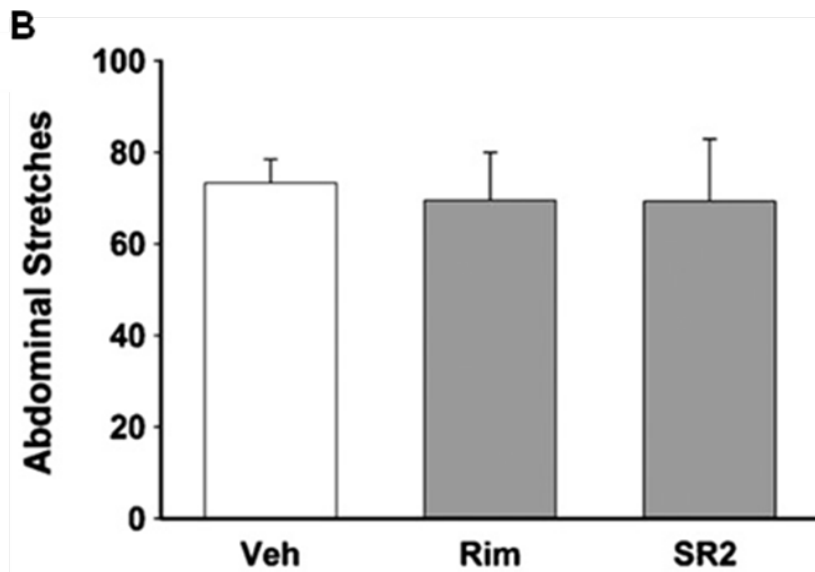
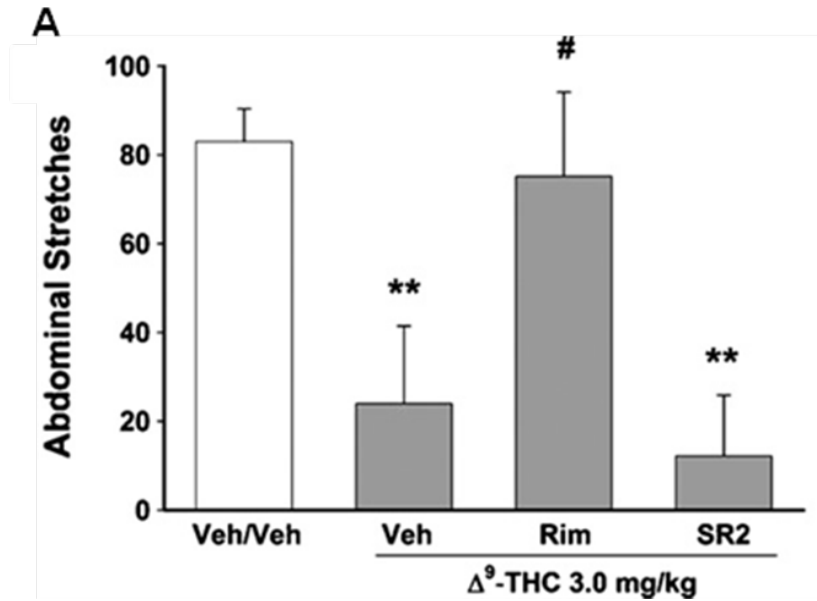


Figure 4. The antinociceptive effects of Δ^9 -THC in the acetic acid model of visceral nociception are mediated through a CB1 receptor mechanism of action. (A) The CB1 receptor antagonist, rimonabant (Rim; 3.0 mg/kg, i.p.), but not the CB2 receptor antagonist, SR144528 (SR2; 3.0 mg/kg, i.p.), blocked the antinociceptive effects of Δ^9 -THC (3 mg/kg, s.c.). # indicates significant difference from Vehicle (Veh)/ Δ^9 -THC control $p < 0.01$; **Significant

difference from Vehicle/Vehicle control $p < 0.01$. (B) Neither rimonabant nor SR144528 given alone affected acetic acid-induced abdominal stretching. $n = 6-8$ mice/group. Data reflect the mean \pm SEM number of abdominal stretches.

3.2 Evaluation of other phytocannabinoids in the acetic acid abdominal stretching model

The question of whether other prevalent naturally occurring marijuana constituents also possess antinociceptive properties was addressed by administering either vehicle, CBC, CBD, CBN, or THCV, 1 h before the administration (i.p.) of acetic acid. As shown in Figure 5A, CBN produced a significant reduction in acetic acid-induced abdominal stretching, [$F(6, 35) = 9.5, p < 0.001$]. According to post hoc analysis, CBN produced significant antinociceptive effects at 50 mg/kg ($p < 0.01$), but not at 20mg/kg ($p = 0.27$). In contrast, high doses of CBC or CBD did not produce antinociceptive effects in this assay. CBN (50 mg/kg) failed to inhibit locomotor activity when administered 60 min prior to recording spontaneous activity (Table 4). As shown in Figure 5B, the antinociceptive effects of CBN (50 mg/kg) were blocked by rimonabant, but not by SR144528 [$F(3, 24)=17.5, p < 0.001$]. While THCV (50 mg/kg) administered alone had no effect on the frequency of stretching behavior (Figure 6), it blocked the antinociceptive effects of Δ^9 -THC, [$F(3, 26) = 9.52, p < 0.001$].

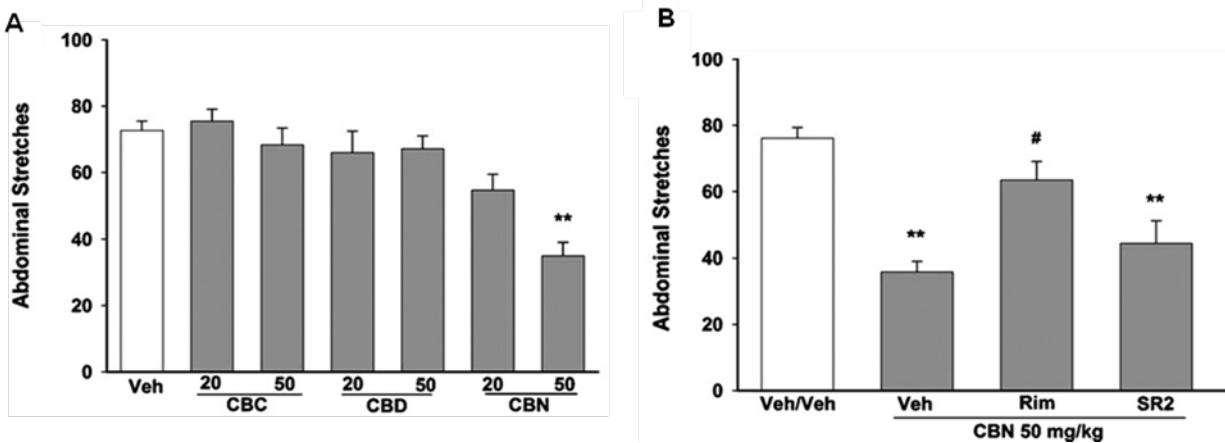


Figure 5. Evaluation of prevalent marijuana constituents in the acetic acid abdominal stretching model. (A) The marijuana constituents, CBC and CBD, did not produce antinociceptive effects. However, CBN (50 mg/kg) significantly suppressed the stretching response compared to vehicle (Veh), $**p < 0.01$ vs. Vehicle. (B) The CB1 receptor antagonist, rimonabant (Rim), but not by the CB2 receptor antagonist, SR144528 (SR2), significantly blocked the antinociceptive effects of CBN (50 mg/kg). $**p < 0.01$ vs. Veh/Veh, and $\#p < 0.01$ vs. Veh/CBN. $n = 6-10$ mice/group. Data reflect the mean \pm SEM abdominal stretches.

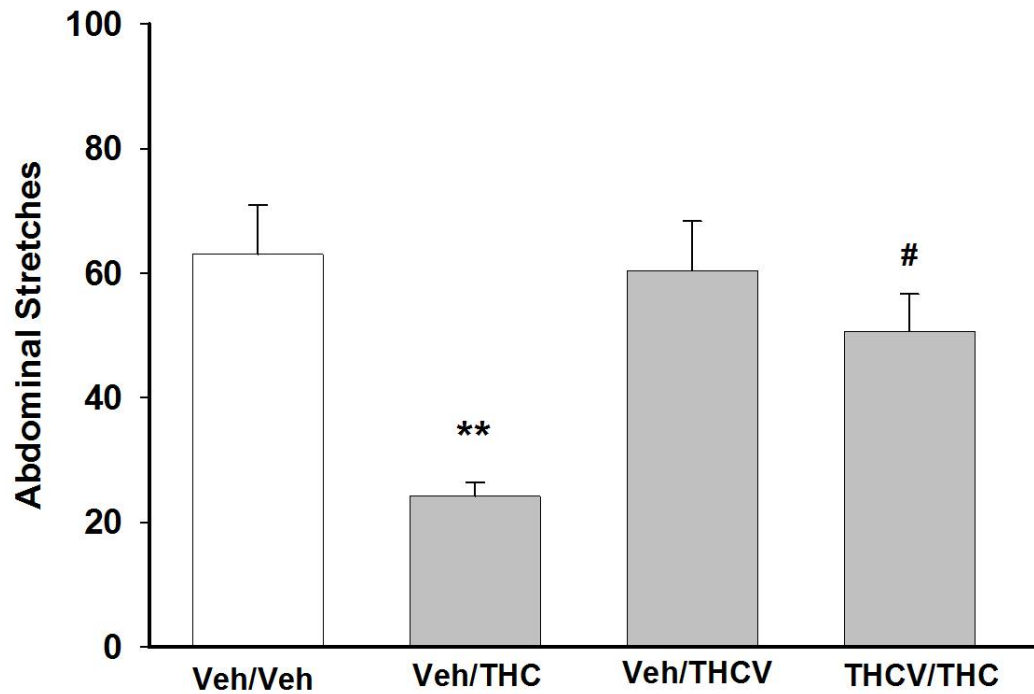


Figure 6. Delta 8-tetrahydrocannabivarin (THCV, 50 mg/kg, s.c.) had no effects on its own, but blocked the antinociceptive effects of THC (3.0 mg/kg, s.c.). ** $p < 0.01$ vs. Veh/Veh group. # $p < 0.05$ vs. Veh/THC. $n = 6-8$ mice/group. Data reflect the mean \pm SEM abdominal stretches.

3.3 Receptor binding affinity of CBN, THC, and THCV

In order to determine whether the prevalent phytocannabinoids were displaying their effects through cannabinoid receptors we conducted binding displacement curves. The binding affinities for THC, CBC, CBD, CBN, and THCV to the cannabinoid receptor were measured by the displacement curve of tritiated CP-55,940 ([³H]-CP-55,940). These data are summarized in Table 5. THC and THCV bound to the CB1 receptor with equal affinity (K_i values \pm SEM = 47.7 ± 4.6 nM and 46.3 ± 6.0 nM, respectively) as illustrated by the similarity of their displacement curves (Figure 7). Rat brains were used due to the increased amount of tissue, however it is noted that there is 99% homology between rat and mouse brains (Chakrabarti et al., 1995). CBN also displaced [³H]-CP-55,940 binding (129.3 ± 12.9 nM), but its affinity was 2–3 fold lower than the affinity of THC at the CB1 receptor. Neither CBD nor CBC showed any affinity for the CB1 receptor (K_i values $> 10,000$ nM).

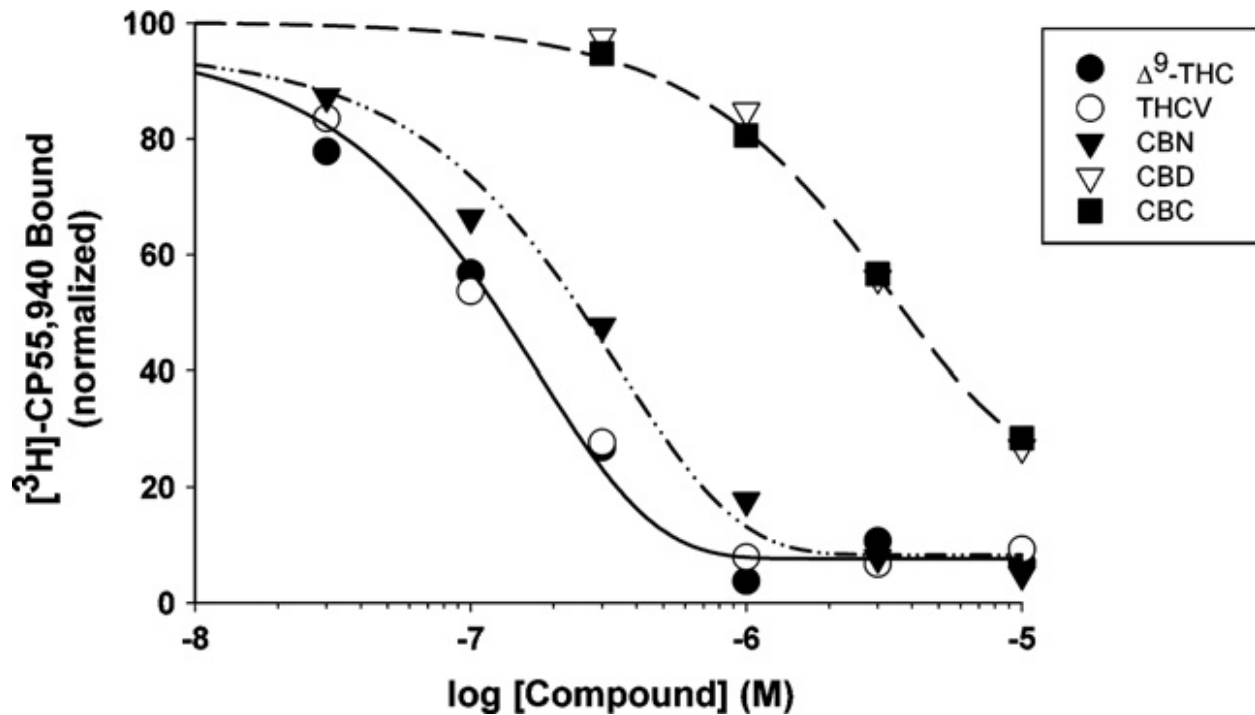


Figure 7. Activity of prevalent phytocannabinoids at rat cannabinoid receptor type 1. The affinity of Δ^9 -THC was determined for rat CB1 receptor (filled circles/solid line), THCV (open circle/solid line), CBN (filled triangle/dash-dotted line), CBD (open triangle/dashed line), and CBC (filled square/dashed line). Details for competition binding experiments are described in the methods section. The points on the graph represent the mean \pm SEM of three independent experiments with duplicate wells on each plate. The data were normalized to the signal in the absence of unlabeled competitor (defined as 100%) and in the presence of excess unlabeled CP-55,940 (defined as 0%).

Table 5. K_i -values for displacement of [3H]-CP-55,940 from mouse whole brain. Δ^9 -THC, CBN, and THCv displaced [3H]-CP-55,940 in the nanomolar range. However, CBD and CBC lacked affinity for the receptor. $n = 3$ brains per drug.

Constituent	K_i (nM)	\pm S.E.
Δ^9 -THC	47.7	4.6
Cannabinol (CBN)	129.3	12.9
THCV (O-4395)	46.3	6.0
Cannabidiol (CBD)	>10,000	
CBC (O-4950)	>10,000	

3.4 Discussion: Phytocannabinoids reduce visceral nociception

In previous years less attention was given to marijuana and its potential use as an analgesic compound, in part, due to its psychoactive properties, which are primarily caused by the actions of Δ^9 -THC. However, other constituents of marijuana may have analgesic properties with minimal psychoactive effects compared to Δ^9 -THC. The results of the present study demonstrate that while Δ^9 -THC and CBN elicited antinociception in the acetic acid abdominal stretching model, other phytocannabinoids (i.e., CBD, CBC, and THCV) did not affect abdominal stretching when given alone, and THCV actually inhibited the antinociceptive effects of Δ^9 -THC. Additionally, the present study determined that the antinociceptive effects of Δ^9 -THC and CBN were mediated through a CB1 receptor mechanism of action.

The results obtained in the present chapter are consistent with the view that Δ^9 -THC is the major phytocannabinoid present in marijuana that produces antinociception in the acetic acid abdominal stretching test. Previous studies reported that Δ^9 -THC dose-dependently suppressed abdominal stretching in the *p*-phenylquinone test (Dewey et al., 1972; Sanders et al., 1979), formic acid test (Welburn et al., 1976), and acetic acid writhing test (Sofia et al., 1975) in mice, with ED50 values ranging between 1.2 and 4.2 mg/kg. In agreement with earlier work, THC dose-dependently reduced abdominal stretching, though we used the s.c. route of administration and the earlier work administered the drug via gavage. The finding that rimonabant completely blocked the antinociceptive effects of Δ^9 -THC indicates a CB1 receptor mechanism of action.

A major goal of the present chapter was to also investigate other prevalent cannabinoid constituents of marijuana in the acetic acid model of visceral pain. These compounds closely resemble Δ^9 -THC structurally and, in some cases, bind to CB1 receptors. Emphasis has been drawn away from naturally occurring compounds, due to their relative low abundance in marijuana compared to Δ^9 -THC (ElSohly et al., 2000). CBN (50 mg/kg) suppressed abdominal stretching through a CB1 receptor mechanism of action. Sofia et al. (1975) also found that high concentrations of CBN were required to elicit antinociceptive effects after gavage administration. Although the binding affinity of Δ^9 -THC is 2–3 folds greater than the binding affinity of CBN, Δ^9 -THC is at least fold 50 fold more potent than CBN in producing antinociception. However, the relationship between binding affinity and *in vivo* activity of the cannabinoids is not linear, but takes on a logarithmic function (Compton et al., 1993). For example, CBN was 90–250 fold less potent than Δ^9 -THC in eliciting Δ^9 -THC-like discriminative cues in pigeons (Jarbe et al., 1977). Thus, the fact that higher doses of CBN than Δ^9 -THC are required to elicit antinociceptive actions in this visceral pain model is consistent with its low binding affinity for the CB1 receptor compared to the affinity of Δ^9 -THC.

These experiments are the first to our knowledge to examine the effectiveness of CBC to displace [3 H] CP-55,940 at the cannabinoid receptor. However, this phytocannabinoid which is also a aromatic hydrocarbon containing 21 carbons, did not bind to CB1 receptors and did not produce antinociceptive effects in the acetic acid model of visceral nociception. The lack of antinociceptive efficacy of CBD in the acetic acid stretching model is consistent with previous

reports (Sanders et al., 1979; Welburn et al., 1976) (Sofia et al., 1975). Moreover, the poor affinity of CBD to the CB1 receptor was consistent with previous research (Showalter et al., 1996; Thomas et al., 2007). Although THCV, the propyl homologue of Δ^9 -THC had equivalent binding affinity as Δ^9 -THC, it failed to elicit antinociceptive effects at doses up to 50 mg/kg. This compound has been reported previously to have competitive antagonist effects with Δ^9 -THC at low concentrations, though it elicited agonist activity at high intravenous doses (Pertwee et al., 2007). Indeed, we report that a high dose of THCV (i.e., 50 mg/kg) significantly antagonized the antinociceptive effects of Δ^9 -THC (3 mg/kg), further supporting the notion that THCV is a naturally occurring CB1 receptor antagonist.

Because cannabinoids are known to elicit hypomotility, which would confound interpretations of the behavioral data in the acetic acid-induced stretching assay, we examined the effects of each active drug on locomotor behavior. Δ^9 -THC did not impair mobility at doses less than 50 mg/kg, suggesting that the dose range used in the acetic acid stretching test did not provoke motor disturbances. Additionally, CBN did not affect mobility at the dose that produced antinociception. Traditionally, cannabinoid agonists have been shown to produce reductions in locomotor activity (Hohmann et al., 2005), which is one of the hallmarks of Δ^9 -THC. The increased potency of Δ^9 -THC in producing antinociception compared to its potency in producing locomotor suppression may be attributed to the fact that the acetic acid assay is particularly sensitive to antinociceptive agents.

CB1 selective and CB2 selective antagonist (rimonabant and SR144528 respectively) failed to increase abdominal stretching when administered alone, suggesting that acetic acid-induced stretching is not affected by endocannabinoid tone. Interestingly, there are considerable *in vitro* and *in vivo* data suggesting that endocannabinoids are produced and released on demand (Jung et al., 2005; Matias et al., 2006; Patel et al., 2003). Walker *et al.* (1999) showed that the electrical stimulation of the periaqueductal gray (PAG) area as well as formalin injected intradermally into the hind paws elevated anandamide levels in the PAG, supporting the role that endocannabinoids are released in response to pain sensing pathways. In addition, Hohmann *et al.* (2005) demonstrated that intracerebral administration of inhibitors of endocannabinoid metabolizing enzymes into the PAG potentiated stress-induced antinociception and led to concomitant release of endocannabinoids within this brain region.

In summary, our results show that Δ^9 -THC dose-dependently suppressed the frequency of acetic acid induced stretching. Its antinociceptive effects were shown to be mediated through a CB1 receptor mechanism of action, without any indication of CB2 receptor involvement. The only other naturally occurring constituent of marijuana evaluated that produced antinociception was CBN, but the required dose (i.e., 50 mg/kg) was substantially higher than the minimal dose of Δ^9 -THC that produced antinociception (i.e., 1mg/kg). CBD, CBC, and THCV failed to produce antinociception in the acetic acid test. Conversely, while THCV given alone did not affect visceral nociception, it antagonized the antinociceptive actions of Δ^9 -THC when both drugs were given in combination. Although this pattern of findings raises the provocative possibility

that other components of this plant can augment (e.g., CBN) or reduce (e.g., THC_V) the antinociception actions of Δ^9 -THC, it should be noted that marijuana contains a far lower abundance of CBN (0.24–1.44%) than Δ^9 -THC (4–20%) (ElSohly et al., 2000). Additionally, the percentage of THC_V is considerably low and varies in samples of marijuana of different origins (Brenneisen and elSohly, 1988). Thus, these specific constituents would not be expected to play a substantial role in marijuana's pharmacological effects. On the other hand, these results suggest that there is potential to develop medications containing various concentrations of specific phytocannabinoids to optimize therapeutic effects (e.g., antinociception) and minimize psychomimetic effects. The results of the present chapter further support the notion that Δ^9 -THC is the predominant constituent of marijuana responsible for eliciting antinociceptive effects and indicate that CB1 receptors play a predominant role in mediating these effects. Moreover, these data suggest that other phytocannabinoids may also be used to modulate the analgesic effects of Δ^9 -THC.

Chapter 4: Evaluation of the endocannabinoid systems role in modulating visceral nociception in the acetic acid abdominal stretching model

4.1 Global activation of the CB1 receptor reduce acetic acid abdominal stretching

In chapter 3, we showed that phytocannabinoids mediated their antinociceptive effects in the visceral pain model through the CB1 receptor, therefore this series of experiments were designed to examine specific components of the endocannabinoid system and its role in modulating visceral nociception. The first objective was to use a selective CB1 and CB2 receptor agonist in an effort to determine cannabinoid receptor involvement.

In the first set of experiments, mice were pretreated (1 h) with either the selective CB1 agonist (ACEA) (Hillard et al., 1999), or the selective CB2 agonist (O-3223) (Kinsey et al., 2011). Afterward mice were given an i.p. injection of 0.6% acetic acid, placed in observation chambers for a 3 min acclimation period, and then scored for abdominal stretching for a total of 20 min.

As illustrated in Figure 8A, ACEA (10 mg/kg) significantly attenuated abdominal stretching ($p < 0.01$). However, the selective CB2 agonist at the low and high dose tested did not affect abdominal stretching, suggesting that this receptor does not play a role in the antinociceptive effects produced by cannabinoids. To confirm the CB1 receptor mechanism, we

employed the selective CB1receptor antagonist, rimonabant. Mice were pretreated with either vehicle or rimonabant (3 mg/kg) for 10 min, followed by a subcutaneous administration of ACEA (10 mg/kg). The antinociceptive effects produced by ACEA in the abdominal stretching model were significantly blocked by the pretreatment of rimonabant ($p<0.01$) Figure 8B.

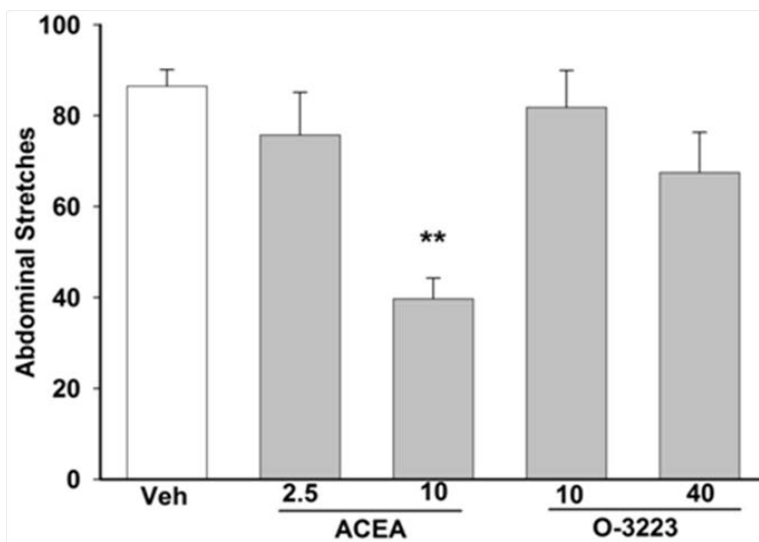
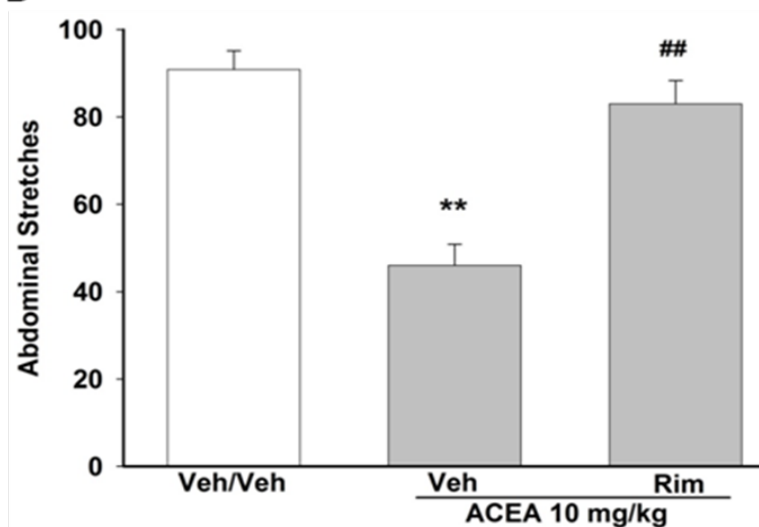
A

Figure 8. Assessment of the selective CB1 agonist, ACEA and the selective CB2 agonist (O-3223) on abdominal stretching. (A) ACEA and not O-3223 reduced abdominal stretching (10 mg/kg, s.c).

B

Rimonabant (3 mg/kg, s.c.) blocked the antinociceptive effects of ACEA. n =6-8 mice per group. ** $p < 0.01$ versus vehicle, ## $p < 0.01$ versus vehicle/ACEA. All data represent the mean ± SEM abdominal stretching

4.2 FAAH knockout mice display an antinociceptive phenotype in the acetic acid abdominal stretching test

While direct-acting cannabinoid receptor agonists such as ACEA possess analgesic properties, their psychomimetic side effects have dampened enthusiasm for their development as therapeutic agents. Conversely, increasing endogenous cannabinoid levels by blocking FAAH represents an attractive alternate approach to elicit antinociception, but without eliciting cannabimimetic effects (Cravatt et al., 2001; Gobbi et al., 2005). Deletion of the FAAH gene increases levels of anandamide, accompanied with CB1 receptor-mediated hypoalgesic phenotypes in models of acute and inflammatory pain (Cravatt et al., 2001). Hence, our aim was to first assess the effect of genetic deletion of FAAH in the abdominal stretching model and determine the receptor mechanism of action underlying the antinociceptive effects caused by FAAH deletion. Unlike our previous experiments, in these studies we used an inbred strain of male mice (C57BL/6) since our genetically modified mice are backcrossed on this background strain.

FAAH (-/-) mice displayed a significant attenuation of acetic acid-induced nociception, $t(10) = 5.0$, $p < 0.001$ Figure 9A. Rimonabant and SR144528, were used to ascertain the involvement of CB1 and CB2 receptors in the antinociceptive phenotype exhibited by FAAH (-/-) mice. Rimonabant (3 mg/kg), but not SR144528 (3 mg/kg), significantly blocked the antinociceptive phenotype of FAAH (-/-) mice, $F(2,33) = 6.1$, $p < 0.01$ (Figure 9B). As we found previously in the outbred ICR strain of mice, the cannabinoid receptor antagonists administered

by itself to wild type mice did not alter the number of abdominal stretches compared with vehicle-treated control mice. These results suggest that the antinociceptive phenotype of FAAH (-/-) mice in the acetic acid model of visceral nociception is mediated through a CB1 cannabinoid receptor. Endocannabinoids are synthesized locally on demand from phospholipid precursors and regulate homeostasis. However, rimonabant or SR144528 administered alone did not alter abdominal stretching suggesting that endocannabinoid tone is not mediating the decrease in abdominal stretching.

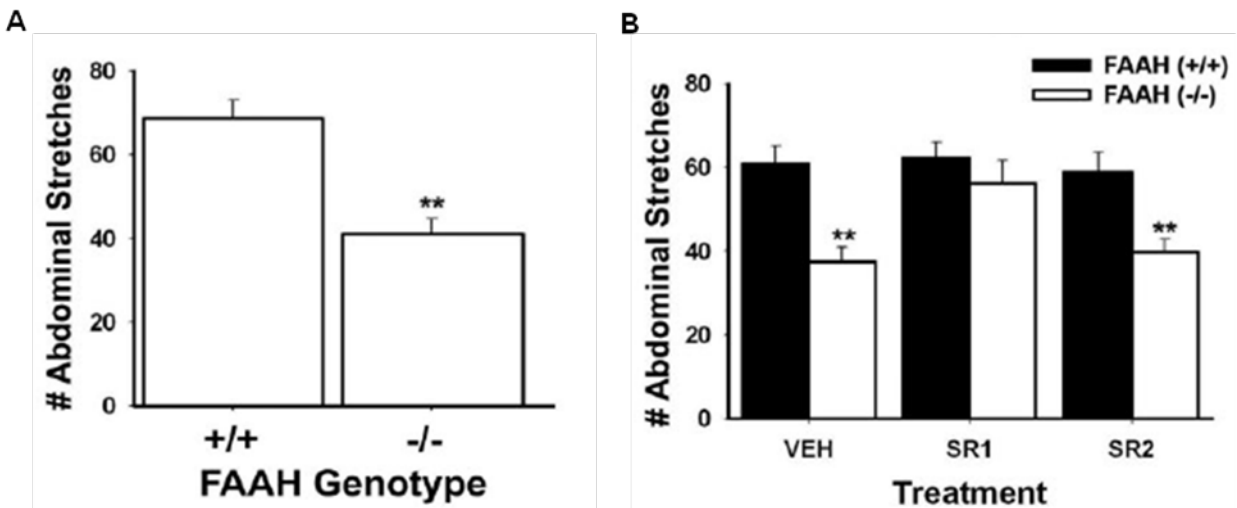


Figure 9. FAAH (-/-) antinociceptive phenotype in the acetic acid model of visceral nociception is mediated through a CB1 receptor mechanism of action. (A) FAAH (-/-) mice exhibit less abdominal stretching than FAAH (+/+) mice. $n = 8$ mice/group. (B) Pretreatment with rimonabant (SR1; 3 mg/kg s.c.), but not SR144528 (SR2; 3 mg/kg s.c.), prevented the FAAH antinociceptive phenotype in acetic acid-treated mice, $n = 12$ mice/group. ** $p < 0.01$ compared with respective control groups; data depicted as means \pm S.E.M.

4.3 FAAH and COX inhibition dose responsively suppress acetic acid abdominal stretching

Our previous study indicated that FAAH (-/-) mice show an antinociceptive phenotype in the abdominal stretching model. Similar to FAAH knockout mice, wild-type mice treated with FAAH inhibitors, such as URB597 (Kathuria et al., 2003) or the reversible FAAH inhibitor, OL-135 (Lichtman et al., 2004a), were previously shown to elicit hypoalgesic effects in acute models of pain that were accompanied with elevations of anandamide in the CNS (Lichtman et al., 2004a). In addition, FAAH inhibitors reduced hypersensitivity to thermal and mechanical hypersensitivity in neuropathic pain models (Chang et al., 2006; Russo et al., 2007). Consequently, we examined whether reversible and irreversible FAAH inhibitors would decrease abdominal stretching in acetic acid induced stretching model. Secondly, we sought to reveal the receptor mechanism of action for the observed antinociceptive effects of FAAH inhibition. To accomplish this goal we pretreated mice with the CB1 and CB2 receptor antagonist, rimonabant or SR144528 respectively, prior to administering each FAAH inhibitor. Additionally, since FAAH inhibitors have been illustrated to have an opioid receptor mechanism of action (Chang et al., 2006), we evaluated whether naltrexone, the opioid receptor antagonist, pretreatment would block the antinociceptive effects of FAAH inhibition.

As shown in Figure 10A, URB597 (3 and 10 mg/kg) significantly reduced acetic acid-induced abdominal stretching, $F(3,32) = 14.8, p < 0.001$. Additionally, diclofenac sodium (10, and 30 mg/kg s.c.) significantly attenuated stretching, $F(3,16) = 12, p < 0.01$. Pretreatment with rimonabant (3 mg/kg), but not SR144528 (3 mg/kg), significantly blocked the antinociceptive

effects of URB597 (Figure 10A). Likewise, administration of OL-135 (30 mg/kg), the reversible FAAH inhibitor produced CB1 receptor-mediated antinociceptive effects in the acetic acid model, $F(3,20)= 19.4, p < 0.001$ (Figure 10B). Furthermore, the antinociceptive effects of URB597 or OL-135 [$F(2,30) = 68, p < 0.001$] were not blocked by the opioid antagonist, naltrexone (1 mg/kg; see Figure 11).

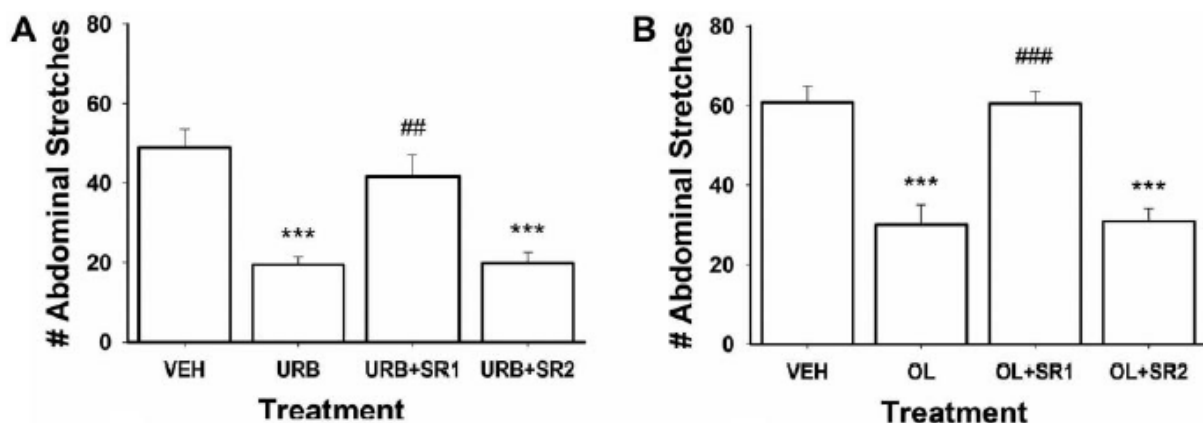


Figure 10. Pharmacological inhibition of FAAH reduces acetic acid-induced visceral nociception through a CB1 receptor mechanism of action. (A) pretreatment with URB597 (URB; 10 mg/kg s.c.), an irreversible FAAH inhibitor significantly reduced the number of abdominal stretches in acetic acid-treated mice. Pretreatment with rimonabant (SR1; 3 mg/kg s.c.), but not SR144528 (SR2; 3 mg/kg s.c.), prevented the antinociceptive effects of URB597. $n = 9$ mice/group. (B) OL-135 (OL; 30 mg/kg s.c.), a reversible FAAH inhibitor, significantly attenuated the number of abdominal stretches in acetic acid-treated mice. Pretreatment with SR1 (3 mg/kg s.c.), but not SR2 (3 mg/kg s.c.), prevented the antinociceptive effects of OL-135. $n = 6$ mice/group. *** $p < 0.001$ compared with the appropriate control group; ## $p < 0.01$ compared with URB-treated group; ### $p < 0.001$ compared with the OL-treated group.

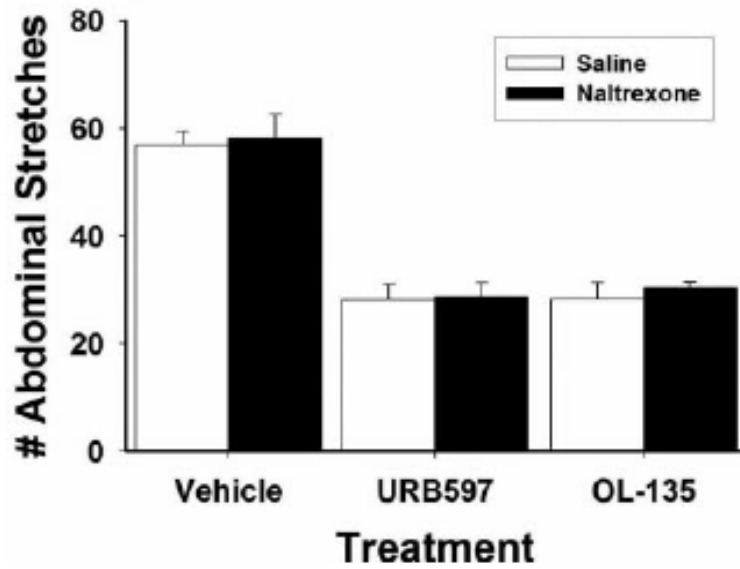


Figure 11. Antinociceptive effects of URB597 (10 mg/kg) or OL-135 (30 mg/kg) were not blocked by naltrexone (1 mg/kg). $n = 6$ mice/group. Data depicted as means \pm SEM abdominal stretches.

Previous studies illustrated that coadministration of an NSAID and a synthetic cannabinoid agonist, WIN55-212 elicited additive analgesic effects in the acetic acid abdominal stretching model (Ulugol et al., 2006). However, WIN55-212 produces THC-like cannabimimetic properties such as hypomotility, catalepsy, and hypothermia. However, FAAH inhibition does not produce these THC like effects. Therefore, we sought to determine if coadministration of a FAAH inhibitor and COX enzyme inhibitor can act synergistically to decrease abdominal stretching. NSAIDs are drugs that target cyclooxygenase (COX) enzymes for which there are two subtypes. COX-1 is constitutively active whereas COX-2 is inducible (Warner and Mitchell, 2004). However, both subtypes regulate the synthesis of prostaglandins (Samad et al., 2001) therefore blocking its activity reduces inflammatory responses. However, chronic use of NSAIDs results in severe gastrointestinal effects such as ulcer formation, thus COX inhibitors although very effective as analgesics, remain a clinical concern (Wallace, 1996). Conversely, there may be beneficial gain to treat pain with drugs of different classes in combination. Combination therapy is beneficial because it can potentiate the desired effects while diminishing unpleasant side effects associated with certain drugs by reducing the dose of each drug. Furthermore, it can reduce the chance of developing rapid tolerance to a single class of drugs. Hence, our next aim was to determine the effect of dual inhibition of FAAH and COX enzymes. Dual inhibition of both enzyme inhibitors may offer an attractive therapeutic approach by increasing analgesia and decreasing untoward side effects associated with NSAIDs alone.

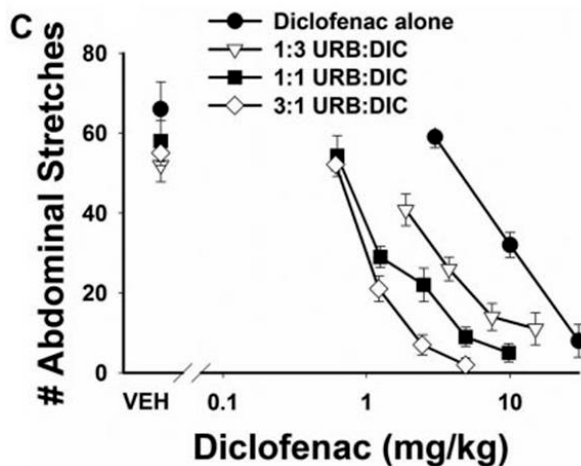
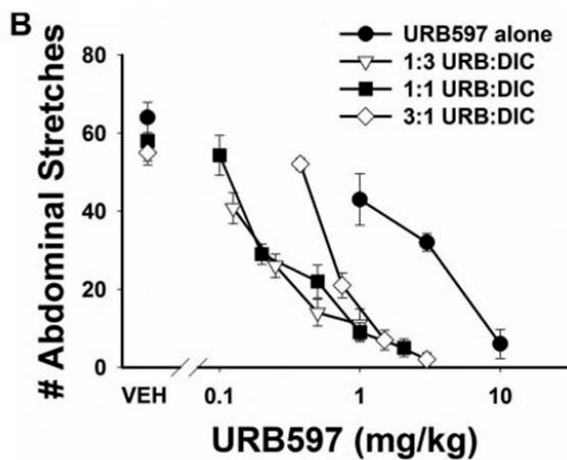
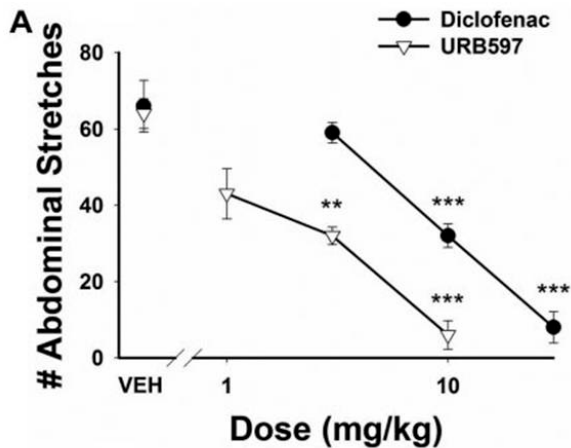


Figure 12. Synergistic effects of FAAH and COX inhibition. (A) FAAH and COX inhibition dose-dependently reduced nociceptive responses, URB597 (1–10 mg/kg s.c.) or diclofenac sodium (3–30 mg/kg s.c.) compared with the control groups. ** $p < 0.01$; *** $p < 0.001$ (B) dose effect curves for URB597 alone and in mixtures with diclofenac and (C) dose effect curves for diclofenac alone and in mixtures with URB597. Data represents the means \pm SEM abdominal stretches. $n=6$ mice/group.

Table 6. Fixed combinations of diclofenac and URB597 produce synergistic analgesic effects in the acetic acid abdominal stretching model. Predictive additive ED50 values (Z_{add}) and experimentally determined ED50 values (Z_{mix}) for mixtures of diclofenac and URB597 in mice. Doses for each drug in combination at the three different ratios are presented in the first column. * $p < 0.05$ as compared with respective Z_{add} (theoretical value) using the Fisher test.

Combination [Mixture (Doses in mg/kg)]	Mixture Ratio	ED ₅₀ mg kg ⁻¹ s.c. (95% Confidence Limit) Diclofenac/URB597 ^a	
		Z_{add} (Theoretical)	Z_{mix} (Experimental)
Diclofenac/URB597 0.6125/0.375 1.225/0.75 2.45/1.5 4.9/3.0	1:3	4.04 (3.37–4.80)	2.01 (1.68–5.49)*
Diclofenac/URB597 0.6125/0.125 1.225/0.25 2.45/0.5 4.9/1.0 9.8/2.0	1:1	5.91 (4.90–7.05)	2.16 (1.69–2.68)*
Diclofenac/URB597 1.875/0.125 3.75/0.25 7.5/0.5 15/1.0	3:1	7.81 (6.44–9.36)	4.14 (2.84–5.49)*

Both URB597 and diclofenac dose responsively reduced acetic acid abdominal stretching (Figure 12A-C). The ED50 values and 95% confidence limit for URB597 and diclofenac were 2.1 (1.5–2.8) and 9.8 (8.2–11.7) mg/kg, respectively. Shown in Figure 12B are the 1:3, 1:1, and 3:1 combinations of URB597 and diclofenac, with the dose of URB597 plotted on the x-axis. The dose response curve of URB597 alone is plotted in this graph for comparison. The same data are also plotted in Figure 12C, with the dose of diclofenac plotted on the x-axis. The dose response curve of diclofenac alone is also included in this graph for comparison. The plots of the combination ED50 values for both fixed ratios (total dose) in relation to the ED50 values of the drugs alone are shown in Figure 13. The isobologram analysis suggests that a synergistic interaction occurs between URB597 and diclofenac because the experimental point falls significantly below the line of additivity. The graphic display of synergism is confirmed mathematically by statistical analysis of the predicted additive ED50 values (Z_{add}) and experimentally derived ED50 values (Z_{mix}) shown in Table 6.

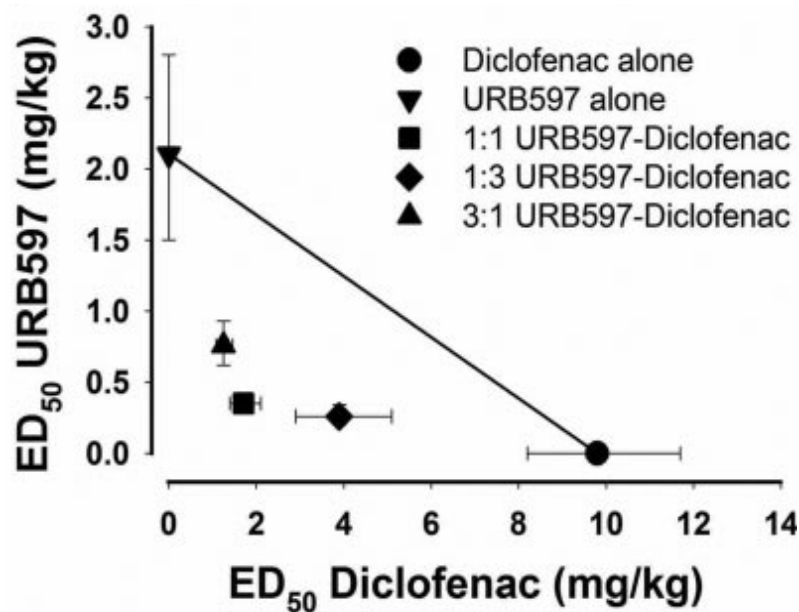


Figure 13. Synergistic interaction between FAAH and COX inhibition. Isobolographic analysis showing the interactions between diclofenac sodium and URB597 in the mouse acetic acid-induced abdominal stretching test. The ED₅₀ values for diclofenac and URB597 are depicted on the x- and y-axes, respectively. The isobole of additivity is shown as a solid line drawn between the ED₅₀ values of diclofenac and URB597. The experimental ED₅₀ values with 95% confidence interval of mixtures of URB597 and diclofenac at the fixed-ratio combinations of 3:1, 1:1, and 1:3 were significantly below the theoretical isoboles of additivity, indicating a synergistic interaction. *n* = 6 mice/group.

4.4 Antinociceptive effects of MAGL inhibition in the abdominal stretching model

Of the two primary endocannabinoid degradative enzymes only FAAH has been characterized for its role in behavioral effects *in vivo*. Recently it was discovered that approximately 85% of 2-AG hydrolysis is attributable to MAGL (Blankman et al., 2007). Therefore, we sought to determine the effects of inhibiting MAGL in the abdominal stretching model of visceral nociception. Although several first generation MAGL inhibitors have been synthesized, their selectivity and efficacy *in vivo* is limited. URB602 was among the first enzymes synthesized to target MAGL although it was later demonstrated that it has low potency (uM range) for MAGL (Makara et al., 2005) and may also target FAAH enzyme. N-arachidonoyl maleimide (NAM), another potential MAGL inhibitor was shown to inhibit up to 80% of MAGL, however it also blocked FAAH mediated effects (Blankman et al., 2007). However, the development of JZL184, a selective MAGL inhibitor shows great selectivity and efficacy *in vivo* (Long et al., 2009a). JZL184 selectively blocks MAGL and elevate 2-AG levels 10-fold in the brain. Conversely, JZL184 also inhibits FAAH at high concentrations, but its inhibition does not significantly elevate AEA levels.

Mice were treated with vehicle or JZL184 (4-40 mg/kg; s.c.) for 120 mins. Afterward, mice were administered acetic acid (0.6%; i.p.) then placed in observation chambers for 3 min acclimation period, followed by a 20 min observation period where they were scored for

abdominal stretching. In a follow-up study, mice were pretreated with rimonabant (3 mg/kg) followed 10 min later by JZL184 (40 mg/kg) and then scored for abdominal stretching.

JZL184 administration dose-dependently blocked acetic acid induced abdominal stretching. JZL184 (16 mg/kg and 40 mg/kg) significantly differed from vehicle (Figure 14). In order to determine whether the analgesic effects of JZL184 were mediated through the CB1 receptor, we pretreated mice with rimonabant followed by JZL184. The medium dose of JZL184 (16 mg/kg) was used to determine this effect since the highest dose resulted in sedative like effects. Rimonabant (3 mg/kg) significantly blocked abdominal stretches in the acetic acid stretching model, $p < 0.01$ versus vehicle. Furthermore, the antinociceptive effect was reversed with the pretreatment of rimonabant $##p < 0.01$ (Figure 15).

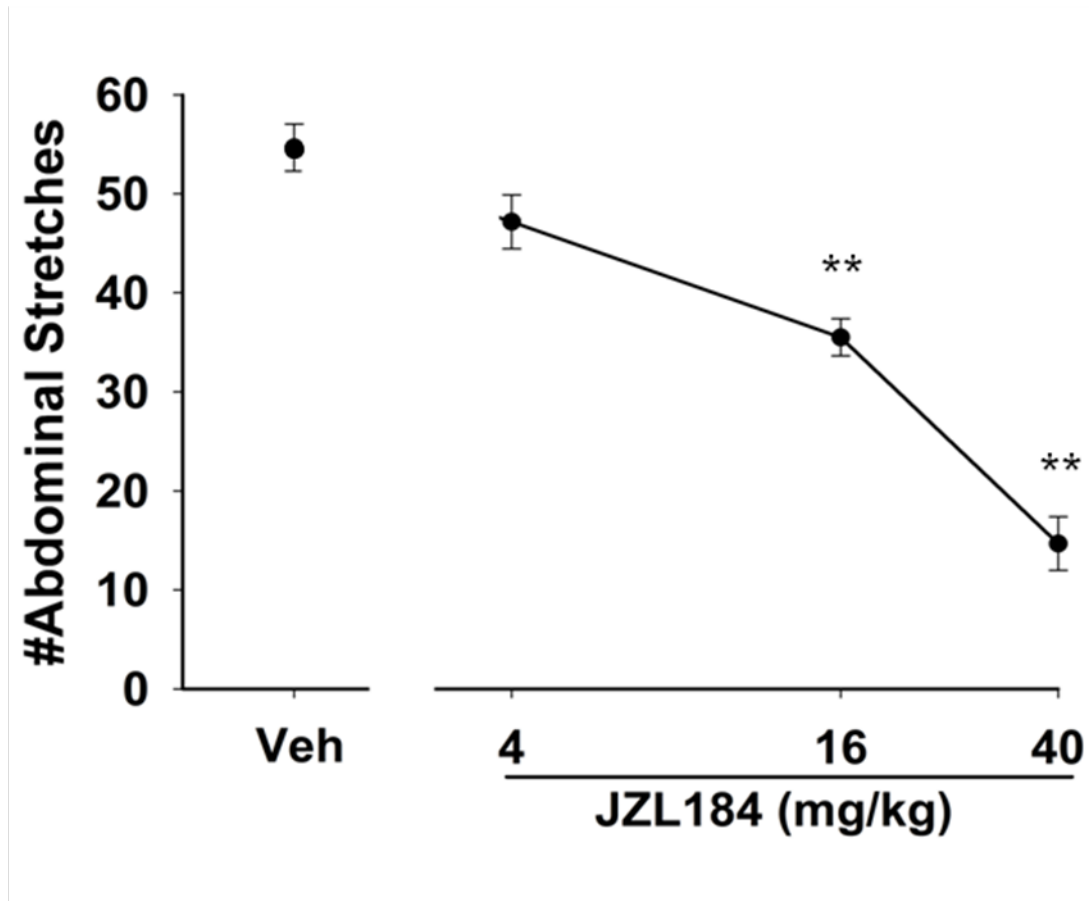


Figure 14. JZL Dose Response in acetic acid abdominal stretching model. 2 h pretreatment with JZL184 dose dependently attenuated the acetic acid abdominal stretches at 16 mg/kg and 40 mg/kg s.c. ****** $p < 0.01$ vs Veh. Data represents the mean \pm SEM abdominal stretches. $n=6$ mice per group.

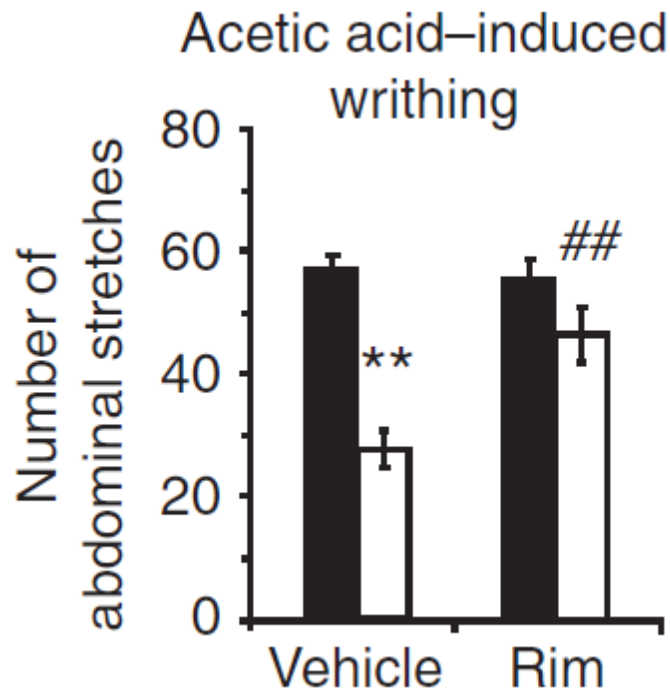


Figure 15. Behavioral effects of JZL184. JZL184 produced antinociceptive effects in the acetic acid abdominal stretching assay of noxious chemical pain sensation (16 mg/kg, intraperitoneally). ** $p < 0.01$ for vehicle-vehicle-treated mice (filled) versus vehicle-JZL184-treated mice (open) ## $p < 0.01$ for vehicle-JZL184-treated mice versus rimonabant-JZL184-treated mice. Data are presented as means \pm SEM; $n = 6-14$ mice per group.

4.5. Dual blockade of FAAH and MAGL in the abdominal stretching model

Due to the fact that FAAH inhibition and MAGL inhibition produced antinociceptive effects in the abdominal stretching model separately, we tested whether dual blockade of both enzymes would enhance the antinociceptive effect of one alone. Recently, JZL184 was described as possessing cannabimimetic effects such as hypothermia, catalepsy, and hypomotility (Long et al., 2009). Conversely, FAAH inhibition does not produce these effects therefore, inhibiting both FAAH and MAGL may potentiate the antinociceptive effects while decreasing the cannabimimetic effects produced by MAGL inhibition alone. To accomplish this objective, FAAH (-/-) mice were treated with JZL184 (16 mg/kg), then assessed in the abdominal stretching model. Complementary pharmacological studies were also conducted to confirm dual enzyme inhibited antinociceptive effects. To accomplish this aim, FAAH wild-type mice were treated with PF-3845, the selective FAAH inhibitor (Ahn et al., 2009), JZL184, or the dual FAAH/MAGL enzyme inhibitor, JZL195 (Long et al., 2009b). Furthermore, to determine the receptor mechanism of action, we evaluated the effect of the dual inhibitor in the presence of rimonabant (3 mg/kg). We hypothesize that dual inhibition of MAGL and FAAH produces an enhancement of effect in the acetic acid stretching test greater than the sum of inhibiting one enzyme. C57BL/6J mice were injected with vehicle, JZL184 (16 mg/kg; s.c.), PF-3845 (10 mg/kg; s.c.), or JZL195 (20 mg/kg; s.c.) 2 h prior to the administration of acetic acid (0.6%; i.p.).

FAAH (-/-) mice which have elevated levels of AEA, show a reduction in abdominal stretches compared to their wild-type match. Additionally, FAAH (-/-) mice administered JZL184 show an enhanced antinociceptive phenotype compared to FAAH (-/-) mice alone $p < 0.01$ (Figure 16). This effect was reversed with rimonabant (3 mg/kg) pretreatment. Pharmacological inhibition was also assessed. FAAH (+/+) mice treated with PF-3845 or JZL184 showed a significant reduction in abdominal stretching $p < 0.01$ vs FAAH (+/+) vehicle. Furthermore, the dual inhibitor JZL195 significantly enhanced the antinociceptive effects compared to PF-3845 or JZL184 alone. Lastly, we determined that the antinociceptive effect was mediated through the CB1 receptor mechanism of action, since rimonabant blocked the antinociceptive effects of JZL195 (Figure 16).

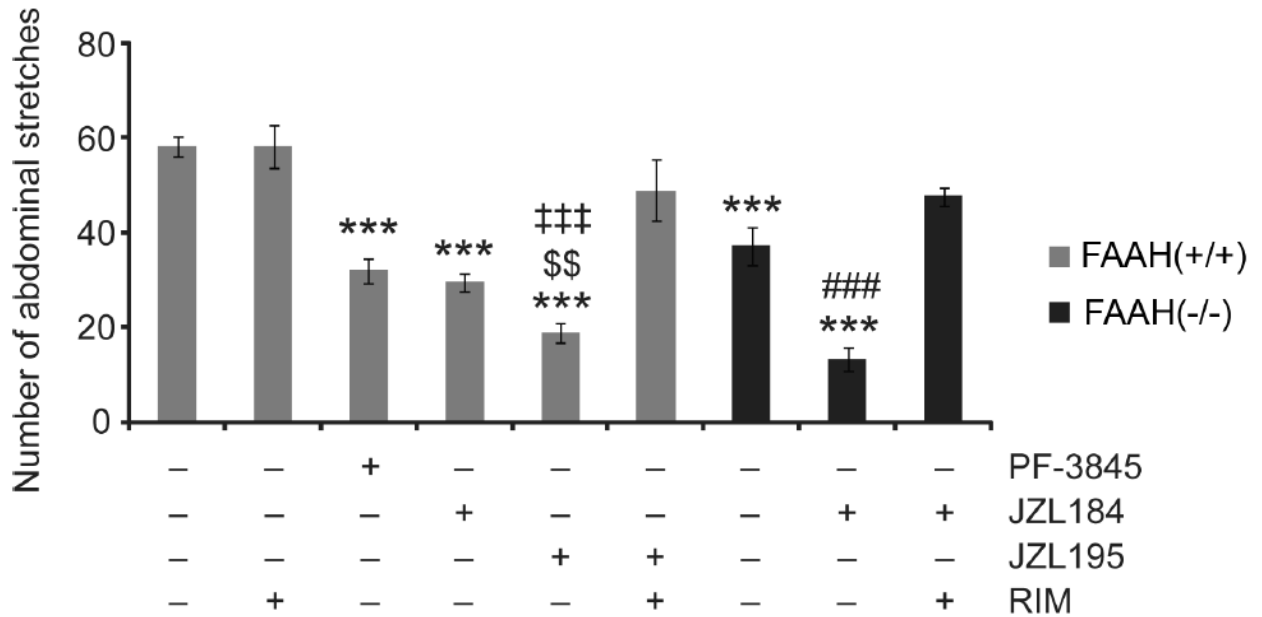


Figure 16. FAAH (-/-) mice show antinociceptive effects in abdominal stretching model.

Dual blockade of FAAH and MAGL produces enhanced antinociceptive effects in the acetic acid model of visceral pain sensation through a CB1 receptor mechanism of action. Subjects were treated with rimonabant (RIM, 3 mg/kg), JZL195 (20 mg/kg), JZL184 (16 mg/kg), or vehicle. *******, $p < 0.001$ vs. FAAH (+/+) vehicle group; **###**, $p < 0.001$ vs. FAAH (-/-), JZL184, and FAAH (-/-) + JZL184 + RIM. **###**, $p < 0.001$ vs. JZL195 + RIM; **\$\$**, $p < 0.01$ vs. JZL184 or PF-3845 (planned comparison). Data are presented as means \pm SEM. $n = 7-10$ mice per group. Data in this figure were compiled from four different experiments (7-23 mice per group), with each experiment consisting of 7-8 mice per group.

4.6 Discussion: Endocannabinoid degradative enzyme inhibition produces antinociception via CB1 receptor

The first goal of the current chapter in this dissertation was to test the hypothesis that CB1 receptor activation reduces visceral nociception. Accordingly, we treated mice with selective CB1 (ACEA) and CB2 agonist (O-3223) and found that only the CB1 receptor agonist blocked the stretching response. Furthermore, we confirmed the receptor mechanism of action by pre-treating mice with the selective CB1 antagonist, rimonabant. Rimonabant blocked the antinociceptive effects of ACEA, implicating that this receptor has a role in the analgesic effects produced by cannabinoids in the abdominal stretching model. These results are consistent with previous data where we showed THC's analgesic effects in the abdominal stretching model are reversed by rimonabant and not SR144528.

The second goal of this chapter was to determine the effects of indirectly elevating anandamide levels by genetic deletion of FAAH or pharmacological inhibition of the FAAH enzyme on abdominal stretching. FAAH (-/-) mice showed an antinociceptive phenotype when treated with acetic acid. This effect was reversed with rimonabant and not SR144528. Previous research has also found that FAAH (-/-) mice and mice treated with FAAH inhibitors possess elevated levels of anandamide in the CNS and periphery that reduce baseline pain thresholds to noxious thermal and chemical stimuli (Lichtman et al., 2004)(Kathuria et al., 2003).

In addition, FAAH (-/-) mice possess increased brain levels of fatty acid amides, including *N*-palmitoyl ethanolamine (PEA) (Cravatt et al., 2001), and *N*-acyl taurines (Saghatelian et al., 2006), which could also contribute to the antinociceptive phenotype. PEA has been revealed to have anti-inflammatory actions in acetic acid test, and formalin test, (Calignano et al., 2001, LoVerme et al., 2005) which were reversed with SR144528 and not rimonabant. Thus, it is probable that elevated levels of PEA or other lipid signaling molecules, in addition to AEA, may contribute to the antinociceptive phenotype observed in FAAH (-/-) mice or mice treated with FAAH inhibitors. The antinociceptive effects in FAAH (-/-) mice were mediated via a CB1 and not a CB2 mechanism of action as previous studies demonstrated (Lichtman et al., 2004). Our pharmacology studies revealed that wild-type mice when treated with either a reversible FAAH inhibitor (OL-135), or an irreversible FAAH inhibitor (URB597) displayed a reduction in the total number of abdominal stretches which were reversed by CB1 antagonism. Conversely, we did not find any involvement of the opioid system in the antinociceptive effects produced by FAAH inhibition as previously illustrated (Chang et al., 2006). These differences may be due to the fact that these previous studies used rats as subjects whereas we assessed these effects in mice. Furthermore, stress induced by the spinal nerve ligation or mild thermal injury model may result in the release of endorphins and subsequent activation of the opioid system.

Due to the fact that NSAIDS and FAAH inhibitors can reduce abdominal stretching independently, we investigated whether blockade of COX and FAAH simultaneously would

enhance the antinociceptive effect of one alone. COX regulates the production of proinflammatory agents such as prostaglandins which have been shown to induce abdominal stretches (Matsumoto et al., 1998) on their own. Therefore, inhibiting COX should reduce abdominal stretching. Diclofenac, a COX inhibitor dose dependently suppressed the abdominal stretching response, although the dose that blocked abdominal stretching was 4 fold higher than the dose of the FAAH inhibitor in this model. Nevertheless, these data suggest that FAAH inhibition is as effective as a therapeutically used NSAID in the acetic acid stretching model. Since both COX and FAAH inhibition reduced stretching when administered alone, we determined whether these two agents would have an enhancement of effect when administered together. Mice were treated with equipotent doses of diclofenac and URB597 in combination in a dose response study. Isobolographic analysis demonstrated that simultaneous administration of FAAH and COX produced synergistic interactions thereby illustrating the potential of combination therapy in the treatment of visceral pain.

Although FAAH has been highly characterized and there are several investigative tools to assess its value, the other primary enzyme inhibitor MAGL has not been investigated in *in vivo* assays. The reason for lack of data on MAGL is that there is a limited availability of selective inhibitors for MAGL. URB602 was the first reported MAGL inhibitor able to elevate 2-AG levels although it also targeted FAAH (Comelli et al., 2007; King et al., 2007; Vandevoorde et al., 2007). URB754 was thought to also be a selective MAGL inhibitor, although the initial characterization of URB754 was recanted due to contaminant issues (Tarzia et al., 2007).

However, the recent development of JZL184, a selective and efficacious MAGL inhibitor made the task of assessing the contribution of MAGL inhibition possible. JZL184 elevates 2-AG levels 8-10 fold but does not modulate levels of anandamide. Furthermore, JZL184 produced analgesia in the tail-flick (Long et al., 2009a), as well as the chronic constriction injury model (Kinsey et al., 2009a). Therefore, we tested the hypothesis that MAGL inhibition blocks acetic acid induced abdominal stretching via a CB1 receptor mechanism. JZL184 dose-dependently blocked acetic acid-induced abdominal stretching. This effect was mediated through the CB1 receptor.

We previously illustrated that FAAH and MAGL inhibition are capable of blocking acetic acid abdominal stretching independent of each other. This information along with the fact that FAAH is located in the postsynaptic terminal and MAGL is located presynaptically, suggests that these lipids may activate different circuits in an effort to maintain homeostasis (on demand synthesis and release). Due to the fact that inhibiting either endocannabinoid degradative enzyme can suppress the nociceptive effect produced by acetic acid, we assessed the effect of increasing both endocannabinoids simultaneously via dual inhibition of FAAH and MAGL degradative enzymes. Using JZL184 in combination with FAAH (-/-) mice, or the dual FAAH/MAGL inhibitor JZL195 significantly enhanced the suppression of abdominal stretches in comparison to blocking one enzyme alone. Both effects were blocked by CB1 antagonism. Although dual inhibition is seemingly beneficial from a therapeutic standpoint, studies from our laboratory illustrated otherwise. Mice treated with the dual FAAH/MAGL enzyme inhibitor

JZL195 (20 mg/kg) produced a greater antinociceptive response in the tail immersion assay of thermal pain compared to FAAH or MAGL inhibitors alone. However, the same mice treated with JZL195 also showed catalepsy and hypomotility which are characteristics of direct acting cannabinoid agonist such as THC. Dual FAAH/MAGL inhibition also caused profound THC-like discriminative stimulus effects as well in the discrimination model. The drug discrimination model serves as an animal model for marijuana intoxication and drugs that substitute for THC in this assay are predicted to have marijuana-like subjective effects in humans (Long et al., 2009b). Therefore this study suggests that simultaneous elevation of anandamide and 2-AG produces untoward psychoactive effects as those associated with direct acting cannabinoid agonist such as THC. Further testing using isobolographic analysis could determine an ideal ratio of FAAH and MAGL inhibitors given in combination that produce optimal antinociceptive effects, with minimal side effects.

Chapter 5: FAAH inhibitors act in the nervous system to reverse lipopolysaccharide-induced mechanical allodynia in mice

5.1 Development of the LPS-induced allodynia model

Inflammatory pain is a debilitating disease that can result in increase pain sensitivity. It can be caused by various mediators such as neuropeptides and cytokines (interleukins: IL-1, IL-2, IL-6, tumor necrosis factor alpha (TNF- α) which cannabinoids have been shown to modulate (Cabral and Marciano-Cabral, 2005; Klein et al., 2003). Unlike the acetic acid abdominal stretching model, cannabinoids in inflammatory pain models favor a CB2 receptor mechanism of action (Ashton, 2007; Guindon and Hohmann, 2008; Gutierrez et al., 2007). Administration of the selective CB2 agonist GW405833 produced anti-inflammatory effects in the carrageenan model of inflammatory pain (Clayton et al., 2002). These effects were reversed with SR144528 suggesting a CB2 receptor involvement. Furthermore, it was demonstrated that exogenous 2-AG administration produced antinociceptive effects in the formalin model of inflammatory pain (Guindon et al., 2007). Inflammation was induced in rat hind paws by intraplantar injection of formalin and subjects were assessed over the next 60 min. Exogenous 2-AG was administered subcutaneously into the dorsal surface of the paw at doses 0.01-100 μ g. 2-AG dose dependently decreased pain behavior however these effects were blocked with the pretreatment of AM630, a selective CB2 receptor antagonist and not AM251, a selective CB1 receptor antagonist.

Therefore, our aim in this chapter is to determine whether genetic deletion or pharmacological

inhibition of the endocannabinoid catabolic enzyme FAAH and MAGL, block LPS-induced allodynia. Additionally, we seek to determine if the anti-allodynic effects are mediated through the CB1 or CB2 receptor.

5.2 Methods

Male C57BL/6 mice were injected intraplantarly in one paw with lipopolysaccharide (LPS) and saline into the opposite paw. This model takes advantage of the fact that each mouse serves as its own control, thereby eliminating the idea that motor deficits were the result of the observed effect. LPS is a bacterial endotoxin derived from the outer cell wall of gram (-) bacteria that induces an inflammatory response when injected into the paw. 24 h after mice are injected with LPS they are examined for a tactile allodynic response. Allodynia is illustrated as a painful response to a non-noxious stimuli, and can be assessed using von Frey filament hairs. These filaments vary in thickness and possess different bending forces ranging from 0.6 g-6.0 g. The bending force at which mice withdraw their paw is determined and recorded as the paw withdrawal threshold (PWT).

Current models of inflammatory pain (e.g. carrageenan, complete Freund's adjuvant, LPS) produce severe edema, which may confound interpreting of the results by decreasing the observation of subtle behavioral effects, or by reducing the ability of mice to move their paw. As a result we modified the previously characterized LPS model of inflammation developed out

of our laboratory (Naidu et al., 2010), in order to induce tactile allodynia without producing overt edema of the paw or eliciting a general malaise (e.g., loss of body weight). To ensure that our model is representative for detecting analgesic compounds, we tested the positive control gabapentin, which possesses efficacy in treating various types of pain (Stahl et al., 2009), and served as the positive control. In addition, we examined the effects of global activation of cannabinoid receptors, using the phytocannabinoid THC which was shown in the acetic acid stretching model to have antinociceptive properties.

Intraplantar LPS elicited profound allodynia in the LPS-treated paw, but not the saline-treated, control paw [$F(3,24)=10.54$, $p < 0.001$; Figure 17A], and occurred at concentrations 10 fold less than those required to produce paw edema [$F(3,24)=11.18$, $p < 0.0001$; Figure 17B]. Whereas the high dose of LPS (25 μg) produced a significant increase in paw thickness, at 2.5 μg , LPS had no effect on edema measurements, as compared to the saline injected paw. In addition to the lack of edematous actions caused by intraplantar injection of 2.5 μg LPS, no weight loss occurred. Thus, this dose of LPS was used in all subsequent experiments.

LPS injection significantly decreased mechanical paw withdrawal threshold (PWT), as compared to saline-treated paws, which remained constant throughout all of the studies. The GABA analogue gabapentin significantly reversed LPS-induced allodynia [$F(3,24)=4.4$, $p < 0.05$; Figure 18A)]. Gabapentin did not affect paw withdrawal threshold in saline-injected control paws ($p=0.64$; Figure 19A). Similarly, THC significantly reversed LPS-induced allodynia in the

LPS-treated paw [$F(3, 28)=5.71, p<0.01$; Figure 18B], but did not modify the saline-treated paw threshold (Figure 19B).

5.3 FAAH (-/-) mice show an anti-allodynic phenotype

In the second series of experiments, we examined whether genetic deletion of FAAH reduces LPS-induced allodynia. FAAH-NS mice, which express the enzyme under a neural specific enolase promoter (Cravatt et al., 2004), were used to distinguish whether inhibiting FAAH expressed in the peripheral and/or nervous system(s) mediates the observed anti-allodynic effects. Third, we examined whether pharmacological blockade of FAAH would reverse LPS-induced allodynia by comparing the anti-allodynic effects of the reversible, α -keto heterocycle FAAH inhibitor OL-135 (Boger et al., 2005; Lichtman et al., 2004), to two irreversible FAAH inhibitors, the carbamate URB597 (Piomelli et al., 2006), and the piperidine urea PF-3845, which carbamylates FAAH's serine nucleophile (Ahn et al., 2009).

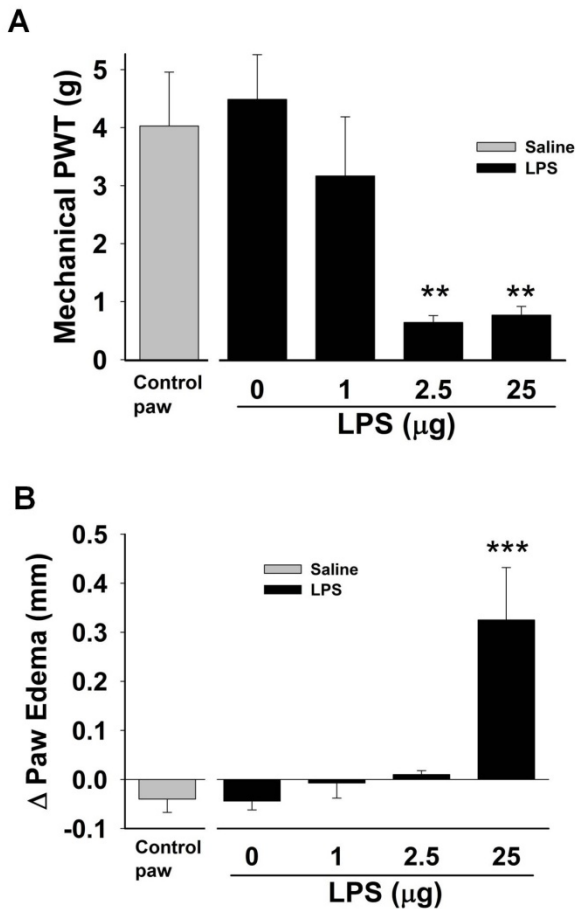


Figure 17. Intraplantar injection of LPS is more potent in eliciting tactile allodynia than in producing paw edema. **(A)** Decreased mechanical paw withdrawal thresholds were reduced in the LPS-injected paw 24 h after injection. LPS injection, at doses of 2.5 μg or 25 μg per paw, increased sensitivity to tactile stimulation that was significantly different from paw withdrawal threshold in the saline injected paw of the same mice. **(B)** Paw edema was significantly increased in hind paws injected with the high dose of LPS (i.e., 25 μg). Control

paw represents the saline-injected paw of LPS treated mice. Values represent the mean (\pm SEM) mechanical paw withdrawal threshold. $n=6-9$ mice/group. ** $p < 0.01$, *** $p < 0.001$ vs. saline-treated paw.

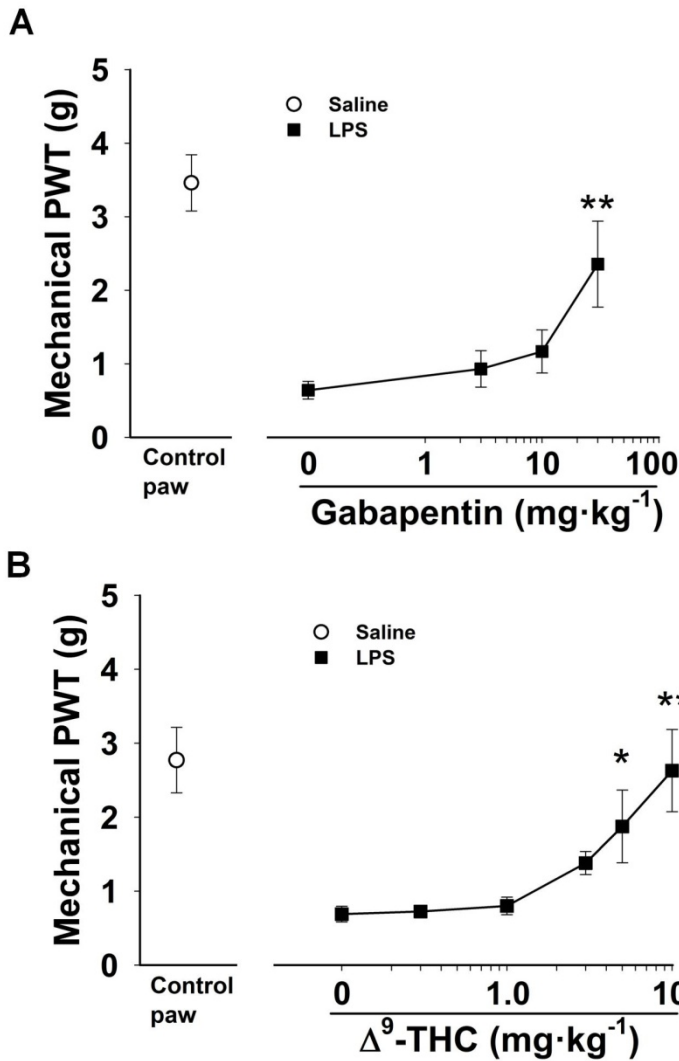
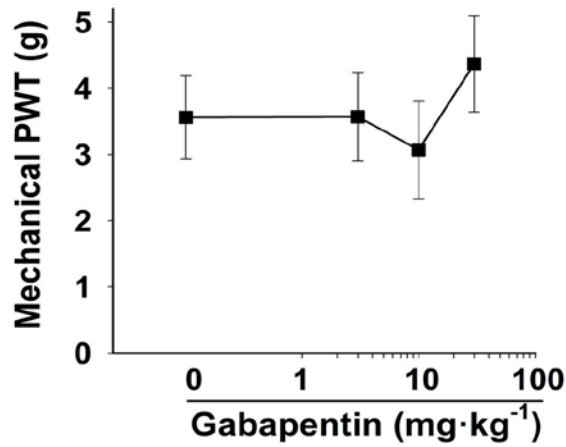


Figure 18. Systemic administration of gabapentin or THC reduces the tactile allodynia caused by intraplantar LPS (2.5 μ g). **(A)** Gabapentin (30 $\text{mg}\cdot\text{kg}^{-1}$) reversed tactile allodynia induced by LPS. **(B)** THC (5 or 10 $\text{mg}\cdot\text{kg}^{-1}$) reversed LPS-induced allodynia in the treated paw. The control paw represents the saline-injected paw of LPS treated mice **(C, D)**. Values represent the mean (\pm SEM) mechanical paw withdrawal threshold. $n=6-10$ mice/group. * $p < 0.05$; ** $p < 0.01$ vs. vehicle in the LPS-treated paw.

A



B

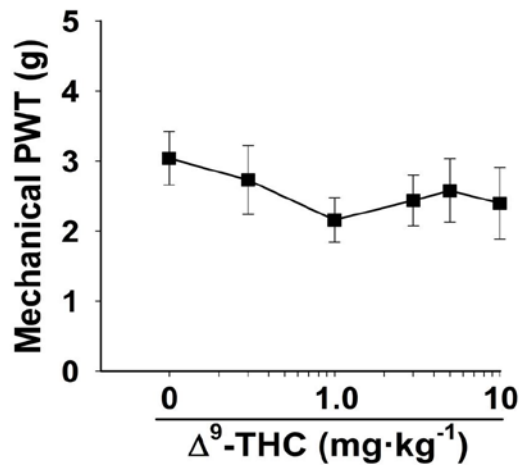


Figure 19. Systemic administration of neither gabapentin (**A**) nor THC (**B**) affected paw withdrawal thresholds in the control saline-injected paw. Values represent the mean (\pm SEM) mechanical paw withdrawal threshold. Data from the LPS-treated paws are depicted in Figure 18. n=6-10 mice/group.

FAAH (-/-) mice displayed significantly less allodynia to LPS treatment than either FAAH (+/-) control mice or FAAH-NS mice [$F(2,21)=8.99, p<0.01$; Figure 20A]. FAAH-NS mice showed wild type responses to LPS-induced inflammatory pain, indicating that the FAAH anti-allodynic phenotype is mediated by elevated FAAs in the nervous system. In addition, there were no genotype differences between groups in the saline-treated paw ($p=0.96$, Figure 21A). Next, we examined the receptor mechanism(s) of action underlying the observed anti-allodynic effects in FAAH-compromised mice. Several studies have implicated a role for CB₁ receptors in reducing hyperalgesia and CB₂ receptors in ameliorating edema (see review (Anand et al., 2009)). A recent study from our laboratory illustrated that FAAH (-/-) mice showed an antiedematous effect, which was prevented by pretreatment by the CB₂ antagonist SR144528 and not the CB₁ antagonist, rimonabant. However, the anti-hyperalgesic effects were blocked with both rimonabant and SR144528 (Naidu et al., 2010). Furthermore, CB₂ (-/-) mice were resistant to the antiedematous effects of FAAH inhibition suggesting a role for CB₂ in modulating edema. Another study demonstrated that in the carrageenan-evoked model of inflammation, the effects of AM1241, a purported CB₂ agonist, were blocked by SR144528, but not rimonabant (Gutierrez et al., 2007)) again reinforcing the theory for CB₂ receptor involvement in edema. Conversely, the antihyperalgesic effects of cannabinoids are shown to be mediated through the CB₁ receptor mechanism of action. We showed in the previous chapters that phytocannabinoids such as marijuana and FAAH inhibitors reduced acetic acid abdominal stretching, which was reversed by rimonabant and not CB₂ antagonism. Furthermore, other reports suggest a similar

role for CB1 in modulating pain. Anandamide administered locally into the rat hind paw reversed pain in the formalin pain model and was reversed by CB1 antagonism and not CB2 (Guindon et al., 2006; Gutierrez et al., 2007). Thus, we sought to determine whether these two cannabinoid receptors play a role in the anti-allodynic effects of FAAH (-/-) mice in our model of LPS-induced allodynia. To determine the receptor mechanism of action by which FAAH inhibition caused anti-allodynic effects, FAAH (-/-) mice were pretreated with the CB₁ receptor antagonist, rimonabant or the CB₂ receptor antagonist, SR144528. Either rimonabant or SR144528 completely reversed the FAAH anti-allodynic phenotype ($p < 0.05$; Figure 20B). No significant effects were found in the saline-treated paw (Figure 21B).

5.4 Pharmacological blockade of FAAH reverses LPS-induced allodynia

Although genetic deletion of FAAH reversed the paw withdrawal thresholds, pharmacological inhibition of FAAH is of clinical importance. Therefore, we examined whether pharmacological blockade of FAAH would reverse LPS-induced allodynia by comparing the anti-allodynic effects of the reversible, α -ketoheterocycle FAAH inhibitor OL-135 (Boger et al., 2005; Lichtman et al., 2004), to two irreversible FAAH inhibitors, the carbamate URB597 (Piomelli et al., 2006), and the piperidine urea PF-3845, which carbamylates FAAH's serine nucleophile (Ahn et al., 2009). In addition, LC/MS/MS analysis was used to quantify endocannabinoid levels after systemic, local, and intracranial ventricular administration of PF-3845. As shown in Figure 22A, administration of each FAAH inhibitor, PF-3845 [$F(3,24)=6.07$,

$p < 0.01$], URB597 [$F(3,30) = 5.95$, $p < 0.01$], or OL-135 [$F(4,41) = 3.82$, $p < 0.01$] significantly reversed LPS-induced tactile allodynia, but did not modify paw withdrawal thresholds in the saline-injected paw (Figure 23A). As shown in Table 7, i.p. administration of PF-3845 (10 mg·kg⁻¹) led to significant increases of AEA levels, but not 2-AG levels, in the brain and spinal cord. In light of the recent report of Clapper et al. (2010) showing that a peripherally restricted FAAH inhibitor reduces pain responses, we next evaluated whether intraplantar administration of PF-3845 reverses LPS-induced allodynia. Intraplantar administration of PF-3845 (1, 3, or 10 µg) reversed allodynia in the LPS administered paw $p < 0.01$ (Figure 22B), but did not affect withdrawal responses in the saline-treated paw (Figure 23B). Additionally, local administration produced a smaller effect when compared to systemic FAAH inhibition. More importantly, an intraplantar injection of PF-3845 (1 µg) to the saline-treated paw did not alter allodynia in the LPS-treated paw (Figure 22C), consistent with the notion that the drug effects were locally mediated. The observation that intraplantar administration of PF-3845 at 1 µg did not affect endocannabinoid levels in the brain or spinal cord, (see Table 7) further supports the idea that these doses of PF-3845 were mediated through a local site of action.

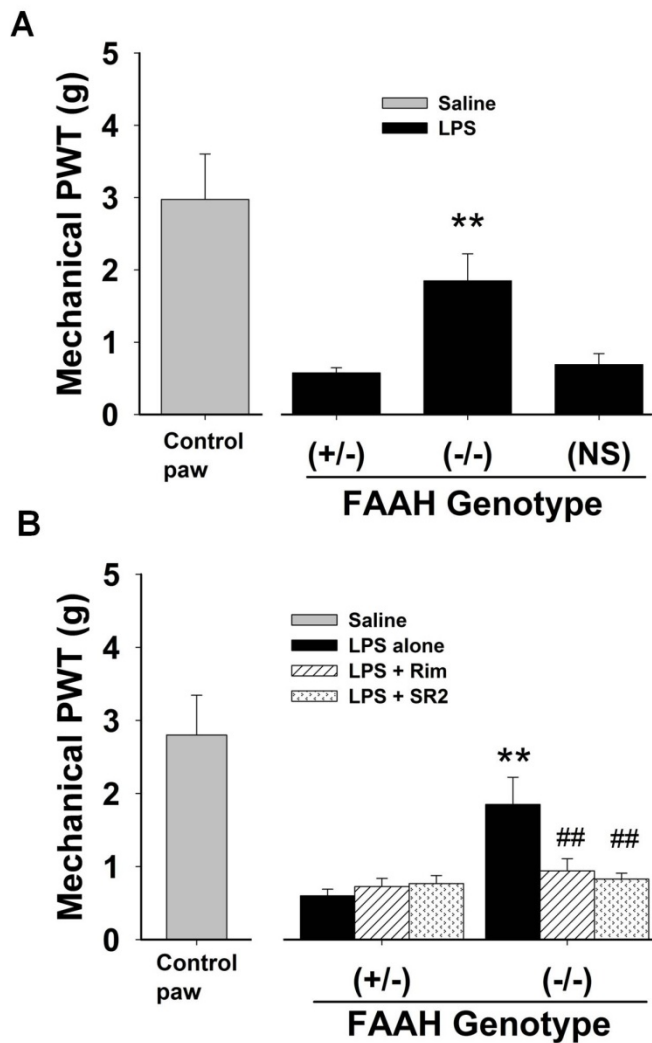


Figure 20. Deletion of FAAH within the nervous system reduces LPS-induced allodynia. (A) Control FAAH (+/-) mice displayed profound allodynic responses to intraplantar LPS (2.5 μg), whereas FAAH knockout mice (-/-) showed an anti-allodynic phenotype. FAAH-NS mice that express FAAH exclusively in neuronal tissue displayed wild type allodynic response to LPS. (B) The anti-allodynic phenotype in FAAH (-/-) mice was suppressed by the pretreatment of rimonabant (3

$\text{mg}\cdot\text{kg}^{-1}$; Rim) and SR144528 (3 $\text{mg}\cdot\text{kg}^{-1}$; SR2). Control paw represents the saline-injected paw of FAAH (+/-) LPS treated mice. All values represent the mean (\pm SEM) mechanical paw withdrawal threshold. $n=6-10$ mice/group. ** $p < 0.01$ vs. FAAH (+/-) mice or FAAH-NS mice (panel A); ## $p < 0.01$ vs. LPS-treated FAAH (-/-) mice that received vehicle.

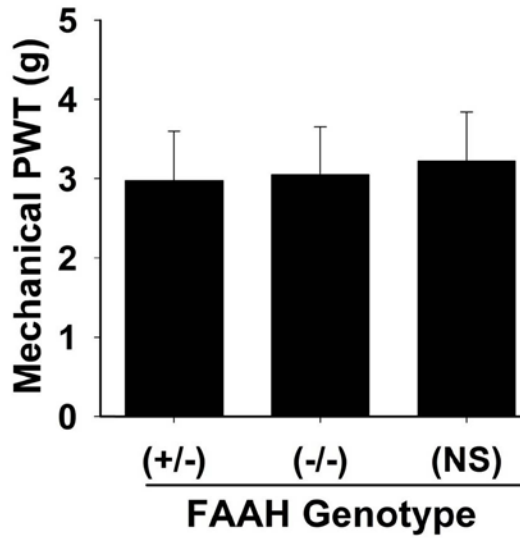
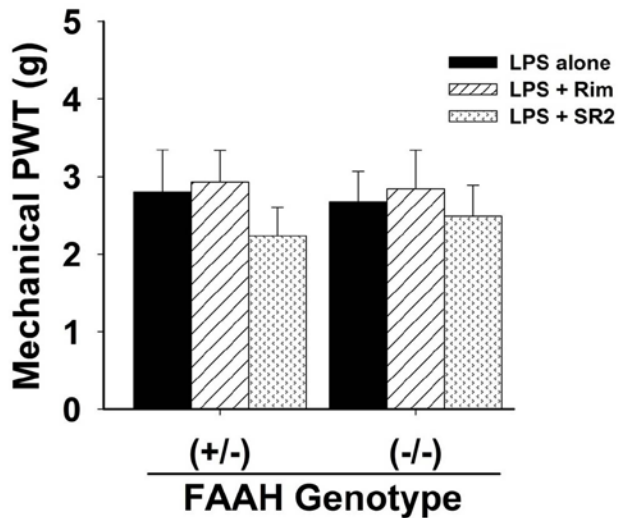
A**B**

Figure 21. (A) FAAH (-/-), FAAH-NS, and FAAH (+/-) mice showed identical paw withdrawal thresholds in the control saline-injected paw. (B) FAAH (-/-) and FAAH (+/-) mice displayed similar withdrawal thresholds in the control saline-injected paw and neither rimonabant (3 mg·kg⁻¹; Rim) nor SR144528 (3 mg·kg⁻¹; SR2) affected control responses. All values represent the mean (\pm SEM) mechanical paw withdrawal threshold. Data from the LPS-treated paws are depicted in Figure 20. n=6-10 mice/group.

In contrast, systemic administration of PF-3845 (10 mg/kg) or intraplantar injection of a much higher dose of drug (i.e., 10 μ g) caused significant increases in AEA, but not 2-AG levels in the brain and spinal cord. However, no differences in paw skin endocannabinoid levels by PF-3845 were detected at the highest concentration tested (see Table 7). Furthermore, in order to determine the location of FAAH inhibited anti-allodynic effects, we administered PF-3845 intrathecally. But first, we confirmed our method of injection by conducting a morphine dose response study in the tail withdrawal assay. Intrathecal morphine dose responsively produced antinociceptive response in the tail withdrawal model suggesting that our method of injection was accurate and reproducible (Figure 24). Subsequently, we administered PF-3845 (1 μ g -10 μ g) in a total volume of 5 μ l intrathecally then assessed mice for their paw withdrawal thresholds following LPS. Mice treated with intrathecal PF-3845 showed a reversal of paw withdrawal thresholds (Figure 25A). Conversely, the dose of PF-3845 that reversed paw withdrawal thresholds also increased brain anandamide levels (Figure 25B) suggesting that drug spread occurred. Due to the fact that intrathecal administration of low doses of PF-3845 resulted in elevated levels of anandamide in the brain, we could not determine with certainty if FAAH located spinally, or supraspinally in the case of intracranial administration mediated the anti-allodynic effects seen in our previous studies with FAAH inhibition.

In order to determine if fatty acid amides were indeed producing the anti-allodynic effect to FAAH inhibition, we examined whether PF-3845 would produce anti-allodynic effects in FAAH-NS mice. As shown in Figure 26, PF-3845 (10 mg/kg) elicited significant anti-allodynic

actions in FAAH (+/-) and FAAH (NS) mice, which significantly differed from mice treated with LPS only [$F(2,52)=4.35, p=0.018$]. In contrast, PF-3845 did not alter the anti-allodynic phenotype of FAAH (-/-) mice, suggesting that the anti-allodynic effects of this drug occurred because of its inhibition of FAAH in the peripheral and/or central nervous system(s). Additionally PF-3845 administration did not affect paw withdrawal in the control paw (Figure 27).

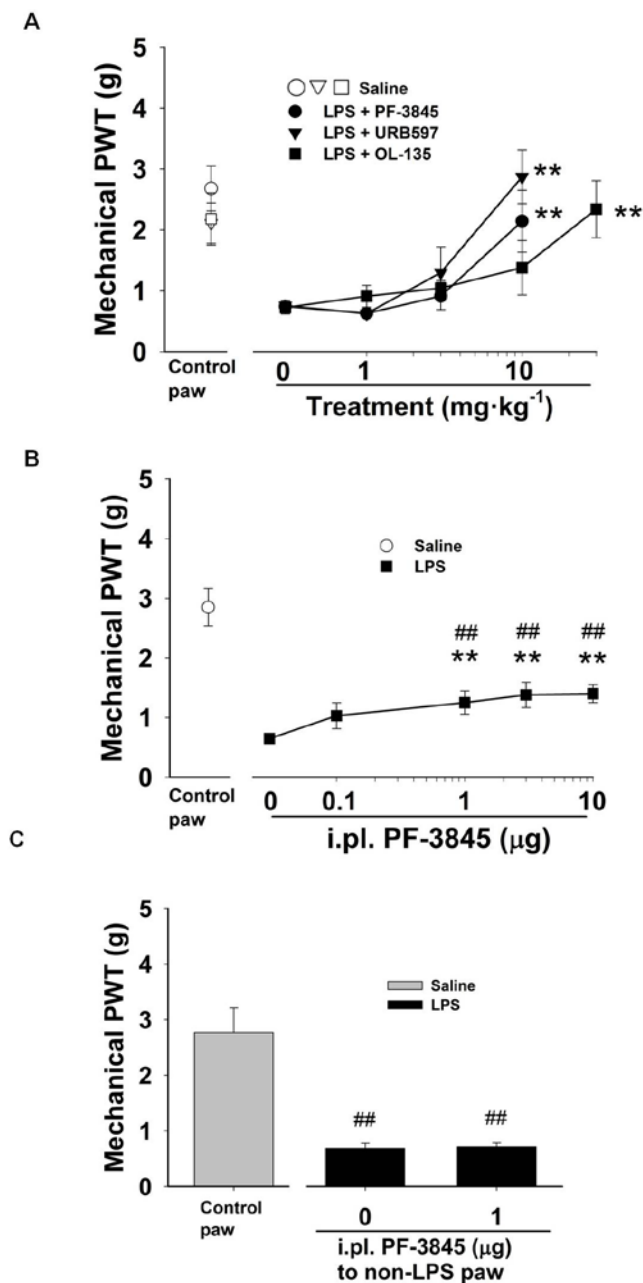


Figure 22. FAAH inhibitors reduce LPS-induced allodynia. **(A)** Three different FAAH inhibitors dose-dependently reversed tactile allodynia 24 h after intraplantar LPS administration. URB597 (10 mg·kg⁻¹; downward triangle), PF-3845 (10 mg·kg⁻¹; circle), and OL-135 (30 mg·kg⁻¹; square), reversed the allodynic response produced by intraplantar injection of LPS (2.5 µg). Open symbols represent the control-saline injected paw of each respective treatment group. **(B)** Intraplantar administration of PF-3845 (1, 3, or 10 µg) partially reversed LPS-induced allodynia. **(C)** PF-3845 administered to the saline-treated, control paw did not reduce allodynic responses in the LPS-treated paw. Control paw represents the saline-injected paw of LPS only treated mice.

Values represent the mean (\pm SEM) mechanical paw withdrawal threshold. $n=7-12$ mice/group.

** $p < 0.01$ vs. vehicle treatment in the LPS-treated paw; ## $p < 0.01$ vs. saline-treated paw.

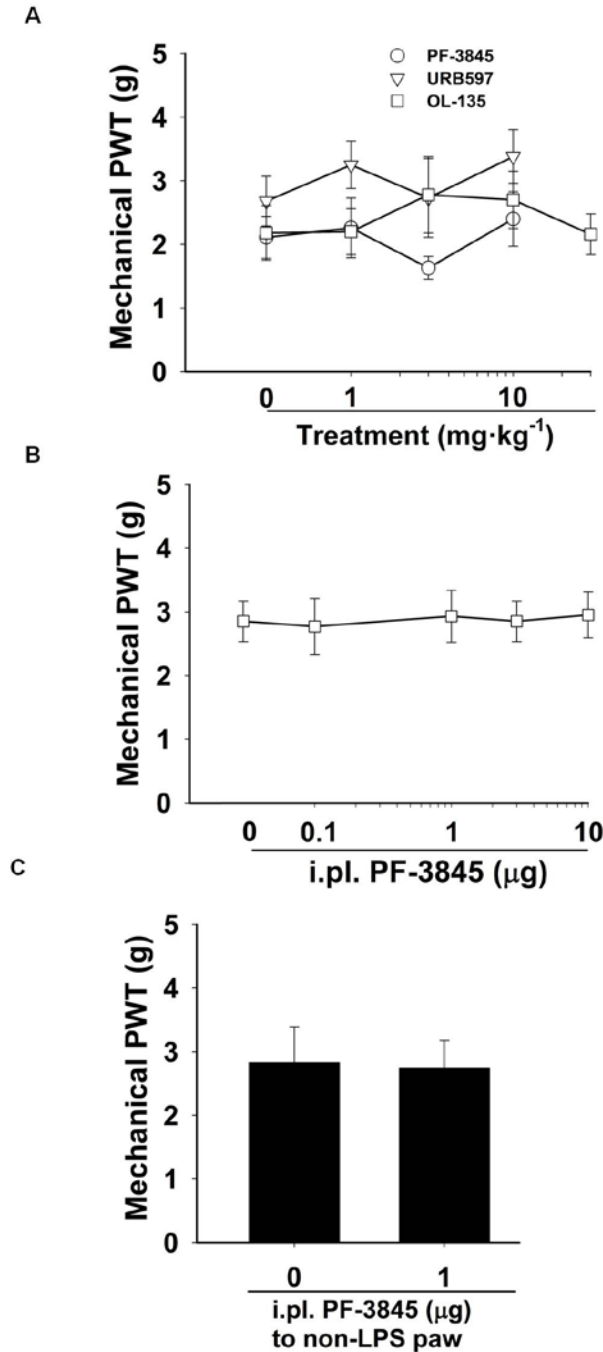


Figure 23. (A) Three different FAAH inhibitors did not affect paw withdrawal thresholds in the control saline-injected paw. (B) Intraplantar administration of PF-3845 into the LPS-treated paw did not affect withdrawal thresholds in the contralateral saline-treated paw. (C) PF-3845 administered to the saline-treated, control paw did not affect withdrawal responses. Values represent the mean (\pm SEM) mechanical paw withdrawal threshold. Data from the LPS-treated paws are depicted in Figure 22. n=7-12 mice/group.

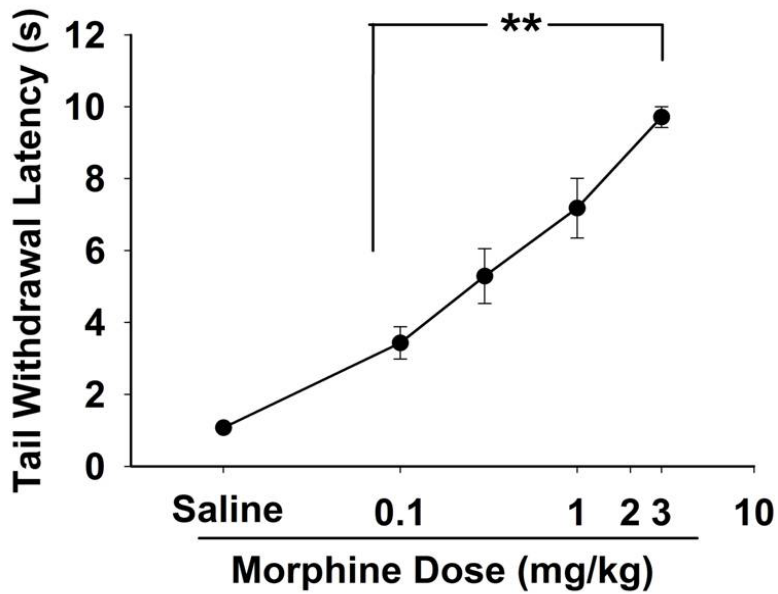
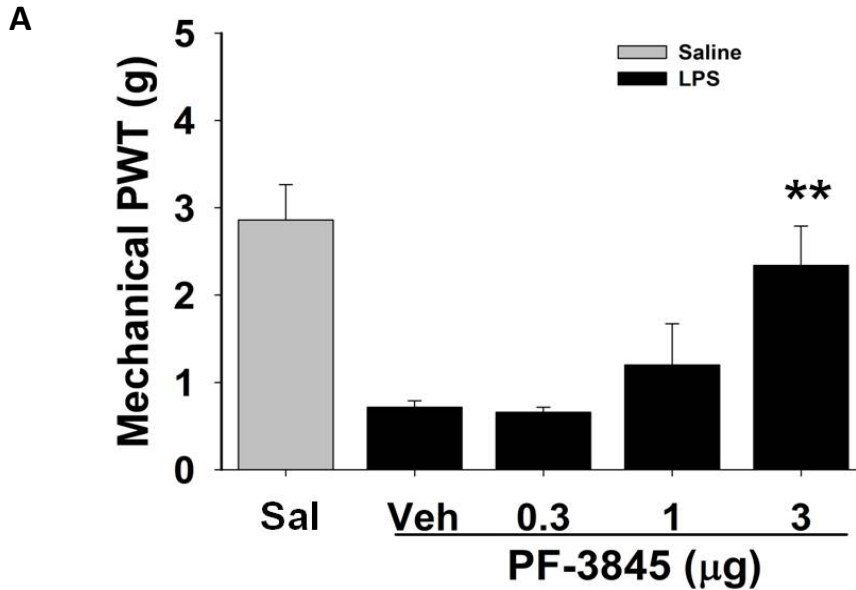


Figure 24. Morphine produces antinociception in the tail withdrawal assay. Intrathecal morphine (0.1 μ g-10 μ g) dose-responsively produces antinociception in the tail withdrawal assay of thermal nociception. Data represents the mean \pm SEM tail withdrawal latency. $p < 0.01$ vs saline. n=6 mice per group.

Figure 25. Spinal PF-3845 produces antinociception. Intrathecal PF-3845 (3 μ g) reverses LPS-induced allodynia (A) and increases brain anandamide (AEA) levels versus vehicle (B). Data represents the mean (\pm SEM) mechanical paw withdrawal. Values in panel B are calculated per wet tissue weight. n=6 mice per group. Data represent the mean (\pm SEM) endocannabinoid content the $**p < 0.01$ vs control. n=6-10 mice/group



B

	AEA (pm/g)	2-AG (nm/g)
Vehicle	4.91 \pm .97	7.78 \pm .76
PF-3845 (3ug)	30.85 \pm 2.01**	6.97 \pm 1.01

Table 7. LC/MS/MS analysis of brain and spinal cord tissues after systemic (10 mg/kg; i.p.) or local (0.1-10 µg; intraplantar) PF-3845 administration in mice. Systemic administration of PF-3845 significantly increased anandamide (AEA) levels in both brain and spinal cord $p<0.001$. Local administration of PF-3845 (10 µg), but not 0.1 or 1.0 µg, increased AEA in the brain and spinal cord tissues. “ND: not determined”. Values are calculated per wet tissue weight. n=6 mice per group. Data represent the mean (\pm SEM) endocannabinoid content.

		Brain		Spinal Cord		Paw Tissue	
Administration route	Injection	AEA (pm/g)	2-AG (nm/g)	AEA (pm/g)	2-AG (nm/g)	AEA (pm/g)	2-AG (nm/g)
i.p.	Vehicle	1.55 \pm 0.11	9.04 \pm 0.89	3.95 \pm 0.18	9.35 \pm 1.23	ND	ND
i.p.	PF-3845 (10 mg/kg)	16.67 \pm 1.15**	9.80 \pm 1.02	37.78 \pm 2.15**	10.32 \pm 2.22	ND	ND
Intraplantar	Vehicle	4.32 \pm 0.84	6.82 \pm 0.74	5.34 \pm 0.60	10.34 \pm 0.61	6.14 \pm 0.50	2.47 \pm 0.23
Intraplantar	PF-3845 (0.1 µg)	5.55 \pm 0.62	7.42 \pm 0.42	6.88 \pm 1.14	12.41 \pm 0.79	ND	ND
Intraplantar	PF-3845 (1.0 µg)	5.69 \pm 0.81	7.00 \pm 0.38	6.50 \pm 0.54	11.84 \pm 0.77	ND	ND
Intraplantar	PF-3845 (10.0 µg)	13.12 \pm 0.40**	5.56 \pm 0.58	28.42 \pm 1.85**	7.34 \pm 0.74	6.61 \pm 0.41	2.48 \pm 0.07

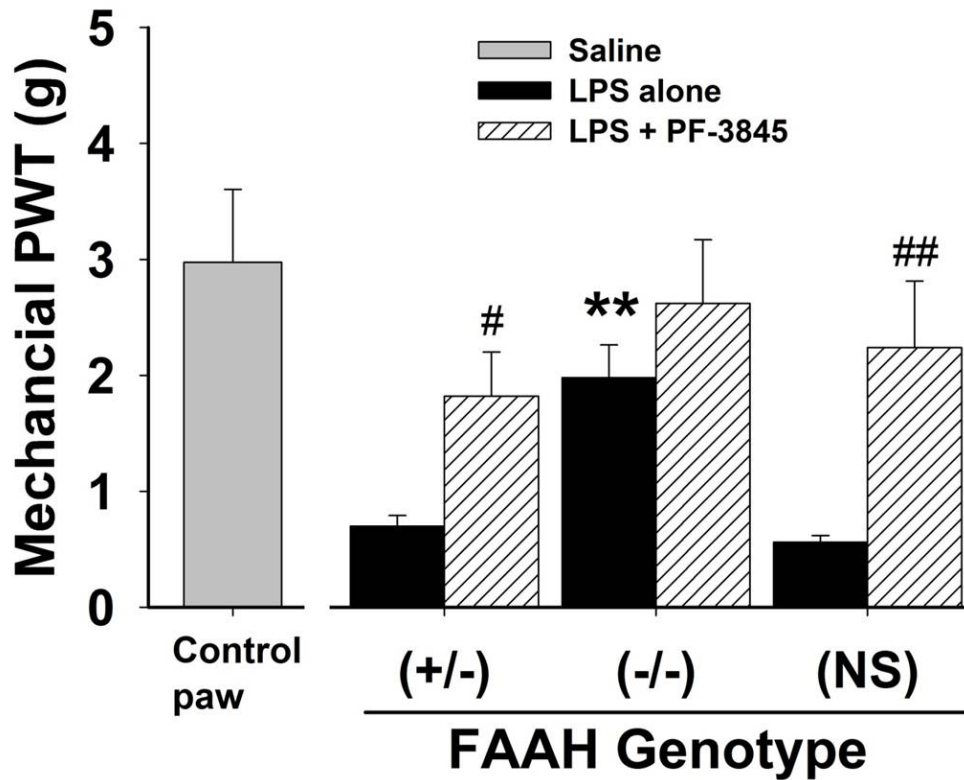


Figure 26. Blocking FAAH in the nervous system mediates the FAAH (-/-) anti-allodynic phenotypic in response to intraplantar LPS. FAAH (-/-) mice displayed an anti-allodynic phenotype that was not present in either the FAAH (+/-) mice or FAAH-NS (i.e., neural specific knock-in) mice. Pretreatment with PF-3845 (10 mg/kg) restored the anti-allodynic phenotype in FAAH (+/-) mice and FAAH-NS mice. Control paw represents the saline-injected paw of FAAH (+/-) LPS only treated mice. All values represent the mean (\pm SEM) mechanical paw withdrawal threshold. $n=8-10$ mice/group. $**p < 0.01$ vs. the vehicle-treated FAAH (+/-) or FAAH-NS mice, $\#p < 0.05$, $\##p < 0.01$ vs. vehicle-treated mice for each respective genotype.

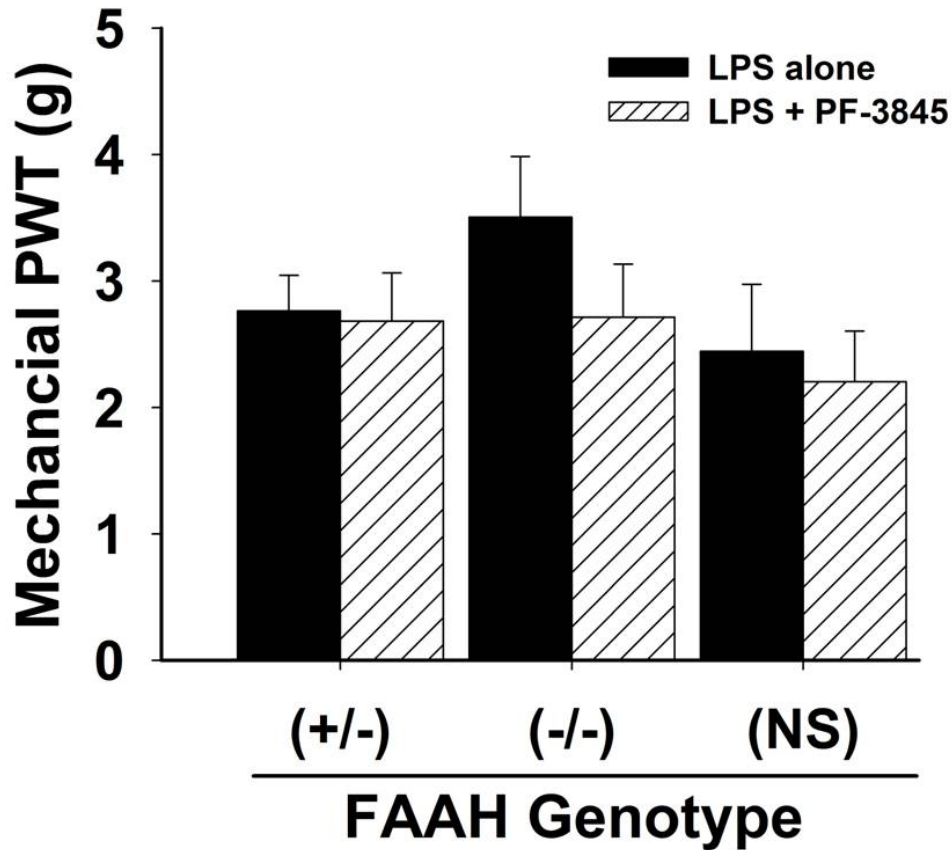


Figure 27. PF-3845 (10 mg/kg; i.p.) did not affect paw withdrawal thresholds in the control saline-injected paw. All values represent the mean (\pm SEM) mechanical paw withdrawal threshold. Data from the LPS-treated paws are depicted in Figure 26. n=8-10 mice/group.

5.5 CB1 and CB2 receptors mediate the anti-allodynic effect of FAAH inhibition

FAAH (-/-) mice and wild type mice treated with FAAH inhibitors display an anti-allodynic effect when administered LPS. These effects were completely reversed with either a CB1 or CB2 receptor selective antagonist. Consequently, we evaluated the effects of PF-3845 in a complementary approach using CB₁, and CB₂, receptor knockout mice. CB1 (-/-) and CB2 (-/-) mice as well as their matched littermates, were administered LPS intraplantarly into one paw and saline into the opposite paw. Twenty-two hrs later PF-3845 (10 mg/kg; i.p) or vehicle was administered and allodynia testing was conducted 2 hrs later. PF-3845 increased the paw withdrawal threshold values in CB₁ (+/+) mice as well as in CB₂ (+/+) mice ($p < 0.01$ for each) after LPS administration, but failed to increase paw withdrawal thresholds in either CB₁ (-/-) mice (Figure 28A) or CB₂ (-/-) mice (Figure 28B). As found previously, PF-3845 administration did not modify paw withdrawal threshold responses to the saline-treated paw (Figure 29A, B).

Since anandamide and other ligands that are targets of FAAH bind to and activate other receptors such as the TRPV1 ion channel, PPAR- α receptors, we evaluated these receptor systems which may also contribute to the anti-allodynic actions of PF-3845. AEA was shown to act as a full agonist at the human vanilloid receptor (Smart et al., 2000). In addition to FAAHs regulation of AEA, FAAH also controls the degradation of non-cannabinoid fatty acid amides, such as N-palmitoyl ethanolamine (PEA) and OEA, each of which possesses anti-inflammatory actions through the PPAR- α receptor (Jhaveri et al., 2008; D'Agostino et al., 2007). Lastly, naloxone has been shown to reduce the antinociceptive actions of the FAAH inhibitor OL-135

(Chang et al., 2006). Thus, we explored whether these non-cannabinoid receptors also play a role in the anti-allodynic effects of PF-3845.

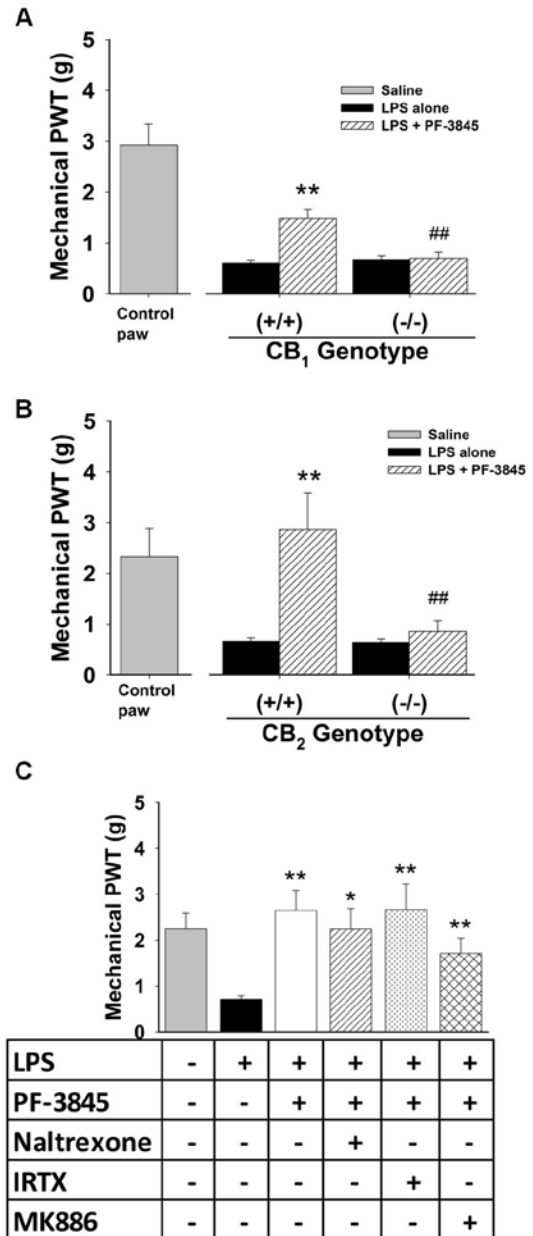
C57BL/6 mice were injected with LPS into one paw and saline into the opposite paw as previously mentioned. 22hrs after LPS administration mice were pretreated subcutaneously with either the opioid receptor antagonist naltrexone (1 mg/kg), the TRPV1 antagonist IRTX (0.5 mg/kg), or the PPAR- α antagonist MK886 (3 mg/kg). Ten minutes after the administration of selective receptor antagonists, mice were injected with PF-3845 (10 mg/kg) and assessed for allodynia after 2 hrs. None of the antagonists modified the anti-allodynic effects of PF-3845 (Figure 28C) and had no effects on LPS-induced allodynia when given alone (Figure 30). As before, no effects of drug treatment were observed on the control paw (Figure 29C).

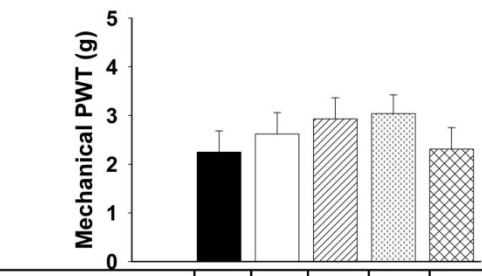
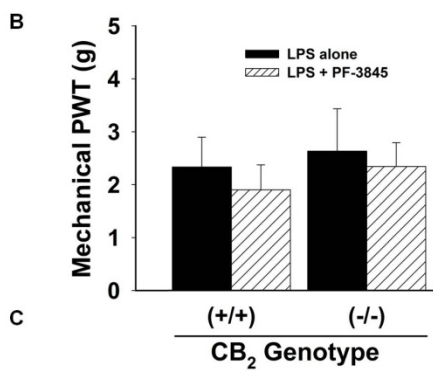
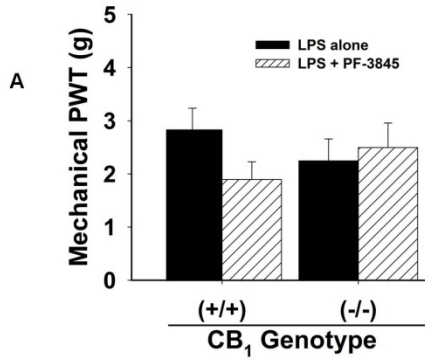
In opposition to FAAH inhibition which produces anti-allodynic effects we found that MAGL inhibition is ineffective. Following the same model for LPS-induced allodynia, we pretreated mice with JZL184 (40 mg/kg), the selective MAGL inhibitor 2 h prior to testing for allodynia. JZL184 treated mice did not show an anti-allodynic response as demonstrated with mice treated with PF-3845 (Figure 31). However, these mice did show increased hyperreflexia at the dose tested.

Figure 28. PF-3845 reduces LPS-induced allodynia through a cannabinoid receptor mechanism of action.

(A) PF-3845 (10 mg/kg) reduced LPS-induced allodynia in CB₁ (+/+) mice, but not in CB₁ (-/-) mice. Control paw represents the saline-injected paw of CB₁ (+/+) LPS only treated mice. (B) PF-3845 (10 mg/kg) reduced LPS-induced allodynia in CB₂ (+/+) mice, but not in CB₂ (-/-) mice. Control paw represents the saline-injected paw of CB₂ (+/+) LPS only treated mice. (C) The anti-allodynic effects of PF-3845 (10 mg/kg) in the LPS model were not blocked by the opioid receptor antagonist naltrexone (1 mg/kg), the TRPV1 receptor antagonist IRTX (0.5 mg/kg), or the PPAR- α antagonist MK886 (3 mg/kg). Data shown in Panel C represent two combined experiments collapsed into a single figure. Control paw represents the saline-injected paw of

LPS only treated mice. n=6-10 mice/group. Values represent the mean (\pm SEM) mechanical paw withdrawal threshold. ** $p < 0.01$ vs. Vehicle-treated mice in the LPS-injected paw; ## $p < 0.01$ vs. PF-3845-treated CB₁ (+/+) or CB₂ (+/+) mice.





LPS	-	-	-	-	-
PF-3845	-	+	+	+	+
Naltrexone	-	-	+	-	-
IRTX	-	-	-	+	-
MK886	-	-	-	-	+

Figure 29. (A) CB1 (+/+) and (-/-) mice show similar paw withdrawal thresholds in the control saline-injected paw and PF-3845 (10 mg/kg; i.p.) had no effects in either genotype. (B) CB2 (+/+) and (-/-) mice show similar paw withdrawal thresholds in the control saline-injected paw and PF-3845 (10 mg/kg; i.p.) had no effects in either genotype. (C) None of the drug treatments from Figure 28 affected paw withdrawal thresholds in the control saline-injected paw. Values represent the mean (\pm SEM) mechanical paw withdrawal threshold. n=6-10 mice/group.

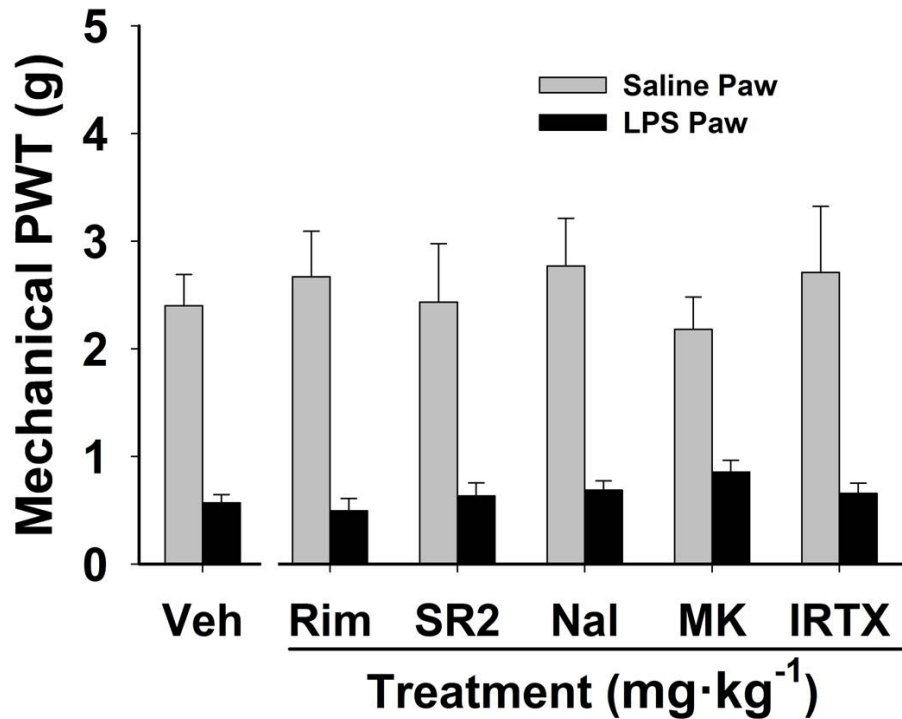


Figure 30. None of the antagonists administered in the absence of a FAAH inhibitor affected tactile paw withdrawal thresholds in either allodynic LPS-injected paws or control saline-injected paws. Values represent the mean (\pm SEM) mechanical paw withdrawal thresholds in LPS- and saline-treated paws. Rimonabant (Rim; 3 mg/kg), SR144528 (SR2; 3 mg/kg), naltrexone (Nal; 1 mg/kg), MK886 (MK; 3 mg/kg), and IRTX (0.5 mg/kg). n=6-14 mice/group.

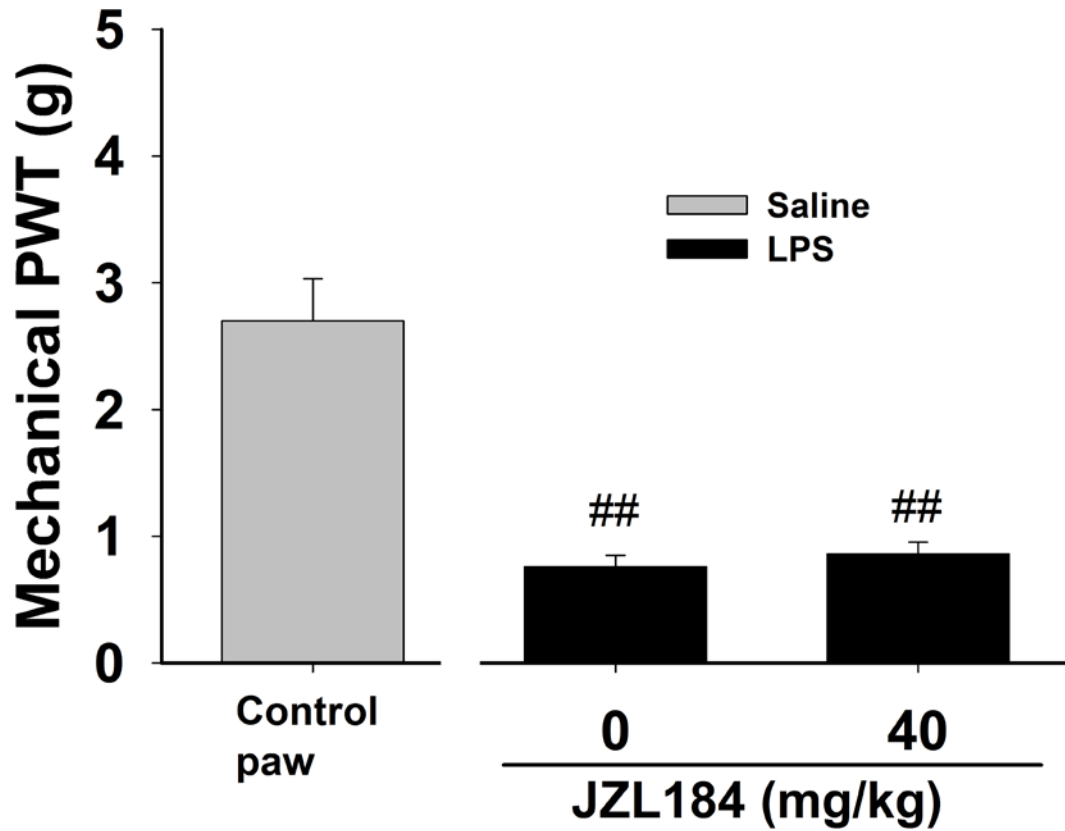


Figure 31. MAGL inhibition in LPS-induced allodynia model. JZL184 (40 mg/kg) failed to produce antinociception in the LPS-induced allodynia model. Values represent the mean (\pm SEM) mechanical paw withdrawal threshold. n=6-8 mice/group

5.6 Discussion: FAAH inhibition reverses LPS induced allodynia via CB1 and CB2 receptors

Allodynia and hyperalgesia are common clinical features of many inflammatory diseases and disorders such as fibromyalgia, postherpetic neuralgia, and mild skin injuries. Whereas hyperalgesia reflects an increased sensitivity to a noxious stimulus, allodynia is a painful response to a typically non-noxious stimulus. In a murine model of inflammatory pain, LPS injected into the hind paw leads to increased sensitivity to thermal nociceptive stimuli (Kanaan et al., 1996; Naidu et al., 2010). In the present chapter, we modified the previously established LPS model (Naidu et al., 2010) by injecting a relatively low concentration of LPS (2.5 µg) into the plantar surface of a hind paw to elicit a profound tactile allodynic response at 24 h, without producing overt edema or weight loss. A previous report from our laboratory showed maximum thermal hyperalgesic responses to 25 µg/paw LPS, though mice in that study also displayed severe edematous effects of the paw and significant weight loss, indicating systemic effects of LPS (Naidu et al., 2010). Gabapentin, a commonly used anti-allodynic GABA analogue, as well as the phytocannabinoid THC, reversed LPS-induced allodynia. However, each of these drugs produces significant motor and cognitive side effects (Backonja et al., 1998; Martin et al., 1991a). In contrast, elevating the endocannabinoid AEA, by blocking its catabolic enzyme FAAH, is well established to reduce nociceptive behavior in a variety of animal models of pain, without eliciting cannabimimetic side effects associated with THC. Therefore, we investigated whether genetic deletion or pharmacological inhibition of FAAH, the primary catabolic enzyme

of AEA, would reduce LPS-induced allodynia. Both approaches reduced tactile allodynia via mechanism(s) that required both CB₁ and CB₂ receptors.

FAAH (-/-) mice displayed a significant anti-allodynic phenotype. However, it is difficult to delineate whether the anti-allodynic responses of FAAH (-/-) mice resulted from increased levels of AEA and other FAAs at the time of testing or because the development of the LPS-induced allodynia was dampened. However, an acute injection of three different FAAH inhibitors reversed the peak allodynic effects of LPS. The reversible (OL-135) and irreversible (URB597, PF-3845) FAAH inhibitors attenuated LPS-induced allodynia. URB597 has anti-hyperalgesic effects in various models of inflammatory pain, such as carrageenan and complete Freund's adjuvant (CFA) models (Holt et al., 2005; Jayamanne et al., 2006). Similarly, the reversible FAAH inhibitor, OL-135 suppressed nociception in thermal pain models (i.e. tail immersion, hot-plate) and the formalin model of pain (Lichtman et al., 2004). Here, we show for the first time OL-135 elicits anti-allodynic effects in an inflammatory pain model. Likewise, PF-3845, which has longer lasting effects than other FAAH inhibitors, also reduced allodynia in the rat CFA model (Ahn et al., 2009).

These results further demonstrate both CB₁ and CB₂ receptors play a necessary role in mediating the anti-allodynic phenotype of FAAH-compromised mice in the LPS model. These data are congruent with other reports showing both CB₁ and CB₂ receptors are required for the anti-allodynic effects of FAAH inhibition, such as the chronic constriction injury model (Kinsey et al., 2009a; Kinsey et al., 2010; Russo et al., 2007) and the partial sciatic and spinal nerve

injury models of pain (Desroches et al., 2008; Jhaveri et al., 2006). The resultant may be attributed to the sequestration of the cannabinoid receptors. *In situ* hybridization revealed that CB₁ receptors are expressed in cells of the dorsal root ganglia inserted on nerve terminals in the periphery and other brain regions associated with pain such as the PAG (Hohmann and Herkenham, 1999). Furthermore, the CB₂ receptor is expressed on activated mast cells (Facci et al., 1995) which infiltrate peripheral nerve tissues during an innate inflammatory response, such as that initiated by LPS exposure. However, the two cannabinoid receptors play differential roles in mediating the antinociceptive actions of FAAH blockade in other pain models. Only the CB₁ receptor mediates the antinociceptive effects of FAAH (-/-) mice in the tail withdrawal and formalin test (Lichtman et al., 2004). In contrast to the data in the present study demonstrating FAAH inhibitors require both cannabinoid receptors to reduce tactile allodynia caused by LPS, we previously reported FAAH inhibition ameliorates thermal hyperalgesia through the activation of CB₁ receptors, only (Naidu et al., 2010). The phenotypic anti-edematous actions caused by FAAH deletion are mediated by the CB₂ receptor, not the CB₁ receptor, in carrageenan (Lichtman et al., 2004) and LPS (Naidu et al., 2010) paw inflammatory assays.

FAAH-NS mice, which express FAAH in the nervous system only (Cravatt et al., 2004), displayed the same allodynic responses to intraplantar LPS as wild type controls. This finding along with the observation that the highly selective FAAH inhibitor, PF-3845, produced anti-allodynic effects in FAAH-NS mice supports the idea that the anti-allodynic effects of FAAH inhibition are mediated in the central and/or peripheral nervous system(s). In contrast, we

recently reported that FAAH-NS mice show an anti-edematous phenotype when given an intraplantar injection of 25 μ g LPS (Naidu et al., 2010). The results of these studies indicate that different pools of FAAs mediate the edematous vs. allodynic effects of LPS. Whereas elevating these lipid signaling molecules in non-neuronal tissue mediates the anti-edematous effects, the anti-allodynic actions appear to be mediated through the nervous system. Of note, a recent study found that a peripherally restricted FAAH inhibitor (URB937) reduced mechanical and thermal hyperalgesia in the carrageenan inflammatory pain model as well as the peripheral nerve injury pain model (Clapper et al., 2010). Thus, targeting FAAH in the peripheral nervous system, as well as in the central nervous system (Suplita et al., 2005), effectively blocks pain-related behavior, possibly by differentially elevating distinct pools of FAAs. In agreement with previous data, systemic administration of PF-3845 increased AEA levels in the brain (Ahn et al., 2009) and spinal cord without increasing levels of 2-AG. Furthermore, we found local administration of PF-3845 into the LPS-injected paw reduces the tactile allodynic response to LPS without affecting levels of AEA in the brain or spinal cord, indicating that the anti-allodynic effects of PF-3845 were not merely due to diffusion into the CNS. That said, the lack of increased levels of AEA in paw skin by PF-3845 may be limited by challenges associated with extracting lipids from mouse skin or possibly low levels of FAAH on discrete sensory neurons relative to the large surface area of the paw. Regardless, the observation that local PF-3845 administration yielded small magnitude, anti-allodynic actions compared to systemic

administration suggests that central as well as peripheral components contribute to maximal anti-allodynic efficacy of FAAH inhibitors.

The endotoxin model used in the present study differs from other models of inflammation, in that LPS is derived from the outer cell wall of gram negative bacteria and is commonly used to model an innate inflammatory response. Unlike the carrageenan model and complete Freund's adjuvant model, there is not severe weight loss or measurable changes in paw edema. Similar to other inflammatory pain models, such as injection of CFA (derived from a mycobacteria) or carrageenan (extracted from red seaweed), LPS mimics an inflammatory response to a non-self immunogen which produces an allodynic response.

The finding that PF-3845 was ineffective in CB₁ (-/-) and CB₂ (-/-) mice confirms the essential role of both the CB₁ and CB₂ receptors in mediating LPS-induced allodynia. CB₂ receptors are highly expressed on immune cells and may play an essential role in modulating the release of inflammatory cytokines/chemokines involved in pain sensitization (Klein et al., 2003; Roche et al., 2006). Therefore, changes in the inflammatory pain response may be attributed to the activation of the CB₂ receptors, which alternatively decrease the release of cytokines and the resulting allodynia. Although the CB₂ receptor is expressed at low levels in the nervous system and much higher levels on mast cells (Facci et al., 1995), previous evidence suggests that it is upregulated in the dorsal horn in neuropathic pain models (Beltramo et al., 2006; Hsieh et al., 2011), hence changes in CB₂ expression during a disease state may be responsible for the strong role of CB₂ in modulating LPS-induced allodynia. Furthermore, a recent report by Hsieh *et al.*,

(2011) also illustrated that in the CFA model of inflammation, CB₂ receptor mRNA, but not CB₁ receptor mRNA, is up-regulated in the dorsal root ganglia and paw tissue, but not in the spinal cord. Conversely, the CB₁ receptor is expressed at much higher levels in the nervous system than in other tissues (e.g., immune cells) and has a more prominent role in modulating neurotransmitter signaling than the CB₂ receptor. However, due to the limitations of the test (e.g. floor effect) it is difficult to determine whether genetic deletion of CB₂, for example, would exacerbate LPS-induced allodynia. Notably, local administration of low doses of PF-3845 partially reversed allodynia, suggesting FAAH in the peripheral nervous system modulates inflammatory pain in the LPS-induced allodynia model.

A growing body of research suggests non-cannabinoid receptors play a role in the anti-inflammatory actions of FAAH inhibitors (Chang et al., 2006; Sun et al., 2006; Tognetto et al., 2001). Although AEA is an agonist at the cannabinoid receptor, it also activates the TRPV1 receptor; albeit its affinity at TRPV1 is lower than its affinity at cannabinoid receptors (Howlett et al., 2002; Zygmunt et al., 1999). The TRPV1 receptor has also been implicated in AEA effects in other models of pain to include the carrageenan-induced thermal hyperalgesia model, and the Hargreaves plantar stimulator test (Horvath et al., 2008; Maione et al., 2006). However, we show that in the LPS-induced inflammatory pain model, the TRPV1 antagonist, IRTX failed to block the anti-allodynic effects of PF-3845. In addition to increasing systemic AEA, FAAH inhibition also increases levels of noncannabinoid FAAs, such as oleoylethanolamide (OEA), oleamide, and palmitoylethanolamine (PEA) (Clement et al., 2003). In particular, the PPAR- α

receptor has been implicated in anti-inflammatory effects of OEA and PEA (Jhaveri et al., 2008). Moreover, PEA attenuates inflammation in the carrageenan paw edema (D'Agostino et al., 2009) and phorbol ester ear edema models (LoVerme et al., 2005), and PPAR- α (-/-) mice are unaffected by exogenous administration of PEA (Lo Verme et al., 2005). To assess whether targets of these other FAAH substrates contribute to the anti-allodynic effects of PF-3845, we tested whether the anti-allodynic effect of FAAH inhibition was mediated through the PPAR- α receptor. The PPAR- α antagonist MK886 did not block the anti-allodynic effects of FAAH inhibition, indicating PPAR- α does not play a necessary role in the anti-allodynic effects of PF-3845. Lastly, it was reported that naloxone reversed analgesia induced by a FAAH inhibitor, OL-135 in the mild thermal injury and spinal nerve injury rat pain models (Chang et al., 2006). Here, we found no evidence of the opioid receptor involvement in the anti-allodynic effects of PF-3845. Additionally, these data support previous work from our laboratory negating the role of opioid receptor involvement with FAAH inhibition in mouse peripheral nerve injury (Kinsey *et al.*, 2009) and visceral pain models (Kinsey et al., 2009b; Naidu et al., 2009).

Inhibiting MAGL did not produce the anti-allodynic response that FAAH inhibition produced. The highest dose of MAGL inhibitor tested (JZL184; 40 mg/kg) was illustrated to produce analgesia in a variety of pain models such as the tail-flick assay, acetic acid abdominal stretching, and chronic constriction injury models (Kinsey et al., 2010; Long et al., 2009a). However, the lack of effect in the LPS model may be due to the fact that MAGL inhibition at the

concentration tested also produced hyperreflexia which is similar to previous studies (Long et al., 2009a).

In summary, these results indicate that genetic deletion or pharmacological inhibition of FAAH reduces LPS-induced pain responses. In particular, we demonstrated that three distinct FAAH inhibitors reverse LPS-induced tactile allodynia. Furthermore, these data reveal that neuronal FAAH inhibition is primarily responsible for the anti-allodynic response to LPS via a mechanism requiring both cannabinoid receptors.

Chapter 6: General Conclusions and Discussion

The overall purpose of this dissertation was to examine the effects of targeting the endocannabinoid system on visceral and inflammatory pain states. We generated two hypotheses to address our purpose. First, we hypothesized that prevalent phytocannabinoids reduce acetic acid induced abdominal stretching via a CB1 receptor mechanism of action. Secondly, we hypothesized that targeting the endocannabinoid system directly by using selective cannabinoid agonist, or indirectly by elevating endocannabinoids via inhibiting their degradative enzymes (i.e. FAAH and MAGL) reduce visceral and inflammatory pain.

6.1 *Phytocannabinoids reduce visceral pain*

To address the hypothesis that phytocannabinoids reduce visceral pain through a CB1 receptor mechanism of action, we carried out a series of pharmacological experiments. Utilizing the five most prevalent cannabinoid constituents found in marijuana (Elsohly and Slade, 2005) we showed that although these prevalent phytocannabinoids are structurally similar to THC, they show different pharmacological properties in modulating visceral pain. For example, THC, THCV, and CBN all showed affinity for the cannabinoid receptor. THC and THCV binding affinities were 3 times greater than that of CBN. Consequently, they were all effective at modulating CB1 mediated effects. THC and CBN reduced the total number of abdominal stretches over the test period, although THC was approximately 50 times more potent than CBN. Additionally, THC was more potent in blocking stretching than in producing motor impairment.

CBC and CBD showed low affinity for the cannabinoid receptor and as a result did not affect abdominal stretching. In an effort to determine if these effects were mediated through CB1 or CB2 receptors we employed selective antagonists for each (rimonabant and SR144528 respectively). Both THC and CBN antinociceptive effects were blocked by the administration of rimonabant but not SR2, proving that this was a CB1 driven effect. Conversely, the concentration of THC and CBN that reduced abdominal stretching was not a result of motor deficits. The concentration of THC needed to suppress motor deficits was 8 fold greater than that dose which was antinociceptive.

Interestingly, we showed that THCV antagonized the effects of THC. THCV has been shown to have both agonist and antagonist properties at the CB1 receptor as a function of dose. Bolognini and colleagues showed that THCV reduced formalin nociceptive behavior at moderate doses up to 5 mg/kg via a CB1 and CB2 mechanism of action (Bolognini et al., 2010). On the other hand, data from other groups show that THCV was able to antagonize the effects of THC in the tetrad when administered intravenously at doses up to 3 mg/kg (Pertwee et al., 2007). The observation that THCV possesses both agonist and antagonist effects may be linked to the fact that it is targeting non-specific targets at high doses, or concentrations such as PPAR receptors or TRP channels (De Petrocellis et al., 2010). More recent studies showed that heterogeneous mixtures of certain phytocannabinoids interact with each other to modulate the overall effect of THC. A recent study from our laboratory showed that a high dose of CBC can augment the pharmacological effects of a threshold dose of THC (DeLong et al., 2010). Another example of

THC modulation by other phytocannabinoids is in the case of a new clinical drug, Sativex (GW Pharmaceuticals, 2003). Sativex was recently developed by GW pharmaceuticals as an oral delivery cannabinoid spray for the treatment of multiple sclerosis which has been approved in the United Kingdom. The oral spray is formulated in a 1:1 mixture of THC and CBD. Although CBD is ineffective on its own in reducing analgesia, it can modulate the effects of THC. Although this mechanism of potentiation is unknown, CBD may interfere with the pharmacodynamic properties of THC rather than pharmacokinetic interactions (Karschner et al., 2011). Studies from our lab showed that high doses of intravenously administered CBD potentiated the antinociceptive effects of a threshold dose of THC in the tail-flick assay compared to CBD alone. Also, THC levels in the brain and blood of mice pretreated with CBD were significantly higher than mice that only received THC suggesting that CBD may block the degradation of THC (Varvel et al., 2006). Additionally, other studies suggest that this non psychoactive compound CBD, can antagonize the reuptake of anandamide in *in vitro* studies (Rakhshan et al., 2000). However, these effects only occurred at high micromolar concentrations which may not be as relevant for *in vivo* studies.

These data strongly suggest that phytocannabinoids and other components that are found in marijuana have a widespread effect on the physiological function of multiple systems and their interaction with each other may contribute to the overall pharmacological effects of *cannabis sativa*.

6.2 Targeting the endocannabinoid system blocks visceral pain

The data presented in this thesis supports the hypothesis that the endocannabinoid system serves as a target for treating visceral pain. THC is a mixed CB1/CB2 agonist therefore the goal of these experiments was to evaluate selective agonist for each subtype. We employed selective CB1 (ACEA) and selective CB2 (O-3223) agonists to determine if stimulating each respective receptor would reduce abdominal stretching. ACEA but not O-3223 reduced nociceptive responses in the acetic acid abdominal stretching model. Furthermore, the effects of ACEA were blocked with pretreatment of rimonabant, confirming a CB1 receptor mediated mechanism.

We further investigated the antinociceptive effects of inhibiting degradative enzymes FAAH and MAGL in the acetic acid stretching model. Initially, we took a genetic approach looking at the effects of FAAH deletion using FAAH (-/-) mice. FAAH (-/-) mice displayed an antinociceptive phenotype as compared to their wild type FAAH (+/+) littermates. This effect was blocked in FAAH (-/-) mice treated with rimonabant, suggesting a CB1 mechanism of action. A more relevant approach and of most importance clinically is whether pharmacological agents can also produce a similar analgesic effect. Therefore, we assessed the effects of irreversible and reversible pharmacological inhibitors of FAAH. Reversible inhibitors in general are clinically important since they do not change the functional aspects of the enzyme like irreversible inhibitors. This is important clinically because reversible inhibitors can be overcome

with the addition of more substrates in the case of an overdose. URB597 the irreversible inhibitor, and OL-135 the reversible inhibitor both reduced abdominal stretches compared to vehicle administration although the dose of URB597 was 3 times more potent than that of OL-135. The antinociceptive effects of both inhibitors were reversed by rimonabant and not the CB2 receptor antagonist. Furthermore we showed that non cannabinoid receptors such as the opioid receptors did not have a factor in modulating these effects. This is a relevant issue with the use of certain drugs in that they tend to reduce nociception, but they also have a non-specific receptor mechanism. A report by Chang *et al.*, 2006 showed that the effects of OL-135 was reversed by naloxone in the spinal nerve ligation (SNL) and mild thermal injury (MTI) models. However, the results from studies in our laboratory do not support the involvement of mu opioid receptors in the antinociceptive effects of FAAH inhibitors in the chronic constriction injury model (Kinsey *et al.*, 2009a; Kinsey *et al.*, 2010), in the acetic acid induced abdominal stretching model, (Naidu *et al.*, 2009), and the LPS-induced inflammatory model (see Figure 28C). The differences may be due in part that Chang *et al.*, (2006) used rats for their test subjects, where as we used mice. Our data indicate that FAAH blockade can reduce nociception, although this effect was below a 50% reduction, therefore we investigated the effects of dual FAAH and COX inhibition on acetic acid abdominal stretching. Isobolographic analysis revealed that dual FAAH/COX inhibition produces synergism in blocking the stretching response, an effect of therapeutic importance. Drug combination therapy is beneficial in that it can reduce the unpleasant side effects of certain drugs while at the same time maintain its therapeutic effects.

Although FAAH inhibited effects have been characterized in several assays to treat pain and inflammation (Schlosburg et al., 2009b) limited data is known about MAGL inhibited effects. This is mostly in part due to the lack of selective MAGL inhibitors available. However, the recent development of JZL184 was described to be highly selective for MAGL (Long et al., 2009a). We showed for the first time behaviorally that MAGL inhibition via JZL184 administration reduced antinociceptive responses via CB1 receptor mechanism of action. Given that FAAH and MAGL regulate different enzymes, and inhibiting either enzyme produces antinociceptive properties, we evaluated the effects dual FAAH and MAGL inhibition. It is possible that elevating both endocannabinoids can potentiate the antinociceptive effects of elevating one. Conversely, elevating one endocannabinoid may suffice to block nociception since both FAAH and MAGL inhibited effects are mediated through the CB1 receptor. To address this idea we employed the use of FAAH (-/-) mice treated with JZL184, and the dual FAAH/MAGL enzyme inhibitor. We demonstrated that by blocking MAGL and FAAH via the dual inhibitor JZL195, or FAAH (-/-) mice treated with JZL184, the antinociceptive effects in the abdominal stretching model are potentiated versus inhibiting one enzyme alone. These effects were also mediated through the CB1 receptor mechanism of action. However it was also discovered in a parallel study from our collaborators that dual elevation of AEA and 2-AG resulted in cannabimimetic effects in THC drug discrimination studies (Long et al., 2009). The drug discrimination model serves as an animal model for marijuana intoxication and drugs that

substitute for THC in this assay are predicted to have marijuana-like subjective effects in humans (Long et al., 2009b). Therefore this study suggests that simultaneous elevation of anandamide and 2-AG produces untoward psychoactive effects as those associated with direct acting cannabinoid agonist such as THC. Conversely, inhibiting one enzyme alone did not produce THC-like effects. These data suggest that activation of the CB1 receptor by AEA and 2-AG are potentiated indicative of endocannabinoid crosstalk.

6.3 Targeting the endocannabinoid system blocks inflammatory pain

As a final goal of this dissertation, we tested whether inhibition of FAAH and MAGL produces an anti-allodynic effect in an inflammatory pain model, and if so, through which receptor mechanism of action. To assess this question we modified an inflammatory model of LPS-induced allodynia that produces increase responsiveness to non-noxious stimuli but does not produce edema, or weight loss. The positive control gabapentin and primary phytocannabinoid THC were capable of reversing paw withdrawal thresholds, which ensured us that our model was capable of detecting drugs that had anti-allodynic properties. After establishing an inflammatory pain model, we tested whether genetically modified FAAH mice would display an anti-allodynic phenotype in this model. To address this question we employed FAAH (-/-) mice as well as FAAH-neurospecific (FAAH-NS) mice. FAAH-NS mice were generated by coupling the FAAH enzyme to the neuro-enolase promoter and mating them with FAAH (-/-) mice. Subsequently, FAAH is only expressed in neuronal tissue (Cravatt et al.,

2004). This creation is effective in deciphering whether FAAH inhibited effects are mediated through the nervous system or in the periphery. FAAH (-/-) mice but not FAAH-NS displayed an anti-allodynic phenotype, which was mediated through the CB1 and CB2 receptor mechanism of action.

In parallel to the anti-allodynic effects with genetic deletion of FAAHs in the LPS model, we also evaluated the effects of pharmacological inhibition of FAAH using reversible and irreversible inhibitors. URB597, PF-3845, and OL-135 all reversed LPS-induced allodynia, although the reversible inhibitor (OL-135) required a higher dose. In addition, we demonstrated that these effects were abolished in CB1 (-/-) and CB2 (-/-) mice. To determine if FAAs were indeed mediating the anti-allodynic effects observed in FAAH (-/-) mice administered LPS, we pretreated FAAH-NS mice with PF-3845. PF-3845 restored the anti-allodynic phenotype in FAAH-NS mice and did not affect FAAH (-/-) mice suggesting that inhibition of FAAH within the nervous system (central and/or peripheral) elicits the anti-allodynic effects in this model.

Since inhibition of neuronal FAAH was mediating the anti-allodynic response, we attempted to elucidate the precise location (e.g. central or peripheral) for its effects. To examine this objective, we took several steps. First, we administered the selective FAAH inhibitor PF-38845 via local, spinal, and supraspinal routes of administration. Local administration of PF-38845 dose responsively reversed paw withdrawal thresholds and was mediated through both CB1 and CB2 receptor mechanism of action. Furthermore, the lowest dose that reversed allodynia when administered to the contralateral saline injected paw and not the LPS treated paw did not

affect the paw withdrawal threshold in the LPS paw suggesting that drug spread did not occur. Since FAAH inhibition elevates AEA we wanted to confirm our local site of action, thus we assessed whether local administration of PF-3845 increased levels of anandamide in the brain, spinal cord, and paw. The highest dose tested (10 μg) increased anandamide in both the brain and spinal cord, but not in the paw indicating that drug spread was occurring at this higher dose. Conversely, local administration of the lower dose of PF-3845 (1 μg) did not affect brain and spinal cord anandamide levels. Although no changes in paw tissue were quantified even at the highest dose, we cannot draw conclusions about this effect. Possible explanations may be linked to the fact that the larger surface area of the paw can dilute the changes in local anandamide levels. An alternative approach in the future would be to look at specific areas of the paw instead of assessing the whole paw. Another explanation is that changes in anandamide after local administration may be below our detectable limit using LC/MS/MS analysis.

To test a spinal mechanism of action the observed FAAH inhibited anti-allodynic effects, we treated mice with LPS followed 22hrs by PF-3845 intrathecally, then assessed paw withdrawal thresholds. Mice treated with intrathecal PF-3845 showed a significant reversal of paw withdrawal thresholds. Conversely, the dose of PF-3845 that reversed paw withdrawal thresholds intrathecally also increased brain anandamide levels suggesting that drug spread in fact occurred. Since intrathecal administration of low doses of PF-3845 resulted in increased brain levels of anandamide, we could not determine with certainty if FAAH located spinally, or supraspinally mediated the anti-allodynic effects seen in our previous studies with FAAH

inhibition. To delineate spinal vs supraspinal FAAH contributions, future studies should conduct dose response and time course studies for intrathecal and intracranial injections and choose a dose/time point that does not increase systemic levels of endogenous cannabinoids. When we find a dose or drug that can produce these effects will we be able to dissociate spinal from supraspinal contributions.

The anti-allodynic effects of systemic and local FAAH inhibition were mediated via both CB1 and CB2 receptors. Furthermore, we showed that FAAH (-/-) mice anti-allodynic effects were also blocked with rimonabant and SR144528. These data are perplexing in that it is believed that cannabinoids mediate their antinociceptive and antihyperalgesic responses via CB1 receptors whereas the anti-edematous effects are mediated through the CB2 receptors. However, our data could not explain how each receptor was mediating FAAHs effects.

Our efforts to assess MAGL inhibition failed due to the fact that we could not complete full characterization of these studies. JZL184 (40 mg/kg) administration resulted in hyperreflexia when administered to mice, therefore presenting a false positive. This property was recently noted in a paper from our laboratory while observing the effects of MAGL inhibition in the tetrad studies (Long et al., 2009b). Full characterization such as dose-response and time course studies should be conducted before further conclusions are made about the effects of MAGL inhibition in the LPS-induced allodynia model.

6.4 Final Discussion

The purpose of this dissertation was to evaluate targeting the endocannabinoid system to reduce nociception. The results reported in this dissertation support the hypotheses that phytocannabinoids as well as elevating endogenous cannabinoids block nociception in the acetic acid abdominal stretching model and the LPS-induced allodynia model of inflammation. The antinociceptive effects were mediated via CB1 receptors in both models of pain, and the CB2 receptor in the LPS model only. Although the mechanism of action for the CB2 receptor mediated effects could not be fully elucidated in these studies there are a couple possibilities for its involvement. Pain pathways are described at three different levels: in the periphery where it originates, in the spinal cord where transmission is integrated, and in the CNS (particularly in the PAG) where pain is processed (Figure 32). The first possibility stems from previous literature where it was illustrated that the CB2 receptor is upregulated in the dorsal root ganglia and paw tissue of rodents administered complete Freund's adjuvant (Hsieh et al., 2011). If this is the case, why do we only see a CB2 receptor mediated component in the inflammatory model and not the visceral pain model? The answer to this question is complex, however one can argue that the LPS model unlike the acetic acid model has a longer development cycle. Acetic acid abdominal stretching is assessed within twenty minutes after acetic acid administration, whereas LPS induced allodynia is assessed 24 h after LPS administration. Consequently, there is more time for the CB2 receptor to be transcribed and transported to the nerve terminals. A similar effect of CB2 receptor involvement is noted in a report from our laboratory demonstrating that the

antinociceptive effects of FAAH inhibition are abolished in CB2 knockout mice in the chronic constriction injury model (Kinsey et al., 2009) which also has a long development period.

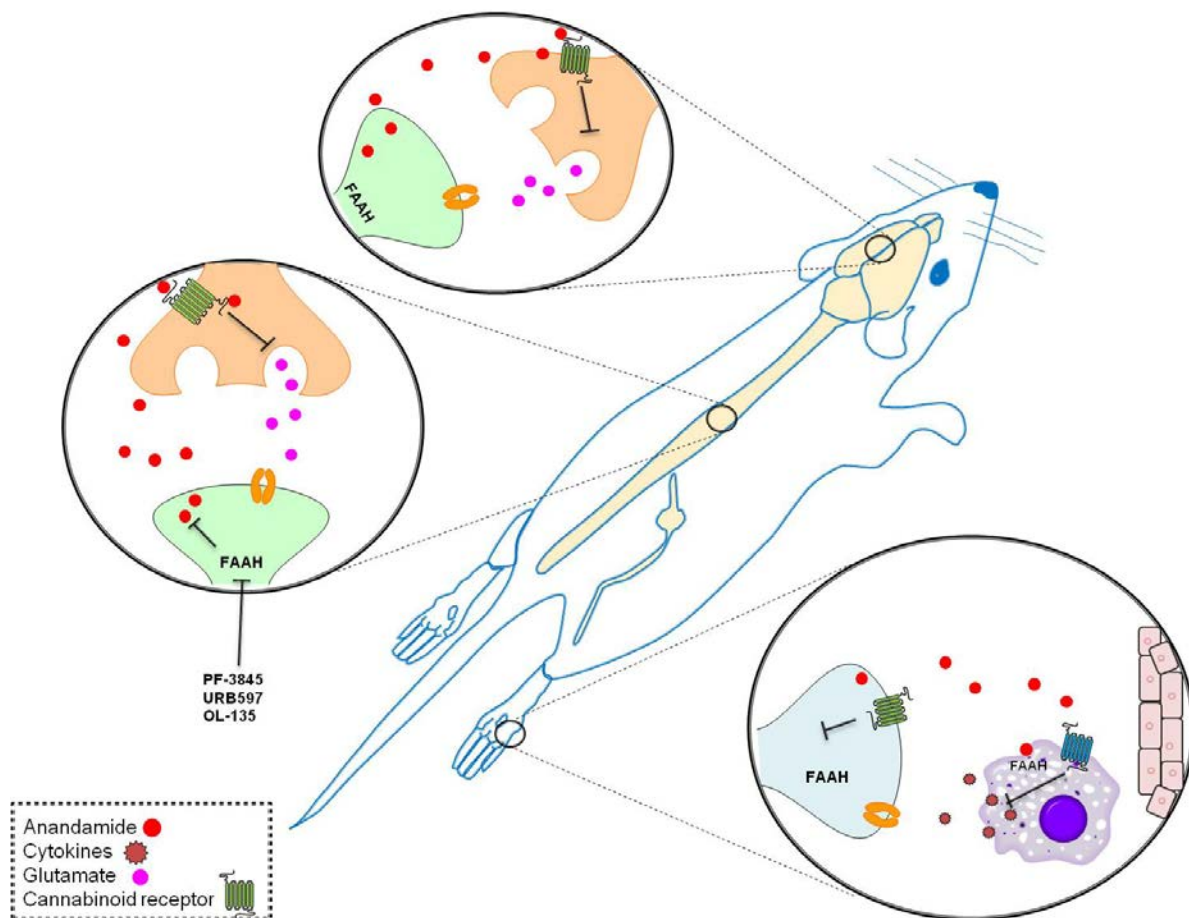


Figure 33. Schematic of FAAH loci of action. The figure illustrates the possible loci of action of FAAH mediated effects. The effects can take place locally in the paw, spinally, or supraspinally. Inhibiting FAAH in either region can increase anandamide levels and prolong the activation of the cannabinoid receptors within these regions.

Another possibility is that allodynia in the LPS induced model of inflammation may be driven by the release of cytokines. It is known that there is an infiltration of macrophages in response to an inflammatory agent such as LPS in our case, which results in the increase release of cytokines local to the site of injection. It is also known that these infiltrating cells contain CB2 receptors, and activation of the receptor can decrease the release of their inflammatory mediators.

Therefore, FAAH inhibition increases anandamide levels, which in turn bind to and activate the CB2 receptor located on the infiltrating macrophages and ultimately decrease the release of cytokines, resulting in a reversal of paw withdrawal thresholds. Although cytokines increase nociceptor sensitivity (Sommer and Kress, 2004) we are not sure if they are still affecting nociceptor functioning at the 24 hr time point after initial induction of inflammation. It is possible that cytokines are no longer being released from the invading cells therefore activation of CB2 receptors on macrophages may or may not affect nociceptor stimulation.

Our studies also illustrated that the FAAH inhibited anti-allodynic effects were completely reversed by either CB1 or CB2 antagonism. These data imply that activation of either receptor alone can drive the anti-allodynic effect. Conversely, since activation of both receptors do not produce an additive effect, may suggest that there is an all of none response of cannabinoid activation beyond a certain endocannabinoid threshold. Another explanation is that the downstream signaling processes due to the activation of the CB1 or CB2 receptors converge with each other to produce similar outcomes.

The question still remain as to where in the neural axis FAAH inhibited anti-allodynic effects are manifested, and how are both cannabinoid receptors mediate different effects in different models. Taken together, the data presented in this dissertation suggest that phytocannabinoids as well as FAAH and MAGL inhibition represents a promising target for the treatment of various types of pain.

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Vitae

Lamont Booker was born September 7, 1981, in Fort Benning, Georgia and is a U.S. citizen. He joined the Department of Pharmacology and Toxicology in 2006 and began his graduate work in the lab of Aron H. Lichtman in the summer of 2007.

EDUCATION

Virginia Commonwealth University, Richmond, Virginia
PhD in Pharmacology & Toxicology, *expected* May 2011
Dissertation Title: Targeting the endocannabinoid system to reduce nociception

North Carolina State University, Raleigh, North Carolina
Masters of Toxicology, May 2005

Fayetteville State University, Fayetteville, North Carolina
B.S in Biology, minor in Chemistry (Cum Laude) May 2003

AWARDS/HONORS

2010	Ruth L. Kirschstein National Research Service Award for Predoctoral Fellowship (F31) to Promote Diversity in Health Related Research
2009, 2010	Travel Award, International Cannabinoid Research Society
2008	Travel Award, Carolina Cannabinoid Collaborative
2007-2008	Graduate Student Mentorship Award
2007-2009	National Institute on Drug Abuse (NIDA) Research Training Fellowship (T32). Principle Investigator: William L. Dewey

PUBLICATIONS

Lamont Booker, Kinsey SG, Abdullah RA, Long JZ, Blankman JL, Cravatt BF, Lichtman AH (2011). Inhibiting Fatty Acid Amide Hydrolase in the Nervous System Reverses Lipopolysaccharide-induced Inflammatory pain. *Br J Pharmacol* (in press)

Schlosburg JE, Blankman JL, Long JZ, Nomura DK, Pan B, Kinsey SG, Nguyen PT, Ramesh D, **Booker L**, Burston JJ, Thomas EA, Selley DE, Sim-Selley LJ, Liu Q, Lichtman AH, Cravatt BF (2010). Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nat Neurosci*. 13(9):1113-9.

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PRESENTATIONS

Scientific Conferences

Poster Presentation. Neuronal FAAH Inhibition via PF-3845 Reverses LPS-Induced Tactile Allodynia. **Booker L.**, Kinsey SG., Abdullah R., Long JZ., Boger D., Cravatt BF., Lichtman AH. International Cannabinoid Research Symposium 2010, Lund Sweden.

Poster Presentation. FAAH Inhibition Modulates Inflammatory Pain in a Lipopolysaccharide-Induced Model. **Booker L.**, Kinsey SG., Ahn K., Cravatt BF., Lichtman AH. International Cannabinoid Research Symposium 2009, St. Charles, IL.

Poster Presentation. Assessment of Phytocannabinoids in the acetic acid induced abdominal stretching model. **Booker L.**, Naidu PS., Lichtman AH. Carolina Cannabinoid Collaboration Conference 2008, Williamsburg, VA.

Poster Presentation. Activating the Endogenous Cannabinoid System Reduces Visceral Nociception. **Booker L.**, Naidu PS., Boger D., Cravatt BF., Lichtman AH. Annual Biomedical Research Conference for Minority Students (ABRCMS) 2008, Orlando, FL.

Oral Presentation. Exploring Home Remedies to Treat the Common Cold-Antibiotic Resistance. **Booker L.**, Richardson S., Raynor JE. National Association for Equal Opportunity in Higher Education (NAFEO) 2002, Washington D.C. (Third Place Winner)

Lectures/Invited Presentations

Guest Lecturer. “Linking the Past to the Future for our Students”. Fayetteville State University Science Colloquium 2010, Fayetteville, NC.

Guest Lecturer. “Marijuana Research Talk”. WVCW Radio Station 2010, Richmond, VA.

Guest Lecturer. Antidepressants and Mood Stabilizers. Course: General Pharmacology and Toxicology 400 Level. Virginia Commonwealth University, Richmond, VA, 2010.

Guest Lecturer. Delta-9 THC and other Phytocannabinoids Reduce Visceral Pain; and Personal Testimony from Undergraduate to Graduate School 2009. Fayetteville State University, Fayetteville, NC.

SERVICE AND MENTORSHIP ACTIVITIES

Volunteer, High School Tutor/Mentor, Armstrong High School 2007-present

Volunteer, VCU Watts Research Symposium 2008-2009

Coordinator: Introduction to Laboratory Techniques and Animal Handling Lecture, 2008

Student Government Representative VCU 2008-2009

Graduate Student Mentorship Program, VCU 2007-2008 (GSMP)

Pharmacology/Toxicology Student Body, Treasurer 2007

Inspirational speaker (Replanting the Seed Ministry)

St. Paul’s Baptist Church Youth College Coordinator (Remote Care Team Leader) 2008-present

Alpha Phi Alpha Fraternity Inc, Recording Secretary 2009-2010, Membership Intake

Coordinator 2010-2011

MEMBERSHIP IN PROFESSIONAL ORGANIZATIONS

2010-present American Society for Pharmacology and Experimental Therapeutics

2008-2010 Society for Neuroscience

2007-present International Cannabinoid Research Society

2006-present Carolina Cannabinoid Research Society

2005-present Alpha Phi Alpha Fraternity Incorporated