

2014

Targeting the Endocannabinoid Metabolic Enzymes to Reduce Inflammatory Pain

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Targeting the Endocannabinoid Metabolic Enzymes to Reduce Inflammatory Pain

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

By Sudeshna Ghosh

01/13/2014

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Acknowledgement

My most sincere thanks go to my advisor Dr. Aron Lichtman. I thank him for introducing me to the wonders and challenges of scientific research. I would also like to thank him for allowing me to work under his guidance. He has been a great influence in this project and the direction the research has taken. He is one of the best examples of an ideal mentor who is always ready to listen, understand the needs of the students, and offer all possible help. Additionally, I would like to thank Dr. Laura Wise for her patience, guidance, encouragement, and support during the development of this project. I am indebted to her for believing in me and giving me the support to achieve my goals. My sincere gratitude goes out to my committee members: Dr. Steve Negus, Dr. Guy Cabral, Dr. Murthy Karnam, and Dr. Sim-Selley for their insight and suggestions, which are precious to me. Thank you to all of the Lichtman past and present lab members specially Tom, Jason, Kim, Travis, Allen, Rehab, Qing, Jenny, Bogna, Divya, Scott, Lamont, Steve, and all others. Without all of your generosity, friendship, help, willingness to teach, and ears to listen, I would not have made it to this point. I believe that our group includes some of the brightest scientists around, and I feel very blessed to have had the opportunity to work with you all. I want to express sincere gratitude to Dr. Hamid Akbarali, and Dr. Dewey for having faith in me and giving me the chance to be a part of this renowned department. Finally, I would like to express my eternal gratitude to my parents, husband, and my 4 months old for their everlasting love and support.

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List of Abbreviations

2-AG, 2-arachidonoylglycerol;

AEA, arachidonylethanolamide (anandamide);

ACEA, arachidonyl-2-chlorethylamide;

ANOVA, analysis of variance;

CB₁, cannabinoid receptor type 1;

CB₁KO, cannabinoid receptor type 1 knock out;

CB₂, cannabinoid receptor type 2;

CB₂KO, cannabinoid receptor type 2 knock out;

cAMP, cyclic adenosine monophosphate;

CCI, chronic constriction injury;

CFA, Complete Freund's adjuvant;

COX, cyclooxygenase;

CNS, central nervous system;

DAGL, diacylglycerol lipase;

FAAH, fatty acid amide hydrolase;

G-protein, guanine nucleotide binding protein;

GPCR, G-protein coupled receptor;

i.p. intraperitoneal;

JZL184, 4-nitrophenyl-4-(dibenzo[d] [1, 3] dioxol-5-yl (hydroxy) methyl) piperidine-1-carboxylate;

JZL195, 4-nitrophenyl 4-(3-phenoxybenzyl) piperazine-1-carboxylate;

JNK, Jun N-terminal kinase;

LPS, lipopolysaccharide;

MAGL, monoacylglycerol lipase;

MAPK, mitogen-activated protein kinase;

NAPE-PLD, N-acyl phosphatidylethanolamine phospholipase D;

PF-3845, N-(pyridin-3-yl)-4-(3-(5-(trifluoromethyl) pyridin-2-yloxy) benzyl) piperidine-1-carboxamide;

Rim, rimonabant, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-1Hpyrazole-3-carboxamide HCl;

SR2, SR144528, N-[(1S)-endo-1, 3, 3,-trimethylbicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-Methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide;

THC, Δ 9-tetrahydrocannabinol;

URB597, [3-(3-carbamoylphenyl) phenyl] N-cyclohexylcarbamate

WIN55, 212-2, (R)-(+)-[2, 3-Dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1, 2, 3-de)-1, 4-benzoxazin-6-yl]-1-naphthalenylmethanone;

WT, wild type;

Abstract

Pain is a debilitating condition that presents a problem of clinical relevance. Common analgesics include opioids and non-steroidal anti-inflammatory drugs (NSAIDs). Despite different degrees of effectiveness, a major drawback of these analgesic classes is their side effects. For example, side effects associated with opioids include pruritus, respiratory depression, hyperalgesia, constipation, dependence. In addition, chronic use of NSAIDs can cause gastric ulcers. Delta-9 tetrahydrocannabinol (THC), the primary psychoactive constituent of marijuana, produces antinociception in various preclinical models of pain. Similarly, many synthetic cannabinoid receptor agonists produce antinociception in preclinical models of pain. However, their psychomimetic effects dampen interest for their therapeutic development. THC and these cannabinoids act upon the endocannabinoid system, which is comprised of the cannabinoid 1 (CB₁) and cannabinoid 2 (CB₂) receptors, endogenous ligands arachidonylethanolamide (anandamide; AEA), 2-arachidonoylglycerol (2-AG), and endocannabinoid biosynthetic and catabolic enzymes. In the present study, we evaluated the impact of inhibiting one of the major biosynthetic enzymes of 2-AG, diacylglycerol lipase- β (DAGL- β), and two primary endocannabinoid catabolic enzymes, monoacylglycerol lipase (MAGL), and fatty acid amide hydrolase (FAAH). MAGL is responsible for degrading 2-AG and FAAH is the principal degradative enzyme for anandamide. We hypothesized that inhibiting these enzymes will produce anti-edematous and anti-allodynic effects in preclinical models of inflammatory pain.

In Chapter 2, we tested whether the selective MAGL inhibitor JZL184 and FAAH inhibitor PF-3845 would reduce nociceptive behavior in the carrageenan test. JZL184 and PF-3845 significantly attenuated carrageenan-induced paw edema and mechanical allodynia (a nociceptive response to normally non-noxious stimuli), whether administered before or after

carrageenan. Complementary genetic and pharmacological approaches revealed that JZL184's anti-allodynic effects required both CB₁ and CB₂ receptors, but only CB₂ receptors mediated its anti-edematous actions. Anti-edematous effects of PF-3845 were mediated through CB₂ receptors. Importantly, the anti-edematous and anti-allodynic effects of JZL184 underwent tolerance following repeated injections of high dose JZL184 (16 or 40 mg/kg), but repeated administration of low dose JZL184 (4 mg/kg) retained efficacy.

Although the data in the first set of studies demonstrate that inhibition of MAGL or FAAH reduces inflammatory pain, JZL184 and PF-3845 only produced partial effects. In an attempt to increase efficacy, Chapter 3 tested whether combined blockade of FAAH and MAGL would produce enhanced anti-edematous and anti-allodynic effects in the carrageenan model of inflammatory pain. Partial blockade of MAGL, with a low dose of JZL184 (4 mg/kg), and full blockade of FAAH, with a high dose of PF-3845 (10 mg/kg), enhanced the anti-allodynic effects, but no further increases in the anti-edematous effects were found. Importantly, repeated administration of this combination did not result in tolerance. A novel FAAH-MAGL dual inhibitor SA-57, which is far more potent in inhibiting FAAH than MAGL, reversed carrageenan-induced allodynia. Taken together, these findings suggest that dual MAGL and FAAH inhibition represents a promising avenue for the treatment of inflammatory pain.

Chapter 4 of this dissertation tested whether inhibition of DAGL- β , a major biosynthetic enzyme of 2-AG, would reverse inflammatory pain. Two DAGL- β inhibitors, KT109, and KT172, which have been previously shown to reduce arachidonic acid, prostaglandins, and TNF- α levels in lipopolysaccharide (LPS)-stimulated murine macrophages, were used to test whether these compounds would elicit antinociceptive effects in the LPS model of inflammatory pain model. Because these drugs also inhibit ABHD6, we assessed KT195, a selective ABHD6

inhibitor that is inactive against DAGL- β . KT109, but not KT172 or KT195, significantly reversed LPS-induced allodynia. Importantly, we found that DAGL- β knockout mice possess an anti-allodynic phenotype, but KT109 did not elicit any further decrease in allodynia in these animals. The anti-allodynic effects of KT-109 did not require cannabinoid receptors. Additionally, the anti-allodynic effects of KT-109 did not undergo tolerance following repeated administration. KT-109 did not produce any gastric hemorrhagic effects when compared to the NSAID diclofenac, which significantly produced gastric hemorrhages. These results suggest that blockade of DAGL- β leads to antinociceptive effects through a cannabinoid receptor independent mechanism of action, with absence of notable side effects.

Collectively, the research presented in this dissertation suggests that the endocannabinoid catabolic enzymes MAGL and FAAH, and the endocannabinoid biosynthetic enzyme DAGL- β , represent promising targets to treat inflammatory pain.

Chapter 1: Introduction

Cannabinoid Discovery and Background

The earliest evidence of the use of cannabis was in 2900 BC on the island of Taiwan, near the mainland of China. Since prehistoric times, cannabis has been used for various purposes, including as a source of fiber for clothes and paper, religious and medicinal purposes as a curative and an anesthetic agent (Mechoulam, 1986). Records also exist of cannabis use in ancient civilizations in Japan, India, Israel, and Egypt. The western world became interested in the plant's therapeutic value beginning in the middle of the 19th century for use in treating ailments such as cough, fatigue, headache, asthma, and rheumatism (O'Shaughnessy et al., 1842; Lowinson et al., 1997). In 1964, Δ^9 -tetrahydrocannabinol (THC) was identified as the primary psychoactive constituent of *Cannabis sativa* (Gaoni and Mechoulam, 1964). The term "cannabinoid" (CB) was defined as a group of structurally related terpenoids responsible for the pharmacological effects of the plant (Gaoni and Mechoulam, 1971, Mechoulam et al., 1987). In addition, more than 100 other phytocannabinoids have been discovered (Appendino et al., 2011). Other prevalent phytocannabinoids, cannabidiol and cannabivarin possess a variety of pharmacological properties, but display fewer central nervous system (CNS) effects than THC and other synthetic cannabinoids (Mechoulam et al., 2002a, Mechoulam et al., 2002b). Subsequent research led to the discovery of cannabinoid receptors and the endocannabinoid system, as described below.

Endocannabinoid System

The endocannabinoid system consists of two G-protein coupled receptors (GPCRs), CB₁ (Devane et al., 1988, Matsuda et al., 1990, Pertwee, 1997) and CB₂ (Munro et al., 1993, Pertwee

et al., 2007) and the endogenous ligands that bind to these receptors, including N-arachidonylethanolamine (anandamide, AEA) (Felder et al., 1996, Martin et al., 1999b) and 2-arachidonoylglycerol (2-AG) (Mechoulam and Deutsch, 2005, Sugiura et al., 1995). In addition to these endogenous cannabinoids, other lipids including virodhamine, (Porter et al., 2002) meadethanolamide (Priller et al., 1995) and noladin ether (Hanus et al., 2001) interact with cannabinoid receptors. Endocannabinoids are enzymatically produced on demand in postsynaptic neurons and travel retrogradely to activate the CB₁ receptors that are located presynaptically (Katona et al., 1999). Stimulation of CB₁ and CB₂ receptors by endocannabinoids or other receptor agonists lead to decreases in cAMP production via blockade of adenylyl cyclase (Howlett et al., 1990), activation of inwardly rectifying potassium (GIRK) channels via Gβγ subunits (Mackie et al., 1995, McAllister et al., 1999), and inhibition of Ca²⁺ channels attenuating presynaptic activity. A detailed description of the cannabinoid receptors and endogenous cannabinoid biosynthesis and degradation is given in the following sections.

Cannabinoid Receptors

Two main cannabinoid receptors CB₁ (Devane, 1988, Matsuda, 1990, Pertwee, 1997) and CB₂ (Munro, 1993, Pertwee, 2007) were discovered in the 1990's. The CB₁ receptor has been cloned from rat (Matsuda, 1990), mouse (Chakrabarti et al., 1995), and human (Gerard et al., 1991) tissues and shows 97–99% amino acid sequence identity across species. CB₁ and CB₂ receptors share approximately 44% identity with each other (Munro, 1993). The transmembrane regions of these two receptors share approximately 68% amino acid similarity (Galiegue et al., 1995). THC and many other cannabinoids activate these receptors. Both CB₁ and CB₂ receptors are G-protein coupled receptors (GPCRs), which are associated with Gi/o G-proteins (Howlett, 1995, Pertwee et al., 2010). Upon interaction with CB₁ and /or CB₂ receptors agonists, these

GPCRs undergo conformational changes. The stimulated receptors facilitate the exchange of GDP for GTP at the α -subunit of the G-protein, leading to its dissociation from the receptor. Subsequently, the α -subunit and $\beta\gamma$ -heterodimer are released and ultimately regulate various downstream effectors. Upon activation, these receptors decrease cAMP production via blockade of adenylyl cyclase (Howlett, 1990), activation of inwardly rectifying potassium (GIRK) channels via $G\beta\gamma$ subunits (Mackie, 1995, McAllister, 1999). Furthermore, activation of the cannabinoid receptors inhibits N- and P/Q-type calcium channels, which reduce synaptic vesicle fusion to the nerve terminal thereby inhibiting the release of excitatory and inhibitory neurotransmitters.

CB₁ receptors are expressed pre-synaptically at both glutamatergic (Huang et al., 2001, Szabo and Schlicker, 2005) and GABAergic interneurons (Katona, 1999). These receptors play a role in short-term synaptic plasticity. CB₁ receptor agonists act as neuromodulators by inhibiting release of glutamate and GABA. Transient suppression of the stimulatory neurotransmitter (e.g. glutamate) is called depolarized-induced suppression of excitation (DSE) (Kreitzer and Regehr, 2001). Conversely, transient suppression of the inhibitory transmission (i.e. GABA) is termed depolarization-induced suppression of inhibition (DSI) (Vincent et al., 1992). Both result in cannabinoid receptor mediated hyperpolarization of a repetitively depolarized neuron, which suppresses subsequent vesicular fusion and release of glutamate or GABA.

The CB₁ receptor was originally described as the “brain type” cannabinoid receptor, because the level of expression was high in the brain (Herkenham et al., 1990). Autoradiography of CB₁ receptors in brain sections from several mammalian species, including rat, mouse and human, reveals a unique and conserved distribution; most dense in the basal ganglia, substantia nigra (pars reticulata), amygdalar nuclei, internal segment of the globus pallidus, caudate and

putamen, and in hippocampus and cerebellum. CB₁ receptors are also present in dorsal horn and lamina X in the spinal cord and in medium and large-sized neurons in dorsal root ganglion. Finally, CB₁ receptors are present in sparse densities in lower brainstem areas such as medulla and pons, periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) (Herkenham et al., 1991a, Herkenham et al., 1991b, Herkenham, 1990, Hohmann and Herkenham, 1999). Most behavioral effects produced by cannabinoids are mediated through CB₁ receptors. For example THC-induced catalepsy, hypolocomotion and hypothermia were completely absent in CB₁ receptor deficient mice indicating that the classical behavioral effects of THC are mediated through CB₁ receptors (Zimmer et al., 1999). Similarly, CB₁ receptors mediate the discriminative stimulus effects of THC in drug discrimination paradigm. In drug discrimination procedure, an animal is trained to perform an operant response paired with vehicle and a different operant response paired with the training drug. For example, the animal learns to press the left lever whenever it receives an injection of the training drug and to press the right lever when it is injected with vehicle. Once a subject has acquired to discriminate between drug and vehicle conditions, novel compounds are evaluated for substitution for the training drug. If a novel drug produces responding on the lever associated with the training drug, it is considered to have discriminative stimulus effects like those of the training drug and would be predicted to produce subjective effects like those of the training drug. The discriminative stimulus effects of cannabinoids in mice, rats, and rhesus monkeys were blocked by the CB₁ receptor antagonist, rimonabant (Rinaldi-Carmona et al., 1994), indicating a CB₁ receptors mechanism of action (Compton et al., 1996, Wiley et al., 1995a, Wiley et al., 1995b).

CB₂ receptors are expressed in both the CNS and periphery. These receptors are associated with immune cells such as B cells, T cells, macrophages, neutrophils (Carayon et al.,

1998, Galiegue, 1995, Pettit et al., 1996, Schatz et al., 1997). They have also been detected in spleen, bone marrow, pancreas, uterus, and lung (Galiegue, 1995). Initially the CB₂ receptors were described as the periphery receptors; however, these are also expressed in the CNS in microglial cells (Cabral and Marciano-Cabral, 2005) and in brainstem neurons (Van Sickle et al., 2005).

Biosynthesis and degradation of Endocannabinoids

The biosynthetic pathways of anandamide and 2-AG have been well-investigated (Di Marzo and Petrosino, 2007). Anandamide was the first endogenous cannabinoid to be isolated and characterized (Devane et al., 1992, Felder et al., 1993). Anandamide belongs to the N-acyl ethanolamine (NAE) family of bioactive lipids. N-acylphosphatidyl ethanolamine (NAPE) is the precursor of anandamide, which is synthesized by transacylation of phosphatidyl ethanolamine (Di Marzo et al., 1994). NAPE is then hydrolyzed by a Ca²⁺ sensitive NAPE-selective phospholipase D (NAPE-PLD), a phosphodiesterase from the metallo- β -lactamase family. NAPE-PLD knockout mice displayed a fivefold reduction in Ca²⁺-dependent conversion of NAPEs to NAEs (Leung et al., 2006). However, NAPE-PLD knockout mice show wild type levels of anandamide (Leung, 2006) indicating the existence of other pathways apart from the transacylation and phosphodiesterase pathways that are involved in anandamide production. In one of the alternate pathways, NAPEs are hydrolyzed to glycerophospho-N-aryl ethanolamine (GP-NAEs) by a serine hydrolase, α/β -hydrolase 4 (ABHD-4). GP-NAE is then hydrolyzed to anandamide by glycerophospho-diesterase-1. In the second alternate pathway, phospho-N-arachidonoyl ethanolamine (pAEA), which is synthesized from N-arachidonoylphosphatidylethanolamine by a phospholipase-C (PLC), is hydrolyzed to anandamide by phosphatases such as PTPN22 (Di Marzo, 2008, 2009, Ligresti et al., 2005).

2-AG and anandamide are metabolized intracellularly to arachidonic acid and other products. Anandamide is hydrolyzed by post-synaptically located fatty acid amide hydrolase (FAAH), which belongs to the serine hydrolase family of enzymes (Cravatt et al., 1996, Patricelli and Cravatt, 1999, 2000, Patricelli et al., 1999, Patricelli et al., 1998). FAAH is a membrane bound serine hydrolase, which is located in the endoplasmic reticulum and acts at an alkaline pH (Cravatt, 1996). The structure of FAAH has been determined by X-ray crystallography (Bracey et al., 2002). FAAH (-/-) mice display 15 fold elevated anandamide levels in the brain (Cravatt et al., 2001, Lichtman et al., 2004). FAAH also hydrolyzes several other bioactive fatty acid amides in vitro (Cravatt, 1996, Desarnaud et al., 1995, Maurelli et al., 1995, Ueda and Yamamoto, 1995), including N-palmitoylethanolamine (Calignano et al., 1998), and the sleep-inducing lipid oleamide (Cravatt et al., 1995). In addition, another FAAH substrate, N-acyl taurines (NATs) were discovered in the nervous system tissues from FAAH (+/+) and (-/-) mice. NATs were elevated more than 10-fold in brains and spinal cords from FAAH(-/-) mice (Saghatelian et al., 2006). Another enzyme of the serine hydrolase family, FAAH-2 is also involved in anandamide hydrolysis (Wei et al., 2006) but the hydrolysis rate is 38-fold slower than FAAH. In humans, FAAH-2 is present in the peripheral tissues such as the liver, but this enzyme is absent in the rodents. FAAH-2 hydrolyzes anandamide in situations where FAAH is inactivated (Wei, 2006). Additionally, a third *N*-acylethanolamine hydrolase termed *N*-acyl-ethanolamine-hydrolyzing acid amidase (NAAA) was discovered in human megakaryoblastic cells (Ueda et al., 1999) and in various rat tissues including lung, spleen, and macrophages (Ueda et al., 2001). This enzyme is active only at acidic pH and is catalytically distinct from FAAH (Ueda and Tsuboi, 2005). This enzyme has shown to primarily hydrolyze N-palmitoyl ethanolamine and anandamide in

macrophages (Solorzano et al., 2009, Sun et al., 2005). Additionally, cyclooxygenase-2 (COX-2) also hydrolyses anandamide to prostaglandin ethanolamides (PGH₂-EA) (Kozak et al., 2002).

The second discovered endogenous cannabinoid, 2-AG is synthesized via a two-step process. Phosphatidyl inositol is converted to Diacylglycerol (DAG) by PLC activity, which is then hydrolyzed by diacylglycerol lipase to 2-AG. Diacylglycerol lipases (DAGLs) are biosynthetic enzymes that are involved in mediating neuronal growth during development and as a retrograde messenger (Tanimura et al., 2010, Williams et al., 2003). Two DAGL enzymes are, DAGL- α (120 kDa) and DAGL- β (70 kDa). DAGL has four transmembrane domains with both the C and N terminus located inside the membrane (Bisogno et al., 2003). This enzyme also belongs to the serine hydrolase family. DAGL- β is predominantly expressed in the developing brain and in the periphery, while the α -isoform is mainly found in the central nervous system (Bisogno and Di Marzo, 2010, Gao et al., 2010). During brain development DAGL, is located on the axonal tract where it produces 2-AG, which mediates the axonal growth (Williams, 2003). In adults, DAGL is located postsynaptically (Williams, 2003). In the hippocampus, the postsynaptic release of 2-AG induced by depolarization results in the transient suppression of GABA-mediated transmission at inhibitory synapses. This form of depolarization-induced suppression of inhibition (DSI) is completely lost in DAGL knockout animals, indicating an essential role for this enzyme in regulating retrograde synaptic plasticity (Gao, 2010). In addition, adult neurogenesis is compromised in the hippocampus and subventricular zone (SVZ) of DAGL- α (-/-) mice and in the hippocampus of DAGL- β (-/-)mice (Gao, 2010). These data indicate that DAGL plays an important role in adult neurogenesis (Gao, 2010). A second pathway for 2-AG production involves an intermediate 2-arachidonoyl-lysophosphatidylinositol (lyso-PI). Phosphatidyl inositol preferring phospholipase A1 converts phosphatidyl inositol to the lyso-PI

intermediate, which in turn gets converted to 2-AG by lysophosphatidyl inositol selective PLC (Di Marzo, 2008, 2009, Ligresti, 2005).

MAGL is the main enzyme responsible for degrading 2-AG (Dinh, 2004, Dinh et al., 2002). It hydrolyses 2-AG to arachidonic acid and glycerol (Fredrikson et al., 1986). The enzyme is expressed presynaptically, and the highest concentrations are found in the cell membrane, and in the cytosolic fraction (Blankman et al., 2007, Dinh, 2002). The physiological roles of MAGL appear to be tissue specific. For example in adipose tissue, monoglycerides formed from stored triglycerides is hydrolyzed by MAGL to fatty acid and glycerol. The released free fatty acids are then used as a source of energy in vivo (Chon et al., 2007). MAGL indirectly controls prostaglandin levels, by being a major enzyme involved in the formation of its precursor arachidonic acid. It has been shown that MAGL exerts control over both basal and LPS-induced arachidonic acid and prostaglandins in liver and lung (Nomura et al., 2011). But cytosolic phospholipase -A2 regulates these lipids in the gut and spleen indicating that MAGL regulates 2-AG degradation in a tissue specific manner (Nomura, 2011). In addition, MAGL controls the duration of retrograde signaling mediated by 2-AG. ABHD6 and ABHD12 also hydrolyze 2-AG to arachidonic acid (Blankman, 2007, Marrs et al., 2010). Additionally, 2-AG can be hydrolyzed by COX-2 to prostaglandin glycerol ester (PGH₂-G) (Di Marzo, 2008, Ligresti, 2005).

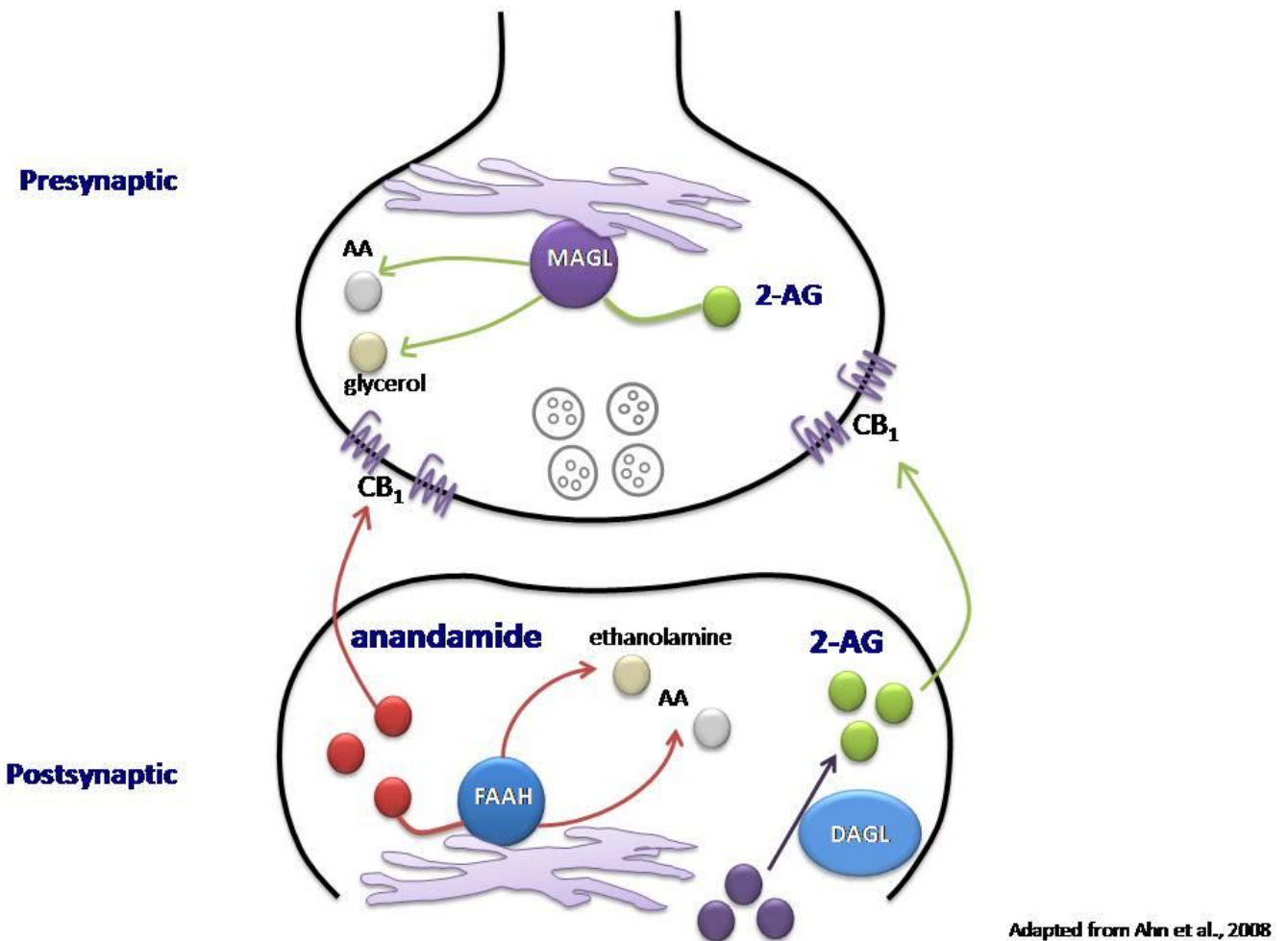


Figure 1: The endocannabinoid system: receptors, ligands and their regulatory pathways

2-AG: 2-arachidonylglycerol; AEA: anandamide; AA: arachidonic acid; MAGL: monoacylglycerol lipase; FAAH: fatty acid amide hydrolase; DAGL: diacylglycerol lipase; CB₁: cannabinoid receptor-1.

Overview of Pain:

As defined by the International Association for the Study of Pain (1979), pain is “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (International association for study of pain, 1979) (Pain,

1979). The sensory components of pain are complex and several distinct types exist, including nociceptive (pain in response to a noxious stimulus), inflammatory (pain in response to tissue damage and inflammation), neuropathic (pain associated with the damage to the nervous system), and functional (pain resulting from abnormal central processing of normal input) (Woolf, 2004). Pain may be evoked by the presence or absence of a stimulus. There are different types of nociceptive stimuli such as thermal, electrical, mechanical and chemical (Bennett, 2001, Dykstra, 1985, Le Bars et al., 2001, Mogil et al., 2001) that have been used to evoke pain in different pain models. Pathological pain states can produce allodynia, which is defined as pain produced by stimulus that is normally not painful and/or hyperalgesia, which is increased pain sensitivity to a stimulus that is normally painful (International association for study of pain, 1979). Analgesia is absence of pain to a noxious stimulus (International association for study of pain, 1979). Millions of people suffer from pain every year and that has tremendous effects on the US in health care costs, rehabilitation and lost worker productivity, as well as cause emotional and financial burden on patients and their families (American academy of pain medicine). According to a recent Institute of Medicine report, pain is a significant public health problem that costs society at least \$560-\$635 billion annually, this includes the total incremental cost of health care due to pain from ranging between \$261 to \$300 billion and \$297-\$336 billion due to lost productivity (PRWEB 2011).

Pain processing

Peripheral:

While pain normally serves as a warning device and can facilitate recuperation, it can also represent a serious and debilitating condition. The complex physiology of pain begins with activation of primary sensory neurons, also known as nociceptors, by noxious stimuli (Burke,

2007). Nociceptors have two different kinds of axons rapidly conducting A- δ fibers, and slowly conducting, unmyelinated C-fibers. A- δ fibers elicit the first phase of sharp pain and C-fibers elicit a wave of dull pain (Julius and Basbaum, 2001, Millan, 1999). Both A δ - and C-fibers synapse in the dorsal horn of the spinal cord. Then their sensory inflow transfers to second-order neurons in the spinal cord in topographically organized manner (Costigan and Woolf, 2000, Markenson, 1996, Millan et al., 1996). Then the projection cells with axons in the receiving neuron transmitted to the brain and ultimately activate specific brain regions. (Riedel and Neeck, 2001, Todd et al., 2006, Willis, 1991).

Central:

Spinal Cord

The neuronal organization of the spinal cord determines characteristic features of pain, e.g. the projection of pain into particular tissues (Schaible et al., 2006). Noxious stimuli activate nociceptors, which transmit the signal through the dorsal root ganglia to the dorsal horn of the spinal cord (Todd, 2006, Woolf, 2004). The activation of nociceptive fibers at the dorsal horn results in the release of excitatory neurotransmitter glutamate, aspartate, calcitonin gene peptide (CGRP), which are important mediators of spinal transmission (Urban and Gebhart, 1997) (Todd, 2006, Yaksh, 1999). The two classes of interneurons in the dorsal horn that play roles in the transmission of nociception are the inhibitory neurons that use γ -aminobutyric acid (GABA) or glycine, and excitatory neurons that are glutamatergic (Todd, 2006). The excitatory amino acid glutamate activates the metabotropic G-protein coupled receptor alpha-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid (AMPA)/kainite (K) and the ionotropic receptor, N-methyl-D-aspartate (NMDA) (Morgan et al., 1999, Ren and Dubner, 1999a, b, Todd, 2006). The AMPA/K receptors mediate fast synaptic transmission by activating and deactivating rapidly and

desensitize upon prolonged agonist exposure. On the other hand, NMDA receptors have a significantly slower activation and deactivation time (McBain and Mayer, 1994). NMDA receptor activation plays a role in inflammatory and neuropathic pain states (Price et al., 1994, Sindrup and Jensen, 1999) and results in the activation and exacerbation of peripheral hyperalgesia and accountable for the changes seen during central sensitization (Costigan and Woolf, 2000, Morgan, 1999, Ren and Dubner, 1999a, b).

Brain

Nociceptive signals in the dorsal horn project to the supraspinal sites (Almeida and Val-Gallas, 1997, Berkley and Hubscher, 1995, Bester et al., 1995, Calvino and Grilo, 2006, Millan, 1999). Two major pathways that project nociceptive information are the spinothalamic tract and the spinoreticular tract. Glutamate facilitates the transfer of the signal from the spinothalamic tract to the thalamus and from the spinomesencephalic tract to the periaqueductal grey (PAG) while other neuropeptides (i.e. SP) play a role in relaying information to the brain (Azkue et al., 1997) (Jensen and Yaksh, 1992). The spinothalamic tract first innervates the thalamus and consequently the postcentral gyrus of the cortex (Apkarian et al., 1992, Berkley and Hubscher, 1995, Casey et al., 1996, Millan, 1999). The thalamus is a major relay station in the emotional and cognitive processing of pain that relays information to various cortical regions via a complex pattern of connections (Millan, 1999). On the other hand, spinoreticular tract fibers ascend to reach the brainstem reticular formation and then project to thalamus and hypothalamus. This pathway is involved in the affective/motivational aspects of pain (Almeida et al., 2004).

Inflammation

Inflammation develops in response to tissue injury and is a main contributor in pain perception (Costigan and Woolf, 2000, Pasero, 2004). Inflammation is triggered by macrophages

release at the site of tissue damage (Costigan and Woolf, 2000). Several inflammatory mediators such as bradykinin, prostaglandins, histamine, and cytokines are released in response to tissue injury (Costigan and Woolf, 2000, Millan, 1999). Bradykinin causes activation of second messengers resulting in increased sodium ion channel conductance and sensitization. Prostaglandins enhance activity of bradykinin and contribute to inflammatory responses. These mediators act on various receptors and ion channels of the nociceptors, causing various signal transduction cascades that result in modulation of effector molecules and changes in gene transcription (Costigan and Woolf, 2000). There are two types of inflammatory responses: acute inflammation, which is a short-term process and displays five major signs of inflammation: swelling, redness, heat, pain, and sometimes dysfunction of organs (Costigan and Woolf, 2000, Millan, 1999) and chronic inflammation which is of longer duration and is associated with local infiltration of monocytes, lymphocytes, macrophages, and proliferation of small blood vessels and fibroblast (Chen et al., 2008).

Preclinical pain models

Experimental models to study pain mechanisms necessitate the use of a quantifiable and reproducible stimulus that elicits measurable nociceptive responses. The different types of stimuli used to generate nociceptive responses include thermal, electrical, mechanical and chemical. Therefore, pain models can be classified based on the different kinds of stimuli.

Common pain models that employ thermal stimuli are the tail-flick test, the paw-withdrawal test, and the hot plate test (Le Bars, 2001). The tail-flick test involves immersing the tip of the tail in a warm water bath at a predetermined temperature that is typically above 50° C or focusing a radiant heat source on the tail. The heat provokes abrupt reflexive movement of the tail and sometimes recoiling of the body. The latency for the response is generally determined

with a stopwatch or an apparatus that senses tail movement (Ben-Bassat et al., 1959, Grotto and Sulman, 1967, Janssen et al., 1963). In paw withdrawal test, heat is applied to the planter surface of an already inflamed paw of freely moving animals and the latency of withdrawing the paw from the stimulus is monitored (Hargreaves et al., 1988a). The limitation of this assay is that the position of the limb because the background level of activity in the flexors changes with the position of the animal. In the hot plate test, the animal is put into a cylindrical space with a floor consisting of a heated metallic plate (Leimbach, 1953, MacDonald, 1944, O'Callaghan and Holtzman, 1975). Then the reaction time of withdrawing the paw by licking or jumping is measured.

Mechanical stimuli applied to a hind paw or the tail and the threshold (weight in grams) an evoked behavior (lifting, stretching, and licking) are measured. This type of stimulus has limitations. For example, repeated use of the stimulus can increase the sensitivity of the stimulated area resulting a false positive result (Fennessy and Rattray, 1971). As proposed by Randall and Selitto in 1957, the latter can be improved by comparing the paw withdrawal threshold of a non-inflamed normal paw with an inflamed paw from the same animal (Randall and Selitto, 1957). A mechanical stimulus is used to measure trauma, inflammation or nerve injury evoked pain.

Electrical stimulus is delivered in the paw through the floor of the cage where the animals are free to move (Blake et al., 1963, Bonnet and Peterson, 1975, Crocker and Russell, 1984, Evans, 1961). The animals respond to this noxious stimulus by and the latency is measured.

Thermal, mechanical, and electrical are short duration stimuli that produce phasic pain (Le Bars, 2001) where the receptors respond to punctuate stimuli (Le Bars, 2001). Other tests involve the use of variety of chemicals that produce inflammatory responses such as formalin

(Hwang and Wilcox, 1986, Kellgren, 1939), acetic acid (Teiger, 1976), complete Freund's adjuvant (Iadarola et al., 1988) and carrageenan (Okuda et al., 1984, Otsuki et al., 1986) (Butler et al., 1992, Coderre and Wall, 1987, Tonussi and Ferreira, 1992). These types of stimuli are used as a model of tonic pain where the receptors adapt slowly to the stimulus and convey information about the duration of the stimulus (Le Bars, 2001). Most of these assays invoke nociceptive behavior or increase sensitivity to the stimuli described above. These responses can persist for a prolonged period of time. Complete Freund's adjuvant or carrageenan can elicit pain that lasts from days to weeks (Okuda, 1984, Otsuki, 1986) (Butler, 1992, Coderre and Wall, 1987, Le Bars, 2001, Tonussi and Ferreira, 1992).

In the models described above, a noxious stimulus is used to produce the measured response. The allodynic, hyperalgesic and other nociceptive responses described above are conceptualized as pain-stimulated behavior (Negus et al., 2006). Many preclinical models of pain (acute, inflammatory, and neuropathic) are based on pain-stimulated behaviors. For example, a noxious stimulus produces an increase in frequency and intensity of the dependant measure, which is used to infer pain (Dykstra, 1985, Le Bars, 2001, Negus, 2006, Stevenson et al., 2009). A pitfall of pain-stimulated assays is that analgesics can produce false positives by impairing motor function.

However, pain is also associated with depression of many behaviors such as feeding, locomotor activity and mating (Flecknell, 1994, Karas, 2002, Negus, 2006, Stevenson, 2009). These responses are conceptualized as pain-depressed behaviors. Accordingly, the pain stimulus results in a depression of learned or unconditional behaviors (Stevenson et al., 2006). In the case of pain-depressed behavior, treatment efficacy is inferred by the reinstatement of the behavior (Negus, 2006). An advantage of models measuring pain-depressed behavior is that there is a

decrease of likelihood of a drug producing false positive analgesic effects. In addition, preclinical models measuring pain-depressed behavior possess clinical relevance because restoration of a function is a desired outcome in patients.

It is believed that pain-depressed behavior involves affective or emotional aspect of pain while pain stimulated behavior reflect sensory-discriminative components of pain (Negus, 2006, Stevenson, 2006, Stevenson, 2009). Therefore, pain-stimulated and pain-depressed behavior represent two complementary approaches to study the sensory-discriminative and affective-motivational aspects of pain. The affective component of pain is clinically relevant and it is also studied by using condition place preference (CPP) paradigm. In this assay, the subject is subjected to a chronic pain condition and the effects of analgesics are tested in an apparatus containing two chambers; one chamber that is paired with the analgesic and the other chamber that is paired with vehicle. A drug that produces pain relief will lead to a preference to the chamber associated with it (Cahill et al., 2013, Niikura et al., 2008, Ozaki et al., 2003, Ozaki et al., 2002). It is also important to test the drug in different animals that are in a pain free condition to assess intrinsic rewarding effects of a drug. An analgesic should be more potent in producing CPP in animals under the pain state than to the animals under the pain free condition. Many types of analgesics such as morphine and gabapentin have been shown to produce CPP in pain-subjected animals, suggesting pain relief.

Analgesics

Analgesics are the therapeutic compounds prescribed to control or treat the pain state, which include opiates and non-steroidal anti-inflammatory drugs (NSAIDs). Despite possessing a range in the degree of effectiveness, a major drawback of these analgesic compounds is related to their long-term use. For example, NSAIDs produce their antinociceptive actions by

inhibiting cyclooxygenases, leading to a decrease in prostaglandin production. Untoward side effects of NSAIDs include gastric ulcers, renal failure, allergic reactions, and hemorrhage by inhibiting platelet aggregation (i.e., aspirin). Additionally, they can cause potentially life-threatening liver damage. Opioids produce their pharmacological effects through μ , δ , κ receptors in the CNS and periphery and are very effective analgesics. These drugs are associated with serious untoward side effects both acutely and upon chronic administration. Acute administration of opiates can cause respiratory depression and pruritus and repeated opioid use can lead to tolerance, opiate-induced hyperalgesia, constipation, and the potential of addiction (Hojsted and Sjogren, 2007, Schug et al., 1992). Because of these significant side effects, there are many other drug classes under investigation for alleviating pain. Another example is gabapentin, which is structurally related to γ -aminobutyric acid (GABA), a neurotransmitter that plays a role in pain transmission and modulation, has been shown to be effective in various animal models of chronic neuropathic pain such as chronic constriction injury model of neuropathic pain in rats (Bennett, 1988). Gabapentin administered intraperitoneally doses ranging from 10 to 75 mg/kg produces significant dose-related improvement in heat hyperalgesia and mechanical allodynia (Xiao and Bennett, 1995). Heat hyperalgesia was also significantly reduced following intrathecal gabapentin administration, indicating that the antihyperalgesic properties of gabapentin are at least partially modulated through spinal cord mechanisms (Xiao and Bennett, 1995). In addition, gabapentin was effective in significantly reducing late-phase tactile allodynia in both the rat formalin (Shimoyama et al., 1997) and carrageenan footpad tests and hyperalgesia in the rat streptozotocin model (Parke-Davis Pharmaceutical Research, unpublished data, 1997). However, this drug also produces significant motor and cognitive side effects (Backonja et al., 1998, Martin et al., 1999a).

Cannabis in clinical model of pain:

Studies evaluating smoked cannabis compared to placebo show significant improvements in pain conditions in patients. For example, smoked cannabis has been shown to reduce pain associated with fibromyalgia, post traumatic and postsurgical neuropathic pain, central peripheral neuropathic pain, human immunodeficiency virus–associated sensory neuropathy and cisplatin-induced pain and hyperalgesia (Abrams et al., 2007, Ellis et al., 2009, Fiz et al., 2011, Wallace et al., 2007, Ware et al., 2010, Wilsey et al., 2008). Approximately 15 to 56 patients were involved in these studies and commonly reported side effects observed were dry mouth, headache, dizziness, dry eyes, hypotension, sedation, and concentration deficit. Another cannabinoid drug called Sativex/Nabiximols that consists of THC and cannabidiol (CBD) in 1:1 ratio, has been tested in patients with rheumatoid arthritis (Blake et al., 2006), neuropathic pain (Nurmikko et al., 2007), central and neuropathic pain associated with multiple sclerosis (Rog et al., 2005, Rog et al., 2007). These studies have demonstrated significant reduction in pain compared to placebo, but patients have reported sedation, dizziness, and nausea as side effects. Similarly, high dose of THC produces analgesia in cancer patients at a dose that is equivalent to high dose of codeine (morphine derivative) and the side effects are dizziness, blurred vision, and ataxia (Noyes et al., 1975a, Noyes et al., 1975b). Another synthetic THC called marinol/dronabinol has not reduced chronic gastrointestinal pain or posthysterectomy pain (Buggy et al., 2003, Holdcroft et al., 1997, Naef et al., 2003) but has demonstrated a reduction in pain score in multiple sclerosis patients (Svendsen et al., 2004). These data indicate that these drugs are effective against limited pain conditions. Additionally, THC has shown to produce poor efficacy in reducing acute pain in humans (Beaulieu, 2006, Buggy, 2003, Kraft et al., 2008,

Naef, 2003, Raft et al., 1977). Enzymes regulating endocannabinoids are an emerging target to treat pain with reduced side effects.

Role of direct cannabinoid receptor agonists on pain:

THC and other direct-acting cannabinoid receptor agonists produce analgesic effects in laboratory animals, but without respiratory depressant effects. For example, administration of synthetic cannabinoid receptor agonists (i.e. CP-55940, WIN 55-212-2 and HU210) produces analgesia and reduces edema in the carrageenan-induced inflammatory pain model (Elmes et al., 2005, Nackley et al., 2003, Wise et al., 2008). Additionally, peripherally restricted cannabinoid receptor agonist, naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)-methanone possesses antihyperalgesic activity in sciatic nerve ligation model of neuropathic pain (Dziadulewicz et al., 2007). Compound A, another peripherally restricted potent CB₁/CB₂ receptor agonist, has produced anti-hyperalgesic activity in spinal nerve ligation model of neuropathic pain in rats as well as in formalin model of inflammatory pain in mice (Boyce, 2008). But this compound has also produced cataleptic activity at the same dose at which it has produced antinociception (Boyce, 2008). Two other such compounds are AZD1940 and AZ11713908. AZD1940 has been shown to reduce capsaicin-induced nociception in rats (Groblewski, 2010). Similarly, AZ11713908 has produced antinociceptive effects comparable to WIN 55, 212-2 in the carrageenan model of inflammatory pain and in spinal nerve ligation model of neuropathic pain in rats (Yu et al., 2010). It is also noteworthy that AZ11713908 has produced CNS mediated side effects such as catalepsy (Yu, 2010). The result indicates that this compound crosses blood brain barrier at a high dose. Additionally, ajulemic acid (CT-3), synthetic analogue of THC, has been shown to ameliorate neuropathic and inflammatory pain (Dyson et al., 2005, Mitchell et al., 2005). Local administration of the selective CB₁ receptor agonist, arachidonyl-2-chlorethylamide

(ACEA), in the paw suppresses the carrageenan-evoked mechanical hyperalgesia in rats (Gutierrez et al., 2007). Although such direct acting cannabinoid receptor agonists produce antinociceptive and anti-inflammatory effects, their CB₁ mediated psychomimetic side effects such as catalepsy, hypomotility, hypothermia, hypolocomotion and abuse potential have dampened enthusiasm for developing drugs that act directly at CB₁ receptors (Schlosburg et al., 2009).

On the other hand, the CB₂ receptor agonist AM1241 inhibits tactile hypersensitivity to carrageenan and reduced spinal nerve ligation induced nociception in rats (Nackley et al., 2004), which indicates that targeting CB₂ receptors is a viable alternative to CB₁ agonists. Similarly, AM1241, GW405833, and HU-308 has shown to produce antinociceptive effects in the hind paw incision model in rats (LaBuda et al., 2005). HU-308 has also reduced formalin-induced peripheral pain in mice (Hanus et al., 1999). Additionally, JWH-015 can reduce post-operative pain in rats (Romero-Sandoval and Eisenach, 2007). Other CB₂ agonists JWH-133 and AM1710 have been shown to reduce neuropathic pain in mice (Yamamoto et al., 2008) and in rats (Wilkerson et al., 2012) respectively. Also, L768242 and 1-(4-(pyridin-2-yl)benzyl)imidazolidine-2,4-dione have reduced allodynia elicited by L5-L6 spinal nerve ligation in rats (Beltramo et al., 2006, van der Stelt et al., 2011). However, none of the CB₂ selective agonists that have been developed so far is completely selective for CB₂ receptors. Therefore, they produce CB₂ selectivity only within a specific dose range (Pertwee, 2012). They tend to activate CB₁ receptors at higher doses (Pertwee, 2012). Furthermore, CB₂ receptor agonist AM1241 has been shown to produce very different effects at CB₂ receptors from different species. For example a racemic mixture of AM1241 is an agonist at human CB₂ but functions as an inverse agonist at rodent CB₂ (Atwood and Mackie, 2010). Similarly, these exogenously

administered synthetic cannabinoids and THC cause a persistent inhibition of CB₁ receptors and neurotransmitter release, and do not mimic the localized and transient effects seen with endocannabinoids (Vaughan and Christie, 2005). Thus, exploration of the other targets in the endocannabinoid system, such as endocannabinoid regulating enzymes, may possess promise in the clinical setting with decreased subset of marijuana-like side effects. For example altering endogenous cannabinoid levels by blocking DAGL- β or MAGL and/or FAAH represent an attractive alternate approach to elicit antinociception, but without eliciting cannabimimetic effects (Cravatt, 2001, Gobbi et al., 2005). Hence, this dissertation focuses on the role of endocannabinoid catabolic and anabolic enzymes inhibition on inflammatory pain in mice.

Role of DAGL inhibition in antinociceptive effects

DAGL represents an upstream enzyme in the production of 2-AG and arachidonic acid. Thus, DAGL inhibition produces a reduction of 2-AG synthesis (Gao, 2010) as a result reduces arachidonic acid production (Hsu et al., 2012). Potential therapeutic applications have been proposed to be similar to those of cannabinoid antagonists (Di Marzo, 2008), which have been shown to aid in weight loss (Chaput and Tremblay, 2006, Cota et al., 2006, Di Marzo et al., 2001, Pagotto et al., 2006), inflammation in rats (Lu et al., 2006, McVey et al., 2003), and Parkinson's symptoms such as dyskinesia (slight tremor of the hands to uncontrollable movement of the upper body but can also be seen in the lower extremities) (Brotchie, 2003, Di Marzo et al., 2000). Yet much less is known about the physiological effects of disrupting endocannabinoid production *in vivo* due, at least in part, to a lack of selective inhibitors for the enzymes that biosynthesize endocannabinoids. Non-selective agents such as RHC80267 and tetrahydrolipstatin (THL) are known to inhibit DAGL, but have off target effects on other lipases. For example, RHC-80267 has been reported to inhibit FAAH (Cravatt, 1996) and acts as

a muscarinic receptor antagonist (Hashimotodani et al., 2008). Similarly THL has been found to block ABHD12 (Blankman, 2007) and pancreatic lipases in the intestine (Chevaleyre et al., 2006). Alternatively, other selective inhibitors of the 2-AG biosynthesis, fluorophosphonates O-3841 and O-3640 show limited activity in cells (Bisogno et al., 2006, Bisogno, 2003) and are less potent than tetrahydrolipstatin (THL) (Ortar et al., 2008). In addition, these compounds do not show selectivity between the α and β forms of the enzyme (Min et al., 2010).

Hsu et al 2012 has recently reported the discovery of 1, 2, 3-triazole urea (1, 2, 3-TU) as a versatile chemotype for serine hydrolase inhibitor development. They describe discovery of two compounds KT109 ($IC_{50} = 82$ nM) and KT172 ($IC_{50} = 71$ nM) that potently and selectively inactivated DAGL- β *in vitro* and *in vivo*. These compounds as well as DAGL- β (-/-) mice (Gao, 2010) were used to show that DAGL- β is the principal 2-AG biosynthetic enzyme in peritoneal macrophages. The pharmacologic or genetic inactivation of DAGL- β produced a remarkable array of metabolic changes in macrophages that included not only reductions in 2-AG levels, but also decreases in arachidonic acid and prostaglandins. Interestingly, inactivation of DAGL- β also attenuated lipopolysaccharide-stimulated TNF- α release from macrophages, implicating a DAGL- β regulated endocannabinoid-eicosanoid network as an important modulator of proinflammatory responses in macrophages (Hsu, 2012).

KT109 and KT172 have a known off-target, ABHD6 (IC_{50} values of 16 and 5 nM, respectively). Hence, a negative-control probe, KT195 has been generated to address that problem. KT195 has shown to be a potent ($IC_{50} = 10$ nM) selective inhibitor of ABHD6 and structurally related to KT109 and KT172, but inactive against DAGL- β (Hsu, 2012). Although the findings indicate that DAGL- β inhibitors, KT-109 and KT-172 reduce 2-AG, AA, prostaglandins as well as reduce TNF- α from the macrophages (Hsu, 2012), the role of this

inhibitors on the prolonged model of inflammatory nociception is yet to be examined. Hence, in the present study we have tested the role of KT-109, KT-172 and KT-195 (as a control for ABHD6) in the LPS model of inflammatory pain in mice. LPS is a bacterial endotoxin derived from the outer cell wall of gram (-) bacteria. When 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 stimulus) (Rowbotham and Fields, 1989, Staud and Domingo, 2001). Hence, in this thesis, I test the hypothesis that genetic or pharmacological inhibition of 2-AG synthesizing enzyme DAGL- β , blocks mechanical allodynia associated with inflammation.

Role of FAAH inhibition in antinociceptive effects

As shown in table 1, the bulk of research examining the role of endocannabinoid catabolic enzymes in nociception has focused on FAAH (Booker et al., 2011, Chang et al., 2006, Clapper et al., 2010, Jayamanne et al., 2006, Kinsey et al., 2011a, Naidu et al., 2008, 2009, Naidu et al., 2010, Suplita et al., 2005), largely because of a greater availability of selective FAAH inhibitors than selective MAGL inhibitors. Irreversible (PF-3845, URB597) and reversible (OL-135) inhibitors of FAAH have been demonstrated to elevate anandamide levels in the brain (Ahn et al., 2009, Boger et al., 2005, Fegley et al., 2005, Kathuria et al., 2003, Lichtman, 2004) and produce analgesia in a wide variety of animal models of pain (Schlosburg, 2009). Systemic administration of the FAAH inhibitor URB597 reduces carrageenan-induced paw edema (Holt et al., 2005). URB597 also reduces both plantar thermal and mechanical threshold sensitivity in a dose-dependent manner in complete Freund's adjuvant-induced

inflammatory pain. Repeated oral administration of URB597 attenuates chronic constriction injury of sciatic nerve-induced thermal hyperalgesia and mechanical allodynia (Russo et al., 2007). The reversible FAAH inhibitor, OL-135, reverses mechanical allodynia in a spinal nerve ligation model (Chang, 2006), chronic constriction injury (CCI) model (Kinsey et al., 2009) and acetic acid (Naidu, 2009), hot plate, tail-immersion, formalin (Lichtman, 2004) and LPS (Booker, 2011) models of pain. Peripherally restricted FAAH inhibitor URB593 has been shown to reduce carrageenan-induced inflammatory pain (Okine et al., 2012) and has also reduced neuropathic pain in spinal nerve ligation model (Clapper, 2010). Another FAAH inhibitor JNJ-1661010 has been shown to attenuate tactile allodynia in rat mild thermal injury model of acute tissue damage, in the rat spinal nerve ligation model of neuropathic pain as well as in carrageenan model of inflammatory pain (Karbarz et al., 2009). The long-lasting FAAH inhibitor PF-3845 has produced anti-allodynic effects in the complete Freund's Adjuvant (CFA) model (Ahn, 2009) and has partially suppressed the hyperalgesia in the LPS mouse model of inflammatory pain (Booker, 2011). Similarly, deletion of the FAAH gene increases levels of anandamide, accompanied with CB₁ receptor-mediated hypoalgesic phenotypes in models of acute and inflammatory pain and acetic acid-induced nociception (Booker, 2011, Chang, 2006, Cravatt, 2001, Lichtman, 2004).

Recently, a novel compound ARN272 was found to block AEA binding to FAAH like anandamide transporter (FLAT). As a result it increased plasma anandamide levels which in turn has produced antinociception in the formalin model of pain (Fu et al., 2012). It should be noted that Leung et al 2013, were unable to detect the expression of FLAT using RT-PCR and western blotting, in dorsal root ganglion, spinal cord, liver, and kidney. FLAT may exhibit a low tissue expression profile that is below the detection limit of these approaches (Leung et al., 2013),

which raises serious questions about this purported anandamide transporter. The reason behind these two contrasting results is unclear and they question the role of ARN272 as well as FLAT on antinociception.

The beneficial effects caused by FAAH blockade in these models are predominantly mediated through the activation of CB₁ and/or CB₂ receptors, though noncannabinoid mechanisms of actions can also play contributory or even primary roles. The two major noncannabinoid receptors that play a role in antinociception are transient receptor potential vanilloid (TRPV1) and peroxisome proliferator-activated receptors (PPAR). Pharmacological data and receptor binding data show that anandamide binds to the TRPV1 receptors in dorsal root ganglion (Tognetto et al., 2001) and in the sensory nerve (Zygmunt et al., 1999). Similarly, FAAH regulated N-acyl taurines have been shown to activate TRPV1 receptors (Saghatelian, 2006). TRPV1 antagonists capsazepine blocked the anti-hyperalgesic effects of anandamide indicate the role of these receptors in antinociception (Horvath et al., 2008). Additionally, administration of capsazepine and CB₁ receptor antagonist AM251 partially blocked the antinociceptive effects of URB597 in the periaqueductal gray (Maione et al., 2006). Similarly, anti-allodynic and anti-hyperalgesic effects of another FAAH substrate palmitoylethanolamide, were mediated by CB₁ and TRPV1 receptors suggesting the involvement of these receptors in mediating the antinociceptive effects (Costa et al., 2008). However, other data suggest that anandamide plays opposing roles on pain through its actions at cannabinoid and TRPV1 receptors (Singh Tahim et al., 2005). It has been shown that elevation of anandamide by inhibiting FAAH, at the periphery produces a TRPV1-mediated, exacerbation of visceral hyperactivity (Dinis et al., 2004, Singh Tahim, 2005).

PPAR- α also plays a vital role in mediating the anti-inflammatory and antinociceptive effects of URB597 (Sagar et al., 2008). PPAR- α receptor antagonist GW6471 blocked the anti-hyperalgesic effects of URB597 in the carrageenan model in rats (Sagar, 2008). Additionally, palmitoylethanolamide produced anti-inflammatory effects through PPAR- α . Palmitoylethanolamide also attenuated carrageenan-induced paw edema and phorbol ester-induced ear edema in PPAR- α wild type animals but had no effects in PPAR- α deficient mice (Lo Verme et al., 2005). Another naturally occurring PPAR- α agonist oleoylethanolamide and synthetic PPAR- α agonist GW7647 have replicated these effects in a PPAR- α dependent manner (Lo Verme, 2005). These data suggest that PPAR- α plays a role in mediating the antinociceptive effects of FAAH inhibitors and FAAH substrates. The table shows a review of studies examining FAAH inhibitors in preclinical pain assays.

Review of studies examining FAAH inhibitors in preclinical pain assays.

Compound	Type of assay/Dose	Tests (animal)	Effects	Reference
OL-135	Inflammatory pain (MTI) 10 mg/kg (i.p.)	Paw pressure test (rats)	Anti-allodynic effects	(Chang, 2006)
	Neuropathic pain (SNL) 20 mg/kg (i.v.)	Paw pressure test (rats) Von Frey test	Anti-allodynic effects	
	Thermal nociception 10 mg/kg (i.p.)	Tail immersion test (mice)	Increase in latency	(Lichtman, 2004)
		HPT (mice)	Increase in latency	
	Visceral pain (Formalin) 10 mg/kg (i.p.)	Paw licking test (mice)	Decreased response	(Kinsey, 2009)
	Neuropathic pain (CCI model) 10 mg/kg (i.p.)	Paw pressure test (mice)	Anti-allodynic effects	
Paw lifting test		Anti-allodynic effects		
URB597	Neuropathic pain (CCI model) 10 mg/kg (i.p.)	Paw pressure test (mice) Von Frey test	Anti-allodynic effects	(Kinsey, 2009)
		Paw lifting test (mice) Cold acetone test	Anti-allodynic effects	
	Inflammatory pain (CFA; i.pl.) 0.3 mg/kg (i.p.)	Paw pressure test (rats) Von Frey test	Anti-allodynic effects	(Jayamanne, 2006)
		Plantar test (rats)	Anti-allodynic effects	
	Neuropathic pain (CCI model) 0.3 mg/kg (i.p.)	Paw pressure test (rats) Von Frey test	No effect	(Naidu, 2009)
	Visceral pain (Acetic acid) 1–10 mg/kg (s.c.)	Acetic acid-induced stretching test (mice)	Stretching decreased	
	Inflammatory pain (Lipopolysaccharide; i.pl.) 10 mg per/kg (s.c.)	Hot plate test (mice)	Hyperalgesia decreased	
		Paw thickness test	No reduction in edema	
Paw thickness test		Anti-edematous effects		
Neuropathic pain (CCI model) 10–50 mg/kg (p.o.)	Paw pressure test	Anti-allodynic effects	(Russo, 2007)	
	Electronic Von Frey test	Anti-allodynic effects		
	Neuropathic pain (CCI)	Thermal		Hyperalgesia

URB597	model) 10 mg/kg (p.o.)	hyperalgesia Paw withdrawal latency	decreased	
	Inflammatory pain (Iodoacetic acid-induced osteoarthritis) 5 mg/kg (s.c.)	Paw pressure in capacitance test	19% MPE	(Schuelert et al., 2011)
	Inflammatory pain (Carrageenan i.pl.) 3 mg/kg (i.p.)	Paw edema measurement (mice)	Anti-edematous effects	(Holt, 2005)
	Bone cancer pain 9 µg per bone infusion	Paw pressure test	Anti-allodynic effects	(Khasabova et al., 2008)
	Tail flick test (TFT) 40 mg/kg (i.p.)	TFT (mice)	No effect	(Haller et al., 2008)
URB597 plus anandamide	Tail flick test (TFT) 10 and 40 mg/kg (i.p.)	TFT (mice)	68% MPE	
URB597	Chemotherapy-induced peripheral neuropathy 0.1 and 1 mg/kg	Von Frey test Cold acetone test (rats)	Anti-allodynic effects	(Guindon et al., 2013)
	Chemotherapy-induced peripheral neuropathy 0.1 and 1 mg/kg	Von Frey test Cold acetone test (rats)	Anti-allodynic effects	
URB937 (peripherally restricted FAAH inhibitor)	Inflammatory pain (Carrageenan s.c.) 0.3 mg/kg (i.p.)	Hyperalgesia Paw thickness test	Hyperalgesia decreased	(Okine, 2012)
	Neuropathic pain (SNL) 1 mg/kg (i.p.)	Paw pressure test; Withdrawal latency	Hyperalgesia decreased	(Clapper, 2010)
		Thermal stimulation; Withdrawal latency	Hyperalgesia decreased	
		Paw pressure tests, Von Frey test	Hyperalgesia decreased	
URB937	Neuropathic pain (SNL) 1 mg/kg (i.p.)	Paw pressure and withdrawal latency; Thermal stimulation and withdrawal latency; Paw pressure and Von Frey tests	Long-lasting similar effects (lack of tolerance)	(Clapper, 2010)

JNJ-1661010	Mild thermal injury 20 mg/kg (i.v.)	Paw pressure test (rats); Von Frey test	Anti-allodynic effects	(Karbarz, 2009)
	Neuropathic pain (SNL) 20 mg/kg (i.v.)	Paw pressure test (rats) Von Frey test	Anti-allodynic effects	(Karbarz, 2009)
PF-3845	Inflammatory pain (CFA; i.p.) 0.1–10 mg per kg (p.o.)	Paw pressure test, Von Frey test (rats)	Anti-allodynic effects	(Ahn et al., 2011)
	Osteoarthritis (MIA-induced injury in knee) 0.3 mg/kg and 3 mg/per kg (p.o.)	Joint compression threshold (rats)	Hyperalgesia decreased	
	Inflammatory pain (Carrageenan) 10 mg/kg (i.p.)	Von Frey test (mice)	Anti-allodynic effects	(Ghosh et al., 2013)
	Inflammatory pain (LPS) 10 mg/kg (i.p.)	Von Frey test (mice)	Anti-allodynic effects	(Booker, 2011)
	Neuropathic pain (CCI) 10 mg/kg (i.p.)	Von Frey test (mice)	Anti-allodynic effects	(Kinsey et al., 2010)
ARN272	Inflammatory pain (Formalin) 0.01–1 mg/per kg (i.p.)	Paw withdrawal latency (mice)	Hyperalgesia decreased	(Fu, 2012)

Table modified from Roques et al., 2012

Role of MAGL inhibition in antinociceptive effects

Table 2 shows the pharmacological effects of MAGL inhibitors on different nociceptive assays in the preclinical models. Initial studies investigating the *in vivo* consequences of inhibiting MAGL employed URB602 (Burston et al., 2008, Comelli et al., 2007, Desroches et al., 2008, Guindon et al., 2007a, Guindon et al., 2007b, Guindon et al., 2011, Guindon and Hohmann, 2008, Hohmann et al., 2005, Vandevoorde et al., 2007) and *N*-arachidonyl maleimide (Burston, 2008). For example, systemic or peripheral administration of URB602 suppressed formalin-induced nociception, carrageenan-induced inflammatory nociception, and partial sciatic nerve ligation-induced nociception in rats (Comelli, 2007, Desroches, 2008, Guindon, 2011). Although URB602 (for MAGL $IC_{50} = 28 \mu\text{M}$, for FAAH $IC_{50} = \sim 5 \mu\text{M}$) and *N*-arachidonyl

maleimide (for MAGL IC_{50} = 140 nM; for FAAH IC_{50} = 2.31 nM) inhibit MAGL in the brain, these compounds are nonselective and inhibit other serine hydrolases, including FAAH (Burston, 2008, Hohmann, 2005, Makara et al., 2005, Saario et al., 2005, Vandevoorde, 2007). Thus, it is unclear whether antinociceptive effects of these nonselective MAGL inhibitors are mediated through MAGL blockade, inhibition of other enzymes (e.g., FAAH), or inhibition of multiple enzymes.

The development of JZL184, a piperidine carbamate that preferentially and irreversibly inhibits MAGL, provided the first pharmacological tool that when administered acutely increases 2-AG levels in the brain, without altering anandamide brain levels (Long et al., 2009a). Despite showing greater than 300-fold selectivity for MAGL over FAAH *in vitro*, JZL184 still partially blocked FAAH activity *in vivo*. This partial inhibition, however, did not result in elevated levels of anandamide (Long, 2009a). Systemic administration of JZL184 reduces nociceptive behaviors in the warm water tail withdrawal, formalin and acetic acid stretching tests (Busquets-Garcia et al., 2011, Long, 2009a) and also reduces mechanical and cold allodynia in the CCI model of neuropathic pain in mice (Kinsey, 2009). Intraplantar injection of JZL184 reduces nociceptive behaviors in the formalin test (Guindon, 2011), capsaicin-induced nociceptive behavior and thermal hyperalgesia (Spradley et al., 2010), chemotherapy-induced peripheral neuropathy (Guindon, 2013). KML29 shows increased selectivity for MAGL over FAAH (Chang et al., 2012). This MAGL inhibitor has been shown to reduce carrageenan and CCI-induced nociception in mice (Ignatowska-Jankowska et al., 2013). In Chapter 2 of this dissertation, we report on the effects of JZL184 in the carrageenan model of inflammatory pain. Similar to FAAH inhibitors, inhibition of MAGL predominantly mediates its effects through the activation of CB_1 and/or CB_2 receptors.

Table 2: Review of studies with different MAGL inhibitors on acute and chronic preclinical pain assays.

Compound	Type of Assay/dose	Dose/Route/Species	Effect	Reference
URB602	Mechanical allodynia and thermal hyperalgesia	0.1, 1, 10, 100, 1000 mg (i.pl.); (Rat)	Dose-dependent anti-allodynic effect	(Desroches, 2008)
	radiant-heat, tail-flick test (TFT)	0.1 nmol (i.c.v); (Rat)	Reduced Stress induced analgesia	(Hohmann, 2005)
	λ -carrageenan	1, 5, 10 mg/kg, (i.p.); (Rat)	dose-dependent anti-edematous and anti-nociceptive effect	(Guindon, 2007b)
	Formalin	0.001-600 μ g (i.pl.); (Rat)	composite pain score-weighted scores technique	(Guindon, 2011)
N-arachidonyl maleimide	Tail withdrawal	1, 3, 10 mg/kg (i.p.); (Mouse)	Dose dependant antinociception	(Burston, 2008)
JZL184	Acetic acid stretching	8, 16 mg/kg (i.p.); 8 mg/kg (i.p.) Mouse	Antinociception	(Busquets-Garcia, 2011, Long, 2009a)
	CCI (mechanical & cold allodynia)	16, 40 mg/kg (i.p.); Mouse	Anti-allodynic	(Kinsey, 2010, Kinsey, 2009)
	Carrageenan-induced paw inflammation	4-40 mg/kg (i.p.); Mouse	Partial anti-allodynic and anti-edema	(Ghosh, 2013)
	Bone cancer pain	10 μ g (i.p.); Mouse	Anti-hyperalgesic	(Khasabova et al., 2011)
	Cisplatin-induced peripheral neuropathy (mechanical and cold allodynia)	1, 3, 8 mg/kg (i.p.); Rat	Anti-allodynic	(Guindon, 2013)
	Formalin pain test	16 mg/kg (i.p.); Mouse 0.001-300 μ g (i.pl);	Antinociceptive at both early and late phase	(Guindon, 2011, Long, 2009a)

		Rat		
	Capsaicin-induced	100µg (i.p.); Rat	Antinociception	(Spradley, 2010)
	Postoperative pain	0.016–16 mg/kg (i.p.); Mouse	Mechanical and thermal hyperalgesia	(Martins et al., 2013)
	Carrageenan	25, 50, 100 µg (i.p.); Rat	Mechanical allodynia	(Woodhams et al., 2012)
KML29	Carrageenan	5,20, 40 mg/kg (i.p.); Mouse	Anti-allodynic	(Ignatowska-Jankowska, 2013)
	CCI (mechanical & cold allodynia)	5, 20, 40 mg/kg (i.p.); Mouse	Anti-allodynic	(Ignatowska-Jankowska, 2013)

Table modified from Ramesh et al., 2012

Rational and Hypothesis:

Overall hypothesis:

Inhibition of endocannabinoid regulating enzymes will reduce nociceptive behavior in mouse inflammatory pain models.

Carrageenan model of inflammatory pain

The present study examined the anti-edematous and anti-allodynic effects of FAAH and MAGL inhibitors in the carrageenan model of inflammatory pain which has been used to assess cannabinoids and may other drugs. It is a common preclinical inflammatory pain model (Guay et al., 2004, Hargreaves, 1988a, Henriques et al., 1987). The stimulus is a sulfated glycoprotein derived from red seaweed. This is a neutrophil mediated acute inflammatory model (Fecho et al., 2005). Carrageenan administration leads to biphasic events, the early 1-6 hours phase and late 12 and 24 hours phase. The initial phase of inflammation (0-1 hrs post carrageenan injection) is due to activation of inflammatory agents such as bradykinin, nitric oxide, and histamine in the injected area (Crunkhorn and Meacock, 1971, Garcia Leme et al., 1973, Hargreaves et al., 1988b,

Salvemini et al., 1996, Vinegar et al., 1987). The second sustained phase of swelling is correlated with the activation of neutrophils, cytokines, prostaglandins, and cyclooxygenase-2 (COX-2) (Loram et al., 2007, Seibert et al., 1994, Vinegar, 1987). In addition, immune response such as activation of macrophages, mast cells, endothelial cells which results in release proinflammatory cytokines such as TNF- α , IL-6, IL-1 β (Fecho et al., 2007) are observed in this phase. This phase is usually termed the acute phase, and occurs 2-4 hours after injection (Hargreaves, 1988a). The carrageenan assay involves the administration of carrageenan into the dorsal surface of the hind paw of mice/rats resulting in edema (increased in paw volume) and an increase in sensitivity to mechanical (allodynia) and thermal stimuli (hyperalgesia). Many electrophysiological studies have shown that carrageenan-induced edema and hyperalgesia last for 1 to 4 days (Hargreaves, 1988a, Kayser and Guilbaud, 1987, Vinegar et al., 1969, Vinegar, 1987). Stimulating the paw with mechanical (von Frey filaments) or thermal stimuli (Hargreaves, radiant heat tests) elicits behavioral responses, such as licking, flexing and flinching of the paw (Guay, 2004, Nantel et al., 1999). These responses can be reliably measured in carrageenan assay (Fecho, 2005, Ferreira et al., 1988). Thus, carrageenan is widely used as a model for localized inflammatory pain. Additionally, FAAH (-/-) mice exhibited reduced inflammatory responses compared to wild types in carrageenan model of inflammatory pain (Lichtman, 2004). Similarly, anandamide, palmitoylethanolamide and oleoylethanolamide have been shown to reduce carrageenan-induced edema in FAAH (-/-) mice (Wise, 2008). A nonselective MAGL inhibitor URB602 has been shown to reduce carrageenan-induced edema and allodynia in mice (Comelli, 2007). These studies indicate that carrageenan is a reliable model for inflammatory pain. Therefore, we have chosen carrageenan as a model to assess edema and allodynia.

Selection of DAGL MAGL and FAAH inhibitors

We selected MAGL and FAAH for our study because MAGL and FAAH are the primary degradative enzymes of endocannabinoids (Cravatt, 1996, Dinh, 2004, Dinh, 2002). As Endocannabinoids are produced on demand and are then rapidly degraded. Inhibition of their degradative enzymes prolongs their action at CB₁ and CB₂ receptors. We selected DAGL-β because it is one of the major biosynthetic enzymes for 2-AG. It is present in the periphery and plays an important role in the production of arachidonic acid. Thus, its inhibition reduces arachidonic acid and leads to a reduction in prostaglandin synthesis and other pro-inflammatory mediators. Hence, we wanted to examine the impact of inhibiting this enzyme in an inflammatory pain model. In addition, inhibiting endocannabinoid regulating enzymes avoids cannabimimetic effects of direct cannabinoid receptor agonist. We specifically chose the following compounds: KT-109, and KT-172 for DAGL-β, JZL184 for MAGL, PF-3845 for FAAH, and SA-57 for dual FAAH-MAGL inhibition, since they are amongst the most selective inhibitors presently available (Ahn, 2009, Hsu, 2012, Long, 2009a). The inhibitors are highly selective and irreversible for DAGL-β, MAGL, and FAAH respectively and have a long half-life (up to 24 h). DAGL-β inhibitors have been shown to reduce 2-AG and arachidonic acid in the liver. In contrast, mice treated with KT109 and KT172 or DAGL-β (-/-) mice did not show changes in 2-AG or arachidonic acid in brain tissue. It should be noted that KT-109 and KT-172 inhibit ABHD-6 with respective IC₅₀ values of 16 and 5 nM (Hsu, 2012). Hence, we have used the selective ABHD6 inhibitor KT-195 as a control for this off-target. MAGL and FAAH inhibitors produce profound (up to 10-fold) elevations of the appropriate endocannabinoids. However, JZL184 also inhibits FAAH, although *in vitro* the compound is approximately 500-fold more selective as a MAGL inhibitor (IC₅₀ = 8 nM) than as a FAAH inhibitor (IC₅₀ = 4,000

nM) (Long, 2009a). Nonetheless, acute treatment with JZL184 in mice does not elevate anandamide levels in the brain. However, repeated treatment with JZL184 leads to augmented brain levels of anandamide in mice (Schlosburg et al., 2010).

Overview of Chapter 2. Impact of MAGL and FAAH inhibitors on carrageenan-induced inflammatory pain

In initial studies, we examined the consequences of MAGL and FAAH inhibition on carrageenan-induced inflammatory nociception in mice. We have taken a complementary genetic and pharmacological approach to determine the involvement of cannabinoid receptor system to mediate the effects of MAGL and FAAH inhibitors. We hypothesized that CB₁ and CB₂ receptors mediated the antinociceptive effects of MAGL inhibitors. This prediction is based on the results of previous studies demonstrating the effects of JZL184 has mediated its antinociceptive effects through CB₁ and CB₂ receptors in formalin-induced inflammatory pain (Guindon, 2011) and in bone cancer pain (Khasabova, 2011). JZL184 has also shown to mediate its antinociceptive effects through CB₁ receptors on acetic acid induced stretching (Busquets-Garcia, 2011, Long, 2009a), tail withdrawal assay (Long, 2009a, Schlosburg, 2010), CCI-induced neuropathic pain (Kinsey, 2010, Kinsey, 2009), and formalin-induced inflammatory pain (Long, 2009a). As carrageenan-induced inflammation triggers cytokine release (Loram, 2007, Seibert, 1994, Vinegar, 1987) and CB₂ receptors are predominantly present in the immune cells (Cabral, 2006), we predicted the involvement of CB₁ as well as CB₂ in carrageenan-induced inflammatory pain. Similarly, anti-allodynic effects of FAAH inhibition is predicted to be mediated by CB₁ and CB₂ receptors. This is based on the previous literature showing both CB₁ and CB₂ receptors are required for the anti-allodynic effects of FAAH inhibition in chronic constriction injury model

(Kinsey, 2010, Kinsey, 2009), (Russo, 2007), partial sciatic and spinal nerve injury models of pain (Desroches, 2008, Jhaveri et al., 2006) as well as in LPS-induced inflammatory pain model (Booker, 2011).

Chronic pharmacological blockade or genetic deletion of MAGL, unlike FAAH inhibition, leads to functional antagonism and loss of cannabinoid-mediated effects and produces cross-tolerance to CB₁ agonists in mice. Chronic MAGL blockade with high dose of JZL-184 also causes physical dependence, impaired endocannabinoid-dependent synaptic plasticity, and desensitized brain CB₁ receptors (Chanda et al., 2010, Schlosburg, 2010), but the effects were maintained following repeated administration of low dose JZL184 (Kinsey et al., 2013, Sciolino et al., 2011). Hence, in our next experiments we examined the role of repeated administration of low versus high dose of JZL184 in carrageenan pain model. As genetic deletion or prolonged pharmacological inhibition of MAGL is known to produce CB₁ receptor functional tolerance (Chanda, 2010, Schlosburg, 2010), we assessed the dose-response relationship of JZL184 after acute and repeated administration in the carrageenan assay. We predicted based on previous literature that MAGL inhibition with high of JZL184 (Schlosburg, 2010) but not low dose (Sciolino, 2011), will show tolerance to the antinociceptive effects of JZL-184.

Overview of Chapter 3. Effects of partial MAGL and complete FAAH blockade on carrageenan-induced inflammatory pain

In Chapter 3, we described the role of dual FAAH and MAGL inhibition. Previous work reported that JZL195, a dual MAGL-FAAH inhibitor, that equipotently raised both 2-AG and anandamide levels and produced a greater antinociceptive response in tail immersion acetic acid abdominal stretching tests than inhibitors of either FAAH or MAGL alone (Long et al., 2009b). However, JZL195 also elicits cannabimimetic activity in the tetrad test, causing analgesia,

hypomotility, catalepsy and THC-like drug discrimination responses (Long, 2009b). Additionally, repeated administration of high dose JZL184 or genetic deletion of MAGL, unlike FAAH inhibition, leads to desensitization and down regulation of brain CB₁ receptors (Chanda, 2010, Schlosburg, 2010). On the other hand, the antinociceptive and anxiolytic-like effects of low dose JZL184 are maintained after repeated administration (Kinsey, 2013, Sciolino, 2011). Hence, our experiments evaluated whether differential elevation of 2-AG and anandamide, by partially inhibiting MAGL and completely inhibiting FAAH produced augmented antinociceptive effects compared with individual enzyme blockade. We have used two pharmacological approaches for these studies. First, we have used low dose JZL-184 and high dose PF-3845 and second we have used a novel differential MAGL and FAAH inhibitor SA-57 (Niphakis et al., 2012). We also evaluated whether the simultaneous blockade of MAGL and FAAH would produce cannabimimetic effects in the tetrad test, as well as whether repeated administration of FAAH and MAGL inhibitors produce cross-tolerance to THC. We predicted that elevating endogenous cannabinoids by inhibiting their hydrolytic enzymes offer potential therapeutic benefits, without the undesirable cannabimimetic actions. We investigated the efficacy of these inhibitors to reduce carrageenan-induced edema and allodynia. Selective CB₁ and CB₂ receptor antagonists were employed to assess cannabinoid receptor involvement of the anti-inflammatory effects of combined JZL184 and PF-3845. Anandamide and 2-AG levels were quantified in whole brain following administration of these enzymes inhibitors. Finally, we evaluated the role of prolonged dual blockade of MAGL and FAAH on carrageenan-induced allodynia and edema. Changes in CB₁ receptor binding and G- protein activation were assessed in brains of mice repeatedly that were treated repeatedly with MAGL and FAAH inhibitors. We hypothesize that the combination

of JZL184 and PF-3845 and SA-57 will also enhance the antinociceptive effects without inducing cannabimimetic behavioral effects and receptor adaptations.

Overview of Chapter 4. Effects of DAGL- β , a major 2-AG biosynthesizing enzyme, inhibitors on LPS-induced inflammatory pain

In Chapter 4, we examined the role of selective DAGL- β inhibitors on LPS model of inflammatory pain. In comparison to the consequences of inhibiting FAAH and MAGL in laboratory animal models of pain, much less is known about the physiological effects of inhibiting the endocannabinoid biosynthetic enzymes on pain. As discussed above, 2-AG biosynthesis is regulated by two sequence-related enzymes, DAGL- α and DAGL- β (Bisogno, 2003). Biochemical studies have provided evidence that these are multi-domain, transmembrane serine hydrolases. Recent genetic studies with constitutive knockout mice have confirmed that both DAGL- α and DAGL- β regulate 2-AG production *in vivo*. The relative contribution made by each enzyme depends on tissue type. For example, DAGL- α is responsible for adult neurogenesis in subventricular zone, while both isoforms are responsible for adult neurogenesis in the hippocampus (Gao, 2010, Tanimura, 2010). Recently, two compounds, KT109 and KT172, that potently and selectively inactivated DAGL- β *in vitro* and *in vivo*, were developed. In Chapter 4, we test whether these two compounds reverse LPS-induced allodynia. We predicted that these compounds would reduce allodynia in cannabinoid independent manner. On the other hand, one may predict that decreased 2-AG levels will lead to increased inflammatory pain. But we based our prediction on the *in vitro* findings that demonstrate that KT-109 and KT-172 reduces 2-AG, arachidonic acid, prostaglandins, and TNF - α release from peritoneal macrophages (Hsu, 2012). Reducing the pool of 2-AG results in less available precursor of

arachidonic acid, and consequently decreasing prostaglandin levels. Finally, we examined if DAGL inhibition cause any gastric ulcerogenic effects due to reduction in prostaglandin levels.

Chapter 2. The monoacylglycerol lipase inhibitor JZL184 suppresses inflammatory pain in the mouse carrageenan model

This chapter has been published as a research report in a peer-review journal (Ghosh et al., 2013).

Introduction

The endogenous cannabinoid system consists of two G-protein-coupled cannabinoid (i.e., CB₁ and CB₂) receptors (Gerard, 1991, Matsuda, 1990), the lipid endogenous ligands N-arachidonylethanolamine (anandamide; AEA) (Devane, 1992) and 2-AG (Mechoulam et al., 1995, Sugiura, 1995), and the enzymes that synthesize and degrade anandamide and 2-AG (Ahn et al., 2008). These endogenous cannabinoids are produced and released on demand, and are then rapidly metabolized by their respective degradative enzymes, fatty acid amide hydrolase (FAAH) (Cravatt, 2001, Cravatt, 1996) and monoacylglycerol lipase (MAGL) (Blankman, 2007, Dinh, 2004). The cannabinoid receptors and the endocannabinoid catabolic enzymes represent potential therapeutic targets to treat obesity, psychiatric disorders, neuroinflammatory diseases, cancer, and pain and inflammatory conditions (Pacher, 2006). Accordingly, a growing body of research has focused on investigating whether elevating endogenous levels of anandamide or 2-AG, via inhibition of their degradative enzymes, reduces nociceptive behavior in laboratory animal models of pain.

The bulk of research examining the role of endocannabinoid catabolic enzymes in nociception has focused on FAAH (Booker, 2011, Chang, 2006, Clapper, 2010, Jayamanne, 2006, Kinsey, 2011a, Naidu, 2008, 2009, Naidu, 2010, Suplita, 2005) largely because of a greater availability of selective FAAH inhibitors than selective MAGL inhibitors. Initial studies investigating the in vivo consequences of inhibiting MAGL employed URB602 (Burston, 2008,

Comelli, 2007, Desroches, 2008, Guindon, 2007a, Guindon, 2007b, Guindon, 2011, Guindon and Hohmann, 2008, Hohmann, 2005, Vandevoorde, 2007) and *N*-arachidonyl maleimide (Burston, 2008). For example, systemic or peripheral administration of URB602 suppressed formalin-induced nociception, carrageenan-induced inflammatory nociception, and partial sciatic nerve ligation-induced nociception in rats (Comelli, 2007, Desroches, 2008, Guindon, 2011). Although URB602 and *N*-arachidonyl maleimide inhibit MAGL in the brain, these compounds are nonselective and inhibit other serine hydrolases, including FAAH (Burston, 2008, Hohmann, 2005, Vandevoorde, 2007).

The development of JZL184, a piperidine carbamate that preferentially and irreversibly inhibits MAGL, provided the first pharmacological tool that when administered acutely increases 2-AG brain levels, without altering anandamide brain levels (Long, 2009a). Systemic administration JZL184 reduces nociceptive behaviors in the warm water tail withdrawal, formalin, and acetic acid stretching tests (Busquets-Garcia, 2011, Long, 2009a) and reduces mechanical and cold allodynia in the CCI model of neuropathic pain in mice (Kinsey, 2009). Intraplantar injection of JZL184 reduces nociceptive behaviors in the formalin test (Guindon, 2011), capsaicin-induced nocifensive behavior, and thermal hyperalgesia (Spradley, 2010).

Although these findings indicate that MAGL inhibition produces antinociceptive effects in multiple pain models, the effects of JZL184 have yet to be evaluated in a prolonged model of inflammatory nociception. Thus, in the present study we tested whether JZL184 would attenuate paw edema and mechanical allodynia in the carrageenan model of inflammatory pain. In an initial study, we evaluated the dose-response effects of JZL184 in this assay. For comparison, we tested the nonsteroidal anti-inflammatory diclofenac as well as the FAAH inhibitor PF-3845, which has been shown to possess anti-inflammatory and anti-allodynic effects in complete

Freund's adjuvant (Ahn, 2009), LPS (Booker, 2011), and CCI pain (Kinsey, 2010, Kinsey, 2009) models. In order to determine whether cannabinoid receptors mediate the anti-allodynic and anti-edematous effects of JZL184, we used complementary genetic and pharmacological tools to assess the contribution of CB₁ and CB₂ receptors to JZL184-induced decreases in nociception and paw edema. Repeated administration of JZL184 or genetic deletion of MAGL results in CB₁ receptor downregulation and desensitization as well as CB₁ functional tolerance (Chanda, 2010, Schlosburg, 2010). Therefore, we also tested whether the anti-edematous and anti-allodynic responses elicited by JZL184 in the carrageenan model would be retained after repeated administration of low and high doses of JZL184. Finally, we tested whether systemic administration of JZL184 after intraplantar carrageenan injections reverses edema and allodynia to infer whether this compound possesses efficacy to treat nociceptive behavior and edema following an inflammatory insult.

Methods

Subjects

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) as well as male and female CB₁ (-/-) and CB₂ (-/-) mice and their respective littermate controls, CB₁ (+/+) and CB₂ (+/+) mice from the Center Transgenic Colony at Virginia Commonwealth University served as subjects. CB₁ (-/-) and CB₂ (-/-) mice were backcrossed onto a C57BL/6J background for 13 and 6 generations, respectively. The subjects weighed between 18 and 25 g, and were housed four-five mice per cage in a temperature (20–22 °C) and humidity controlled AAALAC-approved facility. Mice were given unlimited access to food and water in their home cages and were maintained on a 12/12 h light/dark cycle. The sample size for each treatment group was 6 to 10 mice/group and for knockout studies was 4 to 9 mice/group. All animal protocols were approved

by the Virginia Commonwealth University Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (Institute of Laboratory and Animal Resources, 1996). After testing was completed, all mice were humanely euthanized via CO₂ asphyxia, followed by rapid cervical dislocation.

Drugs

JZL184 and PF-3845 were synthesized as described previously (Ahn, 2009, Long, 2009a) by Organix, Inc. (Woburn, MA). JZL184, PF-3845, the CB₁ receptor antagonist rimonabant (SR141716, National Institute on Drug Abuse, Bethesda, MD), and the CB₂ receptor antagonist SR144528 (National Institute on Drug Abuse) were dissolved in a vehicle that consisted of a mixture of ethanol, alkamuls-620 (Rhone-Poulenc, Princeton, NJ), and saline in a ratio of 1:1:18. The nonselective cyclooxygenases (COX) inhibitor diclofenac (DIC; Tocris, Ellisville, MO) was dissolved in saline. Each drug was given via the i.p. route of administration in a volume of 10 µl/g body weight.

Induction of Paw Edema with Carrageenan

Edema was induced by giving an intraplantar injection of 0.3% carrageenan (Sigma, St Louis) in a 20-µl volume using a 30-gauge needle into the hind left paw. Paw thickness was measured with electronic digital calipers (Traceable Calipers, Friendswood, TX), prior to and 5 h following carrageenan administration, which corresponds to peak edema (Wise et al. 2007). This procedure has been used previously by our laboratory (Cravatt, 2001, Cravatt and Lichtman, 2004, Lichtman, 2004, Wise, 2008).

Mechanical Allodynia

The mice were placed inside ventilated polycarbonate chambers on an elevated aluminum mesh table and allowed to acclimate to the apparatus for 60 min before testing. Mechanical allodynia was assessed with von Frey filaments (North Coast Medical, Morgan Hill, CA), using the “up-down” method (Chaplan et al., 1994) 5 h after carrageenan administration. The plantar surface of each hind paw was stimulated five times with each filament (0.16–6.0 g), at a frequency of approximately 2 Hz, starting with the 0.6-g filament and increasing until the mouse responded by licking and/or lifting the paw off the surface of the test apparatus. Three or more responses out of five stimulations were coded as a positive response. Once a positive response was detected, sequentially lower weight filaments were used to assess the sensory threshold for each paw.

Testing Procedures

Mice were transported to the testing room, weighed, randomly assigned to the different treatment regimens, and allowed to acclimate for at least 1 h before injections. The time course for carrageenan-induced paw edema was assessed in an initial experiment. Peak edema occurred at 1 h, which was sustained for 5 h (figure 2). Mechanical allodynia was assessed at the 5 h time point. For consistency with our previous studies, the 5 h time point was selected to assess paw edema and mechanical allodynia. The pre-treatment times for each drug were as follows: 30 min for diclofenac (5 mg/kg), 2 h for PF-3845 (1, 3, or 10 mg/kg), and 2 h for JZL184 (1.6, 4, 16, or 40 mg/kg). In experiments assessing cannabinoid receptor mechanism of action, the CB₁ receptor antagonist rimonabant (1 mg/kg) and the CB₂ receptor antagonist SR144528 (3 mg/kg) were administered 30 min prior to JZL184 (16 mg/kg) or vehicle. It should be noted that in initial experiments, 3 mg/kg rimonabant reduced paw edema. In contrast, 1 mg/kg rimonabant administered alone did not affect the dependent measures and was employed for the antagonism

studies. Previous studies have shown that these doses of rimonabant (Lichtman et al., 1996, Lichtman and Martin, 1991, Lichtman, 2004) and SR144528 (Lichtman, 2004, Malan et al., 2002) block the pharmacological effects of cannabinoid receptor agonists. The anti-edematous and anti-allodynic effects of 16 mg/kg JZL184 were evaluated in CB₁ (+/+), CB₁ (-/-), CB₂ (+/+), and CB₂ (-/-) mice to further assess receptor involvement. In addition, we determined whether administration of the antagonists after carrageenan would reverse the anti-edematous and anti-allodynic effects JZL184. In this experiment, rimonabant (1 mg/kg) or SR144528 (3 mg/kg) was injected 4 h after carrageenan and edema and allodynia were measured at 5 h.

In order to assess the impact of repeated administration of JZL184 on paw edema and mechanical allodynia, the following groups of mice were tested: (Group 1) vehicle for 6 days, (Groups 2-5) vehicle for 5 days and challenged with 1.6, 4, 16 or 40 mg/kg JZL184 on day 6, and (Groups 6-9) 1.6, 4, 16 or 40 mg/kg JZL184 for 6 days. Mice were administered their respective treatments 2 h before carrageenan was injected. Edema and mechanical allodynia were then assessed 5h later. In the final experiment, JZL184 (16 mg/kg) was administered 3 h after carrageenan to examine whether carrageenan-induced edema and allodynia would be reversed at 5 h.

Data Analyses

Paw edema data are expressed as the difference in paw thickness between the 5 h and pre-injection measures. Paw withdrawal thresholds to the von Frey filaments in the carrageenan-injected and contralateral (i.e., control) paws at the 5 h time point were used to assess mechanical allodynia. All data are depicted as mean \pm standard error of the mean (SEM). Data were analyzed using t-tests, one-way analysis of variance (ANOVA), or two-way ANOVA. Dunnett's test was used for post hoc analysis in the dose-response experiments in which the effects of each drug

dose were compared to those of vehicle. Tukey-Kramer post hoc analysis was used for all tests comparing different treatment groups. Bonferroni planned comparisons were used to assess genotype differences. Differences were considered significant at the $p < 0.05$ level.

Results:

Induction of Edema and allodynia by carrageenan

Intraplantar administration of carrageenan induced paw edema and allodynia over an extended time. In the time course study, carrageenan induced edema peaked at 1 h, which was sustained for 5 h [$F(4, 25) = 536.5$, $p < 0.001$; Figure 2A]. Allodynia was first tested at 5 h time point and it was found to persist at least 24 h after carrageenan [$F(5, 30) = 9.009$, $p < 0.001$] (Figure 2B). As shown in Figure 2C, 3 mg/kg rimonabant produced anti-edematous effects when administered alone [$F(2, 14) = 47.85$, $p < 0.001$]. On the other hand, administration of 1mg/kg rimonabant did not alter paw thickness [$p = 0.09$].

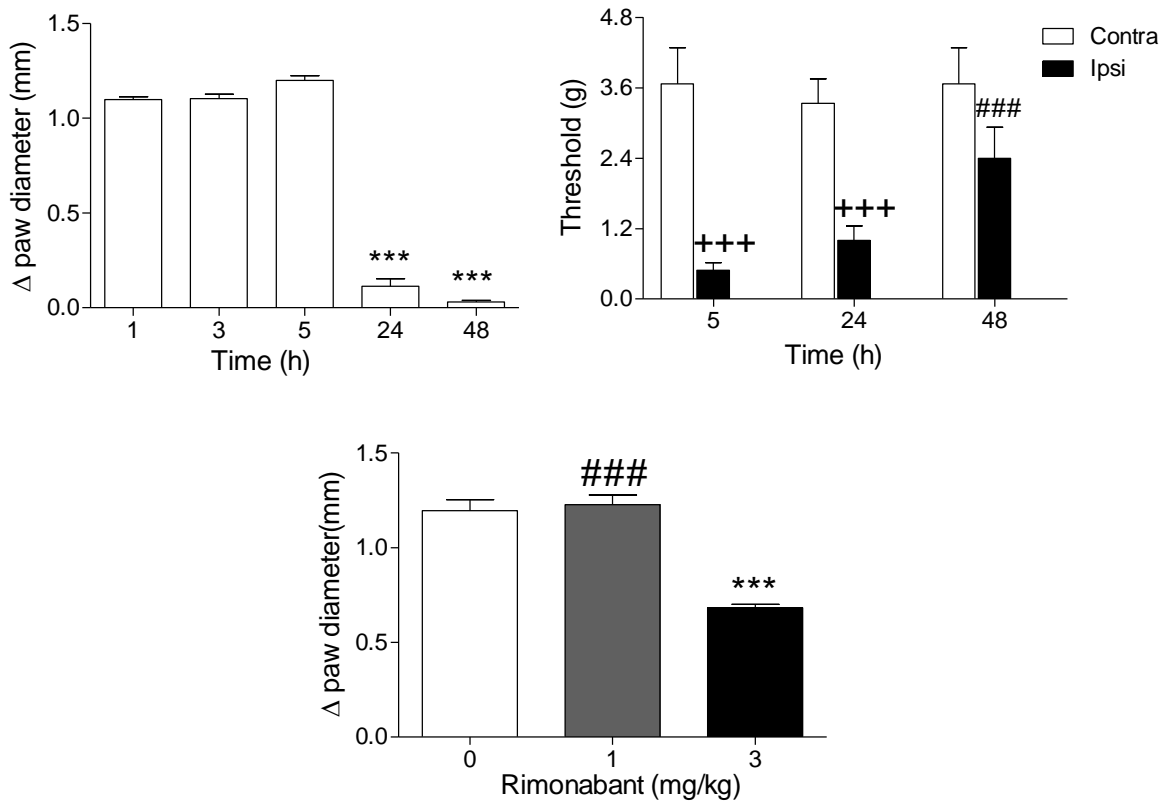


Figure 2: Time course for the edematous and allodynic effects of carrageenan.

Edema peaked at 1 h and persisted for at least 5 h (**Panel A**), ***, $p < 0.001$ versus 1, 3, and 5 h. Allodynia reduced in 48 h (**Panel B**), +++, $p < 0.001$ versus contralateral paw; ###, $p < 0.001$ versus 5 h. Values represented the mean (\pm SEM) mechanical paw withdrawal threshold and difference in paw thickness. $N=6$ /group. **Panel C** showed that the CB₁ antagonist rimonabant produced an effects of its own at a high dose (3mg/kg) but 1 mg/kg did not have an effect of its own ***, $p < 0.001$ versus vehicle; ###, $p < 0.001$ versus 3mg/kg rimonabant. $N = 5-6$ /group.

Anti-edematous and anti-allodynic effects of diclofenac, JZL184, and PF-3845 in the carrageenan model

Intraplantar administration of carrageenan induced paw edema and decreased paw withdrawal threshold over an extended time (Figure 3). In contrast, the withdrawal threshold for

the control paw remained constant throughout all of the studies (Table 3). As shown in Figure 3A, JZL184 [F (4, 33) = 24.64, $p < 0.001$], PF-3845 [F (3, 20) = 25.76, $p < 0.001$], and diclofenac [t (12) = 5.51, $p < 0.001$] significantly attenuated carrageenan-evoked edema. As shown in Figure 3B, carrageenan-induced allodynia was significantly attenuated by JZL184 [F (4, 37) = 11.95, $p < 0.001$], PF-3845 [F (3, 20) = 6.596, $p < 0.05$] and diclofenac [t (12) = 6.03, $p < 0.001$]. None of the treatments altered the paw withdrawal threshold in the contralateral paw (Table 3).

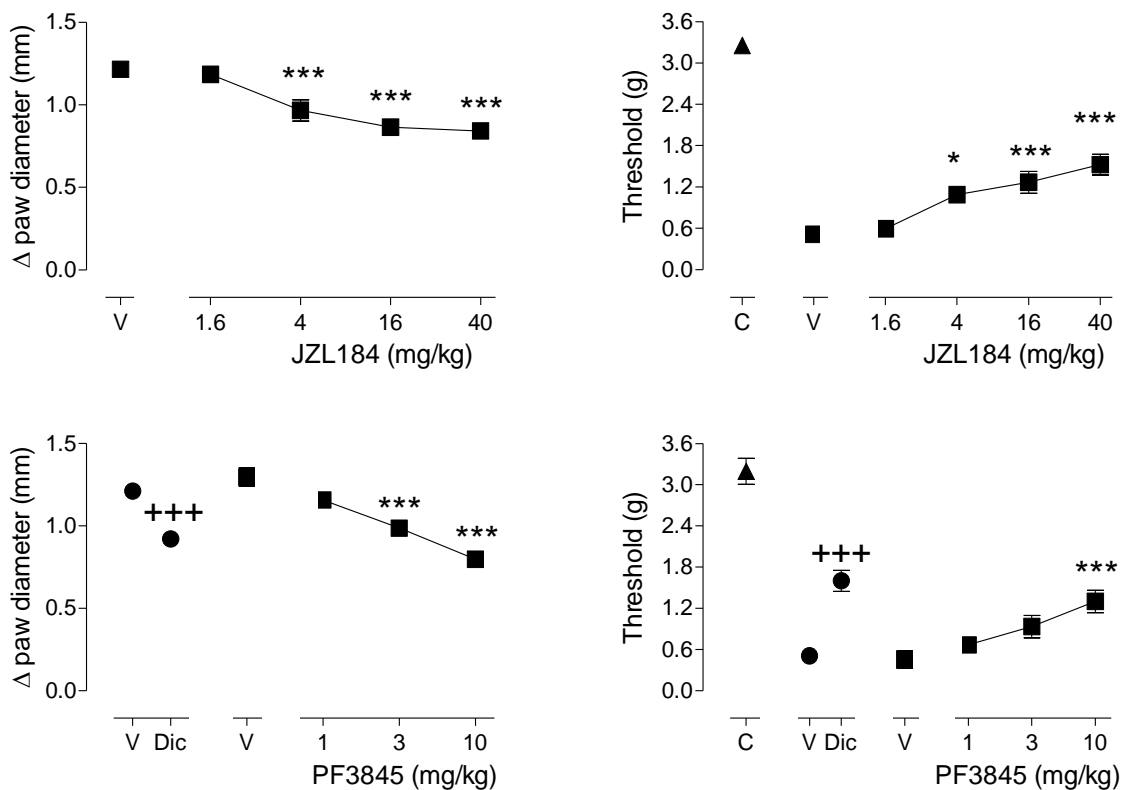


Figure 3: JZL184, PF3845, and diclofenac (5 mg/kg) partially reduced edema and allodynia in the carrageenan model.

JZL184 significantly reduced carrageenan-induced paw edema (A) and allodynia (B). Diclofenac (Dic) and PF-3845, shown on the same graph and both significantly reduced edema (C). Diclofenac (Dic) and PF-3845, shown on the same graph and both of them partially reduced

allodynic (**D**). Values represented the mean (\pm SEM) mechanical paw withdrawal threshold and difference in paw thickness. ***, $p < 0.001$ versus vehicle, +++, $p < 0.001$ versus vehicle. N=6-7 mice/group.

Cannabinoid receptors mediate the anti-allodynic and anti-edematous of JZL184

To determine whether CB₁ and CB₂ receptors mediate the anti-allodynic effects of JZL184, mice were pretreated with rimonabant (1 mg/kg), SR144528 (3 mg/kg), or vehicle 30 min prior to JZL184 (16 mg/kg) injection. As shown in Figure 4A, rimonabant [F (1, 20) = 10.07, $p < 0.001$] and SR144528 [F (1, 20) = 19.21, $p < 0.001$; Figure 4B] completely blocked the anti-allodynic effects of JZL184. There was a significant interaction between JZL184 and pretreatment with the rimonabant and SR144528 on mechanical allodynia, indicating the involvement of CB₁ and CB₂ receptors. Neither rimonabant nor SR144528 significantly affected allodynia when given alone. CB₁ (-/-) [F (1, 19) = 5.73, $p < 0.001$; Figure 4C] and CB₂ (-/-) [F (1, 24) = 8.74, $p < 0.01$; Figure 4D] mice were resistant to the anti-allodynic effects of JZL184. In the absence of drugs, both CB₁ (-/-) and CB₂ (-/-) mice showed similar nociceptive behavior as the wild type control mice. All the F ratios in this section represent the statistical interaction between drug treatment and genotype in the two-ANOVAs.

The data depicted in Figure 5, revealed that CB₂ receptors mediate the anti-edematous effects of JZL184. Whereas rimonabant did not affect the anti-edematous effects of JZL184 [$p=0.22$; Figure 5A]. SR144528 completely blocked the anti-edematous effects of JZL184, as revealed by a significant interaction between JZL184 and SR144528 [F (1, 20) = 8.73, $p < 0.001$; Figure 5B]. Consistent with the cannabinoid receptor antagonist data, JZL184 retained its anti-edematous effects in CB₁ (-/-) mice [$p = 0.84$; Figure 5C], but CB₂ (-/-) mice were completely resistant to the anti-edematous effects of JZL184, as indicated by a significant interaction

between JZL184 and the genotype [$F(1, 21) = 59.97, p < 0.001$; Figure 5D]. As shown in Figure 5E, the anti-edematous effects of PF-3845 were reversed by SR144528 [$F(1, 26) = 74.08, p < 0.001$]. On the other hand, rimonabant did not change the anti-edematous effects of PF-3845 [$F(1, 20) = 24.01, p < 0.001$; Figure 5F].

In the next series of experiments, we examined whether rimonabant or SR144528 injected 4 h after carrageenan would reverse the anti-edematous and anti-allodynic effects of JZL184 (16 mg/kg). The anti-edematous effects of JZL184 were not reversed by either drug [$p=0.53$; Figure 6A], but rimonabant as well as SR144528 completely reversed the anti-allodynic effects of JZL184 [$F(1, 36) = 5.27, p < 0.01$; Figure 6B].

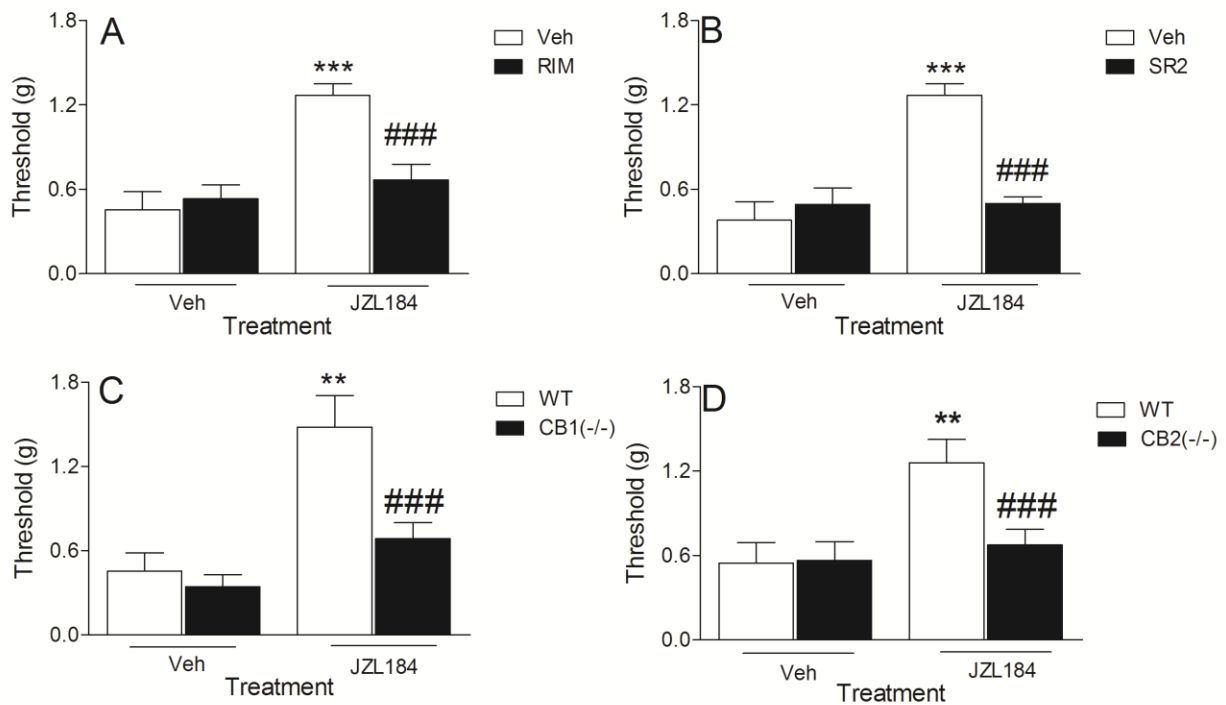


Figure 4: The anti-allodynic effect of JZL184 is mediated by a CB₁ and CB₂ mechanism of action. Rimonabant blocked the anti-allodynic effect of (A) JZL184. ***, $p < 0.001$ versus vehicle/vehicle (veh/veh), ###, $p < 0.001$ versus veh/JZL184. SR144528 also blocked the anti-allodynic effect of JZL184 (B). ***, $p < 0.001$ versus veh/veh. ###, $p < 0.001$ versus veh/JZL184. The anti-allodynic effect of JZL184 did not occur in (C) CB₁ knockout mice (-/-). **, $p < 0.05$ versus WT/veh. ###, $p < 0.001$ versus CB₁ (-/-)/JZL184. The anti-allodynic effect of (D) JZL184 did not occur in CB₂ knockout mice. **, $p < 0.001$ versus wild type or WT/veh. ###, $p < 0.001$ versus CB₂ (-/-)/JZL184. Values represented the mean (\pm SEM) mechanical paw withdrawal threshold. N=6/group.

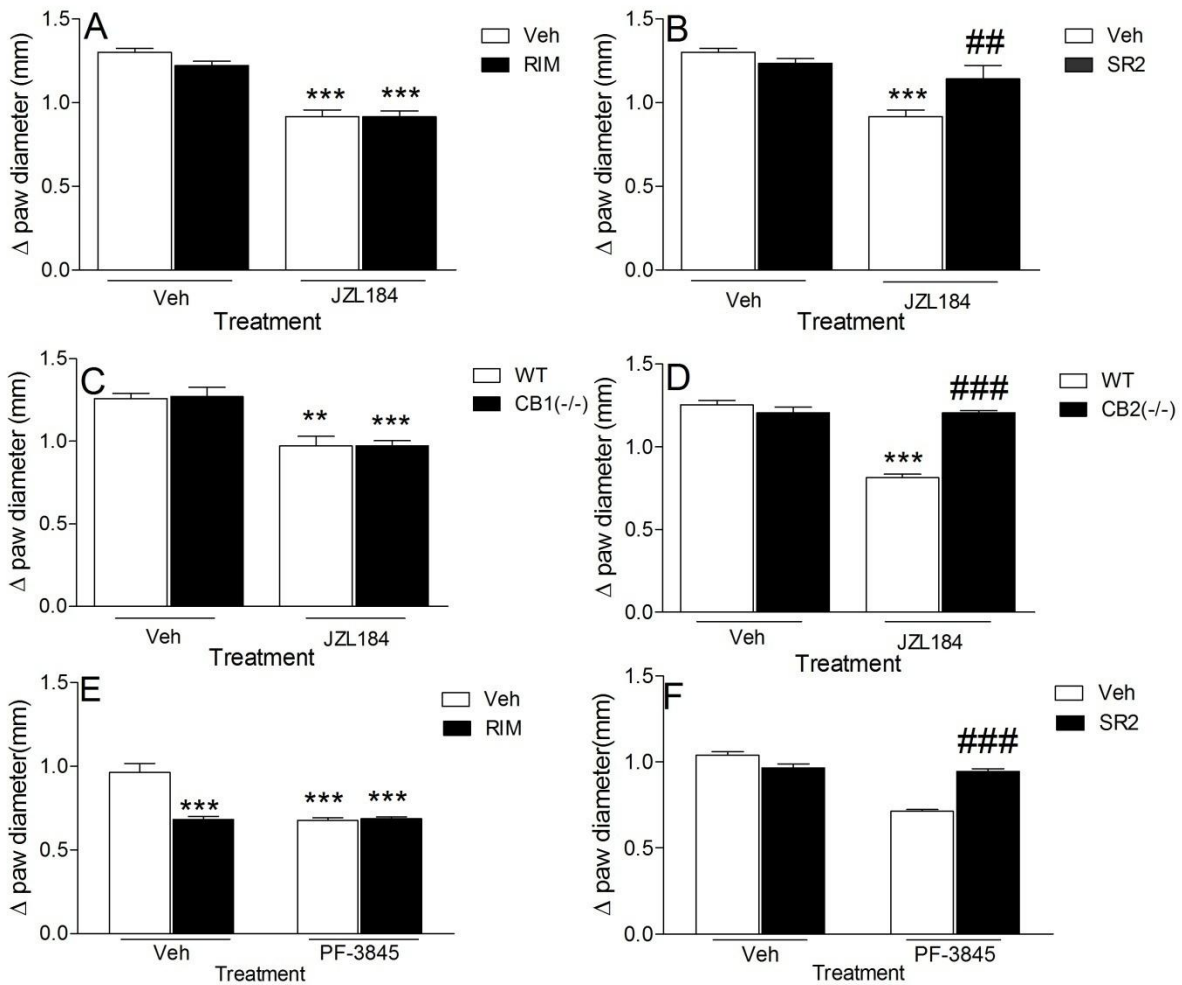


Figure 5: The anti-edematous effect of JZL184 is mediated by a CB₂, but not a CB₁, receptor mechanism of action. Rimonabant did not block the anti-edema effect of JZL184 (A). ***, p < 0.001 versus veh/veh; SR144528 blocked the anti-edematous effect of JZL184. ***, p < 0.001 versus Vehicle/Vehicle (veh/veh). ## p < 0.01 versus veh/JZL184, the anti-edematous effect of JZL184 was present in CB₁ knockout mice (-/-). **, p < 0.01, ***, p < 0.001 versus WT/veh. The anti-edematous effect of JZL184 does not occur in CB₂ knockout mice (D). ***, p < 0.001 versus WT/Veh and ### p < 0.001 versus CB₂ (-/-)/JZL. Values represented the mean (± SEM) difference in paw thickness. N=5-9/group. Panel E showed that anti-edematous effects of PF-3845 were antagonized by SR144528, ***, p < 0.001 versus veh/veh; ###, p < 0.001 versus

Veh/PF-3845 and panel F showed that the anti-edematous effects were maintained in rimonabant-treated animals, ***, $p < 0.001$ versus veh/veh. Rimonabant has produced an anti-edematous effects by itself at 3mg/kg dose ***, $p < 0.001$ versus veh/veh.

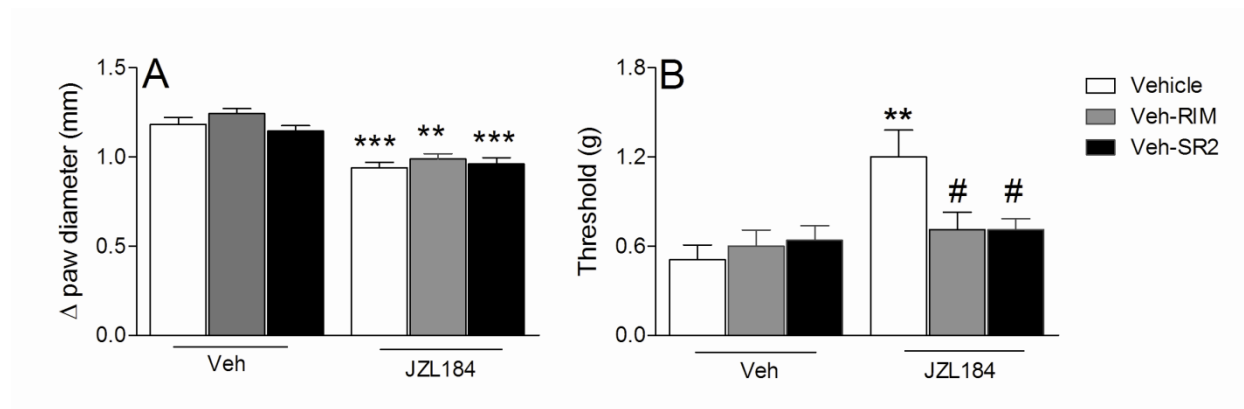


Figure 6: JZL184-induced anti-allodynia occurs independently of its anti-edematous effect.

Neither rimonabant nor SR144528 blocked the anti-edematous effect of JZL184 when injected approximately 4 h after carrageenan (A). Both rimonabant and SR144528 blocked the anti-allodynic effect of JZL184 when injected approximately 1 hour before determining mechanical allodynia (B). Values represented the mean (\pm SEM) mechanical paw withdrawal threshold and difference in paw thickness. **, $p < 0.01$, ***, $p < 0.001$ versus veh/veh; #, $p < 0.05$ versus JZL184/veh. N=6-9/group.

Differential tolerance following repeated administration of low dose and high dose JZL184

As genetic deletion or prolonged pharmacological inhibition of MAGL is known to produce CB₁ receptor functional tolerance (Chanda, 2010, Schlosburg, 2010), we assessed the dose-response relationship of JZL184 (1.6, 4, 16, or 40 mg/kg) after acute and repeated administration in the carrageenan assay. JZL184 dose-dependently attenuated carrageenan-induced paw edema and allodynia. As shown in Figure 7, acute administration of 4, 16, and 40 mg/kg JZL184 significantly attenuated carrageenan-induced edema [$F(8, 65) = 11.62, p < 0.001$] (Figure 7A). While the anti-edematous effects of 4 and 16 mg/kg JZL184 were maintained following repeated dosing, the anti-edematous effects of 40 mg/kg JZL184 underwent tolerance upon repeated administration. Acute administration of 4, 16, and 40 mg/kg JZL184 also

significantly attenuated mechanical allodynia [$F(8, 65) = 7.953, p < 0.001$; Figure 7B]. The anti-allodynic effects of 4 mg/kg JZL184 were maintained after repeated dosing; however, repeated administration of high doses of JZL184 (16 and 40 mg/kg) led to tolerance. The lowest dose of JZL184, 1.6 mg/kg, tested in this study, remained ineffective regardless of whether it was administered acutely or repeatedly. There was no allodynia seen in the contralateral paw (Table 3).

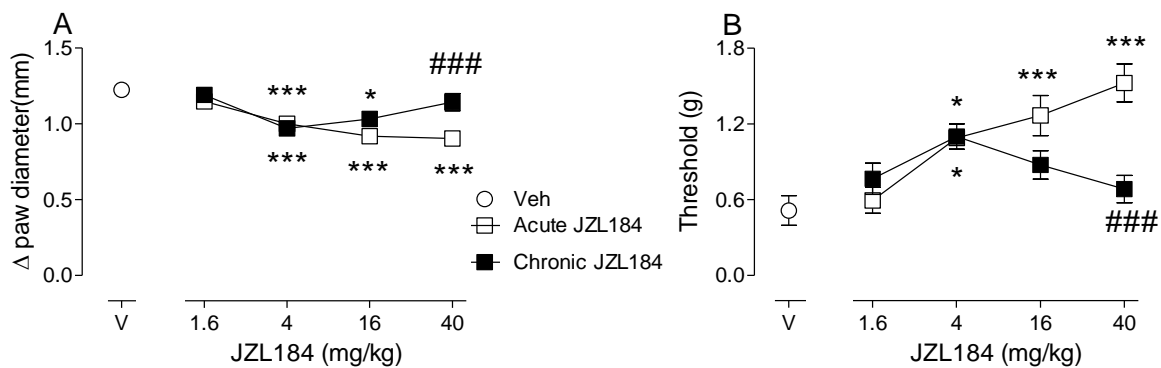


Figure 7: The anti-edematous and anti-allodynic effects of JZL184 undergo tolerance following repeated administration of high dose, but not low dose of the drug. **A.** The anti-edematous effects of low doses of JZL184 remain effective after repeated administration. **B.** The anti-allodynic effects of low doses of JZL184 remain effective after repeated administration, when the high dose produced tolerance. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus veh/veh; ###, $p < 0.001$ versus acute 40 mg/kg JZL184. Values represented the mean (\pm SEM) mechanical paw withdrawal threshold and difference in paw thickness. $N = 6-8/\text{group}$.

JZL184 reverses carrageenan-induced anti-edema and allodynia

To test whether JZL184 retains efficacy when given after the induction of edema, subjects received vehicle or JZL184 (16 mg/kg) 3 h after carrageenan. Edema was measured 3

and 5 h after carrageenan injection and allodynia was assessed at 5 h. As shown in Figure 8A, JZL184 produced a significant partial reversal of carrageenan-induced edema. JZL184 significantly reduced edema at 5 h compared with the 3 h time point [$F(1, 10) = 58.58, p < 0.001$]. JZL184 given after the induction of carrageenan-induced paw edema also significantly attenuated the mechanical allodynia at 5 h [$t(10) = 2.90, p < 0.0$; Figure 8B]. JZL184 did not affect paw withdrawal thresholds in control paws (Table 3).

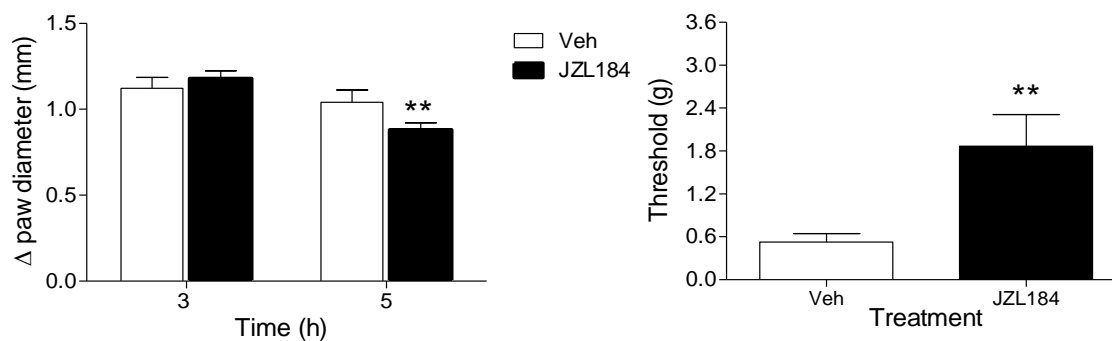


Figure 8: JZL184 given after carrageenan reverses paw edema and mechanical allodynia.

JZL184 reversed edema (A) and allodynia (B) when administered 3 h post carrageenan. Values represented the mean (\pm SEM) mechanical paw withdrawal threshold and difference in paw thickness. **, $p < 0.01$ versus JZL184 at 3 h. T test was done for allodynia. **, $p < 0.01$, versus veh; N=6 /group

Table 3: Paw withdrawal thresholds in the contralateral paw

The paw withdrawal thresholds for the control paw were not altered by any of the treatments as measured with von Frey filaments. Values represent the mean (\pm SEM) mechanical paw withdrawal threshold.

Experiment	Mean (g)	\pm SEM	Sample Size
JZL184 acute dose	3.27	0.142	35
JZL184 chronic dose	3.11	0.119	70
PF-3845 acute dose	3.20	0.276	24
Diclofenac	3.14	0.40	12

Rimonabant	3.35	0.165	27
SR144528	2.90	0.248	26
CB ₁ (-/-) mice	3.37	0.297	24
CB ₂ (-/-) mice	2.96	0.217	23
Rimonabant or SR144528 after carrageenan	3.44	0.189	28
JZL184 after carrageenan	3.50	0.435	12
Combination of low dose of JZL184 and high dose of PF-3845	3.13	0.053	32
Repeated administration low dose of JZL184 and high dose of PF-3845	3.32	0.165	27

Discussion

JZL184 represents the first selective MAGL inhibitor that elevates brain 2-AG, but not anandamide, levels upon acute administration (Long, 2009a). This compound reduces nociceptive behavior in a wide range of laboratory animal models of pain, including warm water tail withdrawal, acetic acid stretching, formalin, mechanical and cold allodynia following chronic constrictive injury of the sciatic nerve, capsaicin-induced mechanical allodynia, and bone cancer tests (Khasabova, 2011; Kinsey, 2010; Kinsey, 2009; Spradley, 2010; Busquets-Garcia, 2011; Guindon, 2011). The present study increases the understanding that MAGL inhibition plays a role in nociception by demonstrating that JZL184 reduces carrageenan-induced paw edema and associated mechanical allodynia. These effects were similar in magnitude to those produced by

the FAAH inhibitor PF-3845, as well as the nonselective COX inhibitor diclofenac. The anti-edematous effects of JZL184 were mediated through CB₂ receptors, while the anti-allodynic effects required both CB₁ receptors and CB₂ receptors. In addition, repeated administration of high dose JZL184 resulted in tolerance to its anti-allodynic and anti-edematous effects, but a low dose of JZL184 retained efficacy after repeated administration.

A key finding in the present study was that JZL184 administration 3 h after carrageenan significantly reversed the magnitude of paw edema and mechanical allodynia. The purpose of administering JZL184 pre versus post carrageenan is to identify the therapeutic significance of the drug. Reversal of anti-allodynic effects by JZL184 indicates that the drug can reverse already exist edema and allodynia. Similarly, URB602 reversed carrageenan-induced paw edema and paw withdrawal latency when administered after carrageenan in mice (Comelli, 2007). These findings suggest that endocannabinoid catabolic inhibitors may possess therapeutic utility in treating clinical symptoms associated with inflammatory disease states.

The observations that JZL184 did not attenuate carrageenan-induced edema in SR144528-treated wild type mice and CB₂ (-/-) mice indicate a necessary role of CB₂ receptors. In contrast, complementary pharmacological and genetic approaches indicated that CB₁ receptors are expendable for the anti-edematous effects of JZL184. Likewise, the anti-edematous effect of URB602, which inhibits FAAH and MAGL with similar potency, has also been reported to be blocked by SR144528, but not by rimonabant. Studies have shown that systemic and local administration of the CB₂ receptor agonist AM1241 suppressed the development of mechanical stimulation in the carrageenan model of inflammation. This suppression was blocked by SR144528, but not by rimonabant. Previous studies have also reported that elevation of 2-AG attenuates inflammatory and immune response in vitro. Thus, inhibiting 2-AG degradation in

vivo offers a strategy to augment levels of this endogenous cannabinoid to elicit anti-inflammatory effects through the stimulation of CB₂ receptors.

Conversely, complementary genetic and pharmacological approaches revealed that the anti-allodynic effects of JZL184 required both CB₁ and CB₂ receptors. Similarly, the suppressive effects of JZL184 on capsaicin-induced thermal hyperalgesia and nocifensive behavior required both CB₁ and CB₂ receptors. Likewise, intra-paw administration of JZL184 or URB602 produced antinociceptive effects in the formalin model, which were blocked by CB₁ and CB₂ receptor antagonists. In contrast, CB₂ receptors did not play a necessary role in the anti-allodynic effects of JZL184 in the chronic constriction injury model. Two related questions are raised by these observations. First, why do both CB₁ and CB₂ receptors play a necessary role in the antinociceptive effects of JZL184 in some types of nociceptive assays (e.g., carrageenan, formalin, and capsaicin), but only CB₁ receptors are required in other models (e.g., warm water tail withdrawal, acetic acid stretching, CCI)? Second, what is the mechanism by which both cannabinoid receptors are required for the anti-allodynic effects of JZL184 in the carrageenan assay? It is unlikely that the method to assess nociception accounts for these disparate findings because von Frey filaments were used to assess mechanical allodynia in both CCI and carrageenan assays. Instead, this differential involvement of CB₂ receptors may be related to the degree to which inflammatory responses contribute to the nociception as well as the concentration of cannabinoid receptors and 2-AG at the critical sites of action. Indeed, these actions could occur at the site of inflammation in the paw, within the dorsal root ganglia or dorsal horn of the spinal cord, or in multiple supraspinal regions (e.g., PAG or the rostral ventromedulla). It has been shown that CB₂ receptors are upregulated in the dorsal root ganglia and paw tissue of rodents administered complete Freund's adjuvant, suggesting that CB₂

receptors in these regions could contribute to the findings reported here. For example, intraplantar carrageenan could lead to the infiltration of immune cells, such as macrophages or neutrophils, at the site of injection, that express CB₂ receptors (Galiegue, 1995). 2-AG activation of CB₂ receptors on infiltrating cells might synergize with the well-described antinociceptive actions of CB₁ receptor stimulation on peripheral nociceptors within the spinal cord and within supraspinal sites of action. It will be important to examine whether JZL184 alters inflammatory mediators (e.g., pro- and anti-inflammatory cytokines and prostaglandins, as well as infiltrating immune cells) caused by carrageenan. In order, to ascertain the relative contribution of CB₁ and CB₂ receptors in these effects, we also evaluated the ability of rimonabant and SR144528 administered 4 h after carrageenan to reverse the anti-allodynic and anti-edematous effects of JZL184. Neither antagonist reversed the anti-edematous effects of JZL184 but both rimonabant and SR144528 reversed the anti-allodynic effects of JZL184, indicating that these effects can be dissociated.

Another important finding in the present study was that the anti-allodynic and anti-edematous effects of JZL184 were maintained following repeated low dose administration, but the effects underwent tolerance after repeated high dose JZL184. Similarly, prolonged inactivation of MAGL via administration of high dose JZL184 (40 mg/kg) results in the loss of analgesic responses in the CCI model, cross-tolerance to exogenous cannabinoid receptor agonists (i.e., THC, and WIN55, 212-2), CB₁ receptor downregulation and desensitization in cingulate cortex, hippocampus, somatosensory cortex, and PAG. Additionally, 2-AG elevation in MAGL (-/-) mice caused tonic activation and partial desensitization of CB₁ receptors (Schlosburg, 2010). The findings that the anti-edematous and anti-allodynic effects of low dose JZL184 were maintained after repeated dosing are consistent with a previous report in which

repeated administration of 8 mg/kg JZL184 maintained its anxiolytic-like effects under high illumination conditions in the rat elevated plus maze assay (Guindon, 2011).

In conclusion, the present study demonstrates that the selective MAGL inhibitor, JZL184, significantly inhibits inflammatory pain, as assessed in the carrageenan assay. More specifically, JZL184 attenuated the development of paw edema and mechanical allodynia and reversed edema and allodynia when administered after carrageenan. Complementary genetic and pharmacological approaches revealed that the anti-allodynic effects of JZL184 required both CB₁ and CB₂ receptors, whereas only CB₂ receptors had a necessary role in mediating its anti-edematous effect. We also found that the anti-allodynic and anti-edematous effects of low, but not high, doses of JZL184 do not undergo tolerance when administered repeatedly. These results indicate that the activation of CB₁ and/or CB₂ receptors by low doses of MAGL inhibitors may have beneficial effects on inflammatory pain that include the ability to prevent inflammation and reverse established inflammatory pain states.

Chapter 3. Combined inhibition of MAGL and FAAH

suppresses edema and produces augmented anti-allodynic effects in the carrageenan mouse model

Introduction:

Direct cannabinoid receptor agonists have long been known to reduce pain and inflammation in acute and chronic preclinical animal models (Kinsey, 2009, Lichtman, 2004, Long, 2009b, Russo, 2007); however, the psychomimetic side effects produced by these drugs dampen enthusiasm for clinical development (Lichtman, 2004). An alternative approach that promises to retain the beneficial effects and to minimize the untoward cannabimimetic side effects associated with CB₁ receptor agonists is to inhibit the primary enzymes responsible for the hydrolysis of the endogenous cannabinoids (endocannabinoids), monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) (Blankman, 2007, Cravatt, 2001, Cravatt, 1996, Dinh, 2002). Pharmacological inhibition of MAGL and FAAH elevates the respective endocannabinoids, anandamide; and 2-AG in brain and spinal cord tissue (Cravatt, 1996, Kinsey, 2009, Long, 2009a). Blockade of these endocannabinoid catabolic enzymes produce analgesic and anti-inflammatory effects in vivo in carrageenan, tail withdrawal, CFA, CCI, LPS and formalin pain assay (Ahn, 2009, Guindon, 2011, Guindon and Hohmann, 2009, Kinsey, 2010, Kinsey, 2009, Lichtman, 2004, Long, 2009a, Long, 2009b, Naidu, 2010, Sasso et al., 2012, Schlosburg, 2009, Sciolino, 2011, Spradley, 2010).

Irreversible (PF-3845, URB597) and reversible (OL-135) inhibitors of FAAH have been demonstrated to elevate anandamide levels in the brain (Ahn, 2009, Boger, 2005, Fegley, 2005)

and attenuate neuropathic pain (Clapper, 2010, Jhaveri, 2006, Kinsey, 2009, Lichtman, 2004, Russo, 2007), inflammatory pain (Booker, 2011), and collagen-induced arthritis (Kinsey, 2011a). Similarly, MAGL inhibitors attenuate nociception to radiant heat and acetic acid-induced abdominal stretching (Long, 2009a), as well as reverse allodynic and hyperalgesic states in neuropathic (Kinsey, 2009) and inflammatory (Ghosh, 2013, Guindon, 2011) pain models. However, these inhibitors are not fully efficacious in producing antinociceptive effects in neuropathic and inflammatory pain models.

It is also important for drugs used to treat chronic pain conditions to maintain their antinociceptive effects following chronic administration. The consequences of prolonged and complete inhibition of MAGL contrast with the consequences of prolonged and complete blockade of FAAH. Repeated administration of high doses of FAAH inhibitors produce sustained analgesia and without loss of CB₁ receptor function (Falenski et al., 2010, Schlosburg, 2010) and FAAH deficient mice display a CB₁ receptor-mediated hypoalgesic phenotype in thermal nociceptive tests, in both phases of the formalin test, thermal anti-hyperalgesic and anti-inflammatory effects in the carrageenan model (except in chronic constriction injury model) (Lichtman et al., 2004). Complete blockade of MAGL leads to tolerance, physical dependence, impaired endocannabinoid-dependent synaptic plasticity, and CB₁ receptor down-regulation and desensitization in select brain regions (Chanda, 2010, Schlosburg, 2010). However, partial MAGL blockade with low dose JZL184 maintains its antinociceptive actions in the CCI of the sciatic nerve neuropathic pain model (Kinsey, 2013) as well as in formalin-induced and carrageenan-induced inflammatory pain model (Ghosh, 2013, Sciolino, 2011). [3H] SR141716A binding reveals that mice show normal CB₁ receptor expression and function following repeated administration of low dose JZL184 (≤ 8 mg/kg) (Kinsey, 2013). Although, antinociceptive

effects of low dose JZL184 are maintained following repeated administration, it produced a partial anti-edematous and anti-allodynic effects in the carrageenan model (Ghosh, 2013). Similarly, the FAAH inhibitor, PF-3845 only partially reduced mechanical allodynia in CCI and carrageenan models (Ghosh, 2013). Thus, it would be advantageous to develop an approach to augment the efficacy of FAAH and MAGL inhibitors in reversing carrageenan-induced allodynia, but without the occurrence of CB₁ receptor functional tolerance, dependence, or cannabimimetic side effects.

Interestingly, the dual FAAH/MAGL inhibitor, JZL195 produced enhanced antinociceptive effects in tail immersion assay of thermal pain sensation, acetic acid writhing test of visceral pain compared with individual inhibition of these enzymes (Long, 2009b). However, JZL195, an equipotent MAGL and FAAH inhibitor also produced hypomotility, catalepsy, substituted for THC in the drug discrimination assay, and impaired spatial memory performance in the Morris water maze task (Long, 2009b, Wise et al., 2012), which are drawbacks for the strategy of completely inhibiting both FAAH and MAGL in combination. Hence, in this chapter, we tested whether partial inhibition of MAGL and full inhibition of FAAH would elicit enhanced antinociceptive effects in mouse models of inflammatory and neuropathic pain. Two approaches were used to produce a differential blockade of these two enzymes. In Chapter 3A, we tested whether co-administration of low dose JZL184 (4 mg/kg) and high dose PF-3845 (10 mg/kg) block nociceptive behavior in the carrageenan model of inflammatory pain and chronic constriction injury model of neuropathic pain. In Chapter 3B, we tested a novel dual FAAH-MAGL inhibitor, SA-57, which is considerably more potent in elevating brain anandamide levels than in elevating brain 2-AG levels (Niphakis, 2012).

Part A.

Endocannabinoid levels in the brain upon acute and repeated administration of JZL184 and PF-3845 given alone and in combination were quantified. The 4 mg/kg dose was chosen for JZL184 because this is the lowest effective dose that produces anti-edematous and anti-allodynic effects and these effects did not undergo tolerance following repeated administration. In addition, this dose maintained its gastro-protective effects upon repeated administration i.e repeated administration of JZL184 (4 mg/kg) produces protective effects in diclofenac-induced gastric hemorrhages (Kinsey, 2013). The 10 mg/kg dose of PF-3845 was chosen because effective in the carrageenan assay (Ghosh, 2013) and its repeated administration did not lead to CB₁ receptor functional tolerance (Schlosburg, 2010). Accordingly, we also evaluated whether the antinociceptive effects of this combination would be retained following repeated administration. Subjects were given repeated administration of this combination and tested for cross-tolerance to THC in the tetrad assay. Moreover, CB₁ receptor expression and function were assessed using [3H] SR141716A binding and CP55, 940-stimulated [35S] GTP γ S-stimulated binding assays as well as inhibition of IPSCs induced by the full CB₁ receptor agonist WIN55, 212-2 in cingulate cortex. Finally, we tested whether the combination of low dose JZL184 and high dose PF-3845 would produce cannabimimetic side effects as assessed in the tetrad.

Methods:

Subjects

Subjects were male C57BL/6J mice from Jackson Laboratory, Bar Harbor, ME. CB₁ (-/-) and CB₂ (-/-) mice and their respective littermate controls, CB₁ (+/+) and CB₂ (+/+) mice from the Center Transgenic Colony at Virginia Commonwealth University served as subjects. CB₁ (-/-) and CB₂ (-/-) mice were backcrossed onto a C57BL/6J background for 13 and 6 generations, respectively. The subjects weighed between 18 and 25 g, and were housed four-five mice per

cage in a temperature (20–22 °C) and humidity controlled AAALAC-approved facility. Mice were given unlimited access to food and water in their home cages and were maintained on a 12/12 h light/dark cycle. The sample size for each treatment group was 6 to 20 mice/group. All animal protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (Institute of Laboratory and Animal Resources, 1996). After testing was completed, all mice were humanely euthanized via CO₂ asphyxia, followed by rapid cervical dislocation except for the binding and endocannabinoid quantification studies where the animals were euthanized by decapitation without anesthesia.

Drugs

JZL184 and PF-3845 were synthesized as described previously (Ahn et al. 2009; Long et al. 2009) by Organix, Inc. (Woburn, MA). JZL184, PF-3845, delta 9-THC, the CB₁ receptor antagonist rimonabant (SR141716) and the CB₂ receptor antagonist SR144528 were obtained from National Institute on Drug Abuse. Previous studies have shown that these doses of rimonabant (Lichtman, 1996, Lichtman, 2004) and SR144528 (Conti et al., 2002, Lichtman, 2004, Malan, 2002) blocked the pharmacological effects of cannabinoid receptor agonists. All the drugs were dissolved in a vehicle that consisted of a mixture of ethanol, alkamuls-620 (Rhone-Poulenc, Princeton, NJ), and saline in a ratio of 1:1:18. Each drug was given via the i.p. route of administration in a volume of 10 µl/g body weight.

Induction of paw edema with carrageenan

Edema was induced by giving an intraplantar injection of 0.3% carrageenan (Sigma, St Louis), in a 20 µl volume using a 30 gauge needle, into the hind left paw. Paw thickness was

measured with electronic digital calipers (Traceable Calipers, Friendswood, TX) prior to and 5 h following carrageenan administration, which corresponds to peak edema (Wise, 2008). This procedure has been used previously by our laboratory (Cravatt and Lichtman, 2004, Ghosh, 2013, Lichtman, 2004, Wise, 2008).

Neuropathic pain model

Mice were subjected to CCI of the sciatic nerve, as described previously (Kinsey et al., 2009). Under isoflurane anesthesia, the right hind leg was shaved and the area swabbed with betadine solution, followed by ethanol. A small incision was made in the skin posterior to the femur, the muscle was separated, and the sciatic nerve was isolated and ligated twice with 5-0 (1.0 metric) black silk braided suture (Surgical Specialties Corporation, Reading, PA). The surrounding muscle and skin were then sutured with 6-0 nylon. Mice were placed in a heated cage to recover from anesthesia before being returned to the vivarium. Mice were tested for allodynia prior to, and 10 days after, CCI surgery to establish baseline levels of allodynia.

Mechanical allodynia

The mice were placed inside ventilated polycarbonate chambers on an elevated aluminum mesh table and allowed to acclimate to the apparatus for 60 min before testing. Mechanical allodynia was assessed with von Frey filaments (North Coast Medical, Morgan Hill, CA), using the “up-down” method (Chaplan, 1994). The plantar surface of each hind paw was stimulated five times with each filament (0.16–6.0 g), at a frequency of approximately 2 Hz, starting with the 0.6-g filament and increasing until the mouse responded by licking and/or lifting the paw off the surface of the test apparatus. Three or more responses out of five stimulations were coded as a positive response. Once a positive response was detected, sequentially lower weight filaments were used to assess the sensory threshold for each paw.

Testing procedures for the carrageenan and CCI studies

Mice were transported to the testing room, weighed, randomly assigned to the different treatment regimens, and allowed to acclimate for at least 1 h before injections. The time course for carrageenan-induced paw edema was assessed previously and peak edema occurred at 1 h (chapter 2 figure 2) and sustained for 5 h (Ghosh, 2013). Hence, all the edema data presented in this study represent the results obtained at the 5 h time point. Mechanical allodynia was assessed at the 5 h time point for carrageenan model and prior to, and 10 days after, CCI surgery to establish baseline levels of allodynia. The pre-treatment times for each drug were as follows: 2 h for PF-3845 (10 mg/kg), and 2 h for JZL184 (4 mg/kg), 2 h for PF-3845 (10 mg/kg) +JZL184 (4mg/kg) except for the reversal study where all the drugs were administered 3 h after carrageenan. In experiments assessing cannabinoid receptor mechanism of action, the CB₁ receptor antagonist rimonabant (1 mg/kg) and the CB₂ receptor antagonist SR144528 (3 mg/kg) were administered 30 min prior to JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) or vehicle. JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) or vehicle were administered 2 h before carrageenan. Edema was measured prior to and 5 h after and allodynia was assessed 5 h after carrageenan. Previous studies have shown that these doses of rimonabant (Lichtman, 1996, Lichtman, 2004) and SR144528 (Conti, 2002, Lichtman, 2004, Malan, 2002) blocked the pharmacological effects of cannabinoid receptor agonists. We have also measured the levels of endocannabinoids in brain, receptor binding, and receptor activity, cross-tolerance to THC as well as electrophysiology after acute as well as repeated administration of the combination. Following all the experiments, the animals were subjected to humane method of euthanasia and their brain tissues were collected for the analysis of brain endocannabinoid levels, binding and activity of the cannabinoid receptors in the same animals.

In order to assess the impact of repeated administration of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) on paw edema and mechanical allodynia in the carrageenan study, the following groups of mice were tested: (Group 1) vehicle for 6 days, (Group 2) vehicle for 5 days and challenged with JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) on day 6, and (Group 3) JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) for 6 days. On test days, mice were administered their respective treatments 2 h before carrageenan was injected. Edema and mechanical allodynia were then assessed 5 h later.

Measurement of brain lipids

Following decapitation, brains were rapidly removed and frozen on dry ice and stored at -80°C. On the day of processing, the pre-weighed tissues were homogenized with 1.4 ml chloroform: methanol (2:1 v/v containing 0.0348g phenylmethylsulfonyl fluoride/ml) after the addition of internal standards to each sample (2-pmol anandamide -d8, 1 nmol 2-AG-d8 and 1 nmol AA-d8). Homogenates were mixed with 0.3 ml of 0.73% w/v NaCl, vortexed and centrifuged for 10 min at 3220 g (4°C). The aqueous phase and debris were collected and extracted again twice 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 cold acetone. The mixtures were centrifuged for 5 min at 1811 g and (4 °C) to precipitate protein. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml methanol and placed in auto sample vials for analysis. LC/MS/MS was used to quantify anandamide, 2-AG, and AA. The mobile phase consisted of methanol (90:10): 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery ® HS C18, 4.6* 15 cm, 3 micron (Supelco, USA). Ions were analyzed in multiple reaction monitoring mode and the

following transitions were monitored in positive mode: (348>62) and (348>91) for anandamide; (356>62) for anandamide-d8; (379>287) and (279>269) for 2-AG ; (387>96) for 2AG-d8; (300>62) and (300>283) in negative mode: (303>259) and (303>59) for AA and (311>267) for AA-d8. A calibration curve was constructed for each assay based on linear regression using the peak area ratios of the calibrators. The extracted standard curves ranged from 0.039 to 40 pmol for anandamide, from 0.0625 to 64 nmol for 2-AG and from 0.1 to 32 nmol for AA.

Agonist-stimulated [35S] GTP γ S binding

Mice were injected with JZL184 + PF-3845 or vehicle for five consecutive days and were humanely euthanized 24 h after the final injection. The brains were removed, hemisected along the midsagittal plane, flash frozen in liquid nitrogen, and stored at - 80°C, as described previously (Selley et al., 1996). One half of each brain was placed in 15 mL of cold TME membrane buffer (50 mM Tris-HCl, 3 mM MgCl₂, 1 mM EGTA, pH 7.4) and homogenized. The other half of each brain was processed for radioligand binding, as described below. The samples were then centrifuged at 50,000 x g for 10 min at 5°C. The supernatant was removed and samples were resuspended in 15 ml of TME membrane buffer. Centrifugation was repeated, the pellet resuspended in TME buffer, and the protein concentration determined. Membranes then were pretreated with adenosine deaminase (10 mU/ml) for 15 min at 30 °C. Membrane protein (10 µg) was incubated in TME/Na (assay buffer with 100 mM NaCl) with 0.1% BSA, 30 µM GDP, 0.1 nM [35S] GTP γ S, and varying concentrations of CP55, 940 (0.01–10 µM) for 2 h at 30°C. Non-specific binding was determined using 20 µM unlabeled GTP γ S. Basal binding was determined in the absence of agonist. The incubation was terminated by rapid filtration through GF/B glass fiber filters and three washes with ice-cold Tris-HCl (pH 7.4). Liquid scintillation spectrophotometry was used to evaluate bound radioactivity after the extraction of filters in

Budget-Solve™ (Research Products International Corp., Mount Prospect, IL) scintillation fluid.

[3H] SR141716A binding

Half of each brain from mice treated with JZL184 or vehicle for five consecutive days, as described above, was placed in 15 mL of cold TME membrane buffer (50 mM Tris-HCl, 3 mM MgCl₂, 1 mM EGTA, and pH 7.4) and homogenized, as described above. Saturation analysis was performed by incubating 30 µg membrane protein with 0.2–3 nM [3H] SR141716A (rimonabant; CB₁ receptor antagonist) in assay buffer A + BSA (0.5 per g) with 2 µM of unlabeled rimonabant for 90 min at 30°C to determine non-specific binding. Total binding was determined in the absence of unlabeled rimonabant. The reactions were terminated using vacuum filtration through Whatman GF/B glass fiber filter pre-soaked in Tris buffer containing 5 g L⁻¹ BSA (Tris–BSA) and three washes with 4 °C Tris–BSA. Bound radioactivity was evaluated by liquid scintillation spectrophotometry at 45% efficiency after extraction in Budget-Solve™ scintillation fluid.

Cannabimimetic behavior assessments

Mice were treated for five consecutive days with vehicle + vehicle or 4 mg/kg JZL184 + 10 mg/kg PF-3845 and tested for cross-tolerance to THC on day six. Prior to testing all mice were acclimated to the test room for at least 1 h (Martin et al., 2003). Baseline nociception and rectal temperature were assessed. Cross-tolerance to THC (3, 10, 30 or 100 mg/kg) was then tested using a within-subjects cumulative dosing design. Specifically, mice were injected with an increasing dose of THC every 40 min and tested 30 min after each injection (Falenski, 2010, Kinsey, 2013) for catalepsy, nociception, and rectal temperature. Catalepsy was assessed using the bar test, in which the forelimbs of each mouse were placed on a horizontal bar during a 60s

test in which time immobile was measured. Hypothermia was determined by change in rectal body temperature from baseline. The tail immersion test was used to measure antinociception. The distal 2 cm of the tail was immersed in 52.0 °C water and the tail withdrawal latency determined. A maximum cutoff of 10 s was used to minimize possible tissue damage. Maximum percent effect was using the formula $[(\text{test value} - \text{base line value}) / (10 - \text{baseline value})] * 100$.

In separate groups of mice, the cannabimimetic behavioral effects of treatment with vehicle + vehicle, 4 mg/kg JZL184 + 10 mg/kg PF-3845, or 40 mg/kg JZL184 + 10 mg/kg PF-3845 were evaluated in the tetrad test. Baseline rectal temperature and nociception were first determined. Two hours after injections, locomotor activity was measured in a 5 min test. Mice were then evaluated for catalepsy, nociception, and rectal temperature as described above. Locomotor activity was quantified using Any-Maze™ software.

Measurements of IPSCs in the cingulate cortex

C57BL/6J mice (~10 weeks old) were intraperitoneally injected with (1) Vehicle + Vehicle (2) Repeated 4 mg/kg JZL184 + vehicle (3) repeated 4 mg/kg JZL184 + 10 mg/kg PF-3845 and (4) repeated 40 mg/kg JZL184 + 10 mg/kg PF-3845 for six days. 2 h after the final injection, mice were anaesthetized by isoflurane inhalation and decapitated. Cortical slices (300 µm thick) were cut using a vibrating slicer (Leica) and prepared as described in our previous studies (Pan et al., 2011, Schlosburg, 2010). Briefly, slices were prepared at 4-6°C in a solution containing (in mM): 220 sucrose, 2.5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂, 7 MgSO₄, 26 NaHCO₃, 10 glucose, and 1 sodiumascorbate. The slices were transferred in artificial cerebrospinal fluid (ACSF) containing (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 glucose and were allowed to recover at least 1 hour at room temperature. All solutions were saturated with 95% O₂ and 5% CO₂.

Whole-cell voltage-clamp recordings were made using patch clamp amplifier (Multiclamp 700B) under infrared-differential contrast interference microscopy. Data acquisition and analysis were performed using a digitizer (DigiData 1440A) and analysis software pClamp 10 (Molecular Devices). Pyramidal neurons in layer V of the cingulate cortex were identified visually based upon pyramidal shaped soma with a prominent apical dendrite. Additionally, the pyramidal neurons exhibit spike frequency adaptation in response to depolarizing current injections (Satake et al., 2008). To evoke IPSCs in layer V pyramidal neurons, the stimulation electrode was placed in layer V of cingulate cortex. The pipettes were filled with an internal solution containing (in mM): 80 K-gluconate, 60 KCl, 10 HEPES, 0.2 EGTA, 2 MgCl₂, 4 MgATP, 0.3 Na₂GTP, and 10 Na₂-phosphocreatine (pH 7.2 with KOH). Glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 20 μM) and D-2-amino-5-phosphonovaleric acid (D-AP-5, 20 μM) were present in the ACSF throughout the experiments. Series resistance (15-30 MΩ) was monitored throughout the recordings, and data were discarded if the resistance changed by more than 20%. All recordings were performed at 32 ± 1°C by using an automatic temperature controller.

Data analyses

Paw edema data are expressed as the difference in paw thickness between the 5 h and pre-injection measures. Paw withdrawal thresholds to the von Frey filaments in the carrageenan-injected and contralateral (i.e., control) paws at the 5 h time point were used to assess mechanical allodynia and prior to, and 10 days after, CCI surgery. All data are depicted as mean +/- standard error of the mean (SEM). Data were analyzed using t-tests, one-way analysis of variance (ANOVA), or two-way ANOVA. Tukey-Kramer post hoc analysis was used for all tests comparing different treatment groups. Bonferroni planned comparisons were used to assess the

data in the mechanism of action study. [3H] SR141716A and [35S] GTP γ S binding data were fit by nonlinear regression analysis to obtain Bmax and KD or Emax values, respectively. The pharmacological effects of THC were analyzed using a mixed design ANOVA vehicle + vehicle and JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) pretreatment was the between subjects variable, and THC dose was the within subjects variable followed by Bonferroni post hoc test to compare each pretreatment with vehicle at individual THC doses. Differences of $p < 0.05$ were considered statistically significant. IPSC amplitude was normalized to the baseline. The depression (percentage) of IPSCs by WIN55, 212-2 was calculated as follows: $100 \times [\text{mean amplitude of IPSCs during the last 5 min treatment}/\text{mean amplitude of baseline IPSCs}]$. Data sets were compared with Student's t-test. All the results were considered significant at $p < 0.05$.

Results:

Co-administration of JZL184 and PF-3845 elevates endocannabinoids without decreasing arachidonic acid in the brain.

To assess the impact of partial MAGL inhibition combined with full FAAH inhibition on brain endocannabinoid levels, male C57BL/6 mice were assigned to one of the following five groups: (1) vehicle + vehicle, (2) vehicle + 4 mg/kg JZL184, (3) vehicle + 10 mg/kg PF-3845, (4) 4 mg/kg JZL184 + 10 mg/kg PF-3845, or (5) 40 mg/kg JZL184 + vehicle (i.e., full MAGL blockade). 40 mg/kg JZL184 was included to compare the impact of low dose high dose JZL184 on the endocannabinoid level. It has been shown in the literature that 40 mg/kg elevates brain 2-AG level by 10 folds (Long, 2009a). Hence, including this condition in our experiment serves the purpose of validating that the assay is working. The mice were sacrificed immediately after assessment of edema and allodynia at 5h that is 7 h after the drug treatment. Brain levels of anandamide, 2-AG, and arachidonic acid (AA) 7 h following the injections are shown in Figure

9A-C. PF-3845 given alone or in combination with JZL184 led to a 10-fold increase in anandamide levels [F (4, 35) = 389.0, $p < 0.001$; **Figure 9A**]. JZL184 (4 mg/kg) given alone or in combination with PF-3845 produced a 3-fold elevation in 2-AG levels [F (4, 35) = 99.10, $p < 0.001$; **Figure 9B**]. The elevation in brain 2-AG was not accompanied by significant reductions in the levels of arachidonic acid (AA) [$p > 0.41$] **Figure 9C**]. On the other hand, full blockade of MAGL using 40 mg/kg JZL184 elevated 2-AG levels eight fold [**Figure 9B**] and reduced brain AA levels by two fold [F (4,35)= 18.92, $p < 0.001$; **Figure 9C**]. Dr. Rehab Abdulla performed this experiment.

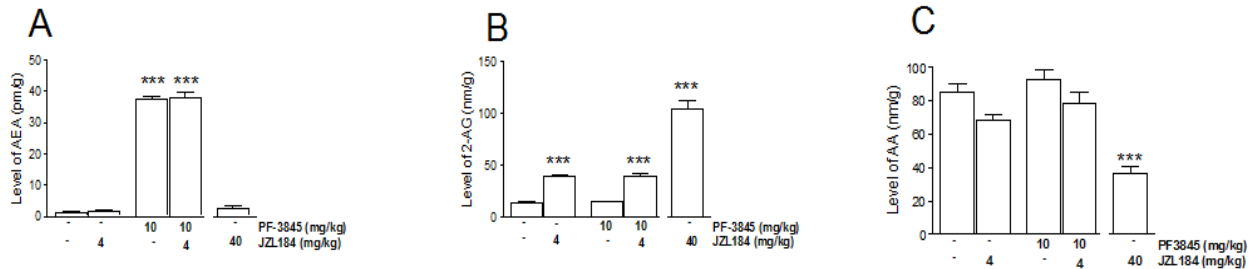


Figure 9. Anandamide, 2-AG levels were elevated by single as well as dual enzyme inhibition. However, arachidonic acid (AA) levels remained unaffected by single enzyme inhibition versus dual enzyme inhibition. Only high dose of JZL184 has decreased the level of arachidonic acid. Acute administration of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) significantly increased brain levels of **(A)** anandamide and **(B)** 2-AG, but did not affect **(C)** arachidonic acid (AA) levels. ***, $p < 0.001$ versus vehicle (Tukey-Kramer post hoc tests). Data presented as mean \pm SEM (N = 7-8).

Partial MAGL and complete FAAH inhibition produce augmented anti-allodynic effects in the carrageenan model of inflammatory pain

We have previously reported that JZL184 (4, 16, or 40 mg/kg) or PF-3845 (3 or 10 mg/kg) (Ghosh, 2013) only partially reversed the anti-allodynic effects in carrageenan and CCI assays (Ghosh, 2013, Kinsey, 2010). Hence, a main goal of this experiment was to test whether the combined administration of low dose JZL184 and high dose PF-3845 would produce augmented anti-allodynic effects compared with each drug given alone. As shown in Figure 10A combined administration of JZL184 (4 mg/kg) and PF-3845 (10 mg/kg) produced augmented the anti-allodynic effects compared with administration of either inhibitor given alone [F (4, 35) = 17.2, $p < 0.001$; **Figure 10A**]. JZL184 (4 mg/kg), PF-3845 (10 mg/kg), or JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) significantly reduced paw threshold values as compared to vehicle. Additionally, the paw threshold responses following combined administration of JZL184 + PF-3845 produced threshold values that did not differ from the contralateral control paw, indicating full reversal of carrageenan-induced allodynia. The combination did not affect paw withdrawal thresholds in the control paws. However, JZL184 (4 mg/kg), PF-3845 (10 mg/kg), or combined administration of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) given 2 h before carrageenan partially reduced paw edema [F (3, 28) = 51.2, $p < 0.001$; **Figure 10B**]. To test whether JZL184 + PF-3845 retains efficacy when given after the induction of edema, subjects received vehicle or JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) 3 h after carrageenan. Edema was measured prior to and 5 h after carrageenan injection and allodynia was assessed at 5 h. As shown in **Figure 10 C**, the combination produced a significant reversal of carrageenan-induced edema at 5 h [F (3, 20) = 15.30, $p < 0.001$]. The combination also significantly reversed mechanical allodynia at this time point [F (4, 25) = 9.21, $p < 0.001$; **Figure 10D**]. JZL184 + PF-3845 did not affect paw

withdrawal thresholds in control paws. Thus, the combination partially reverses carrageenan-induced edema and completely reverses carrageenan-induced allodynia.

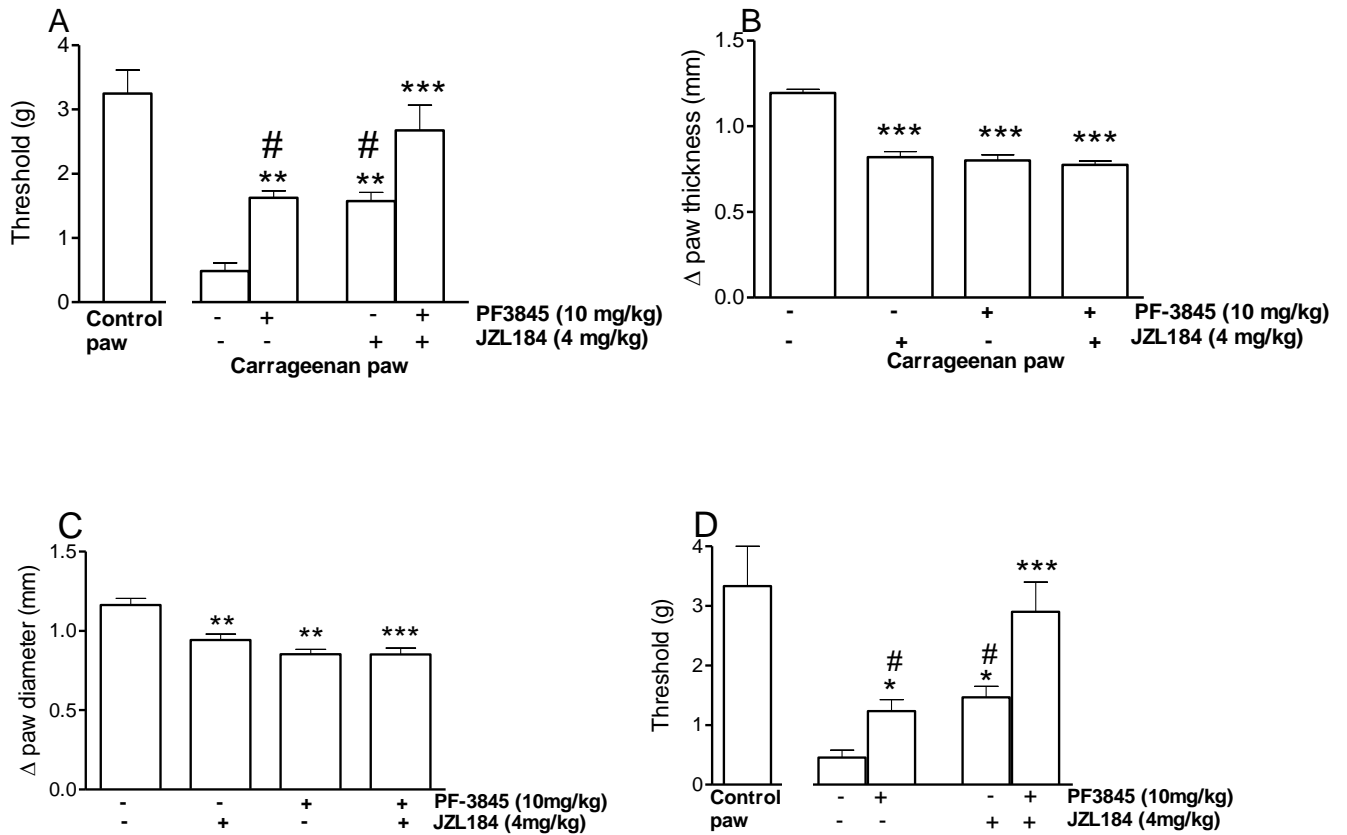


Figure 10. Combined administration of JZL184 and PF-3845 produced enhanced anti-allodynic, but not anti-edematous effects, compared with single enzyme inhibition in the carrageenan model. **(A)** Co-administration of low dose JZL184 (4mg/kg) and high dose PF-3845 (10 mg/kg) augmented the anti-allodynic effects of JZL184 (4mg/kg) and high dose of PF-3845 (10 mg/kg) alone. **(B)** JZL184 (4 mg/kg), PF-3845 (10 mg/kg), and a co-administration of JZL184 (4mg/kg) + PF-3845 (10 mg/kg) produce anti-edematous effects of similar magnitude. JZL184 and/or PF-3845 were administered 2 h before carrageenan. Paw thickness was measured prior to and 5 h after carrageenan injection and mechanical allodynia was assessed at 5 h post carrageenan injection. **, $p < 0.01$, ***, $p < 0.001$ versus vehicle; # $p < 0.05$ versus control paw (Tukey-

Kramer post hoc tests). Data presented as mean \pm SEM (n = 8). Administration of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) reversed (C) edema and (D) allodynia when administered 3 h post carrageenan. Paw thickness was measured prior to and 5 h after carrageenan administration and mechanical allodynia was measured 5 h after carrageenan. *, p < 0.05, **, p < 0.01, ***, p < 0.001 versus vehicle; #, p < 0.05 versus control paw (Tukey-Kramer post hoc tests). Data presented as mean \pm SEM (N = 6).

Partial MAGL and complete FAAH inhibition produce augmented anti-allodynic effects in the chronic constriction injury model of neuropathic pain

Similarly, we have tested the effects of this combination in chronic constriction injury model of neuropathic pain. Each of the inhibitors injected 2 h before testing produced significant anti-allodynic effects in the CCI model of neuropathic pain [F(4,29) = 27.52; p < 0.001; **Figure 11**]. While combined administration of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) produced augmented anti-allodynic compared with JZL184 (4 mg/kg) or PF-3845 (10 mg/kg) alone. Allodynia was partially reversed in this assay. In addition, JZL184 + PF-3845 did not affect paw withdrawal thresholds in control paws. Dr. Steven Kinsey performed this experiment.

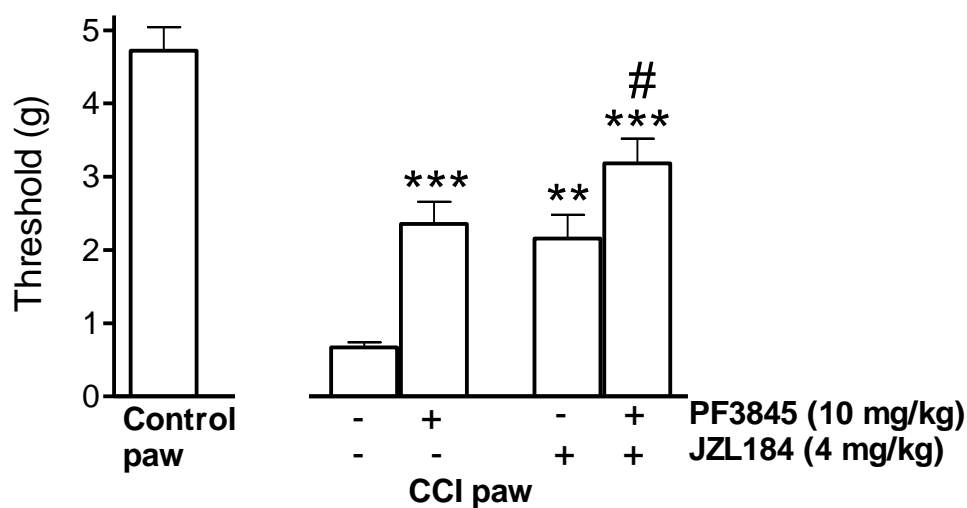


Figure 11. Combined administration of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) produced enhanced anti-allodynic effects in the CCI model of neuropathic pain. Low dose JZL184 (4mg/kg) and high dose PF-3845 (10 mg/kg) produced augmented anti-allodynic effects when administered together as compared to either JZL184 or PF-3845 alone. Mechanical allodynia was assessed 2 h post drug administration. **, $p < 0.01$, ***, $p < 0.001$ versus vehicle; #, $p < 0.05$ versus JZL184 (Tukey-Kramer post hoc tests). Data presented as mean \pm SEM (N = 20).

Combination JZL184 and PF-3845 mediates its anti-allodynic effects through cannabinoid receptors

To determine cannabinoid receptors mediate the anti-allodynic effects of JZL184 + PF-3845, mice were pretreated with rimonabant (1 mg/kg), SR144528 (3 mg/kg), or vehicle 150 min prior to carrageenan/ JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) was administered 120 min before carrageenan. The data depicted in **Figure 12 A**, reveal that CB₂ receptors mediate the anti-edematous effects of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg). Whereas rimonabant was without effect [$p = 0.73$], SR144528 completely blocked the anti-edematous effects of the combination as

revealed by a significant interaction between JZL184+ PF-3845 and SR145528 [$F(1, 21) = 23.0$, $p < 0.001$]. In contrast, either rimonabant [$F(1, 21) = 21.9$, $p < 0.001$] or SR144528 [$F(1, 21) = 20.5$, $p < 0.001$] completely prevented the anti-allodynic effects of combined administration JZL184 + PF-3845, indicating that both CB_1 and CB_2 receptors were required (**Figure 12B**). Neither rimonabant nor SR144528 significantly affected carrageenan-induced edema or allodynia in the absence of an endocannabinoid catabolic enzyme inhibitor.

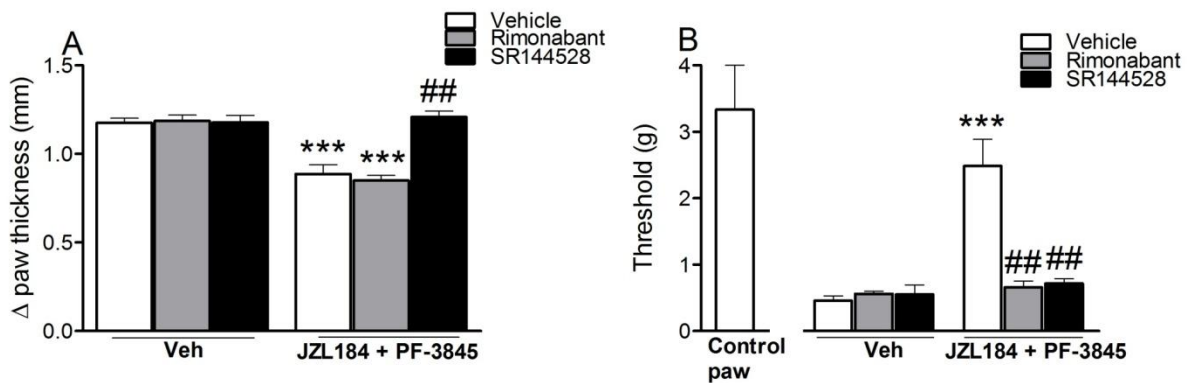


Figure 12. The anti-edematous effect of JZL184 and PF-3845 was mediated by CB_2 but not CB_1 receptors and the anti-allodynic effect of the combination was mediated by CB_1 and CB_2 mechanism of action. **(A)** Rimonabant (1 mg/kg) did not block the anti-edematous effects of JZL184 (4 mg/kg) and PF-3845 (10 mg/kg). ***, $p < 0.001$ versus vehicle/vehicle. SR144528 (3 mg/kg) blocked the anti-edematous effects of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg). Paw diameter thickness was measured prior to and 5 h after carrageenan administration. ##, $p < 0.05$ versus vehicle/JZL184 + PF-3845. **(B)** Rimonabant (1 mg/kg) as well as SR144528 blocked the anti-allodynic effects of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg). ***, $p < 0.001$ versus vehicle/vehicle, ###, $p < 0.001$ versus vehicle/JZL184 + PF-3845. Mechanical allodynia was measured 5 h after carrageenan. JZL184 + PF-3845 or vehicle was injected 2 h before

carrageenan. Rimonabant or SR144528 was injected 30 min prior to JZL184 + PF-3845 or vehicle. Bonferroni post hoc tests were used. Data presented as mean \pm SEM (N = 6).

Repeated administration of the combination of JZL184 and PF-3845 elevate endocannabinoids and decrease arachidonic acid in the brain.

Brain endocannabinoids and arachidonic acid levels were assessed in the brain after 6 days of repeated administration of the following treatments: (1) vehicle + vehicle, (2) vehicle + 4 mg/kg JZL184, (3) vehicle + 10 mg/kg PF-3845, (4) 4 mg/kg JZL184 + 10 mg/kg PF-3845, or (5) 40 mg/kg JZL184 + vehicle. On day 6, animals were sacrificed 2 h following the injection.

Figure 13A-13C shows the levels of anandamide, 2-AG, and AA in the whole brain. As in the case of acute administration, PF-3845 (10 mg/kg) elevated anandamide levels 10-fold whether given alone or in combination with JZL184 (4 mg/kg) [F (4, 31) = 233.3, $p < 0.001$]. Brain 2-AG levels were elevated 6 fold irrespective of whether given alone or in combination with PF-3845 [F (4, 31) = 123.7, $p < 0.001$]. In comparison, 40 mg/kg JZL184 elevated 2-AG levels 8-fold and reduced brain levels of arachidonic acid by 4-fold [F (4, 31) = 31.95, $p < 0.001$]. Repeated administration of high dose of JZL184 (40 mg/kg) has shown a small but significant elevation in anandamide level $p < 0.05$. Dr. Rehab Abdulla conducted this experiment.

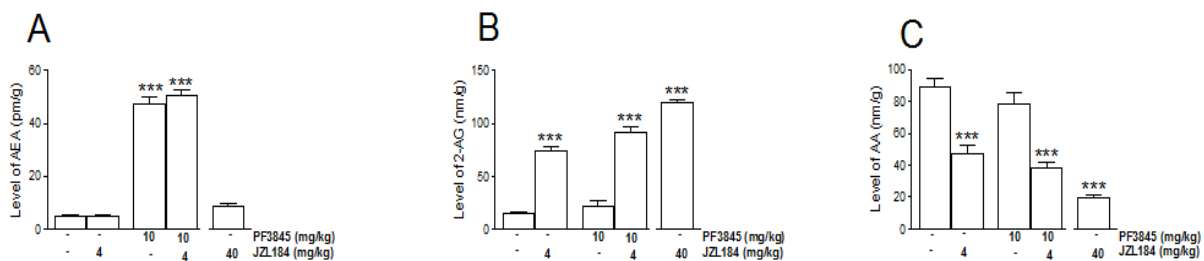


Figure 13. Repeated administration of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) increased (A) anandamide (AEA) and (B) 2-AG levels in the brain as well as decreased (C) arachidonic acid

(AA) levels in the brain.***, $p < 0.001$ versus vehicle (Tukey-Kramer post hoc tests). Data presented as mean \pm SEM (N = 7-8).

Consequences of combined partial MAGL inhibition and full FAAH inhibition given repeatedly

In this experiment, we assessed the consequences of daily injections of JZL184 (4mg/kg) + PF-3845 (10mg/kg) given for six days. Our goal is to determine whether the anti-edematous and anti-allodynic effects of JZL184 + PF-3845 treatment in the carrageenan model would be maintained following repeated injections. As shown in Figure 14 acute and repeated administration of the combination, JZL184 (4mg/kg) + PF-3845 (10mg/kg), produced similar decreases in edema [F (2, 22) = 8.47, $p < 0.01$; Figure 14A] and anti-allodynic effects [F (5, 44) = 5.85, $p < 0.001$; Figure 14B] in the carrageenan model.

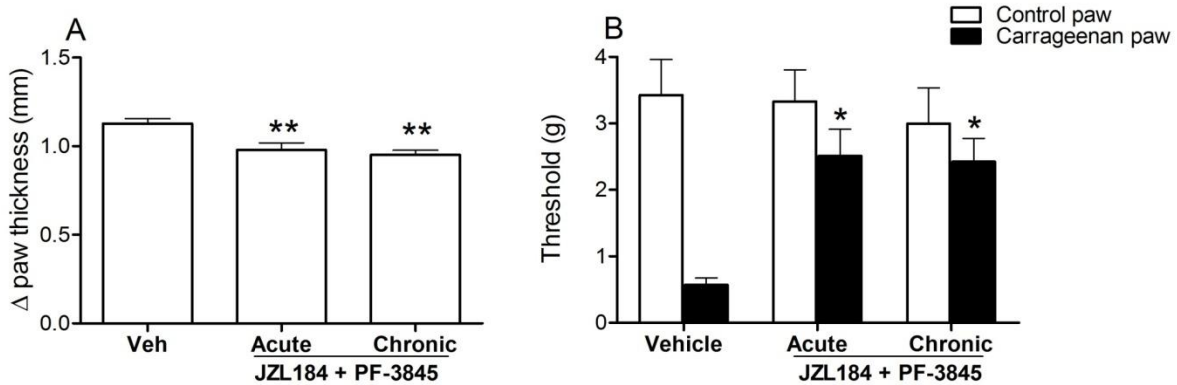


Figure 14. The anti-edematous (A) and anti-allodynic effects (B) of combined administration of JZL184 and PF-3845 did not undergo tolerance following repeated administration. Mice were administered vehicle (1.1.18) injections once daily for 5 days and challenged with vehicle or combined low dose JZL 184 (4 mg/kg) and high dose PF-3845 (10 mg/kg) on day 6 or repeatedly treated with low dose JZL 184 (4 mg/kg) and high dose PF-3845 (10 mg/kg) once

daily for 6 days. Drug treatments on day 6 were given 2 h prior carrageenan injections. Paw thickness was measured prior to and 5 h after carrageenan administration and mechanical allodynia was tested 5 h after carrageenan. *, $p < 0.05$, versus vehicle; ** $p < 0.01$ versus vehicle paw (Tukey-Kramer post hoc tests). Data presented as mean \pm SEM (N = 8-9).

JZL-184 (4mg/kg) with PF-3845 10 (mg/kg) do not elicit cannabimimetic effects, as assessed in the tetrad assay

As indicated in Table 4 low dose JZL184 + high dose of PF-3845 did not produce of THC-like effects in the tetrad assay. Mice were injected with low dose of JZL184 (4 mg/kg) with high dose PF-3845 (10 mg/kg), and then (A) catalepsy, (B) analgesia (C) body temperature and (D) locomotor activity were assessed after 2 h of drug treatment. The data show that JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) does not produce catalepsy, antinociception, hypothermia, and hypolocomotion.

Treatment		Catalepsy (s)	Tail withdrawal latency (s)	Body Temperature ($^{\circ}$ C)	Catalepsy (s)	Tail withdrawal latency (s)	Body Temperature ($^{\circ}$ C)	Distance travelled (m)
		Baseline			After 2 h			
Vehicle/Vehicle	Mean	0	2.03	38.18	0	2.07	38.19	285.25
	\pm SEM	0	\pm 0.103	\pm 0.182	0	\pm 0.235	\pm 0.132	\pm 29.228
JZL184 (4 mg/kg) + PF-3845 (10 mg/kg)	Mean	0	1.81	38.31	0	4.51	37.80	259.75
	\pm SEM	0	\pm 0.091	\pm 0.194	0	\pm 1.171	\pm 0.133	\pm 22.754

Table 4: Administration of low dose JZL184 + high dose of PF-3845 did not produce THC-like effects in the tetrad assay. Mice were injected with low dose of JZL184 (4 mg/kg) in combination with high dose PF-3845 (10 mg/kg), and then tested for (A) catalepsy, (B) analgesia

(C), body temperature, and (D) locomotor activity were assessed after 2 h of drug treatment. Tukey Kramer post hoc test was used. N = 8-12/group.

Partial MAGL and complete FAAH blockade does not cause cross-tolerance to the pharmacological effects of THC

We next tested whether mice given combination of JZL184 and PF-3845 produces cross-tolerance to the cataleptic, antinociceptive, and hypothermic effects of THC. Accordingly, mice were treated for five consecutive days with vehicle + vehicle or 4 mg/kg JZL184 + 10 mg/kg PF-3845. These mice did not display cross-tolerance to the pharmacological effects of THC (3, 10, 30 or 100 mg/kg) on day 6. Specifically, the potency of THC in eliciting catalepsy, antinociception (i.e., tail immersion test), and hypothermia was retained following repeated drug administration. THC significantly increased catalepsy [F (3, 42) = 52.61, $p < 0.0001$; Figure 15A], tail withdrawal latency [F (3, 42) = 65.26, $p < 0.0001$; Figure 15B], and hypothermia [F (3, 42) = 60.15, $p < 0.0001$; Figure 15C] in both treatment groups. Additionally, there were no effects of repeated treatment with vehicle + vehicle or 4 mg/kg JZL184 + 10 mg/kg PF-3845 on catalepsy ($p = 0.40$), tail withdrawal latency ($p = 0.34$), or body temperature ($p = 0.27$) indicating that the combination did not produce cross-tolerance to THC.

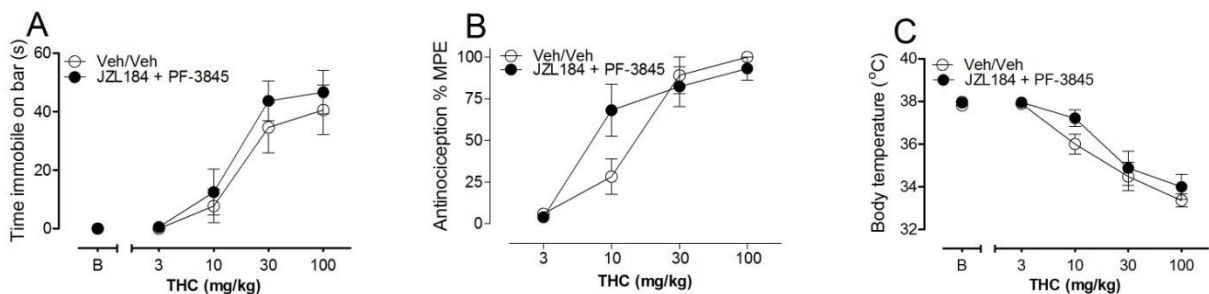


Figure 15. Mice treated repeatedly with vehicle + vehicle or JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) displayed similar (A) catalepsy, (B) antinociception, and (C) body temperature when

tested for cross tolerance to increasing doses of THC. Mice were treated with vehicle + vehicle or JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) once daily for 5 days. On the sixth day cross-tolerance to THC (3, 10, 30, or 100 mg/kg) was tested using a cumulative dosing regimen. Bonferroni post hoc tests were used. Data presented as mean \pm SEM (N = 8).

CB₁ receptor activity and expression are maintained following repeated administration of JZL184 + PF-3845 combination

As previously reported, repeated treatment, injection of 40 mg/kg JZL184 reduced whole brain CB₁ receptor levels by 33.3% and produced a 56.3% reduction in maximal CP55, 940-stimulated G-protein activity. However, simultaneous repeated administration of high dose PF-3845 (10 mg/kg) and low dose JZL184 (4 mg/kg) as well as each treatment alone did not significantly alter CB₁ receptor levels (**Figure 16A**) or CP55, 940-stimulated G-protein activity (**Figure 16B**) as compared to the vehicle + vehicle control group. Dr. Qing Tao did this experiment.

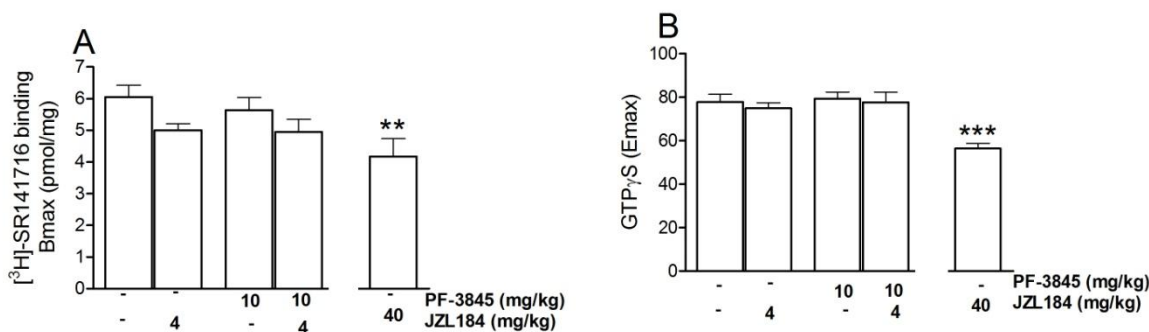


Figure 16. CB₁ receptor expression and activity were reduced following repeated injections of high dose JZL184 (40 mg/kg), but remained unaffected following repeated co-administration of low dose JZL184 + high dose PF-3845. Mice were treated for 6 days with vehicle + vehicle, vehicle + JZL184 (4 mg/kg), vehicle + PF-3845 (10 mg/kg), JZL184 + PF-3845 or vehicle +

JZL184 (40 mg/kg). On day 6, animals were euthanized and then the brain homogenates were used to test **(A)** membrane-specific CB₁ receptor binding by the antagonist [³H]SR141716A (B_{max} values). The B_{max} (maximum receptor binding) and K_d (ligand concentration at which half maximal receptor binding is observed at equilibrium) for each conditions are as follows; for vehicle + vehicle, B_{max} = 6.06, K_d = 1.48; vehicle + JZL184 (4 mg/kg), B_{max} = 5.0, K_d = 1.58; vehicle + PF-3845 (10 mg/kg), B_{max} = 5.64, K_d = 1.58; JZL184 + PF-3845, B_{max} = 4.94, K_d = 1.52 or vehicle + JZL184 (40 mg/kg) B_{max} = 4.18, K_d = 1.58. and **(B)** CP55,940-stimulated [³⁵S]-GTPγS binding. ** p < 0.01, *** p < 0.001 vs. vehicle (E_{max}). Tukey-Kramer post hoc test was used. Data presented as mean ± SEM (N = 8).

CB₁ receptor function in the cingulate cortex is maintained following chronic partial MAGL and complete FAAH blockade

Finally, we examined whether repeated administration of the combination for 6 days induce CB₁ receptor desensitization in the cingulate cortex, a brain region critically involved in the regulation of pain (Zhuo, 2006). For this experiment, the following groups were tested (1) vehicle + vehicle, (2) vehicle + 4 mg/kg JZL184, (3) 4 mg/kg JZL184 + 10 mg/kg PF-3845, or (4) 40 mg/kg JZL184 + 10 mg/kg PF-3845. Bath application of the CB₁ receptor agonist WIN55,212-2 (2 μM) induced similar depression of IPSCs between JZL184- and vehicle-treated mice (vehicle, 61.9 ± 5.5% of baseline, JZL184, 67.4 ± 5.2% of baseline p > 0.05; **Figure 17A**), suggesting that chronic JZL184 at this low dose did not induce significant CB₁ receptor desensitization. No apparent CB₁ receptor desensitization was detected in mice chronically treated with FAAH inhibitor PF3845 (10 mg/kg for 6 d) (Schlosburg, 2010). Consistent with this finding, we found that the magnitude of WIN55, 212-2-induced depression of IPSCs in the cingulate cortex was not significantly different between mice treated with JZL184 (4 mg/kg) plus

vehicle and JZL184 (4 mg/kg) plus PF3845 (10 mg/kg) ($66.4 \pm 5.4\%$ of baseline, $p > 0.05$; **Figure 17 A, 17 B**). However, WIN55, 212-2-induced depression of IPSCs was significantly attenuated in mice treated with JZL184 (40 mg/kg) plus PF3845 (10 mg/kg) ($86.6 \pm 5.3\%$ of baseline) compared with that in mice treated with JZL184 (4 mg/kg) plus PF3845 (10 mg/kg) ($p < 0.05$; **Figure 17 A, 17 B**). Dr. Qing-Song Liu performed this study.

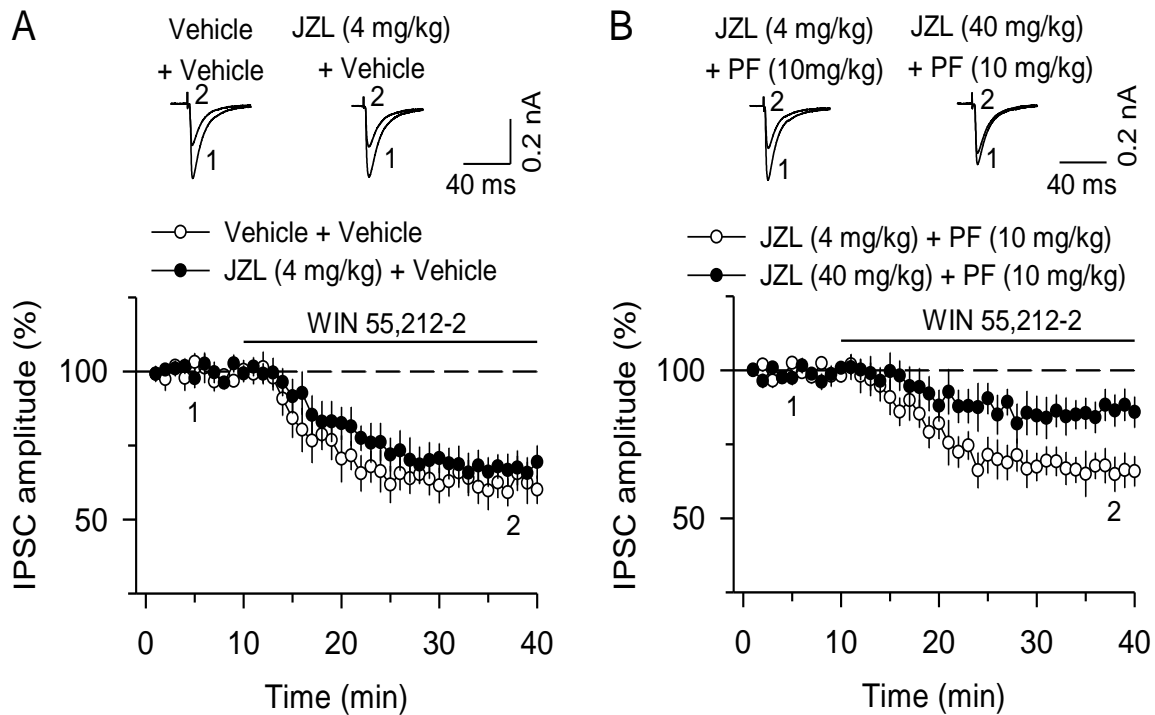


Figure 17. Effects of repeated *in vivo* administration of JZL184 alone or in combination with PF-3845 on WIN55, 212-2-induced depression of IPSCs in the cingulate cortex. WIN55, 212-2-induced depression of IPSCs in cingulate cortex was not significantly reduced by repeated administration of either JZL184 (4 mg/kg) or combination of JZL184 (4 mg/kg) + PF-3845 (10 mg/kg). However, repeated injections of JZL184 (40 mg/kg) + PF-3845 (10 mg/kg) for 6 days led to significant suppression. (C) The magnitude of WIN55,212-2-induced depression of IPSCs was not significantly different between mice treated with vehicle (N=7) and mice treated with JZL184 (4 mg/kg) (N=8), $p < 0.05$ (D) WIN55,212-2 (2 μ M) induced significantly less

depression of IPSCs in mice treated with JZL184 (40 mg/kg) + PF-3845 (10 mg/kg) (N= 7) than that in mice treated with JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) (N=7), $p < 0.05$. Each group of data was obtained from three mice. Dr.Qing-Song Liu has performed this study.

Discussion:

The primary objective of the experiments presented in Part A of Chapter 3 was to test proof of principle that combined blockade of FAAH and MAGL represents a viable strategy to reduce neuropathic and inflammatory pain. Two significant challenges were addressed to test this hypothesis. First, while combined blockade of MAGL and FAAH produces enhanced antinociceptive effects, full blockade of these enzymes in combination also produces THC-like subjective effects in the drug discrimination assay, catalepsy, hypomotility, and impaired performance in a working memory Morris water maze memory task (Long, 2009b, Wise, 2012). Second, repeated administration of MAGL inhibitors or genetic inactivation of MAGL causes profound alterations in the brain endocannabinoid system in mice, as evidenced by a loss of analgesic responses to a MAGL inhibitor, cross-tolerance to exogenous cannabinoid agonists, and CB₁ receptor downregulation and desensitization in specific brain regions such as the cingulate cortex, hippocampus, somatosensory cortex and PAG (Schlosburg, 2010). In contrast, FAAH (-/-) mice or wild type mice given repeated injections of FAAH inhibitors, which maintain an analgesic phenotype and intact CB₁ receptor system (Chanda, 2010, Cravatt, 2001, Lichtman, 2004, Schlosburg, 2010). In order to reduce these cannabimimetic side effects and to circumvent tolerance, we investigated whether a combination of partial MAGL inhibition and complete FAAH inhibition would achieve enhanced efficacy in reversing the allodynia in the carrageenan and chronic CCI rodent models of pain. The major findings of the present study are that partially blocking MAGL in combination with full FAAH blockade produced enhanced anti-

allodynic effects in both assays, but in the absence of cannabimimetic side effects. Specifically, combined administration of low dose of the MAGL inhibitor JZL184 (i.e., 4 mg/kg) and high dose of the FAAH inhibitor PF-3845 (i.e., 10 mg/kg) substantially enhanced the anti-allodynic effects in mice as compared to single inhibition of these enzymes. Interestingly, combined blockade of these enzymes did not produce increased anti-edematous effects in the carrageenan assay. The second major finding was that unlike prolonged MAGL blockade with high dose JZL184 (Schlosburg et al., 2010), repeated administration of JZL184 (4 mg/kg) and PF-3845 (10 mg/kg) did not produce CB₁ receptor functional tolerance and maintained the anti-allodynic and anti-edematous effects. Finally, in contrast to complete blockade of MAGL and FAAH (Schlosburg et al., 2010), combination of JZL184 (4 mg/kg) and PF-3845 (10 mg/kg) did not elicit cannabimimetic side effects.

Experiments were conducted to determine whether the enhanced anti-allodynic effects produced by dual blockade of FAAH and MAGL were mediated by cannabinoid receptors. Accordingly, we used a pharmacological approach to assess whether CB₁ and CB₂ receptors mediate the enhanced anti-allodynic effects of combined administration of JZL184 and PF-3845. The anti-allodynic effects of the combination were completely blocked by both rimonabant and SR144528, indicating that CB₁ and CB₂ receptors play necessary roles in this response. This is in agreement with previous reports showing that JZL184 mediates its anti-allodynic effects through CB₁ and CB₂ receptors in the carrageenan and formalin pain assays (Guindon et al., 2011; Ghosh et al., 2013). In contrast, the anti-edematous effects of the combination were blocked only by SR144528, indicating that anti-edematous effects require CB₂ receptors. Previously it has been shown that anti-edematous effects of JZL184 and PF-3845 were mediated by CB₂ receptors (Ghosh, 2013, Lichtman, 2004, Wise, 2012).

The most striking finding of this study is that simultaneous partial blockade of MAGL with low dose JZL184 and complete blockade of FAAH with high dose PF-3845 produced enhanced anti-allodynic effects as compared to individual blockade of each enzyme in both the carrageenan and CCI models of pain. Several explanations may account for enhanced anti-allodynic effects upon dual FAAH and MAGL inhibition. First, the enhancement may have resulted from simply an increase in the total quantity of endocannabinoid levels in the nervous system to stimulate cannabinoid receptors. However, the observation that 2-AG levels in brain are at least 200-fold higher than anandamide brain levels (Ahn, 2009, Long, 2009a) tends to argue against this idea of mass action. Alternatively, it is plausible that simultaneous inhibition of MAGL and FAAH may produce augmented anti-allodynic effects because distinct CB₁ and CB₂ receptor-mediated circuits are activated by the respective endocannabinoids. Consistent with this idea is that FAAH is predominantly expressed on post-synaptic terminals (Gulyas et al., 2004) and MAGL is expressed on presynaptic terminals (Gulyas, 2004). A third explanation is that along with the increased levels of anandamide and 2-AG, other consequences of FAAH and MAGL inhibition may have contributed to the augmented anti-allodynic effects. These enzymes metabolize other lipid signaling molecules that act through other noncannabinoid receptor targets (Ahn, 2008). In particular, MAGL plays a major role in the biosynthesis of free arachidonic acid (AA) its precursor, 2-AG in brain from (Nomura, 2011). For example, MAGL plays an important role in the biosynthesis of arachidonic acid in brain. Thus, MAGL inhibition results in reduced arachidonic acid, which leads to reduced levels of prostaglandin (Nomura, 2011). However, as 4 mg/kg JZL184 did not alter arachidonic acid levels in whole brain, this explanation tends to argue against the involvement of reduced prostaglandin levels. However, there may be

reductions of arachidonic acid in distinct brain regions or spinal cord associated with pain, which would not be detected from whole brain extracts.

In addition to anandamide, FAAH metabolizes other bioactive fatty acid amides, including N-palmitoylethanolamine, N-oleoylethanolamine, and oleamide (Cravatt, 2001, Cravatt, 1996), as well as N-acyl taurines (Fattore et al., 2005, Leung, 2006), which activate various TRP channels and PPAR- α receptors (De Petrocellis and Di Marzo, 2010, Di Marzo and De Petrocellis, 2010, Jhaveri, 2006). Thus, combined blockade of FAAH and MAGL produces multiple neurochemical alterations that could affect allodynia and edematous responses.

It is interesting that dual inhibition of FAAH and MAGL did not produce increased anti-edematous effects compared with inhibition of either enzyme alone. It is plausible that a “ceiling effect” had occurred following inhibition of FAAH or MAGL and no further reduction in paw edema could be achieved. Another explanation is that anti-edematous effects were mediated by CB₂ receptors only, while the anti-allodynic effects required both CB₁ and CB₂ receptors. Accordingly, increased anti-allodynic effects may be caused by dual inhibition when both CB₁ and CB₂ receptors are involved.

Importantly, the anti-edematous and anti-allodynic effects of low dose JZL184 and high dose PF-3845 in the carrageenan model were maintained following repeated administration. This lack of tolerance is consistent with studies reporting that the pharmacological effects of low doses of JZL184 (4 and 8 mg/kg) were maintained following repeated injections in the carrageenan model of inflammatory pain, CCI model of neuropathic pain (Ghosh, 2013, Kinsey, 2013), and anxiolytic effects in the elevated plus maze in rats (Sciolino, 2011). Additionally, the antinociceptive effects of the FAAH inhibitor PF-3845 in the tail-withdrawal test and CCI model

did not undergo tolerance following repeated administration (Schlosburg, 2010). Likewise, chronic treatment with direct cannabinoid receptor agonists, such as THC or WIN55-212, leads to the development of tolerance (Lichtman and Martin, 2005).

These behavioral phenotypes have been shown to be accompanied by substantial reductions in CB₁ receptor expression and function in the brain (Romero et al., 1997, Sim et al., 1996). Additionally, prolonged elevation of the endogenous cannabinoid 2-AG leads to tolerance to the analgesic effects of acute enzyme inhibition, significant cross-tolerance to cannabinoid receptor agonists, physical dependence, a reduction in CB₁ receptor number and activity, and disruptions in endocannabinoid-dependent synaptic plasticity (Chanda, 2010, Schlosburg, 2010). These findings are in contrast to the observations that with prolonged FAAH and partial MAGL inhibition with low dose JZL184, antinociceptive effects are maintained without producing tolerance, physical dependence, or changes in CB₁ receptor expression or function (Kinsey, 2013, Schlosburg, 2010, Sciolino, 2011). These data suggest that brain CB₁ receptors undergo markedly different adaptations in response to sustained elevations of the two principal endocannabinoids 2-AG and anandamide. The findings that CB₁ receptor number and function in the brain was unaltered by sustained MAGL and FAAH inactivation was confirmed in studies assessing different forms of synaptic plasticity, including DSI, DSE, and long-term depression (Kreitzer and Regehr, 2001, Wilson and Nicoll, 2001). Schlosburg et al 2010, reported attenuation of DSI by chronic high dose JZL184 treatment was consistent with desensitization of CB₁ receptors in the affected neuronal circuits. In contrast, no apparent CB₁ receptor desensitization was detected in mice chronically treated with the FAAH inhibitor PF-3845 (10 mg/kg). In the present study we demonstrated that the magnitude of WIN55, 212-2-induced depression of IPSCs in the cingulate cortex was not significantly different between mice treated

with JZL184 (4 mg/kg) and JZL184 (4 mg/kg) plus PF-3845 (10 mg/kg). However, WIN55, 212-2-induced depression of IPSCs was significantly attenuated in mice treated with JZL184 (40 mg/kg) plus PF-3845 (10 mg/kg) compared with mice treated with JZL184 (4 mg/kg) plus PF3845 (10 mg/kg). Taken together, these results indicate that chronic high dose JZL184 (40 mg/kg) treatment, but not low dose JZL184 (4mg/kg) treatment, induces CB₁ receptor desensitization in the cingulate cortex. These data indicated that sustained partial elevation of 2-AG and complete elevation of anandamide do not alter short-term synaptic plasticity. These results are corroborated by results of acute and repeated administration of JZL184 (4 mg/kg) and PF-3845 (10 mg/kg) on CB₁ receptor expression and activity in brain. The results revealed that CB₁ receptor binding and CP55, 940-stimulated [35S]-GTP γ S binding were decreased by high dose of JZL184 (40 mg/kg). However, low dose JZL184 and high PF-3845 did not alter the CB₁ receptor number or function upon repeated administration. This result is congruent with a recent study showing that repeated administration of low dose JZL184 also did not alter CB₁ receptor expression and function (Kinsey, 2013). Additionally it has been shown that prolonged pharmacological or genetic inactivation of MAGL produces cross-tolerance to exogenous cannabinoid agonists WIN5521-2 (Schlosburg, 2010). Hence we have assessed whether the combination of JZL184 (4 mg/kg) and PF-3845 (10 mg/kg) results in cross tolerance to cannabimimetic side effects, mice were assessed for catalepsy, tail withdrawal test, body temperature . We found that mice treated repeatedly with dose JZL184 (4 mg/kg) and PF-3845 (10 mg/kg) elicited no cross-tolerance to THC in catalepsy, antinociception, and hypothermia tests. These data are supported by the literature demonstrating that prolonged treatment with low dose JZL184 does not lead to cross tolerance to the anti-allodynic, analgesic, and hypothermic effects of THC (Kinsey, 2013). As the manifestation of tolerance after prolonged MAGL

inhibition with high dose of JZL184 may represent a drawback, the aforementioned results suggest that the partial blockade of MAGL and complete blockade of FAAH can be used as a strategy to increase efficacy and circumvent tolerance.

These findings demonstrate that co-administration of JZL184 and PF-3845 elevated brain 2-AG and anandamide levels upon acute (3-4 fold) and repeated (5-6 fold) treatment. However, with acute or chronic dosing regimen, the elevation of 2-AG levels was less than the highest dose of JZL184 (40 mg/kg). This finding is congruent with a previous study showing that high doses of JZL184 (16, 40 mg/kg) elevate 2-AG 8-10 fold in the brain (Long, 2009a). In addition, prolonged inactivation of MAGL with this dose of JZL184 produced CB₁ receptor desensitization and down regulation in mice (Schlosburg, 2010)

Finally, we have assessed if this combination produces the cannabimimetic effects of THC. The results reveals that the combination does not produce catalepsy, hypothermia, antinociception and hypolocomotion in the tetrad assay indicating that this combination does not produce untoward effects of delta-9 THC.

The results of the present studies indicate that JZL184 and PF-3845 given alone produce partial efficacy in CCI and carrageenan models of pain. However, simultaneous inhibition of both enzymes enhances the magnitude of the anti-allodynic effects. Moreover, the antinociceptive effects and CB₁ receptor functional tolerance that occurs following repeated exposure to high dose JZL184 can be avoided by administering low dose JZL184 (4 mg/kg) in combination with high dose PF-3845 (10 mg/kg). These findings also reveal that the dosing regimen investigated in the present study can raise brain 2-AG and anandamide levels, without altering CB₁ receptor expression and function, short-term synaptic plasticity, or causing cross-tolerance to THC. In summary, our data support the hypothesis that simultaneous differential

dual blockade of the endocannabinoid degradative enzymes, MAGL and FAAH, represents a viable strategy to treat inflammatory and neuropathic pain states.

Part B

Anti-allodynic effects of SA-57, dual FAAH, and MAGL inhibitor with differential potencies, in the carrageenan model of inflammatory pain

As shown in Chapter 3A, combined injections of low dose JZL184 (4 mg/kg) and high dose PF-3845 (10 mg/kg), produced significantly enhanced anti-allodynic effects in the carrageenan and CCI assays, without observed cannabimimetic side effects. Moreover, the antinociceptive effects of this drug combination were retained following repeated administration. In this section, we describe the antinociceptive effects of the dual FAAH-MAGL inhibitor SA-57, which is 100 fold more potent in inhibiting FAAH than MAGL (Niphakis, 2012, Ramesh et al., 2013). SA-57 elevated brain anandamide levels ~10-fold at all doses (0.125, 1.25 and 12.5 mg/kg i.p.), but did not increase 2-AG levels at 0.125 or 1.25 mg/kg. , However, this compound increased brain 2-AG levels more than 10-fold at 12.5 mg/kg. In this section, we report on the pharmacological effects of this novel compound in the carrageenan assay and in the tetrad assay to assess cannabimimetic effects. We have also evaluated if the effects of SA-57 are mediated through cannabinoid receptors. Finally, we quantified endocannabinoid levels in whole brain.

Method:

Subjects

The subjects consisted of adult male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME). Adult 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. 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

SA-57 was synthesized in the laboratory of Dr. Cravatt as previously described (Niphakis et al, 2012). SA-57 was dissolved in ethanol, followed by addition of Alkamuls-620 (Sanofi-Aventis, Bridgewater, NJ), and diluted with 0.9% saline to form a vehicle mixture of ethanol, Alkamuls-620, and saline in a ratio of 1: 1: 18. This compound was administered intraperitoneally 3 h after carrageenan. All injections were administered in volume of 10 µl/g body weight. For experiments quantifying brain endocannabinoid SA-57 were administered 2 h before decapitation. The doses were selected based on previous literature (Niphakis, 2012).

Carrageenan pain model

Edema and allodynia were induced by giving an intraplantar injection of 0.3% carrageenan (Sigma, St Louis) in a 20-µl volume using a 30-gauge needle into the hind left paw. Paw thickness was measured with electronic digital calipers (Traceable Calipers, Friendswood, TX), prior to and 1 h, 3 h and 5 h following carrageenan administration. Data showed only 5 h time point, which corresponds to peak edema (Wise, 2008). This procedure has been used

previously by our laboratory (Cravatt and Lichtman, 2004, Lichtman, 2004, Wise, 2008). To test allodynia, the mice were placed inside ventilated polycarbonate chambers on an elevated aluminum mesh table and allowed to acclimate to the apparatus for 60 min before testing. Mechanical allodynia was assessed with von Frey filaments (North Coast Medical, Morgan Hill, CA), using the “up-down” method (Chaplan, 1994) 5 h after carrageenan administration. The plantar surface of each hind paw was stimulated five times with each filament (0.16–6.0 g), at a frequency of approximately 2 Hz, starting with the 0.6-g filament and increasing until the mouse responded by licking and/or lifting the paw off the surface of the test apparatus. Three or more responses out of five stimulations were coded as a positive response. Once a positive response was detected, sequentially lower weight filaments were used to assess the sensory threshold for each paw.

Tetrad

To measure spontaneous activity, the mouse was placed in an Anymaze chamber for 300 seconds and the percentage of time the animal spent immobile was recorded. Catalepsy was assessed in the bar test in which the mouse is placed on a bar oriented parallel to and 1 inch off the ground. The time (s), the mouse remains immobile on the bar in a 60 s time period is recorded. Antinociception was assessed in the tail withdrawal test in which the mouse is immobilized in a bag and its tail is placed into a 52°C warm water bath. The time it takes for the mouse to remove its tail from the water bath is recorded. Hypothermia – body temperature, rectal probe (2 cm) is used to measure body temperature (°C).

Data analyses

Paw edema data are expressed as the difference in paw thickness between the 5 h and pre-injection measures. Paw withdrawal thresholds to the von Frey filaments in the carrageenan-

injected and contralateral (i.e., control) paws at the 5 h time point were used to assess mechanical allodynia. All data are depicted as mean \pm standard error of the mean (SEM). Data were analyzed using t-tests, one-way analysis of variance (ANOVA), or two-way ANOVA. Dunnett's test was used for post hoc analysis in the dose-response experiments in which the effects of each drug dose were compared to those of vehicle. Tukey-Kramer post hoc analysis was used for all tests comparing different treatment groups. All the results were considered significant at $p < 0.05$.

Results

Administration of different doses of SA-57 elevates endocannabinoids and decrease arachidonic acid in the brain

To assess endocannabinoids and arachidonic acid levels in the brain with acute SA-57 treatment, male C57BL/6 mice were administered (1) vehicle (2) 0.125 mg/kg (3) 1.25mg/kg (4) 2.5 mg/kg, (5) 5 mg/kg, and (6) 12.5 mg/kg, and sacrificed immediately after assessment of edema and allodynia at 5h. Brain levels of anandamide, 2-AG, and arachidonic acid (AA) were quantified 2 h following administration of injection is shown in Figure 18A-C. SA-57 (1.25, 2.5, 5, 12.5 mg/kg) led to an 8 -10-fold increase in anandamide levels [F (4, 13) = 50.26, $p < 0.001$; Figure 18A]. SA-57 significantly elevated 2-AG levels [F (4, 13) = 79.43, $p < 0.001$; Figure 18B], but with considerably decreased potency compared with anandamide elevations. 2.5 mg/kg SA-57 elevated 2-AG by 2-3 fold, 5 mg/kg and 12.5 mg/kg dose have elevated 5-5 to 7 folds of 2-AG on the brain. The elevation in brain 2-AG was accompanied by significant reductions in the levels of arachidonic acid (AA) on at the high doses (5, 12.5 mg/kg) [F (4, 13) = 14.91, $p < 0.001$; Figure 18C], but at the lower doses AA level remained unchanged [$p < 0.78$; (dose 1.25 mg/kg); $p < 0.43$; (dose 2.5 mg/kg)] [Figure 18C].

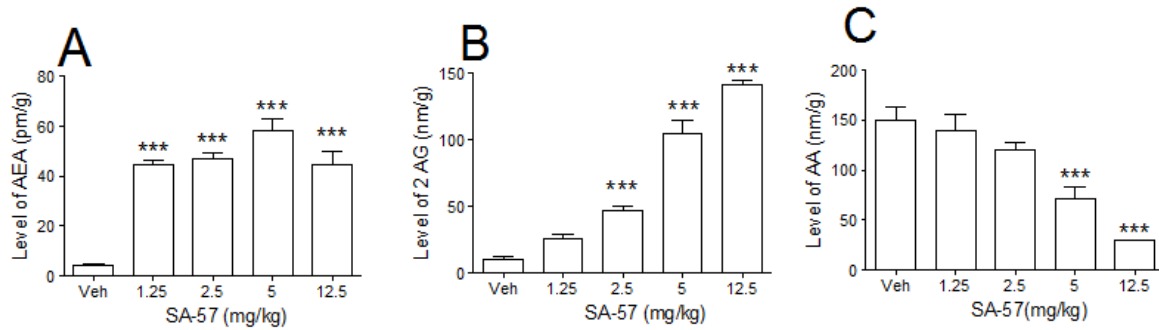


Figure 18. Acute SA-57 elevates anandamide and 2-AG, but decreases arachidonic acid (AA) levels in the whole brain. **(A)** Acute administration of SA-57 (1.25, 2.5, 5, 12.5 mg/kg) led to an 8 -10-fold increase in anandamide. **(B)** SA-57 (2.5, 5, 12.5 mg/kg) produced a 3-10-fold elevation in 2-AG levels. **(C)** AA levels were reduced at higher doses (5, 12.5 mg/kg) of SA-57. ***, $p < 0.001$ versus vehicle (Dunnett's post hoc tests). Data presented as mean \pm SEM (N = 5-6).

Anti-edematous and anti-allodynic effects of SA-57 in the carrageenan model

As shown in Figure 19A SA-57 given 3 h after carrageenan partially reversed paw edema in a dose-related fashion [$F(4,24) = 19.60, p < 0.05$; Figure 19A]. SA-57 also produced a dose-dependent complete reversal of carrageenan-induced allodynia [$F(5, 30) = 6.17, p < 0.05$; Figure 19B]. However, it did not affect paw withdrawal thresholds in the control paws.

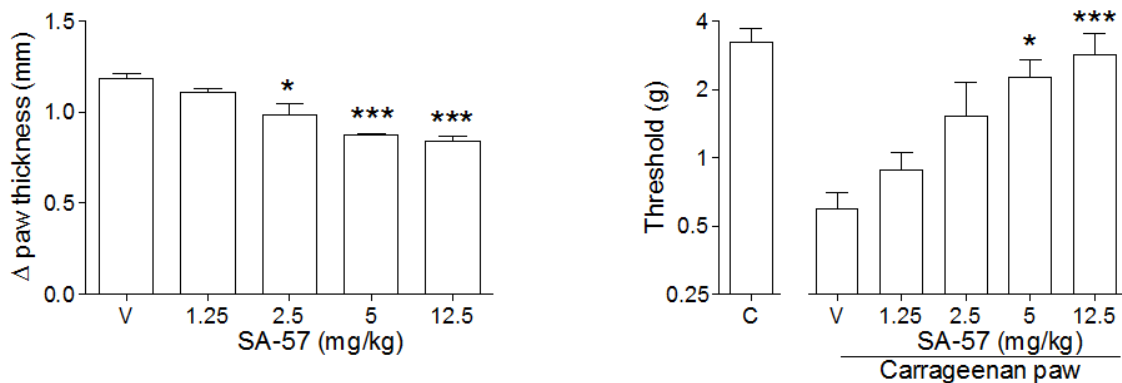


Figure 19. Acute administration of SA-57 reversed carrageenan-induced allodynia and produced anti-edematous effects. **(A)** of SA-57 (2.5, 5, and 12.5 mg/kg) reduced paw edema. **(B)** SA-57 (5 and 12.5 mg/kg) reversed allodynia. *, $p < 0.05$, ***, $p < 0.001$ versus vehicle; (Dunnett's post hoc tests). Data presented as mean \pm SEM (N = 5-6).

Cannabinoid receptors mediate the anti-allodynic and anti-edematous of SA-57

To determine whether CB₁ and CB₂ receptors mediate the anti-edematous and anti-allodynic effects of SA-57, CB₁ (-/-) and CB₂ (-/-) mice were given an intraplantar injection of carrageenan, treated with SA-57 (5 mg/kg) at 3 h, and tested for allodynia and edema at 5h. Edema was measured immediately after allodynia. CB₂ (-/-) mice [F (1, 13) =1.29, $p < 0.001$; Figure 20 A], but not CB₁ (-/-) mice [$p = 0.76$ Figure 20 C] were resistant to the anti-edematous effects of SA-57, indicating that CB₂ receptors are indispensable for mediating the anti-edematous effects.

A different pattern of results was found for the von Frey data. CB₁ (-/-) [F (1, 15) = 23.11, $p < 0.001$; Figure 20 B] and CB₂ (-/-) [F (1, 13) =8.13, $p < 0.001$; Figure 20 D] mice were resistant to the anti-allodynic effects of SA-57. In the absence of drugs, CB₁ (-/-) mice and CB₂

(-/-) mice showed similar nociceptive behavior as the wild type controls.

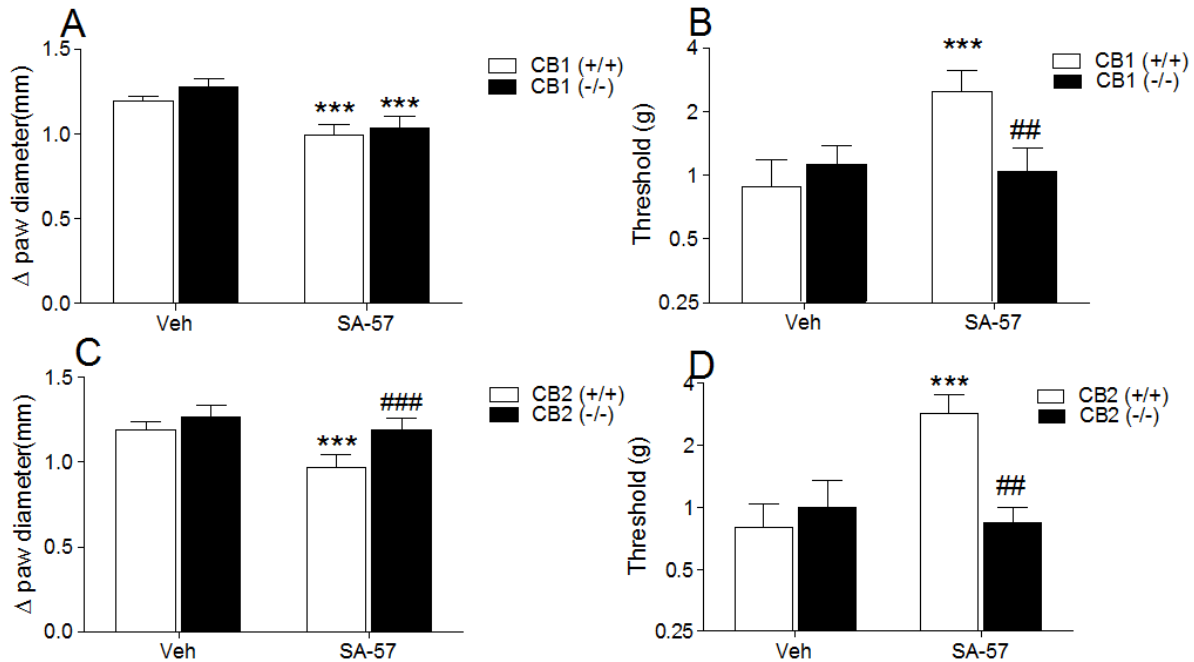


Figure 20. Assessment of the anti-edematous and anti-allodynic effects of SA-57 (5 mg/kg) in CB₁ (-/-) and CB₂ (-/-) mice. (A) SA-57 (5 mg/kg) reduced edema in CB₁ (+/+) and CB₁ (-/-) mice. (B) SA-57 (5 mg/kg) reduced allodynia in CB₁ (+/+) mice but not in CB₁ (-/-) mice and (C) SA-57 (5 mg/kg) reduced edema in CB₂ (+/+) mice but not in CB₂ (-/-) mice (D) SA-57 (5 mg/kg) reduced allodynia in CB₂ (+/+) mice but not in CB₂ (-/-) mice.***, p < 0.001 versus vehicle; #, p < 0.05; ###, p < 0.001 versus CB₁ (+/+) and CB₂ (+/+). (Tukey-Kramer post hoc tests). Data presented as mean \pm SEM (N = 4-5).

SA-57-induced cannabinimetic effects, as assessed in the tetrad assay

Mice were treated with SA-57 (0.125, 1.25, 2.5, 5, 12.5 mg/kg) and were evaluated in the tetrad assay 2 h later. Only the high dose of SA-57 (12.5 mg/kg) produced significant cannabinimetic effects on the tetrad assay. The data show that SA-27 at 12.5 mg/kg produces catalepsy [F (5, 41) = 54.06, p < 0.001; Figure 21A], antinociception [F (5, 41) = 55.85, p <

0.001; Figure 21 B], hypothermia [F (5, 41) = 54.06, $p < 0.001$; Figure 21 C], and hypolocomotion [F (5, 41) = 13.61, $p < 0.001$; Figure 21 D].

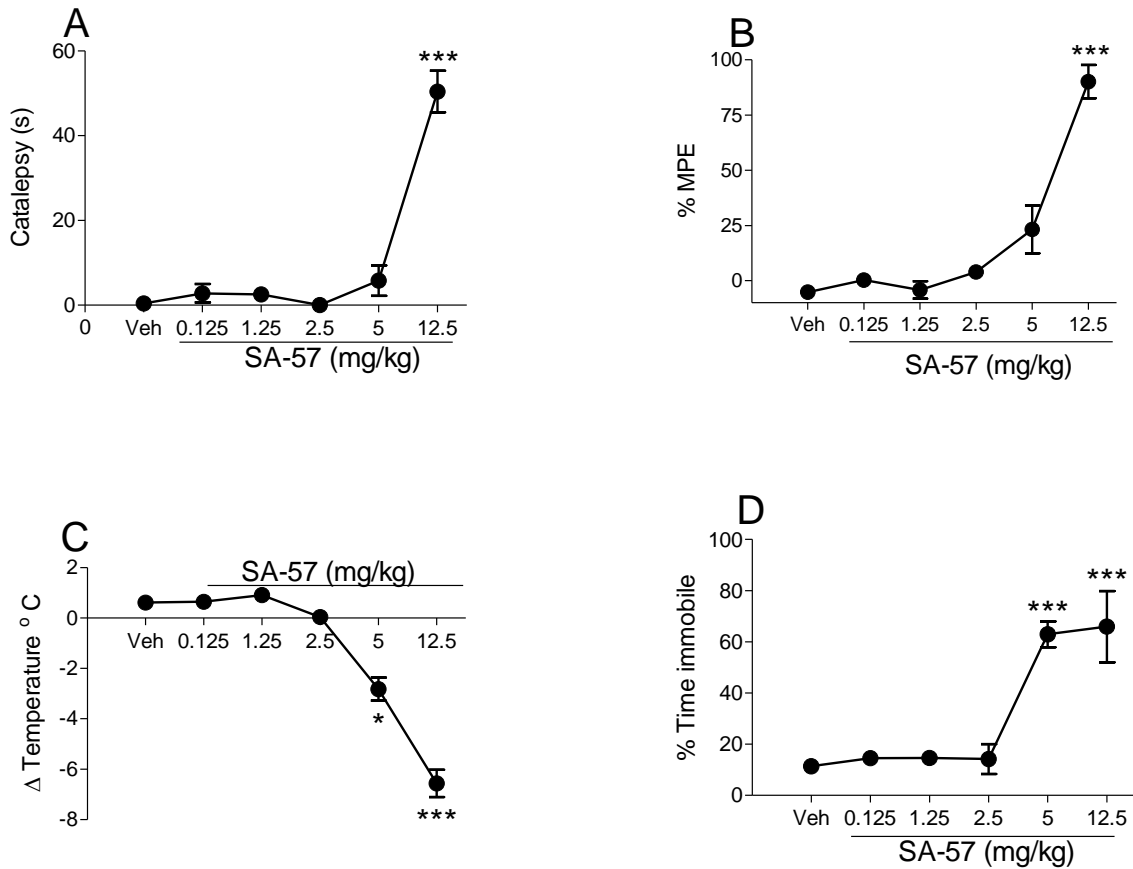


Figure 21: Mice treated with high dose of SA-57 (12.5 mg/kg) displayed (A) catalepsy, (B) antinociception, (C) body temperature, and (D) hypolocomotion in the tetrad assay. *, $p < 0.05$; ***, $p < 0.001$ versus vehicle. Data presented as mean \pm SEM (N = 6).

Discussion:

The dual FAAH-MAGL inhibitor, which shows a differential potency in inhibiting these two enzymes, yielded a similar pattern of results as combination of low-dose JZL184 and high dose PF-3845. 1.25 and 12.5 mg/kg SA-57 elevated 2-AG brain levels by 3- and >10-fold, respectively. On the other hand, SA-57 elevated the FAAH substrates anandamide, PEA, and

OEA by ~10-fold across the entire tested dose-range (0.125 to 12.5 mg/kg). This pattern of results indicates that SA-57 is considerably more potent as a FAAH inhibitor than as a MAGL inhibitor (Niphakis, 2012). The major finding of the present study is that an intermediate dose SA-57 (5 mg/kg), which produced a 10-12 fold increase in anandamide and 5 fold increase in 2-AG substantially reversed carrageenan-induced allodynia in mice compared with single inhibition of these endocannabinoid catabolic enzymes shown in figure 3 from chapter 2 and figure 10 from chapter 3A. These findings are in line with the literature, which shows that SA-57 significantly blocked all spontaneous withdrawal signs in morphine-dependent mice compared with single inhibition of MAGL or FAAH (Ramesh, 2013). JZL195, an equipotent MAGL and FAAH inhibitor, which has been shown to produce enhanced antinociceptive effects, has also produced untoward side effects of THC such as catalepsy, hypothermia and hyperreflexia (Long, 2009b). Hence, we assess if current elevations in anandamide and 2-AG by SA-57 produces CB₁-dependent behavioral effects. The results of this study revealed that SA-57 did not produce observable cannabimimetic effects (except for the highest dose 12.5 mg/kg), as assessed in the tetrad assay. In addition, small but significant hypolocomotion was observed with 5 mg/kg SA-57, the same dose which produces allodynia. Single blockade of MAGL or FAAH only partially attenuated carrageenan-induced allodynia (figure 3 from chapter 2 and figure 10 from chapter 3A) (Ghosh, 2013). On the other hand, low dose of JZL-184 and high dose of PF-3845 completely reversed carrageenan-induced allodynia (figure 10 B and D from chapter 3A). Hence, SA-57, a compound mimics the profile of high dose PF-3845 and low dose JZL184 in elevating anandamide and 2-AG respectively and produced complete anti-allodynic effects in the carrageenan assay suggests that FAAH and MAGL represent promising targets for treating inflammatory pain state.

General discussion for part A and part B

Previous studies have demonstrated direct cannabinoid receptor agonists such as THC or various exogenous cannabinoid agonists (HU210, WIN55212) produced antinociception (Elmes, 2005, Nackley, 2003, Wise, 2008). However, their psychomimetic side effects and abuse potential have dampened enthusiasm for developing drugs that act directly at CB₁ receptors (Schlosburg, 2010). The purpose of the studies in this chapter was to elucidate the impact of elevating both anandamide and 2-AG brain levels by inhibiting their catabolic enzymes, in reducing carrageenan-induced inflammatory pain in mice. As discussed in chapter 2, selective blockade of MAGL or FAAH partially attenuated allodynia and edema in this model. Hence, we tested whether inhibition of both the enzyme together would be more efficacious as compared to individual blockade of each enzyme.

Advantages of combining partial MAGL and complete FAAH inhibition

Previous research reveals that high dose of JZL184 (40 mg/kg) partially substitutes for THC in the drug discrimination paradigm (Long, 2009b). Additionally, repeated administration of high dose JZL184 elicits behavioral and functional tolerance, cannabinoid receptor desensitization, down regulation, cannabinoid receptor cross-tolerance, as well as cannabinoid dependence in mice (Long, 2009b, Schlosburg, 2010). High dose of JZL184 also elicited a subset of cannabimimetic of THC with lower intensity as compared to THC. However, inhibiting FAAH with PF-3845 or MAGL inhibition with low dose of JZL184 (4 or 8 mg/kg) is devoid of side effects of direct cannabinoid agonist. In addition prolonged blockade of FAAH with high dose of PF-3845 or MAGL with low dose of JZL184 maintain the antinociceptive, gastro-protective and anxiolytic effects (Ghosh, 2013, Kinsey, 2013, Schlosburg, 2010, Sciolino, 2011). However, either PF-3845 or JZL184 produces partial efficacy in attenuating edema and allodynia

in the carrageenan model in mice (Ghosh et al., 2013; chapter two, figure 3 A-D). Taken together, these results suggest that while both FAAH and MAGL inhibitors individually represent promising approaches to reducing nociception but they also have limitations. In order to circumvent these limitations, we investigated whether a combination of partial MAGL inhibition and complete FAAH inhibition would elicit enhanced efficacy in attenuating carrageenan-induced nociception in mice, without the side effects associated with complete MAGL inhibition. Low dose of JZL184 (4 mg/kg) was used to achieve partial MAGL inhibition. High dose of PF-3845 led to complete FAAH blockade. 4 mg/kg JZL184 elevated 2-AG levels 2-4 fold and 10-fold anandamide level was elevated by 10 mg/kg. This combination also produced enhanced anti-allodynic effects in the carrageenan assay. In C57BL/6J mice, prolonged exposure to the combination did not induce any CB₁ receptor functional tolerance, antinociceptive tolerance in carrageenan pain assay. These findings are important for the development of compounds, which possess partial MAGL and complete FAAH inhibition and are devoid of the side effects. In Chapter 3B, a novel inhibitor SA-57 that has differential potency at MAGL and FAAH (Niphakis, 2012) was assessed in the carrageenan model of inflammatory pain. A partial blockade of MAGL and complete blockade of FAAH produced increased antinociceptive effects compared with individual enzyme inhibition. Thus, SA-57 offers a useful tool to explore the effects of complete FAAH blockade in conjunction with varying degrees of MAGL blockade.

Chapter 4. KT-109, a selective diacylglycerol lipase beta inhibitor reverses LPS-induced allodynia in mice

Introduction

The previous two chapters focused on the impact of inhibiting endocannabinoid catabolic enzymes FAAH and MAGL in the carrageenan model of inflammatory pain. In the present chapter, we report on the consequences of inhibiting diacylglycerol lipases (DAGLs), one of the major anabolic enzymes of 2-AG that is involved in mediating neuronal growth during development and as a retrograde messenger, (Brittis et al., 1996, Tanimura, 2010, Williams, 2003) in LPS-induced inflammatory pain model.

DAGL was shown to exist in two isoforms α (120 kDa) and β (70 kDa). Human DAGL- α and human DAGL- β are large proteins of 1042 and 672 amino acids, respectively, containing four transmembrane domains with both C and N terminal located inside the membrane (Bisogno, 2003). Sn-1 DAG lipase activity of membranes from cells over-expressing either of the DAGL isoforms was detected with several diacylglycerol. This activity was inhibited by classical serine hydrolase inhibitors and blocked by mutations in DAGL- β serine 443 and aspartate 495, which were identified as residues belonging to the catalytic triad (Bisogno, 2003, Pedicord et al., 2011). The catalytic triad of DAGL- α , consists of serine 472, aspartate 524 and histidine 650 (Bisogno, 2003).

DAGL- β is predominantly expressed in the periphery and controls 2-AG levels in the peripheral tissues such as liver (Hsu, 2012), while the α -isoform is mainly found in the central nervous system (Gao, 2010, Tanimura, 2010). Pharmacological studies indicate that DAGL activity is required for axonal growth in rat brain (Brittis, 1996). Once 2-AG is synthesized, it

activates the CB₁ receptors in the same axon (Williams, 2003). DAGL is located postsynaptically to produce 2-AG from diacylglycerol (Williams et al., 2003; Bisogno et al., 2003), which is produced from rapid hydrolysis of inositol phospholipids by phospholipase C resulting in diacylglycerol (DAG) (Prescott and Majerus, 1983). The development of 1, 2, 3-triazole urea (1, 2, 3-TU) as a versatile chemotype for serine hydrolase inhibitor has been useful for the synthesis of selective DAGL inhibitors (Adibekian et al., 2010). In that study, the screening of DAGL enzymes against a small library of 1, 2, 3-TUs using an activity-based protein profiling (ABPP) assay (Cravatt et al., 2008) culminated in the synthesis of two compounds KT109 and KT172 that potently and selectively inactivated DAGL- β *in vitro* and *in vivo*. Nevertheless, both KT109 (IC₅₀ = 82 nM) and KT172 (IC₅₀ = 70 nM) also inhibit ABHD6 (IC₅₀ values of 16 and 5 nM, respectively). Hence, a negative-control probe, KT195 has been generated. KT195 is a potent (IC₅₀ = 10 nM) selective inhibitor of ABHD6 and structurally related to KT109 and KT172, but inactive against DAGL- β . The pharmacological inhibition of DAGL- β with KT-109 and KT-172 or genetic inactivation of DAGL- β produced a remarkable array of metabolic changes in macrophages that included not only reductions in 2-AG levels, but also decreases in arachidonic acid and prostaglandins levels (Hsu, 2012). Interestingly, inactivation of DAGL- β also attenuated lipopolysaccharide-stimulated TNF- α release from macrophages, implicating a DAGL- β regulated endocannabinoid-eicosanoid network as an important modulator of proinflammatory responses in macrophages (Hsu, 2012). Based on these *in vitro* results, this chapter investigates the consequences of inhibiting this enzyme *in vivo*. Specifically, we test whether inhibition of DAGL- β with KT109 or KT172 will reduce LPS-induced allodynia in mice. We have taken a complementary pharmacological and genetic approach to assess the role of these inhibitors in DAGL-WT as well as DAGL- β deficient mice. In addition, KT195 was

tested in this model to control for ABHD6 inhibition. Additionally, we assessed if these compounds mediate their effects through cannabinoid receptors. Similarly, we examined if these inhibitors possess cannabimimetic effects by interacting with cannabinoid receptors directly. In addition, we have evaluated the effects of repeated administration of KT195.

A major drawback to NSAID drug use is the preponderance of gastrointestinal (GI) side effects. NSAIDs are believed to damage the gastrointestinal tract through the reduction of prostaglandin biosynthesis in stomach via cyclooxygenase inhibition (Musumba et al., 2009). Prostaglandins play a protective role in the stomach by stimulating bicarbonate production (secretes from the mucus layer and neutralizes the digestive acids) and mucus production (Isenberg et al., 1986, Miller, 1983). Additionally, prostaglandin dilates the blood vessels in the stomach to ensure good blood flow (Anderson et al., 1976, Miller, 1983). Prostaglandin reduction leads to increase in stomach acid production and also decreases mucosal secretion (Musumba, 2009) as a result makes the stomach lining susceptible to the action of acid and increase the risk of ulcers. As mentioned before KT-109 and KT-172 have been found to decrease arachidonic acid and prostaglandin level in the peritoneal macrophages (Hsu, 2012). It has been reported that phospholipase A2 (PLA2) enzymes, and cytosolic PLA (cPLA2 or *Pla2g4a*) in particular, is considered to be the principal source of arachidonic acid for cyclooxygenase-mediated prostaglandin production in the gut and spleen (Buczynski et al., 2009, Nomura, 2011). On the other hand, metabolic analysis of *Pla2g4a*^{+/+} mice or *Pla2g4a*^{-/-} mice showed complete loss of prostaglandin D2 and only a modest reduction in PGE2 levels in the peritoneal macrophages (Hsu, 2012). However, treatment with KT109 decreased prostaglandin - E2 level in *Pla2g4a*^{-/-} macrophages as compared to KT109-treated wild-type mice or untreated *Pla2g4a*^{-/-} mice (Hsu, 2012). These data together indicate that DAGL- β and PLA2G4

coordinately regulate the production of this prostaglandin in the peritoneal macrophages. However, it is unclear if these compounds will produce ulcerogenic effects in the stomach. Hence, based on these results we have evaluated the role of DAGL- β inhibitors on gastric hemorrhage. Therefore, in our final study we have examined the role of DAGL- β inhibition on gastric hemorrhage in mice.

Materials and methods

Subjects

The subjects consisted of adult male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME). Subjects weighed between 20-30 g and were housed 4-5 per cage in a temperature-controlled (20-22°C) environment. DAGL β (+/+), DAGL β (+/-), and DAGL β (-/-) mice were on a mixed genetic background of C57BL/6 and 129/SvEv and were obtained from Taconic. Male and female CB₁ (-/-) and CB₂ (-/-) mice and their respective littermate controls, CB₁ (+/+) and CB₂ (+/+) mice from the Center Transgenic Colony at Virginia Commonwealth University served as subjects for the mechanism of action studies. CB₁ (-/-) and CB₂ (-/-) mice were backcrossed onto a C57BL/6J background for 13 and 6 generations, respectively. 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

THC was obtained from the National Institute on Drug Abuse (Bethesda, MD, USA). Diacylglycerol lipase beta inhibitors, KT109, KT172 and the negative control KT-195 were obtained from Scripps institute CA. All the drugs were dissolved in a vehicle that consisted of a mixture of ethanol, alkamuls-620 (Rhone-Poulenc, Princeton, NJ), and saline in a ratio of 1:1:18, except diclofenac, which was dissolved in saline. Each drug was given via the intraperitoneal route of administration in a volume of 10 µl/g body weight.

Lipopolysaccharide (LPS) pain model

Allodynia was induced by injecting 20 µl (2.5 µg) of lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 Sigma (St. Louis, MO) (Naidu, 2010) into the plantar surface of one hind paw of each mouse (Booker, 2011, Kanaan et al., 1996). 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 in chapter 2 and 3 (Kinsey, 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, 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.

Cannabimimetic behavior assessments

Mice were treated with vehicle or 40 mg/kg KT109 or 30 mg/kg THC and tested on the tetrad assay used for assessing cannabimimetic effects of THC and other CB₁ receptor agonists. Prior to testing all mice were acclimated to the test room for at least 1 h (Martin, 2003). Baseline

catalepsy, nociception, rectal temperature, and locomotor activity were assessed. Mice were injected with a 30 mg/kg THC 30 min prior or 40 mg/kg KT-109 120 mins prior and tested for catalepsy, nociception, rectal temperature, and locomotor activity. Catalepsy was assessed using the bar test, in which the forelimbs of each mouse were placed on a horizontal bar during a 60s test in which time immobile was measured. Hypothermia was determined by change in rectal body temperature from baseline. The tail immersion test was used to measure antinociception. The distal 2 cm of the tail was immersed in 52.0 °C water and the tail withdrawal latency determined. A maximum cutoff of 10 s was used to minimize possible tissue damage. Maximum percent effect was using the formula $[(\text{test value} - \text{base line value}) / (10 - \text{baseline value})] * 100$. Locomotor activity was measured in a 5 min test in the activity box.

In separate groups of mice, the cannabimimetic behavioral effects of treatment with vehicle and 40 mg/kg KT-109 were evaluated in the tetrad test. Baseline catalepsy, rectal temperature, nociception, and locomotor activity were first determined. Two hours after injections. Mice were then evaluated for catalepsy, nociception, rectal temperature, and locomotor activity as described above. We used Anymaze boxes to assess locomotor activity.

Gastric inflammatory lesion model

Gastric hemorrhages were induced and quantified as described previously (Kinsey, 2011a, Liu et al., 1998). Male C57BL/6J mice were weighed, then placed on a wire mesh barrier (Thoren Caging Systems, Inc, Hazleton, PN), and food deprived with free access to water. After 24 h, mice were administered KT-109 (40 mg/kg, i.p.), the nonsteroidal anti-inflammatory drug, diclofenac sodium (100 mg/kg, i.p.) or vehicle (1:1:18 parts of EtOH, Emulphor, and normal saline), and returned to the home cage for 6 h. The mice were humanely euthanized via CO₂ asphyxiation, and stomachs were harvested, cut along the greater curvature, rinsed with distilled

water, and photographed on a lighted tracing table (Artograph light pad 1920) and photographed (Canon T3 Rebel digital camera with a 10x close up lens). Basically, each photo has a reference in it, along with the stomach. That ruler is used as a reference to compare with the stomach in each photo. Then photo-shop software is used to trace the length of each hemorrhage and a 1-pixel-wide line is drawn on each photo, using a color that doesn't show up in the photo (like bright green). Then the pixels of bright green are measured in the photo using photo-shop software, thereby adding up all of the hemorrhage lengths together (each line is 1-pixel-wide, so the total number of green pixels is the same as the total length of all the lines. Then the process is repeated on the ruler/reference, tracing a 1 mm segment in another color (like bright pink). Then the same count of bright pink pixels is obtained to find the number of pixels in 1mm. Finally, the, total length of hemorrhages are calculated by $[\text{pixels of hemorrhages}] / [\text{pixels/mm}] = \text{total mm of hemorrhages}$ (Kinsey, 2013, Nomura, 2011). Image files were renamed, and an experimenter blinded to treatment conditions quantified the gastric hemorrhages, relative to a reference in each photo, using Adobe Photoshop (version CS5), as described previously (Kinsey, 2013, Nomura, 2011).

Results:

Anti-allodynic effects of KT-109 is maintained over an extended period

Intraplantar administration of 2.5% lipopolysaccharide induced allodynia. In the time course study, KT-109 produced anti-allodynic effects in 2 h, which was sustained for 28 h and finally gone back in to the baseline level at 48 h [$F(1,80) = 73.37, p < 0.001$; (Figure 22)]. There is no significant interaction between time and the treatment [$p = 0.090$].

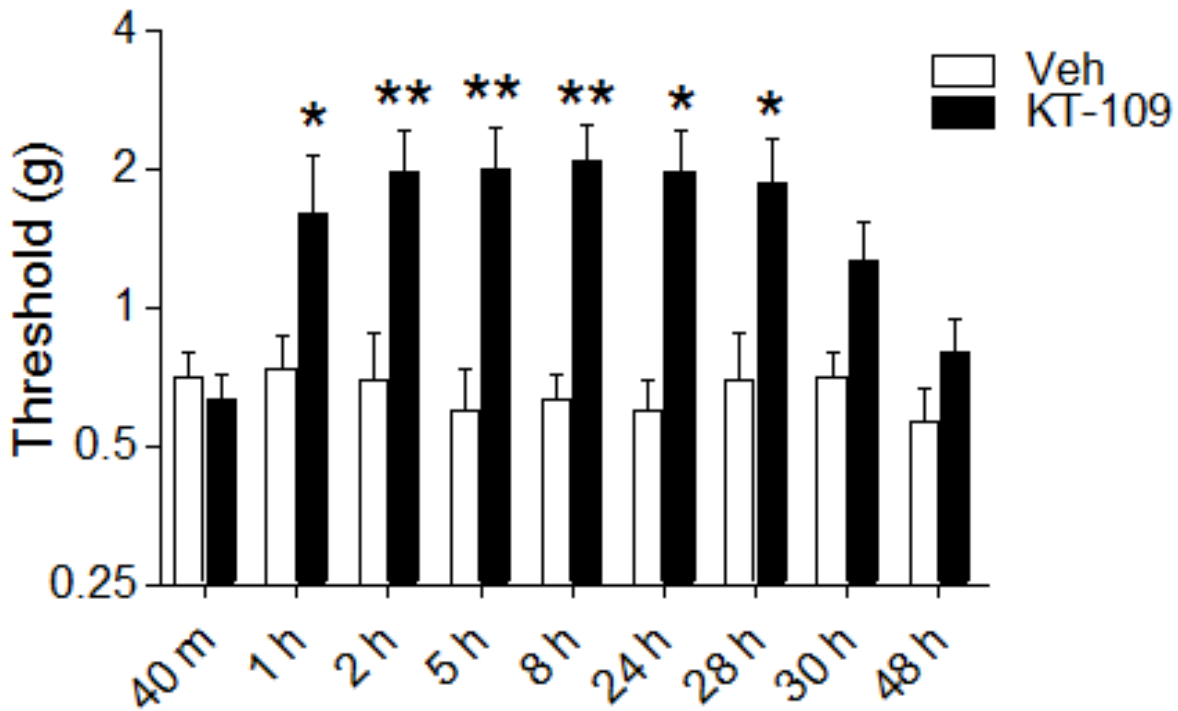


Figure 22: Anti-allodynic effects of KT-109 are maintained over an extended period. (A) In time course study, KT-109 produced anti-allodynic effects in 2 h, which was sustained for 28 h and finally gone back in to the baseline level at 48 h. *, $p < 0.05$; **, $p < 0.01$ versus vehicle (Bonferroni post hoc tests). KT-109 did not produce any anti-allodynic effects in the control paw. Data presented as mean \pm SEM (N = 9).

DAGL- β inhibition with KT-109 but not KT-172 reverse LPS-induced allodynia, negative control probe KT-195 does produce any effect

To test whether DAGL- β inhibitor KT109 and KT172 reverses LPS-induced allodynia , mice were given an intraplantar injection of LPS (2.5 mg) into a hind paw, and 22 h later were given an i.p. injection vehicle (V) , KT109 (1.6, 2.5, 5, 20, 40 mg/kg), or KT172 (5, 20, 40 mg/kg) or KT195 (20 , 40 mg/kg). Subjects were assessed for mechanical sensitivity to von Frey

filaments at 24 h. Figure 23 A shows KT-109 significantly reversed LPS-induced allodynia [$F(6, 56) = 22.98, p < 0.001$; Figure 23A]. However, KT-172 [$p = 0.37$; Figure 23B] did not reverse allodynia. KT195 [$p = 0.47$; Figure 23C] did not produce anti-allodynic effects in this assay. Neither KT-109 or KT172 nor KT-195 affected paw withdrawal thresholds in control paws.

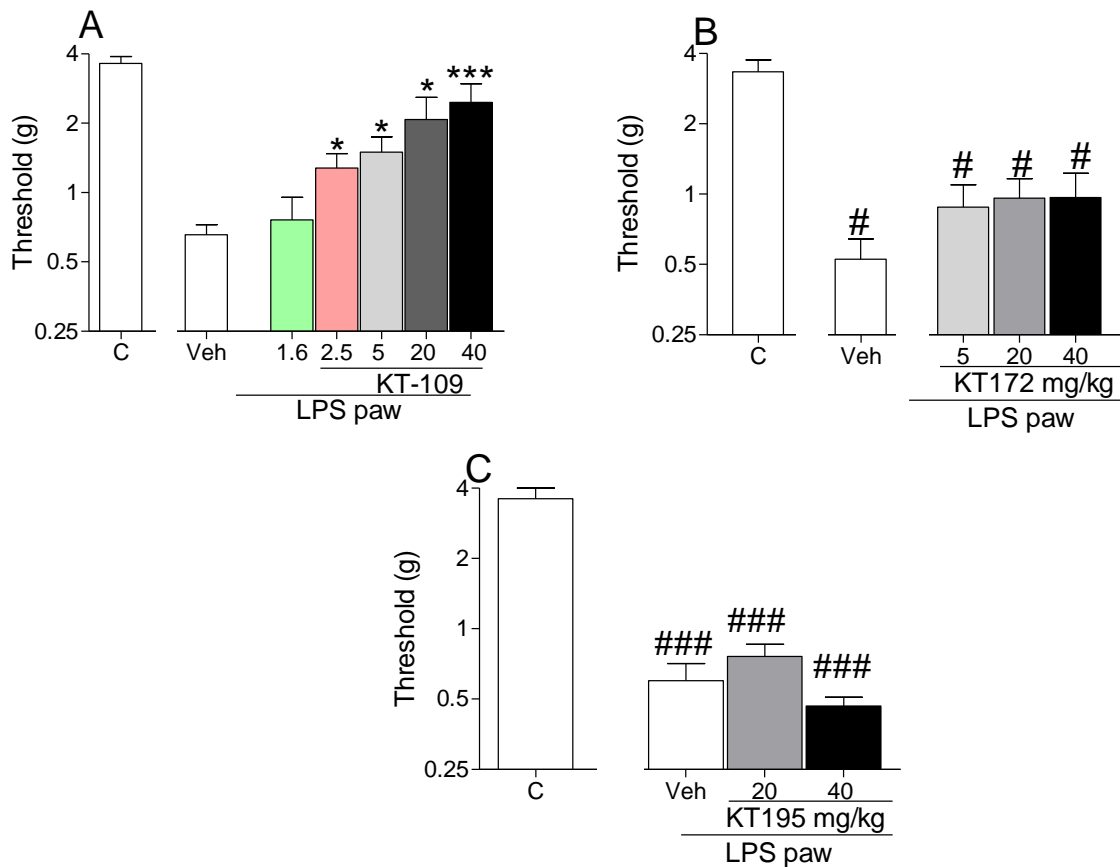


Figure 23. Different doses of KT-109 but not KT-172 reverse LPS-induced allodynia. **(A)** shows KT109 (2.5, 5, 20, 40 mg/kg) significantly reversed LPS-induced allodynia. **(B)** KT-172 (5, 20, 40 mg/kg) could not reverse allodynia. Neither KT-109 nor KT-172 affects paw withdrawal thresholds in control paws. *, $p < 0.05$; ***, $p < 0.001$ versus vehicle; #, $p < 0.05$; ###, $p < 0.001$ versus control paw (Dunnett's post hoc tests). Data presented as mean \pm SEM ($N = 6$).

DAGL knockout mice show anti-allodynic phenotype

Complementary pharmacological and genetic approaches were used to evaluate the impact of DAGL- β blockade in alleviating inflammatory pain. Accordingly, we tested KT-109 in DAGL- β (-/-) and (+/+) mice. DAGL- β (-/-) mice treated with vehicle showed an anti-allodynic. KT-109 reversed allodynia in DAGL- β (+/+) animals but did not further alter nociceptive responses in DAGL- β (-/-) mice. A two-way ANOVA reveal a significant interaction between treatment and genotype [$F(1, 50) = 5.93, p < 0.01$; Figure 24].

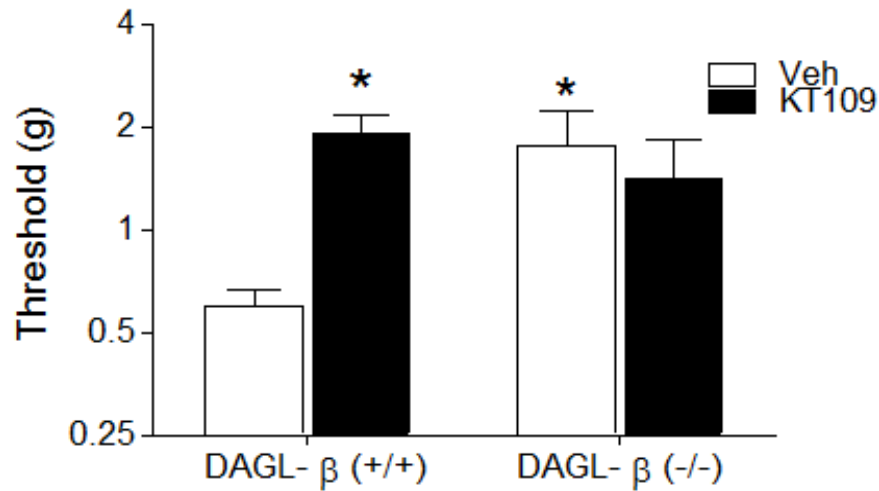


Figure 24: DAGL knockout mice show anti-allodynic phenotype. (A) KT-109 completely reversed allodynia in DAGL- β (+/+) animals. (A) DAGL- β (-/-) mice treated with vehicle show anti-allodynic phenotype but KT109 did not elicit any further decrease in allodynia in DAGL- β (-/-) mice. *, $p < 0.05$ versus vehicle /WT (Bonferroni post hoc tests). Data presented as mean \pm SEM (N = 13-14).

Repeated administration of KT-109 does not lead to tolerance

In this experiment, we examined the effects of repeated administration of high dose KT-109 (40 mg/kg) in the LPS model. As shown in Figure 25, repeated administration 40 mg/kg KT-

109 significantly attenuated LPS-induced allodynia [$F(2, 13) = 5.68, p < 0.001$] indicating that prolonged inhibition of DAGL- β does not lead to tolerance. Repeated drug treatment did not affect the paw withdrawal threshold of the contralateral paw. As expected, no allodynia was observed in the contralateral paw.

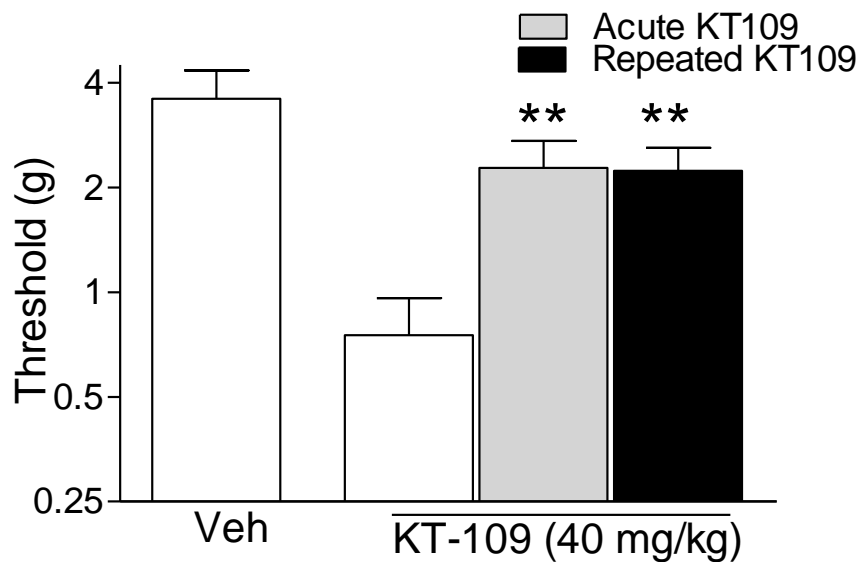


Figure 25: Repeated administration of KT-109 does not lead to tolerance. (A) Repeated administration 40 mg/kg KT-109 significantly attenuated LPS-induced allodynia. There was no allodynia seen in the contralateral paw. **, $p < 0.01$ versus vehicle (Tukey-Kramer post hoc tests). Data presented as mean \pm SEM (N = 5-6).

Anti-allodynic effects of KT-109 are not mediated by cannabinoid receptors

As KT-109 inhibits the biosynthetic enzyme of 2-AG in macrophages (Hsu et al., 2012), it is unlikely that its anti-allodynic effects involve cannabinoid receptors. Nonetheless, to assess direct or indirect interaction of KT-109 with cannabinoid receptors effects, we tested it in CB₁ and CB₂ knockout mice. KT-109 produced anti-allodynic effects in CB₁ (+/+) and CB₂ (+/+) mice. Its anti-allodynic effects persisted in CB₁ (-/-) mice [$p = 0.90$; Figure 26A] and CB₂ (-/-)

mice [$p = 0.20$; Figure 26B]. Thus, the anti-allodynic effects of KT-109 do not require cannabinoid receptors. In the absence of drugs, both CB_1 (-/-) and CB_2 (-/-) mice displayed similar nociceptive responses to intraplantar LPS as the wild type controls.

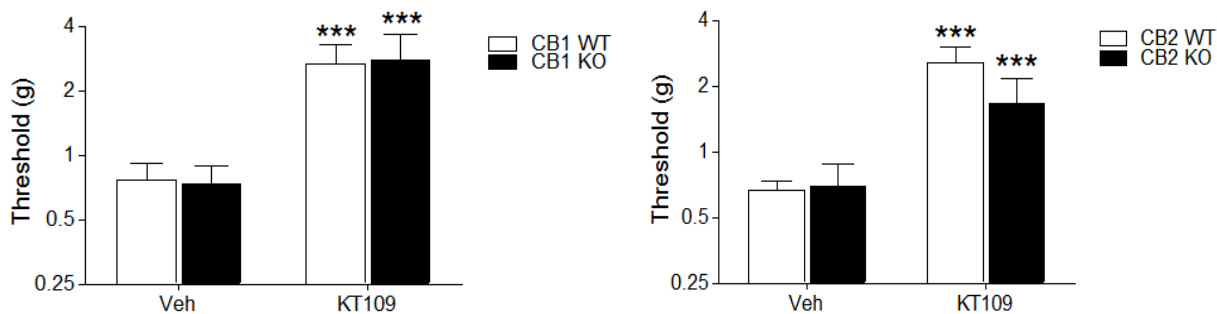


Figure 26: Anti-allodynic effects of KT-109 are not mediated by cannabinoid receptors. KT-109 produced anti-allodynic effects in (A) CB_1 (+/+) and (B) CB_2 (+/+) mice. The anti-allodynic effect was not reversed in (A) CB_1 (-/-) and (B) CB_2 (-/-) mice. In the absence of drugs, both CB_1 (-/-) and CB_2 (-/-) showed similar nociceptive behavior as the wild type controls. ***, $p < 0.001$ versus vehicle (Bonferroni post hoc tests). Data presented as mean \pm SEM (N = 6).

KT-109 did not produce cannabinimetic effects in tetrad

Additionally, we examined if KT-109 produces cannabinimetic effects in the tetrad assay. C57/BL6J mice were administered a high dose KT-109 (40mg/kg) and assessed 2 h later in tetrad test, which includes: spontaneous locomotor suppression, antinociception to noxious thermal stimuli, catalepsy, and hypothermia (Compton et al., 1993, Martin et al., 1991). KT-109 (40 mg/kg) did not produce catalepsy [Figure 27A], increased tail withdrawal latencies ($p = 0.79$) [Figure 27B], hypothermia ($p = 0.44$) [Figure 27C] or hypolocomotion (i.e., defined as time spent immobile; $p = 0.86$) [Figure 27D] indicating that KT-109 is devoid of common pharmacological effects produced by THC and other CB_1 receptor agonists. To verify that the tetrad assay used here can be used to detect CB_1 receptor agonists, we tested THC as a positive

control and THC has shown to produce catalepsy, hypothermia, antinociception, and hypolocomotion.

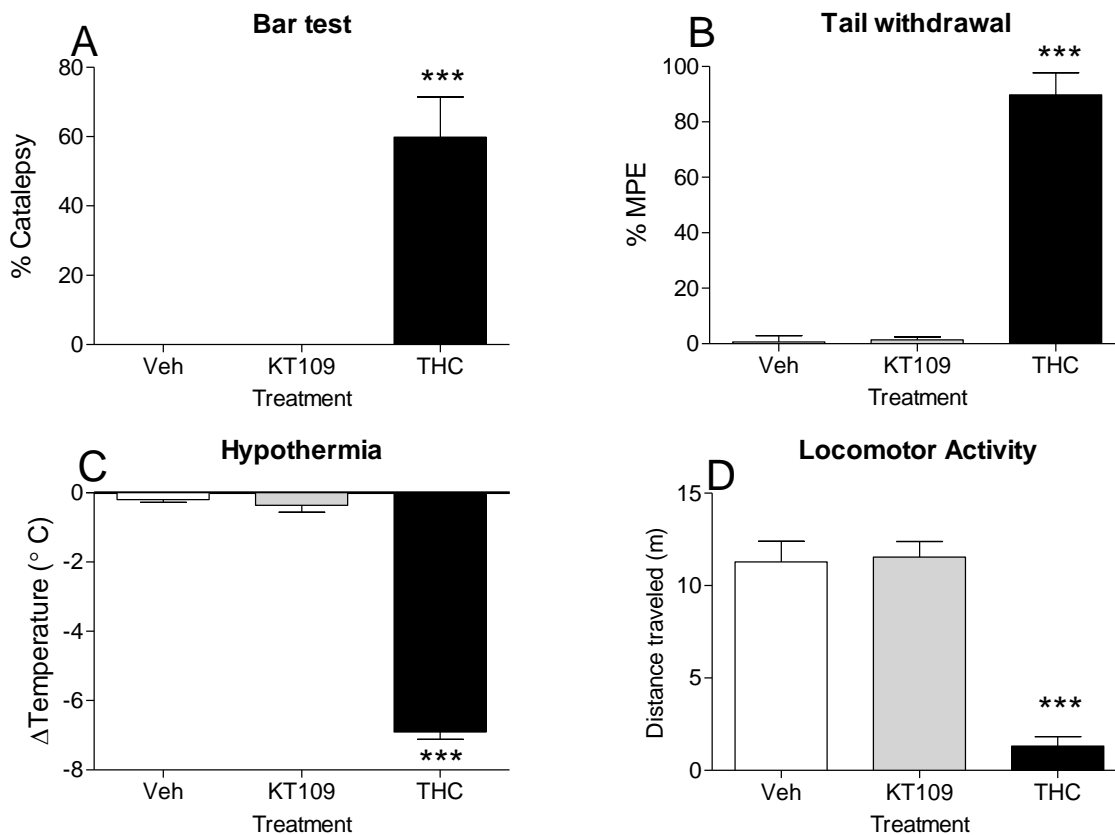


Figure 27: KT-109 did not produce cannabimimetic effects in tetrad. KT-109 (40 mg/kg) did not produce (A) catalepsy, (B) tail withdrawal latency, (C) body temperature, or (D) hypolocomotion. To ensure that our model can detect compounds that produce effects on tetrad, we tested the d-9 THC as our positive control. ***, $p < 0.001$ versus vehicle (Tukey-Kramer post hoc tests). Data presented as mean \pm SEM (N = 5-6).

DAGL- β inhibition with high dose KT-109 does not elicit gastric ulcers in mice

NSAID is believed to damage the gastrointestinal tract through the reduction of prostaglandin biosynthesis in stomach via cyclooxygenase inhibition, which leads to increased

stomach acid production and decreased mucosal secretion (Musumba, 2009). Levels of arachidonic acid and prostaglandin decrease in parallel with 2-AG. Hence, in this study we evaluated whether KT-109 produces gastric hemorrhages. As shown in Figure 28, high dose of KT-109 (40 mg/kg) [p= 0.80] did not cause any significant hemorrhagic effects in the mice. In contrast, the classical NSAID, diclofenac (100 mg/kg) produced a significant increase in gastric ulcers [F (2, 22) = 6.20, p < 0.001].

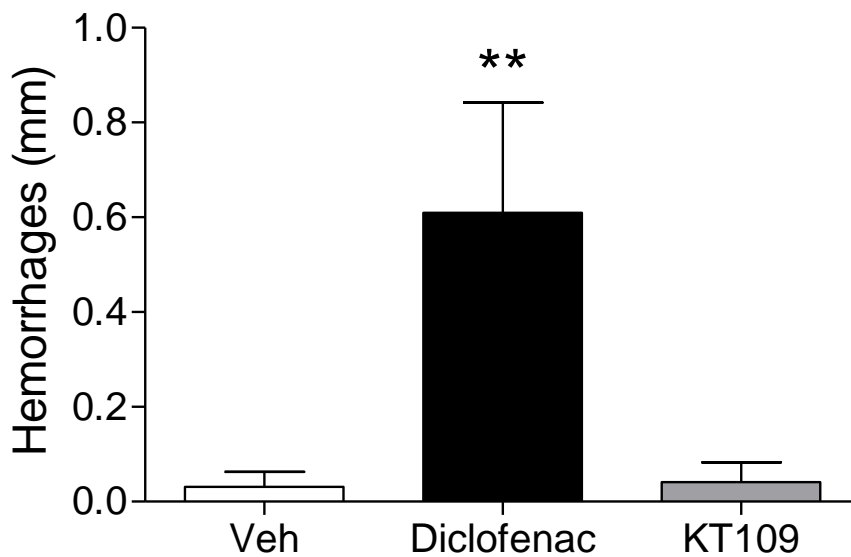
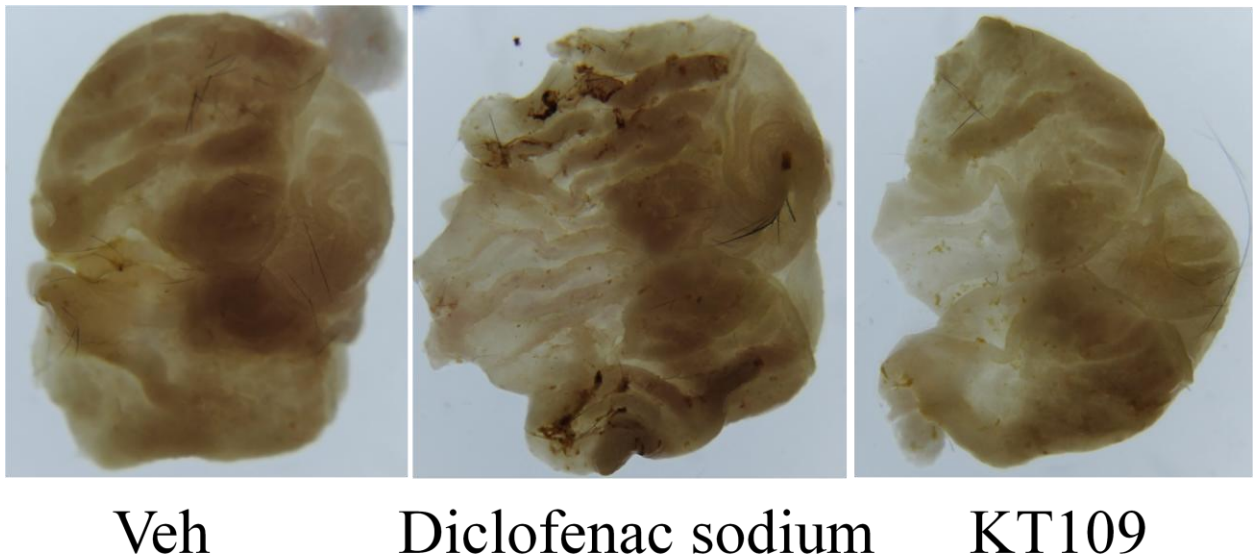


Figure 28: DAGL- β inhibition with high dose KT-109 does not elicit gastric ulcers in mice. The photographs show gastric hemorrhage in mice treated with high dose of diclofenac but stomach treated with KT109 does not show any hemorrhage. Unlike a classical NSAID, diclofenac (100 mg/kg), high dose of KT-109 (40 mg/kg) did not cause any significant hemorrhagic effects in the mice. **, $p < 0.01$ versus vehicle (Tukey-Kramer post hoc tests). Data presented as mean \pm SEM (N = 8-9). Dr. Steven Kinsey has performed these experiments.

Discussion:

KT-109 has high selectivity for DAGL- β over other serine hydrolases, including DAGL- α , but also inhibits ABHD6. To control for ABHD6 inhibition, we have employed KT195, which is a selective inhibitor of this enzyme (Hsu, 2012). KT-109 has decreased 2-AG levels in peritoneal macrophages upon acute administration (Hsu, 2012). This compound reduces levels of 2-AG and the downstream lipids AA and prostaglandins in peritoneal macrophages as well as in Neuro 2A cell lines (Hsu, 2012). Concomitant with these changes in lipid metabolism, DAGL- β inhibitors have significantly reduced the LPS-stimulated production of the pro-inflammatory cytokine TNF- α (Hsu, 2012) in macrophages. The present study increases the understanding that DAGL- β inhibition plays role in nociception by demonstrating that KT-109 reduces LPS-induced mechanical allodynia in mice. A complementary genetic approach using DAGL- β (-/-) mice reveals an anti-allodynic phenotype indicating the DAGL- β plays an essential role in attenuating LPS-induced inflammatory pain. In addition, KT-109 did not elicit further anti-allodynic effects in the KO animals, which indicates that KT-109 is selective for the enzyme DAGL- β . The anti-allodynic effects of KT019 were independent of both CB₁ and CB₂ receptors. In addition, KT-109 did not produce cannabimimetic effects in the tetrad assay

indicating that the compound is devoid of common behavioral side effects produced by THC and other CB₁ receptor agonists. In addition, the anti-allodynic effects of KT-109 did not undergo tolerance following repeated administration. Finally, unlike the classical NSAID, diclofenac, administration of high dose of KT-109 did not elicit gastric ulcers. As this is an acute assay a very high dose of diclofenac was used to ensure that gastric hemorrhages are produced. Diclofenac is capable of producing antinociceptive effects at a lower dose hence, it is important to do an experiment to observe hemorrhagic effects of diclofenac at a the lower doses.

The results reported in this chapter represent the first study to demonstrate the in vivo effects of these selective DAGL- β inhibitors, as described previously we have also tested the role of the negative control probe in the LPS-induced inflammatory pain model. Here, we have shown that high dose KT-109 (40 mg/kg) completely reversed LPS-induced allodynia, low and intermediate doses (1.6, 2.5, 5, 20 mg/kg) produce partial attenuation. On the other hand, KT-172 (5, 20, 40 mg/kg) has failed to attenuate allodynia in mice. These data were surprising because KT172 showed near-equivalent activity against DAGL- β compared to KT109. In addition, equivalent potencies have been shown by KT109 and KT172 for DAGL- β (Hue et al 2012). Additionally, the structural similarity between the compounds suggests that they might produce similar effects in vivo, though it has not been confirmed whether these two compounds bind in the same pocket of the enzyme. Although KT109 and KT172 have shown structural similarity with each other and similar potency for DAGL- β but the downstream effectors for both the inhibitors are yet to be elucidated. Activation of different downstream signaling pathway could be responsible for KT172 inability to produce anti-allodynic effects in the LPS-induced inflammatory pain. In addition to pharmacological inhibition, we confirmed a role for DAGL- β as a target for inflammatory pain by demonstrating that DAGL- β (-/-) mice displays an

anti-allodynic phenotype in the LPS model of inflammatory pain. Another important finding is that repeated administration of KT-109 did not lead to tolerance of its anti-allodynic effects. This effect is consistent with knockout the data. The present study also shows that cannabinoid receptors are dispensable in mediating anti-allodynic effects of KT-109. Although the mechanism of action for KT-109 is not completely understood, we hypothesize that involves a reduction in the LPS elicited production of prostaglandin. We predict that inhibition of 2-AG synthesis will results in reduction of arachidonic acid, which is a precursor for prostaglandin. As a result, the prostaglandin levels will go down. However, suppression of TNF- α production that was observed upon selective pharmacologic or genetic disruption of DAGL- β by Hsu et al 2012 is less understood from a mechanistic perspective. Regardless of the precise mechanism by which DAGL- β inhibitors reduce TNF- α release, this finding has potential clinical relevance, given the demonstrated clinical value of TNF- α blockers for treating inflammatory disorders like rheumatoid arthritis (Parameswaran and Patial, 2010).

The tetrad data indicate that KT-109 does not produce cannabimimetic activity, further confirming the finding that there is no direct interaction of KT-109 with the cannabinoid receptors. In addition, as 2-AG is reduced, there is likely to be decreased endocannabinoid tone. We require more studies to confirm this. Finally, we have shown that high dose KT-109 does not elicit gastric ulcers in mice. NSAID is believed to damage the gastrointestinal tract through the reduction of prostaglandin biosynthesis in stomach via cyclooxygenase inhibition, which leads to increased stomach acid production and decreased mucosal secretion (Musumba, 2009). The impact of DAGL- β inhibition reflects a direct effect on DAG which is not converted to 2-AG in absence of DAGL- β activity. This reduction in 2-AG in turn decreases the production of arachidonic acid and prostaglandins. As reduction of prostaglandin production leads to increases

in stomach acid production and decreased mucosal secretion (Musumba, 2009), we have evaluated in this study the role of KT-109 on gastric hemorrhage. Our result reveals that unlike NSAIDs, KT-109 does not produce any gastric hemorrhage in mice upon acute administration indicating that despite reduction in prostaglandin levels KT-109 is devoid of ulcerogenic effects of classical NSAID. Although it is very promising that high dose of KT-109 does not produce gastric hemorrhage upon acute administration, the effect of prolonged blockade of DAGL- β on gastric hemorrhage is yet to be evaluated. The biochemical and the behavioral data together support the idea that DAGL- β can be a promising target for the development of analgesic therapeutics. It is relevant that this isoform does not play a major role in the production of 2-AG in neurons. Thus, DAGL- β inhibitors would not be expected to disrupt neuronal function or interfere with endocannabinoid-mediated neuronal processes.

Chapter 5. General Discussion

Direct cannabinoid receptor agonists have long been known to reduce pain and inflammation in acute and chronic preclinical animal models (Kinsey, 2009, Lichtman, 2004, Long, 2009a, Russo, 2007); however, the psychomimetic side effects produced by these drugs dampen enthusiasm for clinical development (Lichtman, 2004). An alternative approach that promises to retain the beneficial effects and to minimize the untoward cannabimimetic side effects associated with CB₁ receptor agonists is to inhibit the primary enzymes responsible for the biosynthesis and hydrolysis of the endogenous cannabinoids (Blankman, 2007, Cravatt, 2001, Cravatt, 1996, Dinh, 2002). Pharmacological inhibition of MAGL and FAAH elevates the respective endocannabinoids, anandamide, and 2-AG in brain and spinal cord tissue (Cravatt,

1996, Kinsey, 2009, Long, 2009a). Blockade of FAAH and MAGL produces antinociceptive and anti-inflammatory effects in vivo in carrageenan, tail withdrawal, CFA, CCI, LPS and formalin pain assay (Ahn, 2009, Guindon, 2011, Guindon and Hohmann, 2009, Kinsey, 2010, Kinsey, 2009, Lichtman, 2004, Long, 2009a, Naidu, 2010, Schlosburg, 2009, Sciolino, 2011, Spradley, 2010) (Sasso, 2012).

Irreversible (PF-3845, URB597) and reversible (OL-135) inhibitors of FAAH have been demonstrated to elevate anandamide levels in the brain (Ahn, 2009, Boger, 2005, Fegley, 2005) and attenuate neuropathic pain (Clapper, 2010, Jhaveri, 2006, Kinsey, 2009, Lichtman, 2004, Russo, 2007), inflammatory pain (Booker, 2011), and collagen-induced arthritis (Kinsey et al., 2011). Similarly, MAGL inhibitors attenuate nociception to radiant heat and acetic acid-induced abdominal stretching (Long, 2009a), as well as reverse allodynic and hyperalgesic states in neuropathic (Kinsey, 2009) and inflammatory (Ghosh, 2013, Guindon, 2011, Guindon and Hohmann, 2008) pain models. However, these inhibitors are not fully efficacious in producing antinociceptive effects in neuropathic and inflammatory pain models.

It is also important for drugs used to treat chronic pain conditions to maintain their antinociceptive effects following chronic administration. The consequences of prolonged and complete inhibition of MAGL contrast with the consequences of prolonged and complete blockade of FAAH. Repeated administration of high doses of FAAH inhibitors produce sustained antinociception and without loss of CB₁ receptor function (Falenski, 2010, Schlosburg, 2010) and FAAH deficient mice display a CB₁ receptor-mediated hypoalgesic phenotype in thermal nociceptive tests, in both phases of the formalin test, thermal anti-hyperalgesic and anti-inflammatory effects in the carrageenan model (except in chronic constriction injury model) (Lichtman, 2004). On the other hand, complete blockade of MAGL leads to tolerance, physical

dependence, impaired endocannabinoid-dependent synaptic plasticity, and CB₁ receptor down-regulation and desensitization in select brain regions (Chanda, 2010, Schlosburg, 2010). However, partial MAGL blockade with low dose JZL184 maintains its antinociceptive actions in the CCI of the sciatic nerve neuropathic pain model (Kinsey, 2013) as well as in formalin-induced and carrageenan-induced inflammatory pain model (Ghosh, 2013, Sciolino, 2011). [3H] SR141716A binding reveals that mice show normal CB₁ receptor expression and function following repeated administration of low dose JZL184 (≤ 8 mg/kg) (Kinsey, 2013). Although, antinociceptive effects of low dose JZL184 are maintained following repeated administration, it produced a partial anti-edematous and anti-allodynic effects in the carrageenan model (Ghosh, 2013). Similarly, the FAAH inhibitor, PF-3845 only partially reduced mechanical allodynia in CCI and carrageenan models (Ghosh, 2013). Thus, it would be advantageous to develop an approach to augment the efficacy of FAAH and MAGL inhibitors in reversing carrageenan-induced allodynia, but without the occurrence of CB₁ receptor functional tolerance, dependence, or cannabimimetic side effects.

DAGL- β is one of the main biosynthetic enzymes for 2-AG. Inhibition of DAGL- β is expected to reduce 2-AG levels with therapeutic application similar to those of cannabinoid antagonists (Di-Marzo et al.,2008) which have been shown to aid in weight loss (Chaput and Tremblay, 2006, Cota, 2006, Di Marzo, 2001, Pagotto, 2006), inflammation in rats (Lu, 2006, McVey, 2003), and Parkinson's symptoms such as dyskinesia (slight tremor of the hands to uncontrollable movement of the upper body but can also be seen in the lower extremities) (Brotchie, 2003, Di Marzo, 2000). Yet much less is known about the physiological effects of disrupting endocannabinoid production *in vivo* due, at least in part, to a lack of selective inhibitors for endocannabinoid biosynthetic enzymes. Two DAGL- β inhibitors, KT109 and

KT172, have been developed recently (Hsu, 2012). These compounds have shown to reduce arachidonic acid and prostaglandin levels parallel with 2-AG in peritoneal macrophages. In addition, these compounds have shown reduce TNF- α level in the macrophages. We have found that these compounds produce anti-allodynic effects in LPS model of inflammatory pain. The in vitro data showing reductions in 2-AG, AA, PGE2 and TNF- α from macrophages (Hsu, 2012) and in vivo data showing the antinociceptive effects in mice, make the upstream enzyme DAGL- β a promising target to for the development of analgesic therapeutics.

Inhibition of FAAH or MAGL reduces carrageenan-induced inflammatory pain

The first goal of this dissertation was to test whether inhibition of FAAH or 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 have employed an inflammatory model of carrageenan-induced allodynia that produces increase responsiveness to non-noxious stimuli and edema, an accumulation of fluid in the paw. The positive control diclofenac, a classical NSAID, attenuated edema as well as paw withdrawal thresholds, demonstrating that this model can be used to detect drugs that produce anti-edematous and anti-allodynic properties. After establishing an inflammatory pain model, we tested selective the MAGL inhibitor JZL-184 and FAAH inhibitor PF-3845. We hypothesized that elevating endogenous cannabinoids following inhibition of their appropriate catabolic enzymes would inhibit carrageenan-induced edema and allodynia. Inhibition of these respective enzymes partially attenuated edema and allodynia. These findings are in line with previous literature showing that irreversible (PF-3845, URB597) as well as reversible inhibitors of FAAH elevate anandamide levels 8 to 10 fold in the brain (Ahn, 2009, Fegley, 2005). Importantly, PF-3845 is highly selective for FAAH, unlike URB597 that also binds to other serine hydrolases in peripheral tissues, including several

carboxylesterases (Alexander and Cravatt, 2005, Lichtman, 2004, Zhang et al., 2007). Less off target effects make PF-3845 a better therapeutic compound than URB597. Acute treatment with PF-3845 results in FAAH inhibition up to 24 h and maximal anandamide elevation up to 12 h in mice. In pharmacological assays, PF-3845 has analgesic effects in the LPS, CCI, and acetic acid stretching models of pain as well as anxiolytic-like effects/ digging behaviors in marble-burying assay (Booker, 2011, Kinsey, 2010, Kinsey, 2011a). Similarly, the MAGL inhibitor JZL184 reliably reduces nociceptive behavior in inflammatory and neuropathic models of pain (Booker, 2011, Ghosh, 2013, Kinsey, 2009), blocks NSAID-induced gastric inflammation (Kinsey et al., 2011b), and attenuates anxiety-like behaviors (Kinsey et al., 2011c, Sciolino, 2011).

Having established that inhibitors of FAAH and MAGL produce anti-allodynic and anti-edematous effects, we tested cannabinoid receptor involvement in mediating these effects of JZL-184 and PF-3845. Our pharmacological (inhibitors) as well as genetic (CB₁ and CB₂ knockout) data indicated that anti-edematous effects are mediated by CB₂ receptors and anti-allodynic effects require both CB₁ and CB₂ receptors. It was interesting that the combination mediates its anti-allodynic effects via CB₁ and CB₂ receptors. Previously, PF-3845 has been shown to mediate its anti-allodynic effects via both cannabinoid receptors in the LPS model (Booker., 2011) and JZL184 has been shown to mediate its anti-allodynic effects predominantly via CB₁ receptors in acetic acid-induced abdominal stretching, tail withdrawal assay, CCI (Kinsey, 2009, Long, 2009a). In our studies, either rimonabant or SR144528 have completely blocked anti-allodynic effects and several explanations may account for this observation. As shown in Figure 28, one possibility is that administration of JZL184 or PF-3845 elevates endocannabinoid levels in the CNS as well as in the periphery. The antinociceptive effects of these endocannabinoids require activation of CB₂ present in peripheral tissues (Hohmann and

Herkenham, 1999) and CB₁ present on CNS and PNS regions associated with pain. CB₂ receptors on immune cells, triggered by endogenous cannabinoids, may suppress inflammatory responses in the peripheral tissues such as paw or dorsal root ganglion (DRG). Indeed, CB₂ receptors are expressed on activated mast cells (Facci et al., 1995), which infiltrate peripheral nerve tissues during an innate inflammatory response, such as that initiated by carrageenan. On the other hand, elevated endocannabinoid levels in brain regions such as periaqueductal gray (PAG), rostral ventromedial medulla (RVM), associated with pain activate the CB₁ receptors to activate descending pain inhibitory mechanisms. *In situ* hybridization showing CB₁ receptor expression on dorsal root ganglia nerve terminals and CNS regions associated with pain such as the dorsal horn of the spinal cord and PAG (Hohmann and Herkenham, 1999) supports this idea. Moreover, 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. These enzyme inhibitors prevent the rapid degradation of endocannabinoids, which allow prolonged action at these sites.

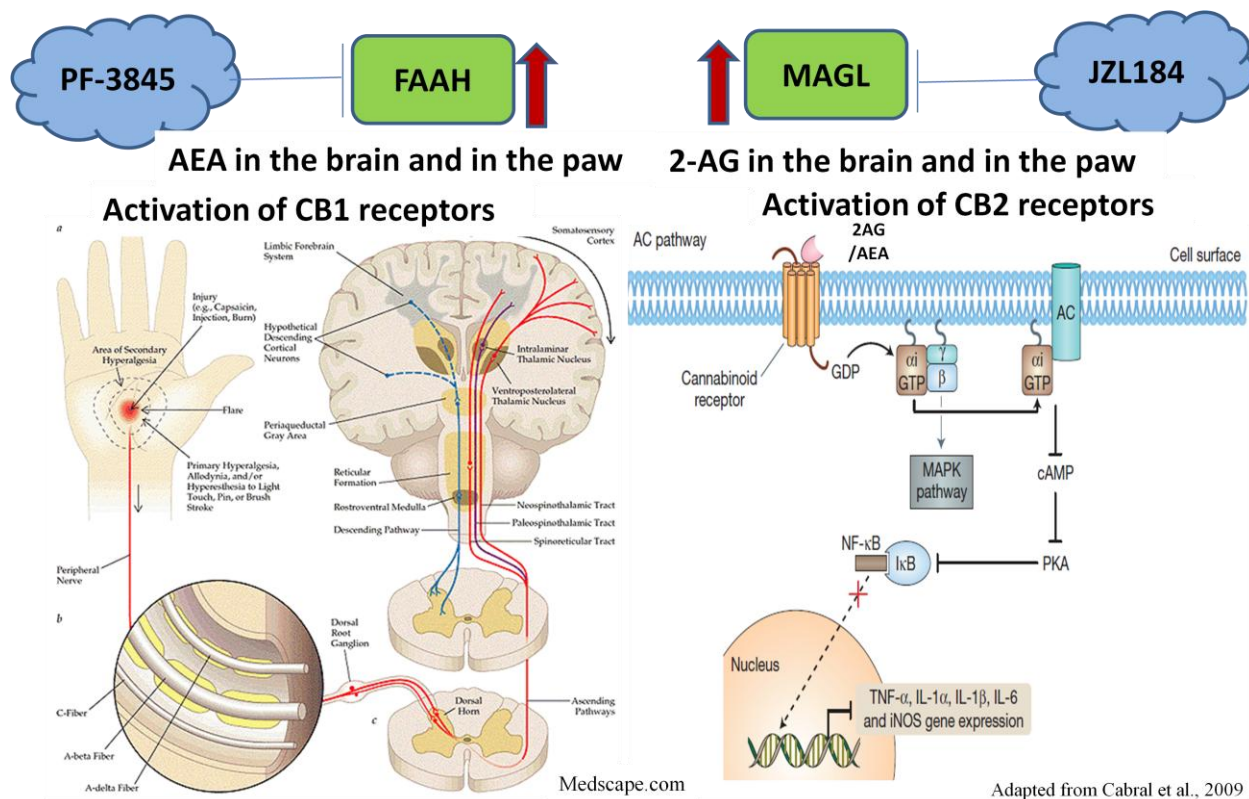


Figure: 29 proposed mechanism of action for anti-allodynic effects of MAGL and FAAH inhibitors.

JZL184 and PF-3845 inhibit MAGL and FAAH and elevate 2-AG and anandamide level respectively. Endocannabinoids are released on demand in regions associated with pain but are rapidly degraded. These enzyme inhibitors prevent endocannabinoid degradation and thereby reduce nociception by stimulation CB₂ receptors in the immune cells (neutrophils, macrophages) and CB₁ receptors in the dorsal horn of the spinal cord, and in the brain (PAG, RVM).

Simultaneous partial MAGL and complete FAAH inhibition reducing carrageenan-induced inflammatory pain

Another major observation of this dissertation (see Chapter 3) is that partial inhibition of MAGL combined with full inhibition of FAAH produced enhanced anti-allodynic effects. Two

pharmacological approaches were used to test the impact of dual FAAH and MAGL blockade in the carrageenan model. First, we have used combined administration of 4 mg/kg JZL184 and 10 mg/kg PF-3845. This dose of JZL184 partially blocked MAGL and elevated 3-4 fold 2-AG level in brain (Chapter 3A Figure 9). This dose of PF-3845 fully blocked FAAH and increased AEA brain levels 8-10 fold. In the second approach, we used a novel inhibitor, SA-57 that is 25 fold more potent in inhibiting FAAH than MAGL (Niphakis, 2012). Both JZL184 (4 mg/kg) + PF-3845 (10 mg/kg) and SA-57 have produced enhanced anti-allodynic effects compared with each enzyme inhibition. On the other hand, these inhibitors produced a relatively small magnitude of anti-edematous effects produced by intraplantar carrageenan.

As reported earlier, prolonged blockade of MAGL and FAAH produced differential analgesic tolerance. MAGL inhibition over six days with repeated administration of high dose JZL184 produced tolerance to its analgesic effects. PF-3845, on the other hand, maintained its analgesic effects upon repeated administration in the CCI model (Schlosburg, 2010). Hence, in the present study, we selected a dose of JZL184 that partially inhibited MAGL and a dose of PF-3845 that completely inhibited FAAH. It has been reported that JZL195, a dual MAGL-FAAH inhibitor, that equipotently raises both 2-AG and anandamide levels, produces greater antinociceptive responses in tail immersion assay and in acetic acid abdominal stretching test of visceral pain than inhibitors of either FAAH or MAGL alone (Long, 2009b). However, this dual inhibitor also produced cannabimimetic activity similar to THC and other exogenously administered cannabinoids, such as spontaneous locomotor suppression and catalepsy (Long, 2009b).

To the contrary, partial blockade of MAGL combined with complete blockade of FAAH in the present study did not produce psychomimetic effects as assessed in the tetrad assay

(Chapter 4, Table 4). Similarly, SA-57 did not produce effects on tetrad at a physiologically active dose (5 mg/kg that produces anti-allodynic and anti-edematous effects) except mild hypolocomotion. Importantly, this combination when given repeatedly did not produce tolerance to its anti-edematous or anti-allodynic effects. These findings consistent with previous findings showing that repeated administration of low doses of JZL184 (4 and 8 mg/kg) maintain anti-allodynic effects in the carrageenan and CCI pain model (Ghosh, 2013, Kinsey, 2013) and also maintain anxiolytic-like effects in rats (Sciolino et al., 2011). Additionally, it has been reported that chronic exposure to a high dose of the FAAH inhibitor PF-3845 did not produce tolerance to its antinociceptive effects in the acute thermal tail-withdrawal test or the chronic constrictive injury of the sciatic nerve (CCI) model (Schlosburg, 2010). As tolerance produced by complete and prolonged MAGL inhibition with high dose of JZL184 may represent a drawback, the aforementioned results suggest that the partial blockade of MAGL and complete blockade of FAAH can be used as a tool to increase efficacy and circumvent tolerance. The observations that combined inhibition of FAAH and MAGL is more effective compared to inhibiting each enzyme alone and the prolong inhibition of partial MAGL and complete FAAH maintain the anti-allodynic and anti-edematous effects in the carrageenan model, suggest that this strategy can serve as a powerful pharmacological approach to treat inflammatory pain. To test this idea, a novel inhibitor SA-57, which has differential potency at MAGL and FAAH (Niphakis, 2012), was characterized in vivo. The other major finding of Chapter 3 is that SA-57 completely reversed carrageenan-induced allodynia, though produced partial anti-edematous effects in mice similar to single inhibition of these endocannabinoid catabolic enzymes. Similarly, SA-57 significantly blocked all spontaneous withdrawal signs in morphine-dependent mice compared with single inhibition of MAGL or FAAH (Ramesh et al., 2013). Although SA-57 produced

antinociceptive effects in the carrageenan assay and blocked spontaneous withdrawal in morphine-dependant mice, at higher doses (12.5 and 5 mg/kg), it produced a subset of cannabimimetic effects in the tetrad assay. It will be important to have a separation of dose ranges for the antinociceptive and anti-edematous effects and cannabimimetic effects. In addition, the effects of repeated administration of SA-57 have not been evaluated and should be assessed in future studies. An alternative approach in the future would be to examine whether repeated administration of a dose, which is inactive upon acute administration, produces antinociceptive effects following repeated dosing.

Why are inhibiting MAGL and FAAH in combination more efficacious in reducing inflammatory nociception than blocking these enzymes individually? A possible explanation is that MAGL and FAAH inhibition elevate anandamide and 2-AG, which not only activate CB₁ and CB₂ receptors but also activate non-cannabinoid receptor targets. In particular, MAGL has a major role in the biosynthesis of free arachidonic acid in brain from its precursor, 2-AG (Nomura, 2011). In addition, FAAH metabolizes other bioactive fatty acid amides, including N-palmitoylethanolamine, N-oleoylethanolamine, and oleamide (Cravatt, 2001, Cravatt, 1996), as well as N-acyl taurines (Fattore, 2005, Leung, 2006). These different lipids activate various transient receptor potential cation (TRPV) channels and peroxisome proliferator-activated receptors (PPAR- α) receptors (De Petrocellis and Di Marzo, 2010, Di Marzo and De Petrocellis, 2010, Jhaveri, 2006). Thus, combined blockade of FAAH and MAGL triggers multiple neurochemical alterations that could affect the magnitude of reducing inflammatory pain. Another explanation is that dual enzyme inhibition produces enhanced anti-allodynic effects by merely increasing total endocannabinoid brain levels to stimulate cannabinoid receptors. However, the fact that 2-AG levels in brain are at least 200-fold higher than anandamide brain

levels (Ahn, 2009, Long, 2009a) tends to argue against this idea of mass action. Alternatively, it is plausible that simultaneous inhibition of MAGL and FAAH may produce augmented effects by activating distinct cannabinoid receptor circuits in CNS and PNS associated with pain and antinociception (PAG, RVM, dorsal horn of spinal cord), as well as in immune cells. Consistent with this idea is that FAAH is predominantly expressed on post-synaptic terminals (Gulyas, 2004) and MAGL is expressed on presynaptic terminals (Dinh, 2002). Also, these enzymes may be expressed in different neuronal circuits which can result in augmented anti-allodynic effects by interactions between two separate synapses i.e., via crosstalk between distinct anandamide and 2-AG-regulated neuronal circuits. Regardless of the precise mechanism for the augmented antinociceptive effects upon dual FAAH and MAGL inhibition, our findings of Chapter 3 taken together indicate that this approach offers a promising strategy to treat inflammatory pain.

Inhibition of DAGL- β , a major biosynthetic enzyme of 2-AG

It has been well established that FAAH and MAGL are the primary hydrolytic enzymes of anandamide and 2-AG (Cravatt, 1996, Dinh, 2004, Dinh, 2002), both of which activate CB₁ and CB₂ receptors. The final goal of this dissertation was to assess the effects of blocking DAGL- β , one of the two major enzymes responsible for 2-AG synthesis. KT-109 represents the first selective DAGL- β inhibitor and decreases 2-AG levels in peritoneal macrophages and in Nero 2A cells (Hsu., 2012). This compound reduces the level of 2-arachidonyl glycerol and the downstream lipids AA and prostaglandins in macrophages as well as in Neuro 2A cells. Concomitant with these changes in lipid metabolism, DAGL- β inhibitors significantly reduced the LPS-stimulated production of the pro-inflammatory cytokine TNF- α (Hsu., 2012). The suppression of TNF- α production by selective pharmacologic or genetic disruption of DAGL- β could be due to changes in endocannabinoids, other arachidonic acid-derived metabolites that

are not biosynthesized by COX enzymes, such as leukotriene B4 (LTB₄), or a partial versus a complete reduction in prostaglandins. The results presented in Chapter 4 demonstrate that DAGL- β inhibition produces antinociceptive effects in the LPS model of inflammatory pain. Specifically, KT-109 reversed LPS-induced mechanical allodynia in mice. These data are corroborated by the complementary genetic approach in which DAGL- β (-/-) mice treated displays an anti-allodynic phenotype. In addition, KT-109 did not elicit any further decrease in allodynia in DAGL- β (-/-) mice. This finding indicates that KT-109 most likely mediates its effects through DAGL- β enzyme. The anti-allodynic effects of KT109 were independent of both CB₁ and CB₂ receptors. Importantly, KT-109 did not produce cannabimimetic effects in the tetrad assay. In addition, repeated administration of high dose KT-109 did not undergo tolerance to its anti-allodynic effects.

Finally, unlike the NSAID diclofenac, administration of high dose of KT-109 did not elicit gastric ulcers. The mechanism by which KT-109 reversed inflammatory pain is yet to be elucidated. As depicted in figure 30 the plausible mode of action can be the reduction in arachidonic acid, which serves as the precursor of prostaglandins, leukotriene, and other related lipids (Kite., 1983). Accordingly, LPS is predicted to increase prostaglandin levels through induction of COX2 activity and KT-109 is hypothesized to reduce the available pool of free arachidonic acid in the paw and hence reduce prostaglandins and other mediators of inflammation. We will measure 2-AG, AA, and prostaglandin levels in the paw as well as in the spinal cord in future studies. Nevertheless, cyclooxygenase inhibitors or NSAIDs the most widely used medications in reducing pain and inflammation, exert their effects by inhibiting COX-2 which is the enzyme responsible for converting arachidonic acid to prostaglandins.

Prostaglandins are produced in the gastric mucosa where they exert a cytoprotective function. Hence, decrease in prostaglandin cause serious GI adverse events. In our study, we have shown that the DAGL- β inhibitor KT-109, despite reducing prostaglandin does not produce gastric hemorrhage in mice (Nomura, 2011). The in vitro data showing reductions in 2-AG, AA, PGE2 and TNF- α from macrophages ((Hsu, 2012) and in vivo data showing the antinociceptive effects in mice, make the upstream enzyme DAGL- β a promising target to for the development of analgesic therapeutics.

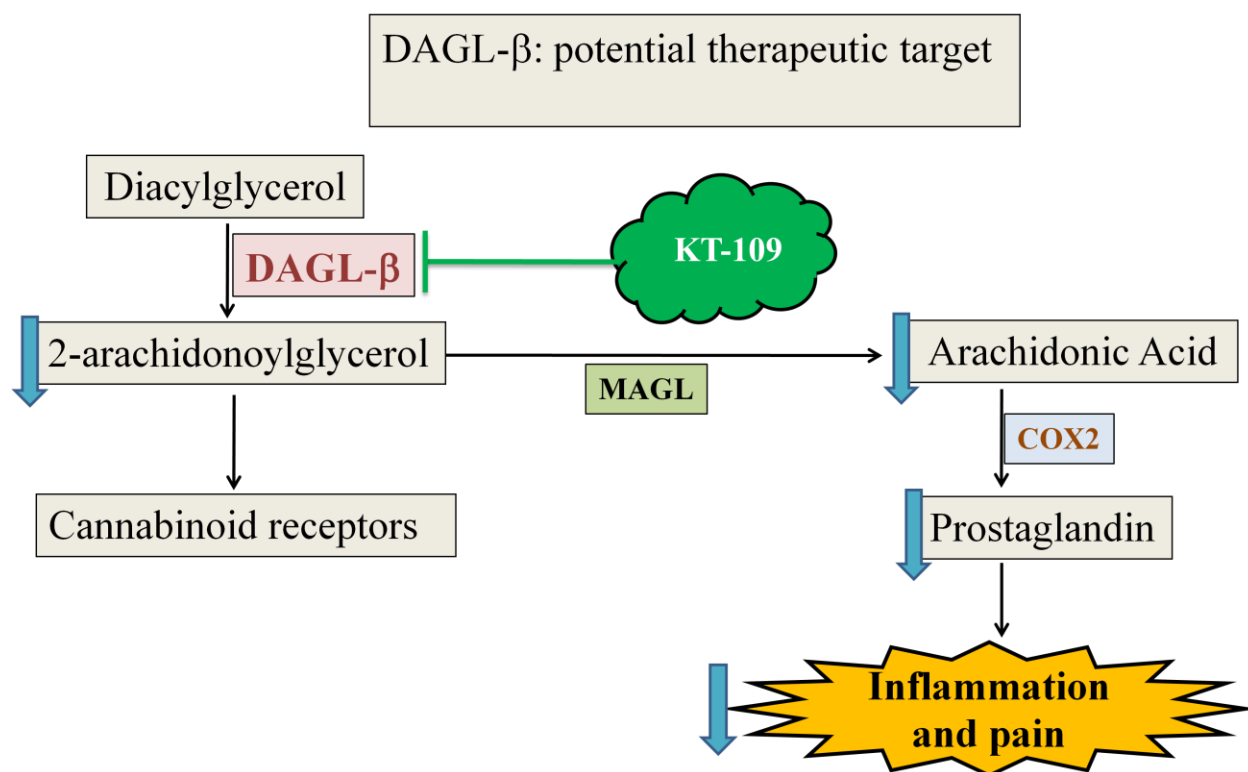


Figure 30: Proposed mechanism of action: Anti-allodynic effects of DAGL- β inhibitor, KT-109. KT019 inhibits DAGL- β and consequently reduces 2-AG production, which in turn leads to a reduction in arachidonic acid. Reduction in arachidonic acid in turn affects the prostaglandin production in the peritoneal macrophages. Although the suppression of TNF- α production by

KT109 or DAGL- β knockout mice is less understood but it could be due to changes in endocannabinoids, other arachidonic acid-derived metabolites such as leukotriene B4 (LTB4), or a partial versus a complete reduction in prostaglandins. Thus, upstream blockade of biosynthetic enzymes regulating AA production leads to a reduction in prostaglandins and other pro-inflammatory mediators underlying inflammatory pain.

Integration of endocannabinoid degradative and biosynthetic enzyme inhibitors

The purpose of this dissertation was to test whether inhibition of enzymes regulating endocannabinoids biosynthesis and degradation would reduce nociceptive behavior in mouse inflammatory pain models. The results reported in this dissertation support the following hypotheses: 1) inhibition of the endocannabinoid catabolic enzymes MAGL and FAAH blocks carrageenan-induced edema and allodynia; and 2) the 2-AG biosynthetic enzyme DAGL- β reverses LPS-induced allodynia. In the first part of the dissertation, we have shown that inhibition of MAGL and FAAH increased 2-AG and anandamide, respectively. These elevated endocannabinoids reduced inflammatory pain through CB₁ and CB₂ receptor mechanisms of action. Hence, elevation of 2-AG and AEA mediate the anti-inflammatory effects through the cannabinoid receptors. The second part of this dissertation shows that inhibition of DAGL- β produces antinociception, despite the fact that it reduces 2-AG levels. Although these two approaches are seemingly in contradiction, DAGL inhibition produces antinociceptive effects through a distinct mechanism related to reductions in arachidonic acid. Arachidonic acid is a major precursor for prostaglandins (Vahouny et al., 1978) and other eicosanoids. Therefore, reduction in arachidonic acid leads to reduction in prostaglandin levels and reduction in prostaglandins play a major role in attenuating inflammatory pain (Ricciotti and FitzGerald,

2011). Another question that arises is that if reduction in arachidonic acid occurs following either MAGL inhibition or DAGL inhibition, why does antagonizing cannabinoid receptors completely block the anti-inflammatory (allodynia and extravasation) JZL184 and PF-3845? A plausible explanation is related to the different magnitudes of reduction of arachidonic acid levels in animals treated with JZL184 + PF-3845 or KT-109. Inhibition of DAGL- β KT109 inhibits arachidonic acid by 90% (Hsu, 2012) but partial MAGL inhibition and complete FAAH inhibition did not reduce arachidonic acid levels in whole brain (Figure 9) upon acute administration. In contrast, repeated administration of low dose JZL184 + high dose PF-3845 reduced arachidonic acid level by 50%. Therefore, a different magnitude of arachidonic acid reduction might be responsible for involving two different mechanisms in attenuating inflammatory pain. However, these results strongly suggest that alteration in 2-AG biosynthesis or degradation play a major role in reducing inflammatory pain (Figure 31) and support our hypothesis that endocannabinoid regulating enzymes represent a promising target to treat inflammatory pain state.

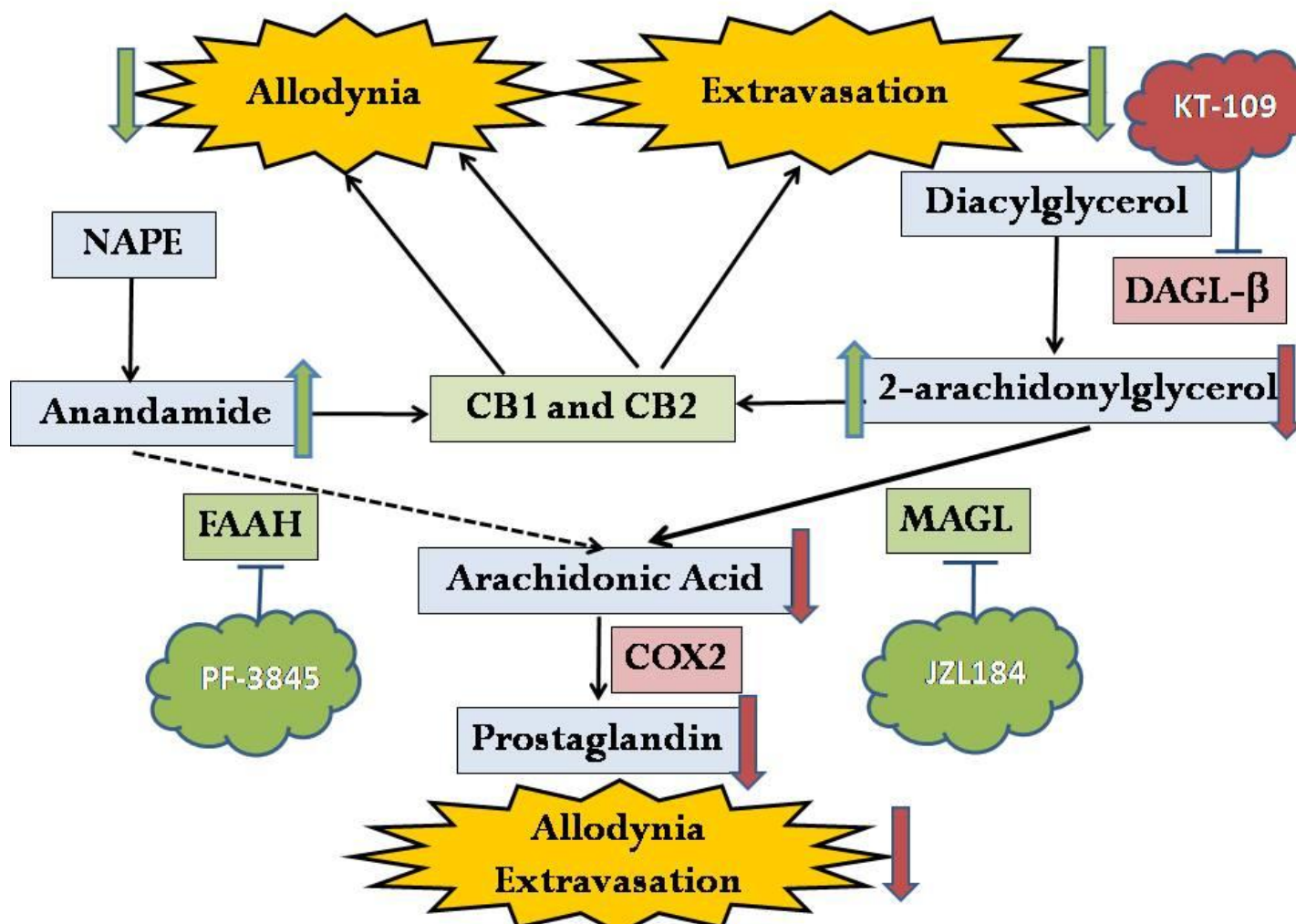


Figure 31: Integration of endocannabinoid degradative and biosynthetic enzyme inhibitors. JZL184 and PF-3845 block MAGL and FAAH respectively. 2-AG and anandamide levels are elevated. They in turn activate the cannabinoid receptors and reduce allodynia and extravasations. On the other hand, KT-109 inhibits DAGL- β , reduces 2-AG level, arachidonic acid level, and prostaglandins level, and as a result reduces allodynia and extravasations.

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