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Targeting the Endocannabinoid System to Reduce Inflammatory Pain

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

By Sudeshna Ghosh

Master of Science, Bangalore University, 2006

VCU PHTX

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2-AG, 2-arachidonoylglycerol;

AEA, arachidonoylethanolamide (anandamide);

ACEA, arachidonyl-2-chlorethylamide;

ANOVA, analysis of variance;

CB_1, cannabinoid receptor type 1;

CB_1KO, cannabinoid receptor type 1 knock out;

CB_2, cannabinoid receptor type 2;

CB_2KO, 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) piperdine-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, Δ9-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-napthalenylmethanone;

WT, wild type;

Abstract

The endogenous cannabinoids (endocannabinoids) anandamide (AEA) and 2-arachidonylglycerol (2-AG) exert their effects predominantly through cannabinoid CB_1 and CB_2 receptors, but these actions are short-lived because of rapid hydrolysis by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively. Selective inhibition of either enzyme elevates CNS levels of the appropriate endocannabinoid and produces analgesic effects with fewer psychomimetic side effects than Δ^9-tetrahydrocannabinol (THC), the primary active constituent of marijuana. While cannabinoid receptor agonists and FAAH inhibitors reliably produce anti-inflammatory and anti-hyperalgesic effects in the carrageenan test and other inflammatory pain models, much less is known about the consequences of inhibiting MAGL in these assays. Here, we tested whether the selective MAGL inhibitor JZL184 would reduce nociceptive behavior in the carrageenan test. JZL184 significantly attenuated carrageenan-induced paw edema and mechanical allodynia, whether administered before or after carrageenan. Complementary genetic and pharmacological approaches revealed that JZL184's anti-allodynic effects required both CB_1 and CB_2 receptors, but only CB_2 receptors mediated its anti-edematous actions. 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. Interestingly, the anti-allodynic effects of the combination of low dose of JZL184 (4mg/kg) and high dose of the selective and long-acting FAAH inhibitor PF-3845 (10 mg/kg) was augmented compared with each drug alone. On the contrary, the combination treatment did not reduce edema more than either JZL184 or PR-3845 given alone. These results suggest that low doses of MAGL inhibitors alone or in combination with FAAH inhibitors, reduce inflammatory nociception through the

activation of both CB_1 and CB_2 receptors with no evidence of tolerance following repeated administration.

Introduction

1. Endocannabinoid system

The endogenous cannabinoid system consists of two G-protein-coupled cannabinoid receptors CB_1 and CB_2 (Matsuda et al. 1990; Gerard et al. 1991), the lipid endogenous ligands N-arachidonoylethanolamine (anandamide or AEA) (Devane et al. 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al. 1995; Sugiura et al. 1995), and the enzymes that synthesize and degrade AEA and 2-AG. CB_1 receptors are heterogeneously distributed in high concentrations throughout the central nervous system (Matsuda et al. 1990; Hohmann and Herkenham 2000) and are believed to mediate marijuana's psychomimetic effects, as well as play a role in modulating nociception. CB_1 receptors are also expressed in the periphery (Felder et al. 2006) where they play a role in energy regulation and nociception. In contrast, CB_2 receptors are expressed predominantly on immune cells (Cabral and Marciano-Cabral 2005) as well as in the CNS on microglial cells (Carlisle et al. 2002; Nunez et al. 2004) and in some cortical neurons, pyramidal neurons of hippocampal allocortex, and interneurons of the stratum oriens and stratum radiatum (Van Sickle 2005; Onaivi et al. 2006). Stimulation of these receptors is believed to reduce inflammatory responses. CB_1 and CB_2 receptors share approximately 44% homology with each other (Munro et al. 1993) and are coupled with Gi/o proteins (Howlett et al. 2002). Activation of these receptors decrease cAMP production via blockade of adenylyl cyclase (Howlett et al. 1990), facilitates phosphorylation of p42/p44 mitogen-activated protein kinase (MAPK) (Bouaboula et al. 1995; Derkinderen et al. 2001; Galve-Roperh et al. 2002), stimulates inward rectifying K+ (GIRK) channels via Gβγ subunits (Mackie et al. 1995; Vasquez et al. 2003), inhibits Na+ channels (Nicholson et al. 2003), stimulates phospholipases C and A2 (PLC, PLA2) (Hunter et al. 1986), activates c-Jun N-terminal kinase (JNK) and p38 kinase (Rueda et

al. 2002). Furthermore activation of the cannabinoid receptors inhibit N- and P/Q-type calcium channels, which reduces synaptic vesicle fusion to the nerve terminal, thereby inhibiting the release of excitatory and inhibitory neurotransmitters. The consequence of this effect leads to a decrease in post-synaptic depolarization (Howlett 2002).

2-AG and anandamide are produced and released on demand (Ahn et al. 2008). Different enzymes are responsible for the synthesis of 2-AG and anandamide. Although the enzymes regulating AEA biosynthesis remain under investigation, the most accepted pathway of its synthesis is via hydrolysis of N-acyl phosphatidylethanolamine or NAPE by NAPE-phospholipase D. However, a previous study (Leung et al. 2006) showed that NAPE-PLD knockout mice still possess wild-type levels of AEA, which suggests that this enzyme is not necessary for AEA synthesis. Alternatively, it has been shown that AEA is synthesized via phophodiesterase or phopholipase C cleavage (Liu et al. 2006). On the other hand, 2-AG is synthesized by the cleavage of diacylglycerol (DAG) by DAG lipase-alpha (DAGLα) (Gao et al. 2010; Tanimura et al. 2010).

Both AEA and 2-AG are promptly metabolized by their degradative enzymes, fatty acid amide hydrolase (FAAH) (Cravatt et al. 1996; Cravatt et al. 2001) and monoacylglycerol lipase (MAGL) (Dinh 2004; Blankman et al. 2007), respectively (Figure 1). FAAH is located in the post synaptic terminal and is responsible for the degradation of other amides such as palmitoylethanolamide (PEA), oleoylethanolamide (OEA) and oleamide (Cravatt et al. 1995). In contrast, 2-AG is degraded in the presynaptic terminal where MAGL is located (Figure 1). Two other enzymes, alpha/beta hydrolase 6 and 12 (ABHD) account for approximately 15% of 2-AG degradation (Blankman et al. 2007). Blockade of FAAH and MAGL leads to the increase of AEA and 2- AG in brain and in other tissues (Long et al. 2009a).

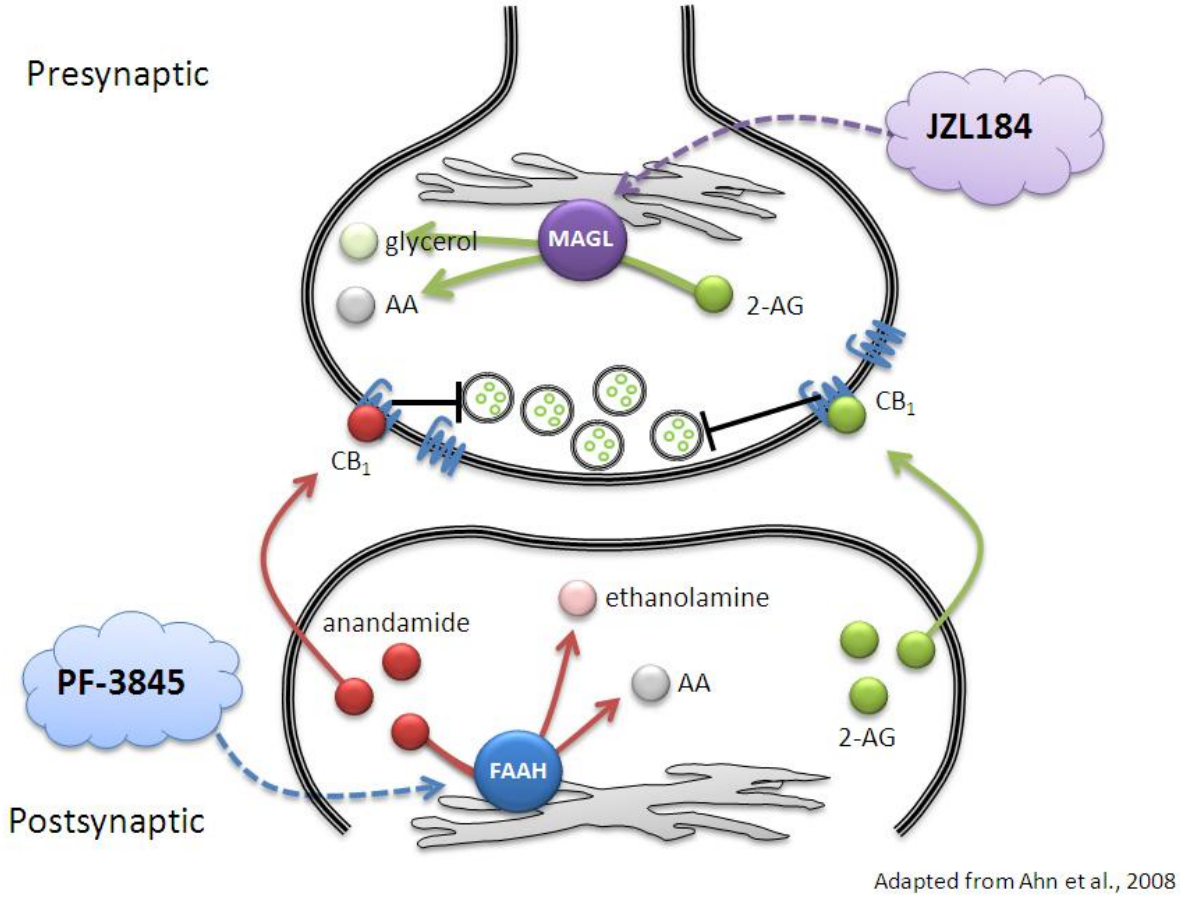

Figure 1: The endocannabinoid system (CNS)

Cannabinoid receptors, AEA, 2-AG and their regulatory pathways. JZL184 and PF-3845 are selective inhibitors of MAGL and FAAH which degrade 2-AG and AEA respectively(Ahn et al. 2008).

2. Carrageenan Assay

Carrageenan-induced inflammatory pain is one of the most common preclinical models of pain. Carrageenan is a family of linear sulphated polysaccharides extracted from red seaweeds. There are three main types of carrageenan; lambda, kappa, and iota. The lambda carrageenan can be administered by injection to induce an inflammatory response. Intraplanter administration of carrageenan produces edema, which is defined as abnormal accumulation of fluid that produces swelling. Carrageenan also induces allodynia, which is a painful response to non painful stimuli and hyperalgesia, an increased sensitivity to pain. In our project we have administered intraplanter carrageenan to induce inflammatory pain in the murine model.

3. Direct acting cannabinoids on edema and allodynia

Administration of synthetic cannabinoid receptor agonists (i.e. WIN 55-212-2 and HU210) produce analgesia and reduce edema in the carrageenan-induced inflammatory pain model (Nackley et al. 2003a; Elmes et al. 2005; Wise et al. 2008). Local administration of the selective CB_1 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 psychomimetic side effects and abuse potential have dampened enthusiasm for developing drugs that act directly at CB_1 receptors. On the other hand, the CB_2 receptor agonist AM1241 inhibits tactile hypersensitivity to carrageenan in rats (Nackley et al. 2004), which indicates that targeting CB_2 receptors is a viable alternative to CB_1 agonists. But at the same time these exogenously administered synthetic cannabinoids and THC cause a persistent inhibition of neurotransmitter release, and do not mimic the localized and transient effects seen with

endocannabinoids (Vaughan and Christie 2005). Thus, exploration of the other targets such as the catabolic enzymes of the endocannabinoid system may possess promise in the clinical setting with decreased subset of marijuana-like side effects.

4. Role of FAAH inhibition on antinociceptive effects

The bulk of research examining the role of endocannabinoid catabolic enzymes in nociception has focused on FAAH (Suplita et al. 2005; Chang et al. 2006; Jayamanne et al. 2006; Naidu et al. 2008; Naidu et al. 2009; Clapper et al. 2010; Naidu et al. 2010; Booker et al. 2011; Kinsey et al. 2011) 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 AEA levels in the brain (Boger et al. 2005; Fegley et al. 2005; Ahn et al. 2009) and produce analgesia in a wide variety of animal models of pain, as described below (Schlosburg et al. 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-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 et al. 2006), chronic constriction injury (CCI) model (Kinsey et al. 2009) and acetic acid (Naidu et al. 2009), hot-plate, tail-immersion, formalin (Lichtman et al. 2004) and lipopolysaccharide-induced (LPS) (Booker et al. 2011) models of pain. The long-lasting FAAH inhibitor PF-3845 produced anti-allodynic effects in the CFA model (Ahn et al.

2009) and partially suppressed the hyperalgesia in the LPS mouse model of inflammatory pain (Booker et al. 2011).

In contrast to the extensive amount of research investigating the consequences of FAAH inhibition on nociceptive behavior, few studies have evaluated the consequences of a MAGL inhibitor due to unavailability of selective MAGL inhibitors. Initial studies investigating the *in vivo* consequences of inhibiting MAGL employed URB602 (Hohmann et al. 2005; Comelli et al. 2007; Guindon et al. 2007a; Guindon et al. 2007b; Vandevoorde et al. 2007; Desroches et al. 2008; Guindon and Hohmann 2008; Guindon et al. 2011) and *N*-arachidonyl maleimide (Burston et al. 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 et al. 2007; Desroches et al. 2008; Guindon et al. 2011) Although URB602 and *N*-arachidonyl maleimide inhibit MAGL in the brain, these compound are nonselective and inhibit other serine hydrolases, including FAAH (Hohmann et al. 2005; Vandevoorde et al. 2007; Burston et al. 2008).Thus, it is unclear whether antinociceptive effects of these nonselective MAGL inhibits are mediated through the inhibition of MAGL, inhibition of other enzymes (e.g., FAAH), or a combination of inhibition 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). Systemic administration of JZL184 reduces nociceptive behaviors in the warm water tail withdrawal, formalin and acetic acid stretching tests (Long et al. 2009a; Busquets-Garcia et al. 2011) and also reduces mechanical and cold allodynia in the chronic constriction injury (CCI) model of neuropathic pain in mice (Kinsey et al. 2009). Intraplantar injection of JZL184 reduces

nociceptive behaviors in the formalin test (Guindon et al. 2011), capsaicin-induced nociceptive behavior and thermal hyperalgesia (Spradley et al. 2010). Although the findings described above indicate that MAGL inhibition produces antinociceptive effects in multiple pain models, the effects of JZL184 are yet to be evaluated in a prolonged model of inflammatory nociception. Thus, in the present study we have tested the role of JZL184 in the carrageenan model of inflammatory pain.

Rationale and Hypothesis

The overall goal of this thesis was to investigate the role of the primary endocannabinoid degradative enzymes in carrageenan-induced inflammatory pain. We hypothesized that targeting FAAH and MAGL individually or in combination will suppress edema and allodynia in the carrageenan model through the activation of cannabinoid receptors.

Compound selection

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 agent 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 et al. 2009), LPS (Booker et al. 2011), and CCI pain models (Kinsey et al. 2009; Kinsey et al. 2010). 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 CB_1 and CB_2 receptors to JZL184-induced decreases in nociception and paw edema.CB_1 receptor antagonist rimonabant and CB_2 receptor antagonist SR144528 were used as pharmacological tools and CB_1 and CB_2 knock out (-/-) animals were used as genetic tools to elucidate receptor mechanism of action. On one hand, repeated administration of high dose JZL184 (40mg/kg) or genetic deletion of MAGL results in CB_1 receptor downregulation and desensitization as well as CB_1 functional tolerance (Chanda et al. 2010; Schlosburg et al. 2010). But on the other hand, repeated administration of low dose (8 mg/kg) of JZL184 maintained its anxiolytic-like effects under high illumination conditions in the rat elevated plus maze assay (Sciolino et al. 2011). Therefore, we tested whether the anti-edematous and anti-allodynic responses elicited by JZL184 in the carrageenan model were retained after repeated administration of low and high doses of JZL184. In addition, we tested whether systemic administration of JZL184 after intraplantar carrageenan injections reverses edema and allodynia in order to determine if this compound possesses efficacy to treat nociceptive behavior and edema following an inflammatory insult. The available literature

revealed that prolonged blockade of MAGL and FAAH causes differential analgesic tolerance. Inactivation of MAGL for six days via administration of high dose JZL184 (40 mg/kg) results in the loss of analgesic responses in the CCI model and cross-tolerance to the pharmacological effects of exogenous cannabinoid receptor agonists (i.e., THC, and WIN55,212-2) (Chanda et al. 2010; Schlosburg et al. 2010). In contrast, mice treated repeatedly with PF-3845 maintained hypoalgesic and anti-allodynic effects in the CCI model of pain (Schlosburg et al. 2010). Similarly, it has been shown that anxiolytic effects of JZL184 were maintained following repeated administration of 8mg/kg JZL184 in rats (Sciolino et al. 2011). Based on these findings we hypothesized that combination of partial blockade of MAGL and full blockade of FAAH would augment the anti-allodynic and anti-edematous effects of each drug individually. Hence, we investigated the consequences of low dose of JZL184 and high dose of PF-3845 in the carrageenan model to determine whether the combination is more efficacious than each inhibitor alone.

Materials and Methods

1. Subjects

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) as well as male and female CB_1 (-/-) and CB_2 (-/-) mice and their respective littermate controls, CB_1 (+/+) and CB_2 (+/+) mice from the Center Transgenic Colony at Virginia Commonwealth University served as subjects. CB_1 (-/-) and CB_2 (-/-) 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 the tests were completed, all mice were humanely euthanized via CO_2 asphyxia, followed by rapid cervical dislocation.

2. Drugs

JZL184 and PF-3845 were synthesized as described previously (Ahn et al. 2009; Long et al. 2009a) by Organix, Inc. (Woburn, MA). JZL184, PF-3845, CB_1 receptor antagonist rimonabant (SR141716, National Institute on Drug Abuse) and CB_2 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 cyclooxygenase inhibitor diclofenac (DIC;Tocris, Ellisville, MO) was dissolved in saline. Each drug was given via the i.p. route administration in a volume of 10 µl/g body weight.

3. Induction of Paw Edema with Carrageenan

Edema was induced by administration of 0.3% carrageenan (Sigma, St Louis) in a 20 ul volume using a 30 gauge needle in the hind left paw of the mice. 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. 2008). This procedure has been used previously by our laboratory (Cravatt et al. 2004; Lichtman et al. 2004; Wise et al. 2008).

4. Mechanical Allodynia

Intraplanter carrageenan injection led to the induction of mechanical allodynia in mice. Then 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) after 5 h of 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.

5. Testing Procedure

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 and sustained for 5 h. 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 pretreatment 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), 2 h for JZL184 (1.6, 4, 16, or 40 mg/kg) and 2 h for the combination of JZL184 (4 mg/kg) and PF-3845 (10 mg/kg).

In experiments assessing cannabinoid receptor mechanism of action, the CB_1 receptor antagonist rimonabant (1 or 3 mg/kg) and the CB_2 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 1997; Lichtman et al. 2004) and SR144528 (Conti et al. 2002; Malan et al. 2002; Lichtman et al. 2004) block the pharmacological effects of cannabinoid receptor agonists. The anti-edematous and anti-allodynic effects of 16 mg/kg JZL184 were evaluated in CB_1 (+/+), CB_1 (-/-), CB_2 (+/+) and CB_2 (-/-) mice to assess further receptor involvement. In addition, we assessed if administration of the antagonists after carrageenan would reverse the anti-edematous

and anti-allodyic effects of JZL184. In this experiment, rimonabant (1 mg/kg) or SR144528 (3 mg/kg) was administered 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 JZL-184 on day 6, and (Groups 6-9) 1.6, 4, 16 or 40 mg/kg JZL-184 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 reversal study, JZL184 (16 mg/kg) was administered 3 h after carrageenan to examine whether carrageenan-induced edema and allodynia would be reversed at 5 h. Finally, like all other studies, the combination of low dose of JZL184 (4mg/kg) and high dose PF-3845 (10mg/kg) was administered systemically 2 h before carrageenan. Edema was tested prior to and 5 h after carrageenan and allodynia was assessed at the 5 h time point. Similarly, to assess the effects of repeated administration of the combination on the anti-edematous and anti-allodynic effects, Group 1 received vehicle continuously for 6 days. Group 2 was given vehicle from 1 to 5 days and was challenged with the combination (JZL184, 4 mg/kg and PF-3845,10 mg/kg) on the 6^{th} day. Group 3 received the combination (JZL184, 4 mg/kg and PF-3845,10 mg/kg) for all 6 days, and on the 6^{th} day all the groups received carrageenan 2 h after the drug or vehicle. Edema was tested prior to and 5 h after carrageenan injection and allodynia was assessed 5 h after carrageenan injection.

6. Data Analysis

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 mean (SEM). Data were analyzed using t-tests, one-way 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.

Result: Effects of MAGL and FAAH Inhibition in the carrageenan model of inflammatory pain

1. Induction of Edema and allodynia by carrageenan

Intraplantar administration of carrageenan induced paw edema and allodynia over an extended time period. 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 [$p=0.09$].

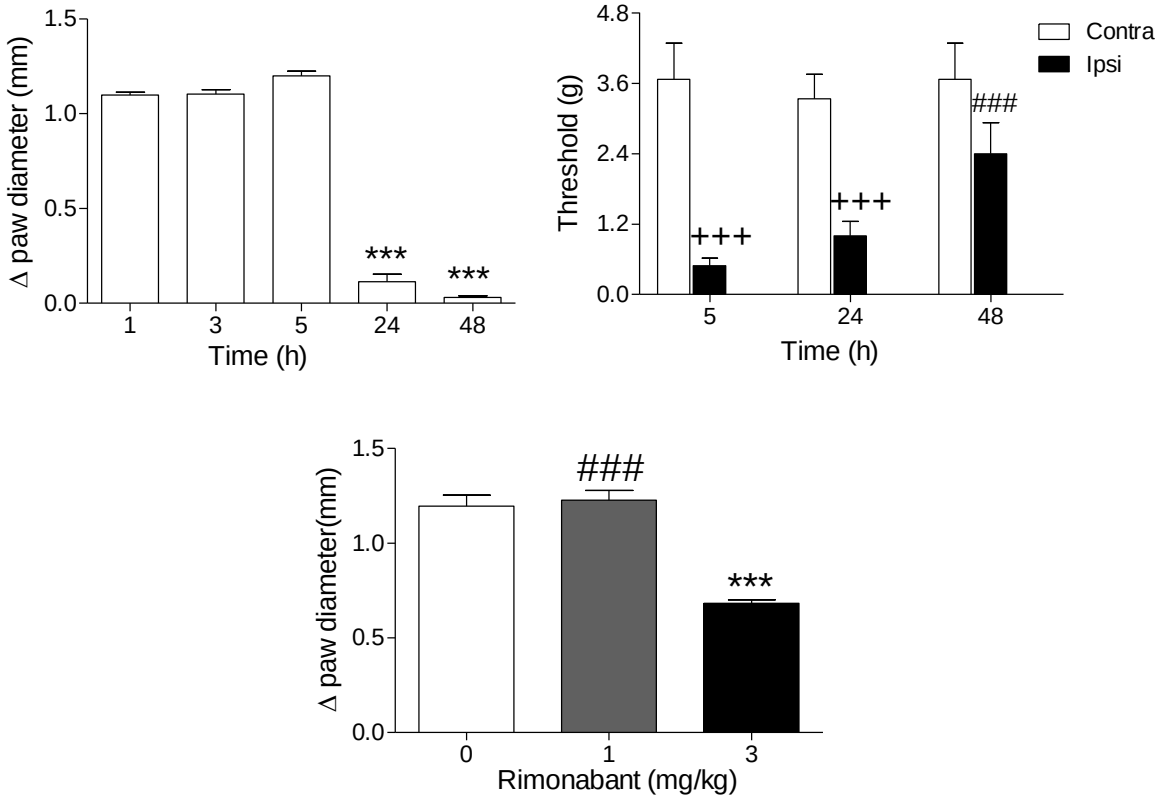

Figure 2: Time course for the edematous and allodynic effects of carrageenan.

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 (± SEM) mechanical paw withdrawal threshold and difference in paw thickness. N=6 /group. **Panel C** showed that CB_1 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.

2. 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 period (Figure 3). In contrast, the withdrawal threshold for the control paw remained constant throughout all of the studies (Table 1). 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 1).

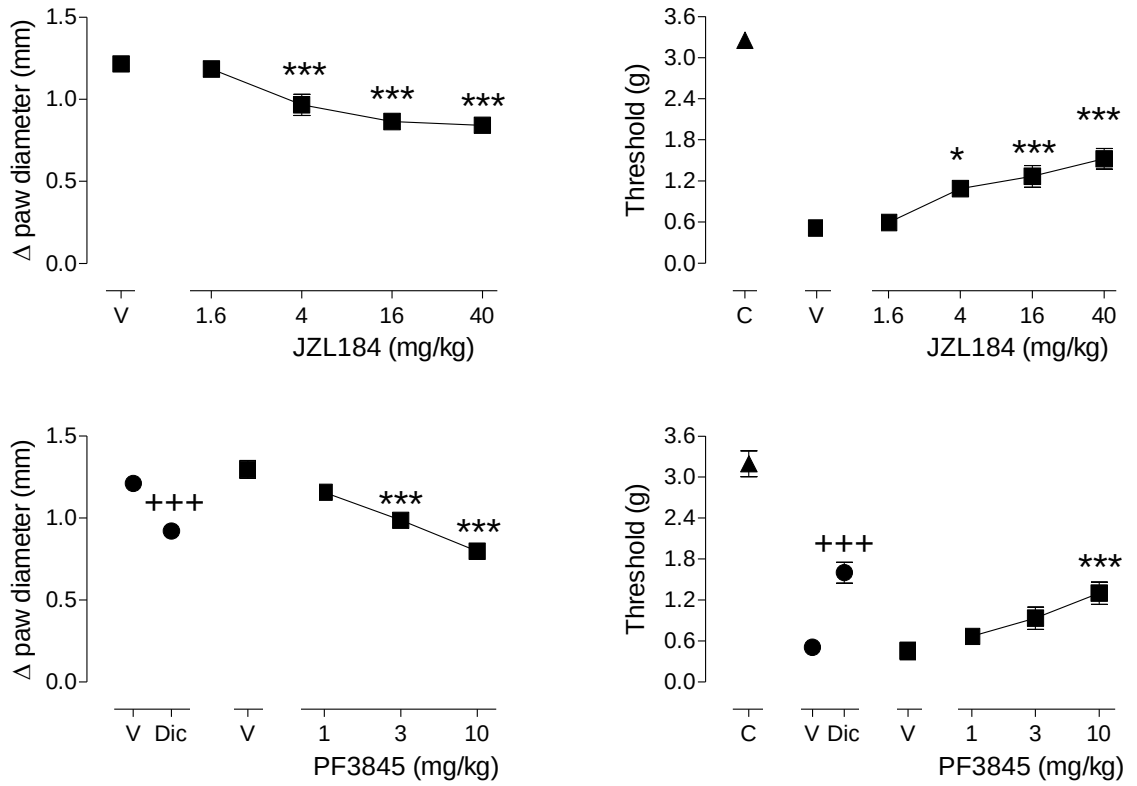

Figure 3: JZL184, PF3845, and diclofenac (5 mg/kg) partially reduced edema and allodynia in the carrageenan model.

JZL184 significantly reduced carrageenan-induced paw edema **(Panel A)** and allodynia **(Panel B).** Diclofenac (Dic) and PF-3845, shown on the same graph and both significantly reduced edema **(Panel C)**. Diclofenac (Dic) and PF-3845, shown on the same graph and both of them partially reduced allodynic **(Panel D)**. Values represented the mean (± 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.

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

To determine whether CB_1 and CB_2 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 CB_1 and CB_2 receptors. Neither rimonabant nor SR144528 significantly affected allodynia when given alone. CB_1 (-/-) [$F(1, 19)= 5.73$, $p<0.001$](Figure 4C) and CB_2 (-/-) [$F(1, 24)= 8.74$, $p<0.01$](Figure 4D) mice were resistant to the anti-allodynic effects of JZL184. In the absence of drugs, both CB_1 (-/-) and CB_2 (-/-) 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 CB_2 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 SR145528 [$F(1, 20) = 8.73$, $p<0.001$] (Figure 5B). Consistent with the cannabinoid receptor antagonist data, JZL184 retained its anti-edematous effects in CB_1 (-/-) mice [$p= 0.84$] (Figure 5C), but CB_2 (-/-) 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).

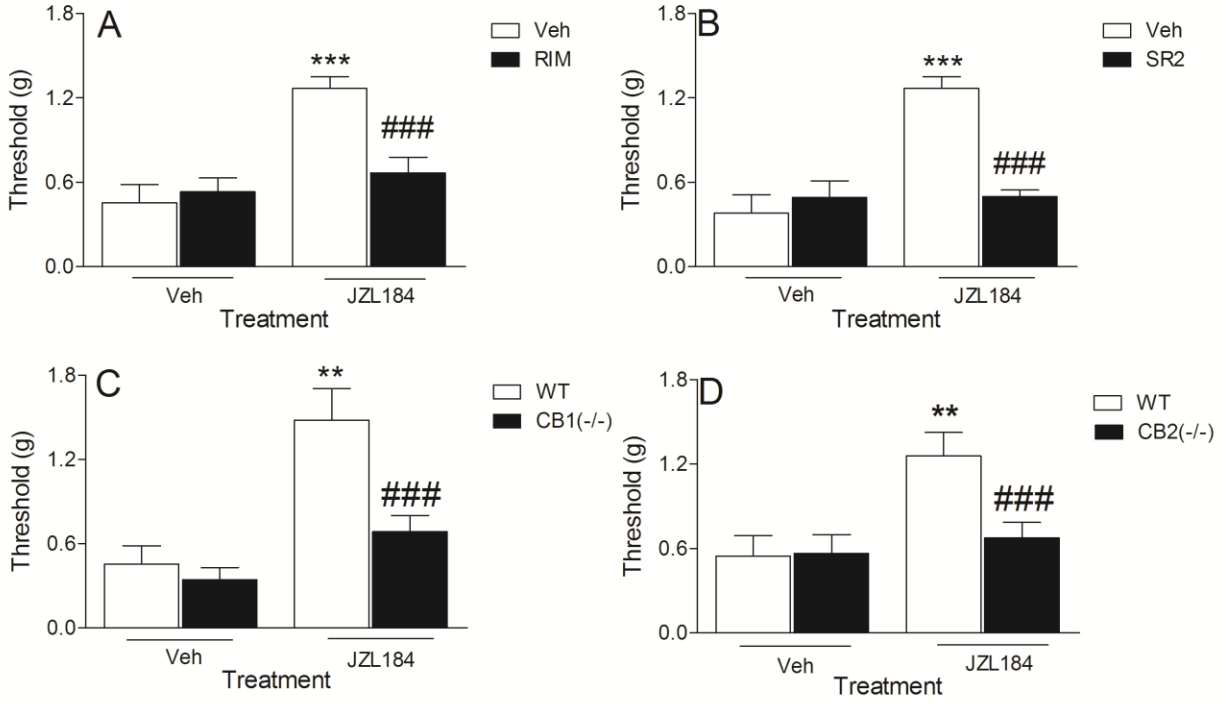

Figure 4: The anti-allodynic effect of JZL184 is mediated by a CB_1 and CB_2 mechanism of action. Rimonabant blocked the anti-allodynic **(Panel A)** effect of JZL184. *** $p < 0.001$ versus Vehicle/vehicle (VEH/VEH), ###, $p < 0.001$ versus VEH/JZL184. SR144528 also blocked the anti-allodynic **(Panel B)** effect of JZL184. ***, $p < 0.001$ versus VEH/VEH. ###, $p<0.001$ versus VEH/JZL184**.** The anti-allodynic **(Panel C)** effect of JZL184 did not occur in CB_1 knockout mice (-/-). **, $p < 0.05$ versus WT/VEH. ###, $p<0.001$ versus CB_1 (-/-)/JZL184. The anti-allodynic **(Panel D)** effect of JZL184 did not occur in CB_2 knockout mice. **, $p < 0.001$ versus wild type or WT/VEH. ###, $p < 0.001$ versus CB_2(-/-)/JZL184. Values represented the mean (± SEM) mechanical paw withdrawal threshold. N=6/group.

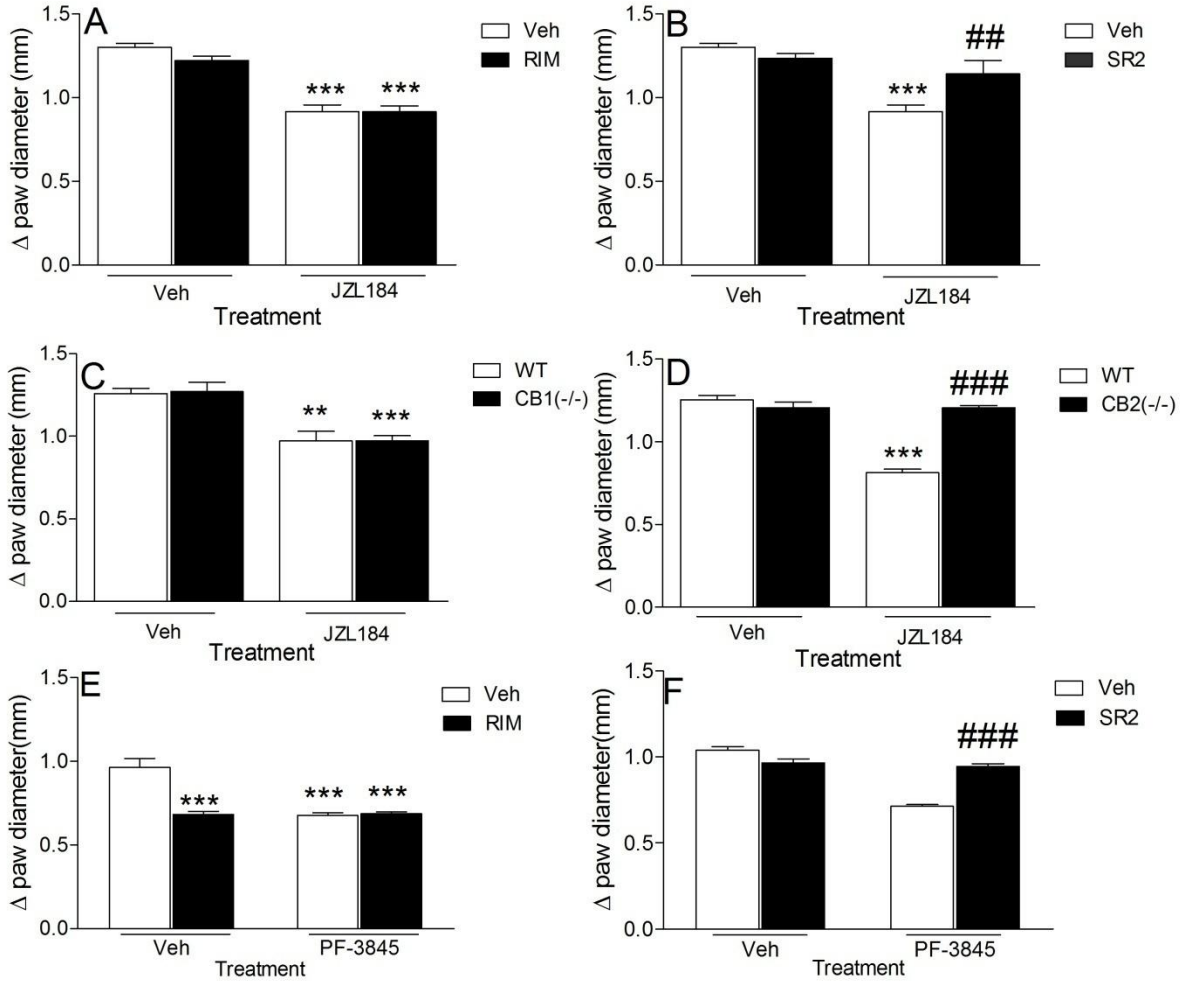

Figure 5: The anti-edematous effect of JZL184 is mediated by a CB_2, but not a CB_1, receptor mechanism of action. Rimonabant did not block the anti-edema effect of JZL184 **(Panel A)**. ***, $p < 0.001$ versus VEH/VEH; SR144528 blocked the anti-edematous effect of JZL184 **(Panel B)**. ***, $p < 0.001$ versus Vehicle/Vehicle (VEH/VEH). ## $p<0.01$ versus VEH/JZL184, the anti-edematous effect of JZL184 was present in CB_1 knockout mice (-/-) **(Panel C)**. **, $p < 0.01$, ***, $p < 0.001$ versus WT/VEH. The anti-edematous effect of JZL184 does not occur in

CB_2 knockout mice **(Panel D)**. ***, $p < 0.001$ versus WT/VEH and ### $p<0.001$versus CB_2 (-/-)/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.

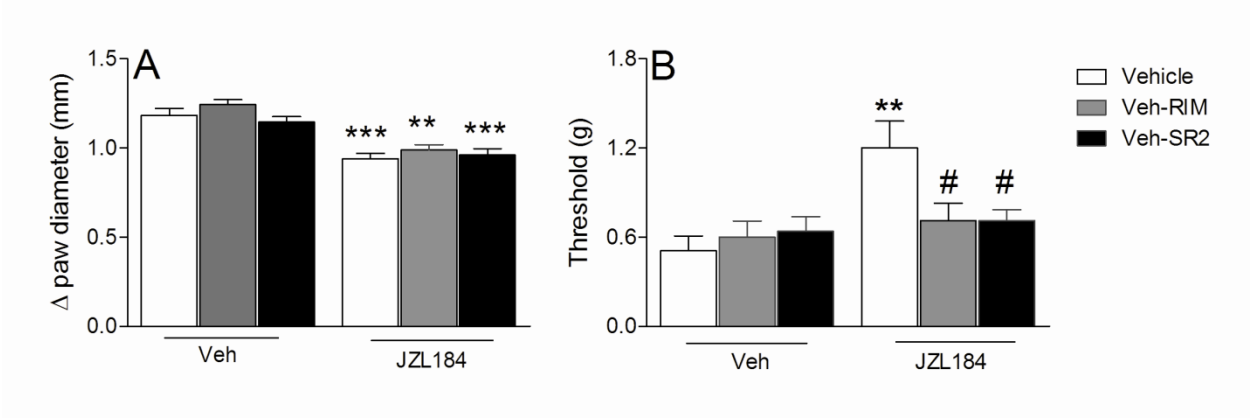

Figure 6: JZL184-induced anti-allodynia occurs independently of its anti-edematous effect. Neither rimonabant nor SR144528 blocked the anti-edematous effect of JZL184 when injected approximately 4 h after carrageenan (**Panel A)**. Both rimonabant and SR144528 blocked the anti-allodynic effect of JZL184 when injected approximately 1 hour before determining mechanical allodynia **(Panel B)**. Values represented the mean (± 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

4. 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 CB_1 receptor functional tolerance (Chanda et al. 2010; Schlosburg et al. 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 1).

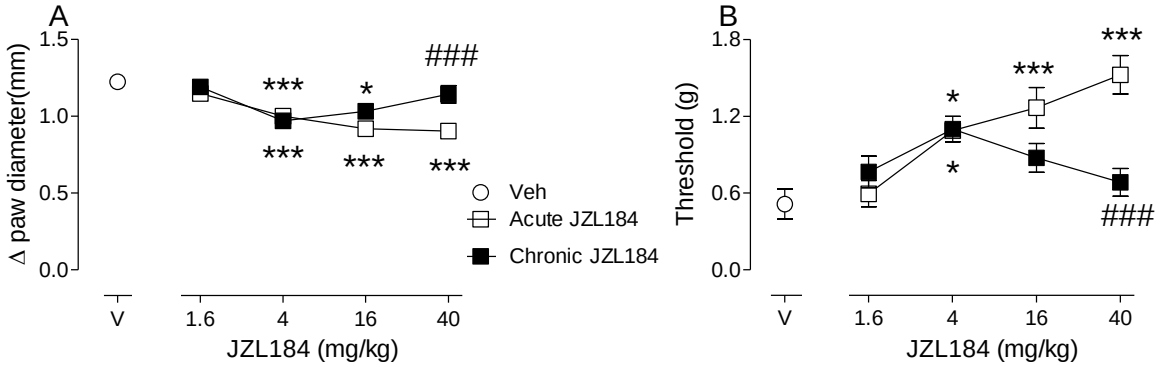

Figure 7: The anti-edematous and anti-allodynic effects of JZL184 undergo tolerance following repeated administration of high dose, but not low dose of the drug. **Panel A** showed that the anti-edematous effects of low doses of JZL184 remain effective after repeated administration. Similarly **Panel B** showed that 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 (± SEM) mechanical paw withdrawal threshold and difference in paw thickness. N = 6-8/group.

5. 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 [$t(10) = 2.90$, $p< 0.01$]; (Figure 8B). JZL184 did not affect paw withdrawal thresholds in control paws (Table 1).

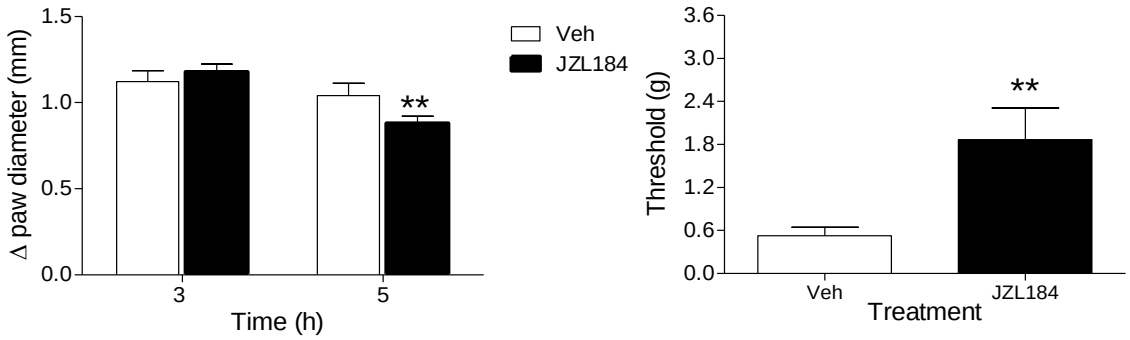

Figure 8: JZL184 given after carrageenan reverses paw edema and mechanical allodynia. JZL184 reversed edema **(Panel A)** and allodynia **(Panel B)** when administered 3h post carrageenan. Values represented the mean (± SEM) mechanical paw withdrawal threshold and difference in paw thickness. **, $p < 0.01$ versus JZL184 at 3 h. T test was done for allodynia. **, $p < 0.01$, versus VEH; N=6 /group

6. Low JZL184 combined with high dose of PF-3845 augments the anti-allodynic effects but anti-edematous effects remain unchanged

The carrageenan-induced edematous and allodynic effects were only partially blocked by all the effective doses of JZL184 (4,16,40 mg/kg) and PF-3845 (3,10 mg/kg) in this study. Hence we tested whether combined MAGL and FAAH inhibition would produce enhanced efficacy. As shown in Figure 9A, combined administration of JZL184 and PF-3845 augmented the anti-allodynic effects compared with individual administration of either inhibitor [$F(4,31) = 18.96$, $p < 0.001$]. In contrast, the combination did not produce augmented anti-edematous effects compared with single administration of JZL184 or PF-3845 [$F(3,28) = 51.19$, $p < 0.001$]; (Figure 9B). The combination did not affect paw withdrawal thresholds in the control paws (Table 1).

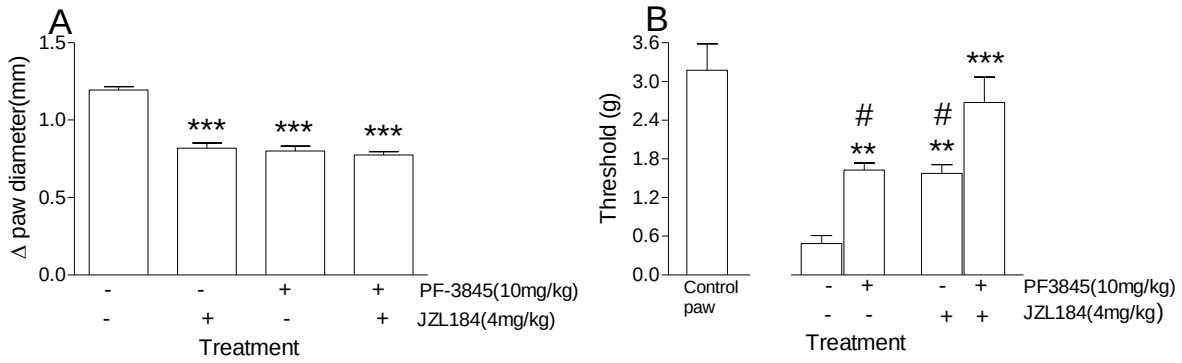

Figure 9: Effects of combination of low dose JZL184 and high dose PF-3845 in the carrageenan assay. The combination of low dose of JZL184 and high dose of PF3845 did not further enhance the anti-edematous of either agent given alone **(Panel A)**. On the contrary, the combination produced enhanced anti-allodynic **(Panel B)** effects. Values represented the mean (± SEM) mechanical paw withdrawal threshold and difference in paw thickness. **, $p < 0.01$,***, $p < 0.001$ versus VEH; #, $p < 0.01$ versus contralateral paw. N= 8/group

7. Anti-allodynic and anti-edematous effects of JZL184 and PF-3845 given in combination did not undergo tolerance when administered repeatedly

Repeated administration of the combination of JZL184 (4mg/kg) and PF-3845 (10mg/kg) maintained the anti-edematous and anti-allodynic effects in this study. Figure 10A showed that chronic administration of the combination maintained augmented anti-allodynic effects [$F(5,44) = 5.85$, $p < 0.001$]. Repeated administration of this combination also maintained the anti-edematous effects in the carrageenan model [$F(2,22) = 8.47$, $p < 0.01$];(Figure 10B).

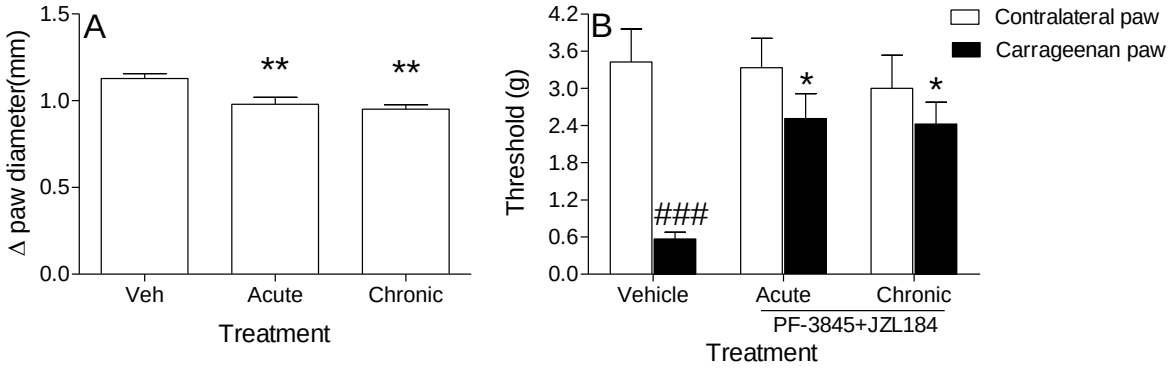

Figure 10: The repeated administration of the combination of low dose of JZL184 and high dose of PF3845 maintained the anti-edematous and anti-allodynic effects. **Panel A** showed that the anti-edematous effects after repeated administration of the combination remained effective. Similarly, **Panel B** showed that the anti-allodynic effects do not undergo tolerance after repeated dosing of the combination. Values represented the mean (± SEM) mechanical paw withdrawal threshold and difference in paw thickness. **, $p < 0.01$, *, $p < 0.05$ versus VEH; ###, $p < 0.001$ versus contralateral paw. N= 8-9/group

Table 1: Paw withdrawal thresholds in the contralateral paw

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

Experiment	Mean (g)	± SEM	Sample Size
JZL184 acute dose	3.27	0.142	35
JZL 184 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
CB1 (-/-) mice	3.37	0.297	24
CB2 (-/-) 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	3.32	0.165	27

of PF-3845			

Discussion

JZL184 represents the first selective MAGL inhibitor that elevates brain 2-AG, but not anandamide, levels upon acute administration (Long et al. 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 (Kinsey et al. 2009; Kinsey et al. 2010; Spradley et al. 2010; Busquets-Garcia et al. 2011; Guindon et al. 2011; Khasabova et al. 2011). The present study increases the understanding that MAGL inhibition plays in nociception by demonstrating that JZL184 reduced 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 and PF-3845 were mediated through CB_2 receptors, while the anti-allodynic effects of JZL184 required both CB_1 receptors and CB_2 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. Similarly, URB602 reversed carrageenan-induced paw edema and paw withdrawal latency when administered after carrageenan in mice (Comelli et al. 2007).The fact that administration of JZL 184 post carrageenan maintained reversed paw edema and mechanical allodynia, suggests that JZL184 possesses therapeutic potential to treat established inflammatory states, rather than just preventing the development of inflammatory pain.

Another major observation of the present study was that the combination of low dose of JZL184 and high dose of PF-3845 produced enhanced anti-allodynic effects compared with each drug alone. On the other hand, the magnitude of the anti-edematous effects remained unchanged compared with single administration of JZL184 or PF-3845. As reported earlier, prolonged blockade of MAGL and FAAH cause differential analgesic tolerance. MAGL inhibition over 6 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 et al. 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 AEA levels produces a greater antinociceptive response in tail immersion assay and in acetic acid writhing test of visceral pain sensation than inhibitors of either FAAH or MAGL alone (Long et al. 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 et al. 2009b). To the contrary, partial blockade of MAGL combined with complete blockade of FAAH in the present study did not produce apparent CB_1 receptor dependent behavioral effects, as assessed in the tetrad assay (unpublished). This battery of four tests was used to assess cannabimimetic activity, and included: spontaneous locomotor suppression, analgesia to noxious thermal stimuli, catalepsy, and hypothermia (Martin et al. 1991; Compton et al. 1993). Importantly, this combination when given repeatedly did not produce tolerance to its anti-edematous or anti-allodynic effects. 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 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.

The observations that JZL184 failed to attenuate carrageenan-induced edema in SR144528-treated wild type mice and CB_2 (-/-) mice, indicate that CB_2 receptors play a necessary role in mediating these anti-edematous effects. In contrast, complementary pharmacological and genetic approaches indicated that CB_1 receptors were expendable in the anti-edematous effects of JZL184. Likewise, the anti-edematous effect of URB602, which inhibited FAAH and MAGL with similar potency, had also been reported to be blocked by SR144528, but not by rimonabant (Comelli et al. 2007). Systemic or local administration of the CB_2 receptor agonist AM1241 suppressed the development of mechanical stimulation in the carrageenan model of inflammation (Nackley et al. 2003a). This suppression was blocked by SR144528, but not by rimonabant (Nackley et al. 2003b). Our study also showed that the anti-edematous effects of PF-3845 were mediated by CB_2 receptors which is supported by the available report in which the FAAH inhibitor URB597 elicited an anti-edema effect in pentobarbital-anesthetized mice that was blocked by the CB_2 receptor antagonist SR144528 (Clayton et al. 2002; Holt et al. 2005). Similarly, SR144528 elicited a partial attenuation of the FAAH (-/-) anti-edema in carrageenan model(Lichtman et al. 2004). Previous studies have also reported that elevation of 2-AG attenuates inflammatory and immune response in vitro (Ouyang et al. 1998; Gallily et al. 2000; Facchinetti et al. 2003). Thus, inhibiting MAGL in vivo offers a

strategy to augment 2-AG levels to elicit anti-inflammatory effects through the stimulation of CB_2 receptors.

In contrast to JZL184-induced anti-edematous effects, complementary genetic and pharmacological approaches revealed that the anti-allodynic effects of JZL184 required both CB_1 and CB_2 receptors. Similarly, the suppressive effects of JZL184 on capsaicin-induced thermal hyperalgesia and nocifensive behavior (i.e., defensive response to pain) required both CB_1 and CB_2 receptors (Spradley et al. 2010). Likewise, intra-paw administration of JZL184 or URB602 produced antinociceptive effects in the formalin model, which were blocked by CB_1 and CB_2 receptor antagonists (Guindon et al. 2007a; Guindon et al. 2011). In contrast, CB_2 receptors did not play a necessary role in the anti-allodynic effects of JZL184 in the chronic constriction injury model (Kinsey et al. 2009; Kinsey et al. 2010).

Two related questions are raised by these observations. First, why do both CB_1 and CB_2 receptors play a necessarily role in the antinociceptive effects of JZL184 in some types of nociceptive assays (e.g., carrageenan, formalin, and capsaicin), but only CB_1 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 play a necessary role in 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 are used to assess mechanical allodynia in both CCI and carrageenan assays. Instead, it 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, of in multiple supraspinal regions (e.g., PAG or the rostral ventromedulla). It has been shown that CB_2 receptors are upregulated in the dorsal root ganglia and paw tissue of rodents administered complete Freund's adjuvant (Hsieh et al. 2011), suggesting that CB_2 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 CB_2 receptors (Galiegue et al. 1995). 2-AG activation of CB_2 receptors on infiltrating cells might serve to enhance the well described antinociceptive actions of CB_1 receptor stimulation on peripheral nociceptors (Agarwal et al. 2007) within the spinal cord (Yaksh 1981; Lichtman and Martin 1991) and within supraspinal sites of action (Lichtman et al. 1996; Martin et al. 1999). 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 CB_1 and CB_2 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. Whereas neither antagonist reversed the anti-edematous effects of JZL184, both rimonabant and SR144528 reversed the anti-allodynic effects of JZL184, indicating that these effects can be dissociated.

Another 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 repeated 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), CB_1 receptor downregulation and desensitization in cingulate cortex, hippocampus, somatosensory cortex, and PAG (Chanda et al. 2010; Schlosburg et al. 2010). Additionally, MAGL (-/-) mice, which possess constitutively elevated levels of 2-AG, show partial desensitization of CB_1 receptors (Chanda et al. 2010; Schlosburg et al. 2010; Pan et al. 2011). 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 (Sciolino et al. 2011). Taken together, these results indicate that repeated administration of low dose of JZL184 can maintain its beneficial pharmacological effects without producing tolerance. Hence this catabolic inhibitor may possess therapeutic utility in treating clinical symptoms associated with inflammatory disease states.

Conclusion

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 also reversed edema and allodynia when administered after carrageenan. Complementary genetic and pharmacological approaches revealed that the anti-allodynic effects of JZL184 required both CB_1 and CB_2 receptors, whereas only CB_2 receptors had a necessary role in mediating its anti-edematous effects. 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. Finally we have reported that the partial MAGL and complete FAAH inhibition produced enhanced anti-allodynic effects. Also the combination administered repeatedly did not produce any tolerance to its anti-edematous or anti-allodynic effects. These results together indicate that the activation of both CB_1 and CB_2 receptors by partial MAGL blockade and complete FAAH blockade may represent a promising strategy to treat inflammatory pain that includes the ability to prevent inflammation and reverse established inflammatory pain states.

Future directions

Based on the results of the present project, future studies demand a thorough investigation of a dual MAGL-FAAH inhibitor. Indeed, the recently developed dual inhibitor O-hydroxyacetamide carbamates, SA-57, possesses considerably greater potency in inhibiting FAAH than inhibiting MAGL (J.Niphakis 2011). For example, at 1.25 mg/kg it elevates AEA and 2-AG 10 and 3 fold, respectively. However, 12.5mg/kg SA-57 elevates AEA and 2-AG 10 and >10 fold, respectively. Thus, it would be interesting to investigate whether intermediate doses of SA-57 that produce large increases in AEA (e.g., 7-10 fold) and relatively small increases in 2-AG (e.g., 2-4 fold) would show high efficacy in reversing mechanical allodynia in the carrageenan assay. It would also be interesting to determine the endocannabonoid levels in brain areas that mediate antinociception, such as cingulate cortex, somatosensory cortex and PAG, following acute as well as chronic administration of the dual inhibitor. Endocannabinoid content varies across different brain regions, with three to five differences in content reported in studies designed for regional comparisons. For example, JZL184, a potent and selective inhibitor of MAGL when administered systemically (16 mg/kg) increases 2-AG levels in a number of regions but the magnitude of 2-AG changes varies significantly between these tissues (Long et al. 2009a). Thus, to provide a comprehensive picture of regional differences, it would be of merit to determine endocannabinoid levels in different regions of the brain. It would also be important to determine whether the pharmacological treatments that reduce mechanical allodynia also reduce the inflammatory mediators triggered by carrageenan. Future studies will be designed to focus on determining the underlying mechanism mediating the anti-allodynic and anti-inflammatory actions of MAGL and FAAH inhibitors. Moreover, future studies will be

designed to investigate the differential role of CB_1 and CB_2 receptors in mediating anti-allodynic effects of JZL184 in inflammatory versus nociceptive pain models. One of the possibilities for the differential involvement of the receptors could be the activation of different molecular mechanisms involved in attenuating neuropathic and inflammatory pain. Identifying those differences by screening for differentially expressed genes in the spinal cord might provide insight into the underlying molecular mechanisms of neuropathic and inflammatory pain.

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