

ENDOCANNABINOID MODULATION OF CAPSAICIN-INDUCED BEHAVIORAL
HYPERSENSITIVITY

by

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(Under the direction of Andrea G. Hohmann)

ABSTRACT

Monoacylglycerol lipase (MGL) and fatty acid amide hydrolase (FAAH) degrade the endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide (AEA), respectively. Pharmacological inhibition of these enzymes in the periphery holds promise for suppressing persistent pain in the absence of centrally-mediated psychotropic side-effects. We compared effects of intraplantar injection of the MGL inhibitor JZL184, the FAAH inhibitor URB597, and the endocannabinoid uptake inhibitor VDM11 on behavioral hypersensitivities produced by capsaicin, the pungent ingredient in hot chili peppers. Intradermal administration of capsaicin (10 μg i.pl.) produced nocifensive behavior, thermal hyperalgesia, and mechanical allodynia in rats. JZL184 (100 μg i.pl.) suppressed capsaicin-induced nocifensive behavior and thermal hyperalgesia without altering capsaicin-evoked mechanical allodynia. URB597 (75 μg i.pl.) suppressed capsaicin-induced mechanical allodynia without altering capsaicin-evoked thermal hyperalgesia or nocifensive behavior. VDM11 (100 μg i.pl.) suppressed capsaicin-evoked hypersensitivity for all three dependent measures (nocifensive behavior, thermal hyperalgesia, and mechanical allodynia), suggesting an additive effect following elevation of both AEA and 2-

AG. Pharmacological inhibition of MGL and FAAH also activates specific cannabinoid receptor subtypes. Both the CB₁ antagonist AM251 (80 µg i.pl.) and the CB₂ antagonist AM630 (25 µg i.pl.) blocked the JZL184-induced suppressions of nocifensive behavior and thermal hyperalgesia. By contrast, AM251 (80 µg i.pl.), but not AM630 (25 µg i.pl.), blocked the anti-allodynic effects of URB597. The VDM11-induced suppression of capsaicin-evoked nocifensive behavior and thermal hyperalgesia was blocked by either AM251 (80 µg i.pl.) or AM630 (25 µg i.pl.), as observed with JZL184. The VDM11-induced suppression of capsaicin-evoked mechanical allodynia was blocked by AM251 (25 µg i.pl.) only, as observed with URB597. In summary, peripheral inhibition of enzymes hydrolyzing 2-AG and AEA suppresses capsaicin-evoked behavioral hypersensitivities in a modality-specific manner. Modulation of endocannabinoids in the periphery suppresses capsaicin-evoked nocifensive behavior and thermal hyperalgesia through either CB₁ or CB₂ receptor mechanisms. By contrast, modulation of endocannabinoids suppresses capsaicin-evoked mechanical allodynia through CB₁ mechanisms only. Inhibition of endocannabinoid transport was more effective in suppressing capsaicin-induced hypersensitivities compared to inhibition of either FAAH or MGL alone. These studies are the first to unveil the effects of pharmacologically increasing peripheral endocannabinoid levels on capsaicin-induced behavioral hypersensitivities.

KEYWORDS endocannabinoid, monoacylglycerol lipase, fatty acid amide hydrolase, capsaicin, pain, endocannabinoid transporter

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DEDICATION

This dissertation is dedicated to Brian, my family, and May.

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CHAPTER 1: INTRODUCTION

1.1. Statement of Purpose

The purpose of this dissertation is to illustrate the roles of peripheral endocannabinoids in a model of inflammatory pain. Endocannabinoids are the body's own naturally-produced cannabis-like compounds. Like cannabis, endocannabinoids are capable of binding cannabinoid receptors and providing pain relief, but also like cannabis they are associated with negative centrally-mediated psychotropic side effects. Thus, studies of the roles of endocannabinoids and their receptors in the periphery are necessary to understand and potentially develop more effective pain treatments without causing any unwanted centrally-mediated side effects.

Many rodent models of inflammatory and neuropathic pain exist, but the capsaicin-induced inflammatory pain model is unique in that it specifically activates TRPV1 vanilloid channels in addition to an injury-induced biological environment rich in inflammatory factors as seen in other pain models. Very little research has been published regarding peripheral endocannabinoids and capsaicin, leaving much work to be done. A better understanding of the interaction between endocannabinoids and capsaicin is crucial in order to determine better therapeutic targets for pathological conditions in which TRPV1 channels are especially activated in response to peripheral damage. Rather than peripherally administering exogenous cannabinoids or cannabinoid-like compounds, pharmacologically increasing the activity of the body's own naturally-produced endocannabinoids may prove to be more therapeutically useful. Thus, the original research presented in this dissertation attempts to uncover the therapeutic

usefulness of pharmacologically elevating peripheral endocannabinoids in a capsaicin-induced TRPV1-activated model of inflammatory pain.

1.2. Overview

The next chapter of this dissertation will introduce and discuss the recent literature regarding endocannabinoids and their receptors, the capsaicin-induced inflammatory pain model, and its mechanisms of action. After a general introduction to endocannabinoids, their receptors, and the capsaicin inflammatory pain model, a more detailed literature review of endocannabinoids in the periphery will follow, detailing the results of relevant research regarding pharmacological manipulations of endocannabinoids and models of pain. Next, a manuscript will be presented containing original data obtained for the purpose of identifying the efficacy of pharmacologically elevated endocannabinoid levels in reducing capsaicin-induced behavioral hypersensitivities in rats. Lastly, a discussion will follow to summarize the main points of this dissertation.

CHAPTER 2: LITERATURE REVIEW

2.1. Endocannabinoids

Cannabis has been used for centuries for its pain-relieving properties. The main active ingredient of cannabis is Δ^9 -Tetrahydrocannabinol, which produces antinociceptive effects by binding its G protein-coupled receptors CB₁ (Matsuda et al., 1990; Ledent et al., 1999; Zimmer et al., 1999) and CB₂ (Munro et al., 1993; for review see Walker and Hohmann, 2005). Endocannabinoids, the body's own cannabis-like compounds, also bind to cannabinoid CB₁ and CB₂ receptors to provide antinociception by altering neuronal signaling processes via regulating ion channels and neurotransmitter release (Devane et al., 1992; for review see Piomelli, 2003). The two major endocannabinoids are Anandamide (AEA, arachidonylethanolamide) and 2-arachidonoylglycerol (2-AG). Anandamide was originally thought to be formed from the cleavage of N-arachidonylphosphatidylethanolamine by phospholipase D (NAPE-PLD), although this is currently being debated due to the production of AEA in NAPE-PLD knockout mice (Liu et al., 2008); after AEA formation, the enzyme fatty acid amide hydrolase (FAAH) then hydrolyzes AEA into arachidonic acid and ethanolamine (Cravatt et al., 1996). 2-AG is formed in a two-step process through activation of the enzymes phospholipase C and diacylglycerol lipase- α (Bisogno et al., 2003; Jung et al., 2007); the enzyme monacylglycerol lipase (MGL) is responsible for the hydrolysis of 2-AG into fatty acid and glycerol (Dinh et al., 2002). However, there are also reports of 2-AG hydrolysis by FAAH (Jhaveri et al., 2006; Maione et al., 2006) as well as the hydrolysis of both AEA and 2-AG by cyclooxygenase type 2 (COX2) (Fowler et al., 1997, 1999; Yu et al., 1997; Kozak et al., 2000, for review see Kozak et

al., 2004). Also, a hypothesized endocannabinoid transporter aids the endocannabinoids in reaching their designated intracellularly-located degrading enzymes (Di Marzo et al., 1994; Hillard et al., 1997; Bisogno et al., 2001).

2.2. Cannabinoid Receptor Subtypes

Cannabinoid CB₁ receptors are mainly located in the central nervous system in regions such as the hippocampus, cortex, cerebellum, basal ganglia, and spinal cord (Herkenham et al., 1991; Farquhar-Smith et al., 2000), but they are also present in the periphery in regions such as adipose tissue, heart, uterus, ovary, testes, bone marrow, and thymus (Galiegue et al., 1995). CB₂ receptors, which were originally thought to exist only in the periphery, are most prevalent in immune tissues (Galiegue et al., 1995). However, Onaivi et al (2006) discovered that CB₂ receptors are indeed widely distributed in the brain, albeit at lower levels than CB₁ receptors, via CB₂ knockouts, RT-PCR, immunoblotting, and a number of other techniques. One noteworthy aspect of CB₁ and CB₂ prevalence in the nervous system is that both receptor subtypes are found at important central and peripheral pain pathway regions (for review, see Hohmann, 2002; Guindon and Hohmann, 2009).

2.3. Capsaicin as a Model for Inflammatory Pain

Due to the presence of cannabinoid receptors in regions important in pain modulation, cannabinoid receptors and their ligands (both endogenous and exogenous) have become therapeutic targets for the treatment of acute, persistent inflammatory, and neuropathic pain (for

review see Guindon and Hohmann, 2009). Peripherally-acting compounds are especially desirable for the treatment of pain in order to avoid any unwanted centrally-mediated side effects. A variety of animal models of inflammatory pain have been developed as tools for examining therapeutic pharmacological manipulations to better understand the mechanisms of inflammatory pain (for review see Guindon and Hohmann 2009). Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the main pungent component of hot chili peppers, causes a burning sensation, pain, and inflammation. When injected intradermally in rats it produces hyperalgesia, defined as a decrease in pain threshold and/or an increase in pain levels in response to a normally painful stimulus (Gilchrist et al., 1996). Capsaicin-induced hyperalgesia is present in response to both radiant heat and mechanical stimulation; also present in capsaicin-injected rats is an increase in nocifensive behavior, characterized by licking, lifting, and guarding the injected paw (Gilchrist et al., 1996). Primary hyperalgesia occurs at the site of injury and can be mediated by C-fiber polymodal mechanoheat peripheral nociceptors (Kenins, 1982; Szolcsanyi et al., 1988; Baumann et al., 1991; LaMotte et al., 1992). Secondary hyperalgesia occurs in response to mechanical stimulation in regions surrounding the injury, and is mediated by central nervous system sensitization (Baumann et al., 1991; LaMotte et al., 1992; Torebjork et al., 1992) and peripheral C-fibers (Serra et al., 2004). Thus capsaicin-induced hyperalgesia serves as a useful animal model for inflammatory pain.

In the spinal cord, capsaicin was found to stimulate the release of CGRP and substance P in *in vitro* superfusion studies, contributing to nociception (Oku et al., 1987). However, capsaicin also produces hyperalgesia through activation of the TRPV1 ion channel (Caterina et al., 1997). TRPV1 is a ligand-gated non-selective cation channel expressed in sensory neurons, and its channel pore is opened by noxious heat (for review see Szallasi and Blumberg, 1999).

Ligands for TRPV1 such as exogenous capsaicin or protons (producing an acidic environment) decrease the temperature threshold for TRPV1 to produce a sensation of noxious heat, even at room temperature (Tominaga et al., 1998). TRPV1 is required for inflammatory sensitization to noxious thermal stimuli: in TRPV1 knockout mice, thermal hyperalgesia induced by the irritant carrageenan was completely absent, while the sensation of normal noxious heat was unaltered (Davis et al, 2000).

2.4. TRPV1 Activation in Pathological Conditions

TRPV1 activation and/or upregulation has been observed in a number of pathological conditions including heat hyperalgesia associated with peripheral nerve injury, diabetic neuropathy, and herpes simplex infection; disorders of the inner ear including hyperacusis, tinnitus, and some forms of vertigo; skin disorders; gastrointestinal tract disorders such as ileitis and irritable bowel syndrome; bladder dysfunction; and respiratory disorders such as asthma (for review see Di Marzo et al., 2002 and Nagy et al., 2004). Tissue acidosis is a noxious condition that is associated with a drop in interstitial pH, inflammation, and/or ischemia, and it may be a source of the pain associated with damage to peripheral nerves, angina pectoris, and malignant tumors (for review see Holzer, 2009). An interstitial pH below 6 in the case of tissue acidosis results from an excess of protons, and these protons also activate TRPV1 (for review see Holzer, 2009). Thus, activated TRPV1 receptors are very relevant to a number of pathological conditions, and better treatments for any or all of these conditions are highly desirable.

Rodent models of inflammatory pain such as local injections of formalin, carrageenan, CFA, and capsaicin, as well as neuropathic pain models such as sciatic nerve ligation, partial

sciatic nerve ligation, and chronic constriction injury, all likely cause tissue acidosis. In the periphery, tissue acidosis is associated with an increase in the release of neuropeptides like CGRP that lead to neurogenic inflammation and induction of pain sensation due to the influx of protons through the opened TRPV1 channels. Thus, all of the models of inflammatory and neuropathic pain mentioned above serve as good models that may actually mimic pathological conditions in humans. However, capsaicin is unique in that in addition to causing tissue acidosis, it directly activates TRPV1, as mentioned above. This quality of capsaicin lends itself as a very useful model for studying pathological conditions that are particularly associated with TRPV1 over-activation or upregulation. Thus, it is important to note that the use of this model of inflammatory pain may help to uncover the roles of particular drugs in over-activated TRPV1 pathological conditions that normally would not be observed in other inflammatory and neuropathic pain models.

2.5. Anandamide is a TRPV1 Agonist

AEA, but not 2-AG, is an endogenous ligand for TRPV1 in a manner similar to capsaicin (Zygmunt et al., 1999; Al-Hayani et al., 2001). Thus, cannabinoid receptor activation by AEA to produce antinociception seemingly contradicts its role as a TRPV1 ligand to produce hyperalgesia. Maione et al. (2006) found that intracranial injections of the FAAH inhibitor URB597 into the periaqueductal gray region of the midbrain increased endocannabinoid concentrations in that region and suppressed or increased thermal nociception through TRPV1 and CB1 mechanisms, depending on the dose. In tests measuring the hypokinesic effects of AEA, de Lago et al. (2004) found that AEA activates TRPV1 on nigrostriatal dopaminergic

neurons and the hypokinesic effects can be completely blocked by the TRPV1 antagonist capsazepine. Indeed, the role of AEA in peripheral antinociception and hyperalgesia was complicated further when Potenzieri et al. (2009) discovered that intraplantar injections of exogenous AEA alone produced nocifensive behaviors, but not thermal hyperalgesia, through activation of TRPV1. These data contradict the results of Calignano et al. (2001) in which a lower dose of peripherally-administered exogenous AEA alleviated nociception in several inflammatory pain models. In summary, it is clear that the effects of AEA are unpredictable due to their ability to agonize both CB₁ receptors and TRPV1 channels.

2.6. Endocannabinoid Uptake Inhibition

AEA and 2-AG are unique neurotransmitters in that they are lipophilic, allowing them to passively diffuse across the lipid bilayer of membranes, likely with the aid of a membrane transporter (Di Marzo et al., 1994; Hillard et al., 1997; Bisogno et al., 2001). However, the idea of a membrane transporter has been a source of debate due to evidence that a transporter may not exist, but rather these inhibitors act via FAAH inhibition (Glaser et al., 2003). However, Ortega-Gutierrez et al. (2004) identified a distinct role for both FAAH and an anandamide transport inhibitor UCM707-sensitive protein in mediating the uptake of AEA. In 2009, Kaczocha et al. identified two fatty acid binding proteins that transport AEA from the plasma membrane to FAAH located on the endoplasmic reticulum membrane to be degraded *in vitro*. Thus, it is likely that both FAAH and a transporter protein aid in AEA and/or 2-AG degradation.

Based on *in vitro* studies of the anandamide transport inhibitor UCM707 (Lopez-Rodriguez et al., 2001), it was discovered that intraperitoneal *in vivo* use in combination with

exogenous AEA was effective at promoting antinociception in the hot plate test, with negligible affinity for CB₁, CB₂, or TRPV1, and is the most potent and selective of the endocannabinoid transport inhibitors (de Lago et al., 2002). In contrast, de Lago et al. (2005) found that repeated intraperitoneal administration of UCM707 increased only 2-AG levels in the brain, while the other endocannabinoid uptake inhibitors OMDM-2 and VDM11 increased both AEA and 2-AG brain levels. The discovery that endocannabinoid uptake inhibitors such as AM404 also activate vanilloid receptors (Zygmunt et al., 2000) led others to construct and identify inhibitors that do not agonize vanilloid receptors but maintain the capability to inhibit the endocannabinoid transporter; thus, VDM11 and similar AEA derivatives were created (De Petrocellis et al., 2000).

2.7. Endocannabinoid and TRPV1 Composition in Peripheral Sensory Nerves

The locations and mechanisms of endocannabinoid formation and degradation are well studied in the brain and spinal cord (for review see Piomelli, 2003; Katona et al., 2006; Nyilas et al., 2009; Pernia-Andrade et al., 2009). Much less is known about endocannabinoid location and modulation in the periphery. Originally cannabinoid receptors were thought to be present in only the central nervous system. However, Munro et al. (1993) first identified the presence of a peripheral cannabinoid receptor in macrophages of the spleen. This discovery led to a number of other studies identifying the presence and transport of cannabinoid receptors in the periphery.

In situ hybridization studies revealed the presence of cannabinoid CB₁ receptor messenger RNA in medium and large sized cells of the dorsal root ganglia; however, the mRNA for substance P, CGRP, and somatostatin were not colocalized with CB₁ mRNA (Hohmann and Herkenham, 1999a). Further studies employing receptor autoradiography determined that CB₁,

but not CB₂ receptors, originated in the dorsal root ganglion cells and accumulated in the sciatic nerve proximal to a nerve ligation (Hohmann and Herkenham, 1999b). These studies suggest that CB₁ receptors are synthesized in the dorsal root ganglion and are transported axonally to peripheral sensory nerve terminals. Additionally, these CB₁ receptors located on peripheral nociceptors were successfully deleted in mice by Agarwal et al., 2007. Without affecting centrally-located CB₁ receptors, these deletion studies by Agarwal et al. revealed the importance of peripheral CB₁ in providing analgesia via local and systemic cannabinoid treatments. Thus, CB₁ receptors are indeed present on peripheral nociceptors and play an important role in nociception.

CB₂ receptors have been identified in the small cells of the dorsal root ganglion as well as in peripheral nerve fibers, via immunostaining and immunoblotting techniques (Anand et al., 2008). In addition, CB₂ receptors are colocalized with TRPV1 channels on these small-sized dorsal root ganglion cells (Anand et al., 2008).

The presence of TRPV1 in small and medium sized dorsal root ganglion cells was first verified in a separate study via in situ hybridization and histochemical studies (Michael and Priestley, 1999). Axotomy led to a downregulation of TRPV1 mRNA in dorsal root ganglion cells, suggesting that compounds from the periphery are responsible for regulating TRPV1 mRNA levels in the dorsal root ganglion (Michael and Priestley, 1999).

Very little research has been published regarding the location and transport of the synthesizing and degrading enzymes of endocannabinoids in the periphery. Lever et al. (2009) identified the presence of FAAH in the soma of dorsal root ganglion neurons of naïve rats via immunostaining, immunoblotting, and PCR. They also found that FAAH mostly colocalizes with TRPV1 (68%), and upon sciatic nerve axotomy, FAAH location shifted from the small-

sized cells to larger-sized cells, suggesting that the endocannabinoid system may be altered upon peripheral nerve damage. Currently there are no published reports regarding the locations of DGL and MGL in the periphery.

2.8. Capsaicin and Endocannabinoids

Peripheral endocannabinoids are known to have antinociceptive effects in a number of pain models (for review see Hohmann, 2002). It is well known that CB₁ receptors on peripheral nociceptors are essential for antinociception: in CB₁ peripheral nociceptor knockout mice, exaggerated inflammatory hyperalgesia was experienced by the mice after the irritant Complete Freund's Adjuvant (CFA) was injected intradermally (Agarwal *et al.*, 2007). Thus, cannabinoid receptors on peripheral nociceptors are essential for antinociceptive effects in inflammatory pain models. In the carrageenan-induced and formalin-induced inflammatory pain models, AEA intraplantar injections significantly reduced hyperalgesia and/or produced antinociception through activation of peripheral cannabinoid receptors (Calignano *et al.*, 1998; Richardson *et al.*, 1998; Guindon *et al.*, 2006a,b). 2-AG intraplantar injections also produced antinociception mediated by peripheral CB₂ in the formalin-induced inflammatory pain model (Guindon *et al.*, 2007).

Due to the presence of endocannabinoids and their receptors in the periphery, the fact that AEA is both a cannabinoid receptor and TRPV1 agonist, and the fact that it is possible to elevate peripheral endocannabinoid activity naturally via pharmacological uptake and enzyme inhibition, the capsaicin model of peripheral inflammatory pain serves as a useful tool for studying peripheral endocannabinoids and their effects on reducing pain behavior.

However, very few published studies exist detailing the effects of peripheral endocannabinoids on a TRPV1-activated capsaicin-induced model of inflammatory pain. More data exists regarding administration of exogenous cannabinoid-like compounds. Peripheral dorsal hindpaw and intraplantar injections as well as intraperitoneal injections of the CB₂ receptor-selective agonist AM1241 significantly reduced flinching behavior, nocifensive behavior, thermal hyperalgesia, and mechanical hyperalgesia in response to capsaicin (Quartilho et al., 2003; Hohmann et al., 2004). Also, the CB₁/CB₂ agonist WIN55,212-2 injected intravenously suppressed thermal hyperalgesia, mechanical hyperalgesia, and nocifensive behavior in the capsaicin model (Li et al., 1999). However, intraplantar injections of WIN55,212-2 resulted in an attenuation of thermal hyperalgesia but not mechanical hyperalgesia or nocifensive behavior, while intrathecal administration of WIN55,212-2 attenuated both thermal and mechanical hyperalgesia but not nocifensive behavior (Johanek et al., 2001). Similarly in a relevant study, intraplantar injections of WIN55,212-2 successfully reduced mechanical and thermal hypersensitivities in response to heat injury (but not capsaicin) via CB₁ and CB₂ (Johanek and Simone, 2004).

2.9. Preventing the Metabolism of Endocannabinoids in Pain Models

Preventing the metabolism of AEA and 2-AG through FAAH, MGL, and uptake inhibition has promise for therapeutic potential in inflammatory pain models (for reviews see Jhaveri et al., 2007; Schlosberg et al., 2009). Low doses of the FAAH inhibitor URB597 in the carrageenan-induced inflammatory model attenuated hyperalgesia (Jhaveri et al., 2008). In the formalin model, the FAAH inhibitor/TRPV1 antagonist N-arachidonoyl-serotonin produced

antinociception (Maione et al., 2007), while ibuprofen (a COX inhibitor) together with exogenous AEA synergistically produced antinociception (Guindon et al., 2006b). Additionally, the non-competitive MGL inhibitor URB602 produced antinociceptive effects during the late phase of the formalin test dose-dependently (Guindon et al., 2007). In a model of neuropathic pain, the MGL inhibitor URB602, the FAAH inhibitor URB597, and exogenous 2-AG all significantly reduced mechanical and thermal hypersensitivities when injected peripherally, and these effects were blocked by both CB₁ and CB₂ receptor antagonists (Desroches et al., 2008). Development of a new potent selective MGL inhibitor, JZL184 (Long et al., 2009), also holds great promise for *in vivo* studies: when injected intraperitoneally in a model of neuropathic pain, both URB597 and JZL184 decreased mechanical and cold allodynia via a CB₁- and/or CB₂-dependent mechanism (Kinsey et al., 2009).

2.10. Summary

The endocannabinoids AEA and 2-AG, their degrading enzymes, and their cannabinoid receptors are thought to be potential therapeutic targets for relieving pain. Many rodent models of inflammatory and neuropathic pain exist to aid in determining endocannabinoids' therapeutic usefulness. However, the capsaicin-induced inflammatory pain model is unique in that capsaicin is a direct agonist of TRPV1, an ion channel commonly activated in many pathological conditions. A close examination of the literature regarding exogenously-administered cannabinoid-like compounds and endocannabinoids in specific models of pain identifies the value of their therapeutic usefulness. However, the effects of pharmacologically inhibiting the degradation of peripheral endocannabinoids particularly in a TRPV1-hyperactivated capsaicin-

induced model of inflammatory pain remain unknown. Based on previous studies in other pain models, the MGL inhibitor JZL184 and the FAAH inhibitor URB597 should prove to be effective at reducing the behavioral hypersensitivities caused by capsaicin, although there is some uncertainty of the role of AEA in a TRPV1-activated pain model. Additionally, the endocannabinoid uptake inhibitor VDM11's ability to increase activity of both endocannabinoids without activating TRPV1 makes it an excellent candidate for determining the effectiveness of elevating peripheral endocannabinoid levels in this model. Thus, the following original research will attempt to uncover the effectiveness of JZL184, URB597, and VDM11 at relieving capsaicin-induced behavioral hypersensitivities.

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**CHAPTER 3: INHIBITORS OF MONOACYLGLYCEROL LIPASE, FATTY-ACID
AMIDE HYDROLASE, AND ENDOCANNABINOID TRANSPORT DIFFERENTIALLY
SUPPRESS CAPSAICIN-INDUCED BEHAVIORAL SENSITIZATION THROUGH
PERIPHERAL CANNABINOID MECHANISMS¹**

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3.1. Abstract

Monoacylglycerol lipase (MGL) and fatty acid amide hydrolase (FAAH) degrade the endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide (AEA), respectively. Pharmacological inhibition of these enzymes in the periphery holds promise for suppressing persistent pain in the absence of centrally-mediated psychotropic side-effects. We compared effects of intraplantar injection of the MGL inhibitor JZL184, the FAAH inhibitor URB597, and the endocannabinoid uptake inhibitor VDM11 on behavioral hypersensitivities produced by capsaicin, the pungent ingredient in hot chili peppers. Intradermal administration of capsaicin (10 µg i.pl.) produced nocifensive behavior, thermal hyperalgesia, and mechanical allodynia in rats. JZL184 (100 µg i.pl.) suppressed capsaicin-induced nocifensive behavior and thermal hyperalgesia without altering capsaicin-evoked mechanical allodynia. URB597 (75 µg i.pl.) suppressed capsaicin-induced mechanical allodynia without altering capsaicin-evoked thermal hyperalgesia or nocifensive behavior. VDM11 (100 µg i.pl.) suppressed capsaicin-evoked hypersensitivity for all three dependent measures (nocifensive behavior, thermal hyperalgesia, and mechanical allodynia), suggesting an additive effect following elevation of both AEA and 2-AG. Pharmacological inhibition of MGL and FAAH also activates specific cannabinoid receptor subtypes. Both the CB₁ antagonist AM251 (80 µg i.pl.) and the CB₂ antagonist AM630 (25 µg i.pl.) blocked the JZL184-induced suppressions of nocifensive behavior and thermal hyperalgesia. By contrast, AM251 (80 µg i.pl.), but not AM630 (25 µg i.pl.), blocked the anti-allodynic effects of URB597. The VDM11-induced suppression of capsaicin-evoked nocifensive behavior and thermal hyperalgesia was blocked by either AM251 (80 µg i.pl.) or AM630 (25 µg i.pl.), as observed with JZL184. The VDM11-induced suppression of capsaicin-evoked mechanical allodynia was blocked by AM251 (25 µg i.pl.) only, as observed with

URB597. In summary, peripheral inhibition of enzymes hydrolyzing 2-AG and AEA suppresses capsaicin-evoked behavioral hypersensitivities in a modality-specific manner. Modulation of endocannabinoids in the periphery suppresses capsaicin-evoked nocifensive behavior and thermal hyperalgesia through either CB₁ or CB₂ receptor mechanisms. By contrast, modulation of endocannabinoids suppresses capsaicin-evoked mechanical allodynia through CB₁ mechanisms only. Inhibition of endocannabinoid transport was more effective in suppressing capsaicin-induced hypersensitivities compared to inhibition of either FAAH or MGL alone. These studies are the first to unveil the effects of pharmacologically increasing peripheral endocannabinoid levels on capsaicin-induced behavioral hypersensitivities

KEYWORDS: endocannabinoid, monoacylglycerol lipase, fatty acid amide hydrolase, capsaicin, pain, endocannabinoid transporter

3.2. Introduction

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the main pungent component of hot chili peppers, causes a burning sensation, pain, and inflammation. These characteristics make it a useful tool for studying inflammatory pain. When injected intradermally in rats, capsaicin produces hyperalgesia, defined as a decrease in pain threshold and/or an increase in pain levels in response to a normally painful stimulus [1]. Intradermal administration of capsaicin also produces nocifensive behavior in rats, characterized by licking, lifting, and guarding the injected paw [1]. Capsaicin-induced hyperalgesia is present in response to both radiant heat and mechanical stimulation. Primary hyperalgesia occurs at the site of injury and is mediated by C-fiber polymodal mechanoheat peripheral nociceptors [2] [3] [4] [5]. Secondary hyperalgesia is

elicited in response to mechanical stimulation in regions surrounding the injury, and is mediated by central nervous system sensitization [4] [5] [6] as well as peripheral mechano-insensitive C-fibers [7]. Capsaicin produces hyperalgesia primarily through activation of the transient receptor potential cation channel, subfamily V, number 1 (TRPV1) ion channel [8]. TRPV1 is a ligand-gated non-selective cation channel expressed in sensory neurons (for review see [9]). Ligands for TRPV1 such as exogenous capsaicin or protons (producing an acidic environment) decrease the temperature threshold for TRPV1 activation, producing a sensation of noxious heat, even at room temperature [10]. TRPV1 is required for inflammatory sensitization to noxious thermal stimuli; TRPV1 knockout mice failed to develop carrageenan-induced thermal hyperalgesia [11].

Cannabis has been used for centuries for its pain-relieving properties. The main active ingredient of cannabis, Δ^9 -tetrahydrocannabinol, produces antinociception by binding to G protein-coupled CB₁ [12] [13] [14] and CB₂ [15] receptors. Cannabinoids produce antinociception in animal models of both acute and chronic pain (for review see [16]). Anandamide (AEA, arachidonylethanolamide) and 2-arachidonoylglycerol (2-AG) are endogenous ligands for the cannabinoid receptors. Activation of cannabinoid receptors by endocannabinoids also produces antinociception [17] (for review see [18]). The enzyme fatty-acid amide hydrolase (FAAH) is responsible for hydrolysis of AEA into arachidonic acid and ethanolamine [19]. By contrast, the enzyme monacylglycerol lipase (MGL) is responsible for hydrolysis of 2-AG into fatty acid and glycerol [20]. AEA, but not 2-AG, is also an endogenous ligand for TRPV1 [21] [22]. Thus, AEA may act as an endocannabinoid to activate cannabinoid receptors to produce antinociception or as an endovanilloid at TRPV1 to produce hyperalgesia. Indeed, elevated AEA levels in the periaqueductal gray have been found to either suppress or enhance thermal nociception through TRPV1 and CB₁ mechanisms, depending on the dose [23].

In the periphery, exogenous AEA either reduces nocifensive behavior produced by capsaicin [24] or induces nocifensive behavior in the absence of capsaicin via TRPV1 activation [25].

Peripheral cannabinoid antinociceptive mechanisms involve cannabinoid CB₁ and CB₂ receptor activation [26] [27] [28] [29] [30] [31]. Less is known about the roles of peripheral CB₁ and CB₂ receptors in modulating capsaicin-induced sensitization. Local hindpaw injections of the CB₂-selective agonist AM1241 reduce capsaicin-induced mechanical allodynia [32], nocifensive behavior and thermal hyperalgesia [32] [33]. Intraplantar injections of the mixed CB₁/CB₂ agonist WIN55,212-2 attenuates thermal hyperalgesia, but not mechanical hyperalgesia or nocifensive behavior [34]. Similarly, intraplantar injections of WIN55,212-2 reduced mechanical and thermal hypersensitivities in response to heat injury (but not capsaicin) via CB₁- and CB₂-dependent mechanisms [35]. However, the impact of elevating endocannabinoids in the periphery on a TRPV1-activated model of inflammatory pain remains unknown.

Pharmacological inhibition of FAAH and MGL exhibits therapeutic potential in inflammatory pain models (for review see [36]) [31] [37] [38]. The recent development of JZL184, a potent selective MGL inhibitor, offers the potential to elucidate the role of peripheral 2-AG in pain modulation [39]. When injected systemically, the MGL inhibitor JZL184 and the FAAH inhibitor URB597 decreased nerve injury-induced mechanical and cold allodynia via CB₁- and/or CB₂-dependent mechanisms [40].

In the present study, we compared effects of pharmacological inhibition of MGL (with JZL184), FAAH (with URB597) and endocannabinoid uptake (with VDM11) at the peripheral level on behavioral hypersensitivity evoked by intradermal administration of capsaicin. Nocifensive behavior, thermal hyperalgesia, and mechanical allodynia were quantified in response to drug- or vehicle-pretreatment and capsaicin administration. The CB₁-selective

antagonist AM251 and the CB₂-selective antagonist AM630 were co-administered with the agonists to evaluate the specific receptor subtypes underlying antihyperalgesic and antiallodynic effects of endocannabinoid modulators. The present studies are the first to unveil the effects of pharmacologically increasing peripheral endocannabinoid levels on capsaicin-induced behavioral hypersensitivities.

3.3. Materials and Methods

3.3.1. Subjects

Two hundred and nineteen male Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 260-350 g were used in behavioral experiments. Rats were allowed unlimited access to food and water, and were housed on a 12 h light/dark cycle. The experimental research protocols were approved by The University of Georgia Animal Care and Use Committee. All procedures followed the guidelines for the treatment of animals according to the International Association for the Study of Pain [41].

3.3.2. Drugs and Chemicals

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved (1 mg/mL) in a vehicle of 7% Tween 80 in 0.9% saline, sonicated, and filtered as described previously [1]. VDM11 [N-(4-hydroxy-2-methylphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide], URB597 [(3'-(aminocarbonyl)[1,1'-biphenyl]-3-yl)-cyclohexyl carbamate], JZL184 [4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxyl)methyl)piperidine-1-carboxylate], AM251 [1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-

pyrazole-3-carboxamide] and AM630 [[6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)-methanone] were purchased from Cayman Chemical (Ann Arbor, MI). Doses of AM251 and AM630 were those used by Guindon et al. [31]. AM251, AM630, VDM11, and URB597 were dissolved in a 1:1:1:17 ratio of DMSO:ethanol:cremophor:saline. JZL184 was dissolved in a 4:1 ratio of polyethylene glycol 300:Tween 80, as described previously [39]. The vehicles employed were those used previously [31] [39] [42]. In order to evaluate pharmacological specificity, the highest doses of VDM11, JZL184, and URB597 were co-administered in cocktails with AM251 or AM630. The volume of drug or vehicle administered in the paw (i.e. either alone or in combination with antagonists) was 50 μ L in all studies.

3.3.3. Behavioral Testing

Responsiveness to thermal and mechanical stimulation was examined in separate groups of rats to prevent development of behavioral sensitization to the stimuli. The same animals were used to evaluate thermal hyperalgesia and nocifensive behavior.

Thermal hyperalgesia was determined using the radiant heat method [43]. Rats were placed on an elevated glass platform in individual plastic cages. Radiant heat was applied through the glass to the midplantar surface of the right and left hindpaws. Rats were allowed to habituate to the apparatus for at least 15 minutes until exploratory behavior was no longer observed. Stable baseline latencies (about 12 seconds) were obtained prior to drug or vehicle administration. A ceiling latency of 20 seconds was employed to prevent tissue damage to the hindpaws. Fifteen minutes prior to capsaicin administration, rats received an intraplantar

injection in the right (ipsilateral to capsaicin injection) hindpaw of one of the following: VDM11 (100 µg i.pl.; n = 8), JZL184 (1, 10, or 100 µg i.pl.; n = 6 – 8 per group), URB597 (5 or 75 µg i.pl.; n = 6 per group), AM251 (80 µg i.pl.; n = 6), AM630 (25 µg i.pl.; n = 6), or an agonist/antagonist cocktail consisting of VDM11 (100 µg i.pl.) coadministered with AM251 (80 µg i.pl.), VDM11 coadministered with AM630 (25 µg i.pl.) (n = 6 per group), JZL184 (100 µg i.pl.) coadministered with AM251 (80 µg i.pl.), or JZL184 (100 µg i.pl.) coadministered with AM630 (25 µg) (n = 6 per group). Separate groups received vehicle consisting of either a 1:1:1:17 ratio of DMSO:ethanol:cremophor:saline (n = 8) or a 4:1 ratio of polyethylene glycol 300:Tween 80 (n = 8). Separate groups of rats received an injection in the left hindpaw (contralateral to capsaicin injection) of one of the following: VDM11 (100 µg i.pl.), JZL184 (100 µg i.pl.), or 4:1 vehicle (50 µL) (n = 5 – 6 per group). At time 0, capsaicin (10 µg) was injected (10 µL) into the plantar surface of the right (ipsilateral) hindpaw. The amount of time (in seconds) that rats spent displaying nocifensive behavior (i.e. guarding, licking, or lifting the injected paw) was quantified for 5 minutes beginning immediately after capsaicin injection [1]. Thermal withdrawal latencies were recorded at 5, 15, 30, 45, and 60 minutes after capsaicin injection. The fifteen minute time interval between drug pretreatment and capsaicin injection was selected based upon previous work documenting peripheral antinociceptive effects of FAAH and MGL inhibitors at the same time point [31] [44].

Paw withdrawal thresholds to mechanical stimulation were measured using an electronic Von Frey device (IITC Life Sciences, Woodland Hills, CA). Rats were placed in individual plastic cages on an elevated wire mesh platform, and were allowed to habituate for at least 15 minutes until exploratory behavior was no longer observed. A rigid tip was applied in duplicate to the midplantar region of the left and right hindpaws before and after capsaicin administration.

Mechanical stimulation was terminated when the paw was withdrawn. Stable baselines were obtained prior to experimental testing. Fifteen minutes prior to capsaicin administration, rats received an intraplantar injection (50 μ L) in the right (ipsilateral) hindpaw of one of the following: VDM11 (100 μ g i.pl.; n = 8), JZL184 (1, 10 or 100 μ g i.pl.; n = 6 – 8 per group), URB597 (5 or 75 μ g i.pl.; n = 6 – 8 per group), AM251 (80 μ g i.pl.; n = 6), AM630 (25 μ g i.pl.; n = 6), or drug/antagonist cocktails (n = 6 per group) consisting of VDM11 (100 μ g i.pl.) coadministered with AM251 (80 μ g i.pl.), VDM11 (100 μ g i.pl.) coadministered with AM630 (25 μ g i.pl.), URB597 (75 μ g i.pl.) coadministered with AM251 (80 μ g i.pl.), or URB597 (75 μ g i.pl.) coadministered with AM630 (25 μ g i.pl.). Separate groups of rats received either vehicle consisting of a 1:1:1:17 ratio of DMSO:ethanol:cremophor:saline (n = 12) or vehicle consisting of a 4:1 ratio of polyethylene glycol 300:Tween 80 (n = 8). Separate groups of rats received an injection in the left (contralateral) hindpaw of either VDM11 (100 μ g i.pl.; n = 6), or URB597 (75 μ g i.pl.; n = 6). Capsaicin (10 μ g/10 μ L) was subsequently injected into the plantar surface of the right (ipsilateral) hindpaw. Mechanical withdrawal thresholds were assessed at 5, 30, 60, and 120 minutes after capsaicin injections.

3.3.4. Statistical Analysis

Mechanical paw withdrawal thresholds and thermal paw withdrawal latencies were determined in duplicate at each time point and averaged for each paw separately. Thermal paw withdrawal latencies and mechanical paw withdrawal thresholds were analyzed separately in the ipsilateral and contralateral hindpaws. Data obtained from thermal and Von Frey testing were analyzed by repeated measures analysis of variance (ANOVA). When sphericity determined by Mauchly's sphericity test was not assumed, the Greenhouse-Geisser correction factor was

applied to all repeated factors. The sources of significant interactions were further evaluated by performing one-way ANOVAs for each individual time point, followed by Tukey post hoc tests. Planned comparisons were performed using independent samples t-tests (one-tailed). Nocifensive behavior was analyzed using univariate ANOVA and planned comparison independent samples t-tests, one- or two-tailed as appropriate. Tukey post hocs were performed on all Univariate ANOVAs. $P \leq 0.05$ was considered statistically significant.

3.4. Results

3.4.1. Control Conditions

Thermal paw withdrawal latencies and mechanical paw withdrawal thresholds were similar between groups prior to capsaicin treatment. Intradermal capsaicin produced nocifensive behavior in animals receiving vehicle (lasting 181.25 ± 11.3 s in response to 1:1:1:17 vehicle pretreatment and 244.25 ± 12 s in response to 4:1 vehicle pretreatment). Capsaicin also decreased thermal paw withdrawal latencies (by 54.2% (1:1:1:17 vehicle) and 65.4% (4:1 vehicle)), relative to baseline, at the time of maximal thermal hyperalgesia. Likewise, capsaicin lowered mechanical paw withdrawal thresholds, relative to baseline, in vehicle-treated animals (by 59.0% (1:1:1:17) and 64.2% (4:1 vehicle)). In all studies, pharmacological manipulations did not alter thermal paw withdrawal latencies or mechanical paw withdrawal thresholds, relative to vehicle, in the hindpaw contralateral to capsaicin treatment ($P > 0.05$), unless specifically described.

3.4.2. MGL inhibition via JZL184

The highest dose of JZL184 (100 µg i.pl.) suppressed capsaicin-evoked nocifensive behavior (by 19.9%) compared to vehicle ($F_{3,24} = 4.637$, $P = 0.011$; ANOVA; Fig. 3.1a), whereas lower doses were without effect. JZL184 produced a dose-dependent suppression of the magnitude ($F_{3,24} = 9.996$, $P = 0.0002$; Fig. 3.1b) and time course ($F_{15,120} = 1.864$, $P = 0.034$) of thermal hyperalgesia. The antihyperalgesic effects produced by the high dose of JZL184 (100 µg i.pl.) outlasted that of the middle dose of JZL184 (10 µg i.pl.) ($P = 0.033$; ANOVA). JZL184 (100 µg i.pl.) increased thermal withdrawal latencies (by 115% compared to vehicle), at the time of maximal capsaicin-induced thermal hyperalgesia. However, intraplantar administration of JZL184 did not alter mechanical withdrawal thresholds, relative to vehicle, at any dose ($P = 0.64$; Fig. 3.1c).

A behaviorally active dose of JZL184 (100 µg i.pl.), administered to the contralateral paw, did not alter capsaicin-evoked nocifensive behavior relative to contralateral paw injections of vehicle ($P = 0.4005$; Fig. 3.2a). However, the vehicle itself reliably increased capsaicin-evoked nocifensive behavior ($P = 0.0052$, two-tailed t-test); nocifensive behavior was higher in groups receiving the 4:1 vehicle in the ipsilateral compared to the contralateral paw ($F_{3,23} = 3.299$, $P = 0.038$, ANOVA, Tukey post hoc). Capsaicin-evoked nocifensive behavior was also similar in groups receiving ipsilateral paw injections of JZL184 and contralateral paw injections of JZL184 or vehicle. Ipsilateral paw injections of JZL184 (100 µg i.pl.) suppressed the magnitude ($F_{2,19} = 12.077$, $P = 0.0004$; ANOVA) and time course ($F_{10,95} = 3.349$, $P = 0.001$; Fig. 3.2b; ANOVA) of capsaicin-evoked thermal hyperalgesia relative to either vehicle ($P = 0$, Tukey post hoc) or contralateral paw injections of JZL184 ($P = 0.017$, Tukey post hoc).

To identify the specific cannabinoid receptor subtype responsible for the effects of MGL inhibition, the CB₁ antagonist AM251 (80 µg i.pl.) and the CB₂ antagonist AM630 (25 µg i.pl.), were co-administered with the highest effective dose of JZL184 (100 µg i.pl.). The JZL184-induced attenuation of nocifensive behavior ($F_{3,24} = 5.994$, $P = 0.003$, ANOVA) and thermal hyperalgesia ($F_{3,24} = 8.869$, $P = 0.0004$; ANOVA) was blocked by either AM251 ($P = 0.011$) or AM630 ($P = 0.004$). Blockade of the antihyperalgesic effects of JZL84 was also time-dependent ($F_{15,120} = 2.26$, $P = 0.008$; ANOVA; Fig. 3.3a and 3.3b). Intraplantar injections of AM251 or AM630 alone had no effect on either nocifensive behavior ($P = 0.64$; ANOVA; Fig. 3.3c) or thermal paw withdrawal latencies ($P = 0.78$; ANOVA; Fig. 3.3d) relative to vehicle. However, AM251 produced a modest decrease in thermal paw withdrawal latencies, relative to vehicle, in the hindpaw contralateral to capsaicin injection ($F_{2,17} = 4.034$, $P = 0.037$, ANOVA; $P = 0.033$ versus vehicle, Tukey post hoc; data not shown).

3.4.3. FAAH Inhibition via URB597

The FAAH inhibitor URB597 did not alter capsaicin-evoked nocifensive behavior at any dose ($P = 0.35$; Fig. 3.4a). Similarly, URB597 did not alter the magnitude ($P = 0.488$) or time course ($P = 0.096$; Fig. 3.4b) of capsaicin-evoked thermal hyperalgesia, relative to vehicle. By contrast, URB597 (75 µg i.pl.) suppressed capsaicin-evoked mechanical hypersensitivity throughout the observation interval; this suppression ($F_{2,21} = 8.915$, $P = 0.002$; ANOVA; Fig. 3.4c) was observed relative to either vehicle ($P = 0.001$) or the low dose (5 µg i.pl.) of URB597 ($P = 0.036$). URB597 (75 µg i.pl.) increased mechanical withdrawal thresholds (by 38.71%), compared to vehicle, at the time of maximal capsaicin-evoked mechanical allodynia.

The anti-allodynic effects of URB597 (75 µg i.pl.) were mediated by a local site of action. URB597, administered to the capsaicin-injected paw, suppressed capsaicin-evoked mechanical allodynia ($F_{2,21} = 12.136$, $P = 0.0003$; Fig. 3.5a) relative to either vehicle ($P = 0.002$, Tukey post hoc), administered to the same paw, or URB597 ($P = 0.0001$, Tukey post hoc), administered to the opposite paw.

The URB597-induced suppression of capsaicin-evoked mechanical allodynia was blocked ($F_{3,26} = 6.291$, $P = 0.002$; Fig. 3.5b) by a CB₁ but not a CB₂ antagonist. URB597 suppressed mechanical allodynia relative to either vehicle ($P = 0.003$) or URB597 coadministered with AM251 ($P = 0.006$). By contrast, the CB₂ antagonist failed to block the anti-allodynic effects of URB597 ($P = 0.184$). When administered alone, neither AM251 nor AM630 significantly altered capsaicin-evoked mechanical allodynia relative to vehicle ($P = 0.24$; ANOVA; Fig. 3.5c).

3.4.4. Endocannabinoid Uptake Inhibition via VDM11

VDM11 (100 µg i.pl.), administered to the capsaicin-injected (ipsilateral) paw, decreased ($F_{2,19} = 56.41$, $P = 0$; ANOVA) capsaicin-evoked nocifensive behavior (by 73.5%) compared to either vehicle ($P = 0.0001$; Fig. 3.6a) or the same dose administered to the contralateral paw ($P = 0.0001$). VDM11, administered to the capsaicin-injected paw, also suppressed the magnitude ($F_{2,19} = 5.334$, $P = 0.015$) and time course ($F_{10,95} = 5.421$, $P = 0.0001$; ANOVA; Fig. 3.6b) of thermal hyperalgesia. The suppressive effect of VDM11 was maximal from 5 – 15 minutes post-capsaicin. VDM11 normalized thermal withdrawal latencies relative to baseline at five minutes post-capsaicin ($P = 0.57$). At 30 min post-capsaicin, VDM11 still suppressed thermal hyperalgesia; thermal paw withdrawal latencies were higher following VDM11 administration

ipsilateral, as opposed to contralateral, to the capsaicin-injected paw ($P < 0.05$; Tukey post hoc). VDM11 increased thermal paw withdrawal latencies (by 136.2%), relative to vehicle, at the time of maximal capsaicin-evoked thermal hyperalgesia.

VDM11 (100 μg i.pl.), administered to the capsaicin-injected paw ($F_{2,23}=19.076$, $P = 0.0001$; ANOVA; Fig. 3.6c), also suppressed mechanical allodynia; this suppression was observed relative to either vehicle ($P = 0.0001$, Tukey post hoc) or VDM11 administered to the contralateral paw ($P = 0.001$). The VDM11-induced suppression of capsaicin-evoked mechanical allodynia was also time-dependent ($F_{8,92} = 3.837$, $P = 0.001$); effects of VDM11 were maximal at 5 ($F_{2,25} = 20.641$, $P = 0.0001$) and 30 ($F_{2,25} = 9.105$, $P = 0.001$) minutes post-capsaicin. VDM11 (100 μg i.pl.), administered to the ipsilateral paw, increased capsaicin-evoked mechanical paw withdrawal thresholds (by 95.1%), relative to vehicle, at the time of maximal capsaicin-evoked mechanical allodynia (Fig. 3.6c).

The VDM11-induced suppression of capsaicin-evoked nocifensive behavior ($F_{3,24} = 19.511$, $P = 0.0001$; ANOVA; Fig. 3.7a) was blocked by either the CB_1 antagonist AM251 ($P = 0.0001$) or the CB_2 antagonist AM630 ($P = 0.0001$). Similarly, both AM251 and AM630 blocked the VDM11-induced suppression of thermal hyperalgesia ($F_{3,24} = 5.851$, $P = 0.004$; ANOVA). This blockade was maximal between 5 and 30 min post-capsaicin ($P < 0.05$ for all time points; Fig. 3.7b).

A transient but reliable decrease in thermal paw withdrawal latencies was observed in the contralateral paw ($F_{12,112} = 1.399$, $P = 0.02$; ANOVA; data not shown) at 30 min post injection in groups receiving AM630 coadministered with VDM11 ($P = 0.02$; Tukey post hoc). The VDM11-induced suppression of capsaicin-evoked mechanical allodynia ($F_{3,28} = 8.205$, $P = 0.0004$; ANOVA) was blocked by a CB_1 ($P = 0.026$; Tukey post hoc) but not a CB_2 antagonist (P

= 0.796; Tukey post hoc; Fig. 3.7c). All groups exhibited a time-dependent blockade of capsaicin-evoked mechanical allodynia ($F_{3,31}=13.322$, $P = 0.0001$; ANOVA) that was maximal at 5 min post capsaicin (Fig. 3.7c).

3.5. Discussion

3.5.1. Summary of study

Local injection of the MGL inhibitor JZL184, the FAAH inhibitor URB597, and the endocannabinoid uptake inhibitor VDM11 differentially suppressed capsaicin-evoked behavioral hypersensitivities. Moreover, these suppressions were mediated by distinct cannabinoid receptor subtypes and occurred in a modality-specific manner. JZL184 suppressed both capsaicin-evoked nocifensive behavior and thermal hyperalgesia via either CB₁- or CB₂-receptor mechanisms. By contrast, URB597 suppressed capsaicin-evoked mechanical allodynia via a CB₁-mediated mechanism only. URB597 did not alter capsaicin-evoked nocifensive behavior or thermal hyperalgesia and JZL184 did not alter capsaicin-evoked mechanical allodynia. Finally, VDM11 suppressed all three dependent measures with pharmacological specificity consistent with that observed following inhibition of both FAAH and MGL.

In our study, intraplantar injections of capsaicin produced nocifensive behavior, thermal hyperalgesia, and mechanical allodynia in the injected paw, as described previously [1]. Local injections of JZL184, URB597, and VDM11 ipsilateral, but not contralateral, to the site of injury suppressed capsaicin-evoked behavioral hypersensitivities compared to control conditions. Thus, effects of the drug manipulations employed here were mediated by a peripheral mechanism. Moreover, inhibitors of endocannabinoid degradation and uptake selectively

suppressed capsaicin-induced hypersensitivities without producing analgesia. On the whole, responses to mechanical and thermal stimulation in the paws contralateral to capsaicin were rarely different between groups. Minor exceptions may be attributed to changes in weight-bearing resulting from capsaicin injection, or by normal variation between rats. These data are in agreement with previous studies demonstrating the importance of peripheral mechanisms of cannabinoid antihyperalgesic action in other pain models [26] [27] [28] [29] [30] [31] [45] [46].

3.5.2. Effects of the MGL inhibitor JZL184

The MGL inhibitor JZL184 produced a dose-dependent suppression of capsaicin-evoked thermal hyperalgesia, presumably by elevating endogenous levels of 2-AG. These data are in agreement with previous studies demonstrating antihyperalgesic effects of MGL inhibitors and exogenous 2-AG, administered locally in the paw [44]. However, mechanical allodynia is also suppressed by MGL inhibitors, administered systemically, in neuropathic pain models [40]. Neuropathic pain may elevate levels of endocannabinoid tone [47] [48] and produce regulatory changes in endocannabinoid hydrolyzing enzymes and their receptors [49]; such changes may facilitate cannabinoid-mediated attenuations of nerve-injury induced mechanical allodynia. However, in the present study, JZL184 failed to suppress capsaicin-evoked mechanical allodynia. It is noteworthy that the vehicle used here to dissolve JZL184 also produced edema and enhanced capsaicin-induced nocifensive behavior (see [39] for vehicle description). Edema in conjunction with capsaicin may further lower mechanical withdrawal thresholds, masking JZL184-induced suppression of mechanical allodynia. However, it is important to emphasize that any pronociceptive effects produced by this vehicle did not prevent detection of JZL184-induced suppression capsaicin-evoked nocifensive behavior. Moreover, mechanical withdrawal

thresholds and thermal withdrawal latencies were similar in capsaicin-treated animals receiving the vehicle for either JZL184 or URB597; thus, the choice of vehicle employed for JZL184 is unlikely to confound interpretation of antihyperalgesic effects of MGL inhibition. Doses of JZL184 employed here were determined based upon dose-response studies performed in the formalin test (unpublished data). These latter studies validated the choice of doses administered to the paw in the present studies. Further dose escalation was prevented by limitations in drug solubility. It is unlikely that a higher dose of JZL184 is necessary to observe suppression of mechanical allodynia because thermal hyperalgesia was profoundly suppressed by JZL184 at the same time points.

The exact anatomical localization of MGL in the periphery remains unknown. Our studies suggest that MGL is unlikely to reside on primary afferents in naïve rats that contribute to capsaicin-evoked mechanical allodynia at the site of injury. Following peripheral nerve damage, FAAH is known to transition from small-sized cells to large-sized cells of dorsal root ganglia [49]. It is, therefore, possible that MGL undergoes similar phenotypic switches in response to long term injury. However, the development of capsaicin-induced hypersensitivity follows a rapid time course which likely precludes such downstream changes from contributing to the pattern of results observed here. Nonetheless, phenotypic switches in MGL expressing cells following nerve injury could contribute to differences observed between effects of MGL inhibitors in neuropathic pain models [40] [44] and the present capsaicin model. In the present studies, JZL184 suppressed capsaicin-evoked nocifensive behavior, a dependent measure that has not previously been examined for responsiveness to MGL inhibition. Our observation that both CB₁ and CB₂ receptor subtypes are implicated in the suppressions of capsaicin-evoked nocifensive behavior and thermal hyperalgesia induced by endocannabinoid modulators is

consistent with the findings of previous studies [32] [33] [34]. Thus, the present experiments describe a modality-specific effect of MGL inhibition in response to capsaicin that is distinct from that observed with neuropathic pain.

3.5.3. Effects of the FAAH inhibitor URB597

The FAAH inhibitor URB597 suppressed capsaicin-evoked mechanical allodynia, presumably by elevating endogenous levels of AEA. However, URB597, administered locally in the paw, did not alter capsaicin-evoked thermal hyperalgesia or nocifensive behavior under identical conditions. Local inhibition of FAAH in the periphery suppresses mechanical allodynia in neuropathic pain model [44]. Moreover, systemically-administered URB597 also suppresses thermal hyperalgesia in a model of neuropathic pain [44]. Transition of FAAH from smaller to larger cell sizes in dorsal root ganglia following peripheral nerve injury [49] may explain the differences observed between neuropathic pain and capsaicin models. Exogenous AEA both suppresses and induces nociception in a variety of pain models via CB₁ and TRPV1 activation, respectively [24] [25] [50]. The failure of peripheral FAAH inhibition to suppress capsaicin-evoked thermal hyperalgesia or nocifensive behavior may perhaps be accounted for by the dual role of AEA as an endocannabinoid and endovanilloid [21] [22]. Alternatively, capsaicin activation of TRPV1 receptors may render agonist effects of AEA at TRPV1 insignificant; thus, only agonistic effects at cannabinoid receptors on mechanoreceptors are observed, resulting in suppression of mechanical allodynia. Due to limits in solubility, we did not evaluate doses of URB597 higher than 75 µg i.pl. Previous studies have verified endocannabinoid-modulating activity with similar doses of URB597; thus, this local dose is unlikely to be too low to effectively suppress thermal hypersensitivity and nocifensive behavior [44] [51]. TRPV1

activation is required for thermal hyperalgesia [11]. Capsaicin-induced TRPV1 activation, in combination with elevated AEA levels produced by FAAH inhibition, may prevent suppression of thermal hyperalgesia by AEA. Studies demonstrating colocalization of FAAH and TRPV1 [49] provide anatomical support for the behavioral observations presented here.

The present studies demonstrate that URB597-induced suppressions of mechanical allodynia were mediated by a peripheral CB₁ mechanism. Previous anatomical studies reveal the presence of CB₁ in dorsal root ganglion cells of heterogeneous size [48] [52] [53].

Colocalization of TRPV1 with CB₂ on small cells of the dorsal root ganglion has also been reported [54]. It is possible that CB₂ activation is masked by capsaicin-induced TRPV1 activation on small cells, whereas mechanoreceptors localized to medium- and larger-diameter cells are suppressed by FAAH inhibition-induced activation of CB₁, independently of TRPV1 activation. Of course, biological conditions (e.g. severe tissue acidosis) exist where TRPV1 is tonically activated (for review see [55]). Our studies suggest that in situations where TRPV1 is activated, AEA reduces mechanical allodynia via CB₁ receptor activation only.

3.5.4. Effects of the endocannabinoid transport inhibitor VDM11

The existence of an endocannabinoid membrane transporter has remained controversial. It has been argued that endocannabinoid transport inhibitors produce physiological effects via passive diffusion or FAAH inhibition [56]. However, endocannabinoid uptake inhibitors produce pharmacological effects in FAAH knockout mice, suggesting that FAAH does not mediate endocannabinoid uptake [57]. The recent identification of two fatty acid binding proteins that transport AEA across cell membranes [58] provide further evidence that transport inhibitors do indeed inhibit endocannabinoid uptake. The endocannabinoid uptake inhibitor VDM11 was

specifically employed in these experiments because it has very little agonist activity at TRPV1 [59], unlike other uptake inhibitors such as AM404 [60]. Moreover, VDM11 may be employed *in vivo* to increase levels of both AEA and 2-AG [44]. In the present studies, VDM11 suppressed capsaicin-evoked nocifensive behavior and thermal hyperalgesia as well as mechanical allodynia. VDM11 suppressed nocifensive behavior and thermal hyperalgesia via a CB₁/CB₂-mediated mechanism, similar to that observed following local administration of the MGL inhibitor JZL184 in the paw. Moreover, VDM11 additionally suppressed capsaicin-evoked mechanical allodynia via a CB₁-mediated mechanism, similar to that observed following intraplantar administration of the FAAH inhibitor URB597. It is reasonable to speculate that MGL inhibition increases 2-AG whereas FAAH inhibition elevates AEA, and that inhibition of endocannabinoid transport, by elevating levels of both AEA and 2-AG, suppresses all three dependent measures. Our data suggest that VDM11, administered locally in the paw, prevents the uptake of both 2-AG and AEA. Elevation of each endocannabinoid has distinct effects in modulating the behavioral response to capsaicin and both sets of effects are mimicked by administration of VDM11. Our studies also suggest that capsaicin-induced mechanical allodynia is more effectively suppressed with VDM11 than with URB597. However, additional dose-response studies are required to definitively test this hypothesis. This latter observation may be accounted for by recruitment of both 2-AG and AEA following inhibition of endocannabinoid uptake and by the ability of VDM11 to block AEA's ability to cross the membrane and bind TRPV1 at its intracellular site [61].

3.5.5. Conclusion

In conclusion, pharmacological elevation of endocannabinoids in the periphery suppresses capsaicin-induced behavioral hypersensitivity via distinct mechanisms. Inhibition of MGL effectively suppresses capsaicin-induced nocifensive behavior and thermal hyperalgesia, presumably by increasing accumulation of 2-AG. By contrast, inhibition of FAAH suppresses capsaicin-induced mechanical allodynia only, presumably by increasing accumulation of AEA. Elevation of both endocannabinoids via inhibition of endocannabinoid uptake displays an additive effect, mimicking actions of both the FAAH and MGL inhibitor in combination. Moreover, the present experiments suggest specific roles for endocannabinoids in activating peripheral CB₁ and CB₂ receptors. MGL inhibition activates CB₁ and CB₂ receptors to reduce capsaicin-evoked nocifensive behavior and thermal hyperalgesia, whereas FAAH inhibition activates CB₁ receptors only to reduce mechanical allodynia. Inhibition of endocannabinoid uptake suppresses capsaicin-evoked behavioral sensitization with a profile of pharmacological specificity that is, again, mimicked by inhibition of both MGL and FAAH. These studies are the first to document that pharmacological inhibition of endocannabinoid uptake and degradation suppresses capsaicin-induced behavioral hypersensitivities. Thus, elevating levels of both 2-AG and AEA at the site of injury in the periphery may prove to be more therapeutically beneficial than targeting only one endocannabinoid via inhibition of FAAH or MGL alone.

3.6. References

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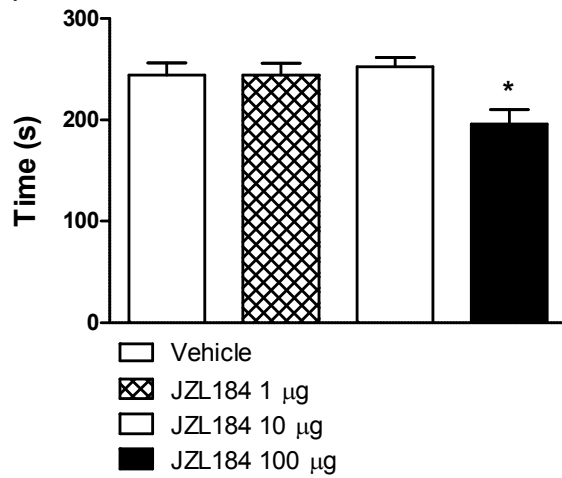
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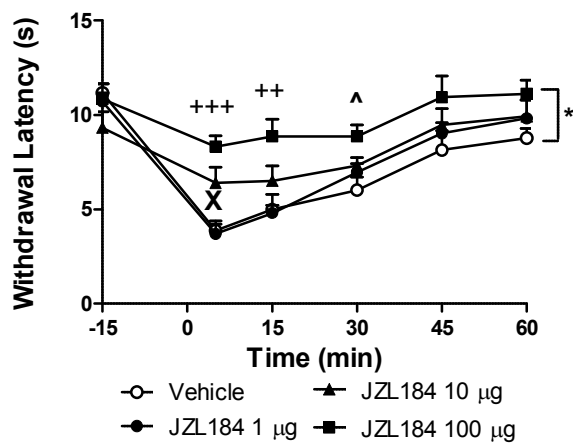
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Figure 3.1

(a)



(b)



(c)

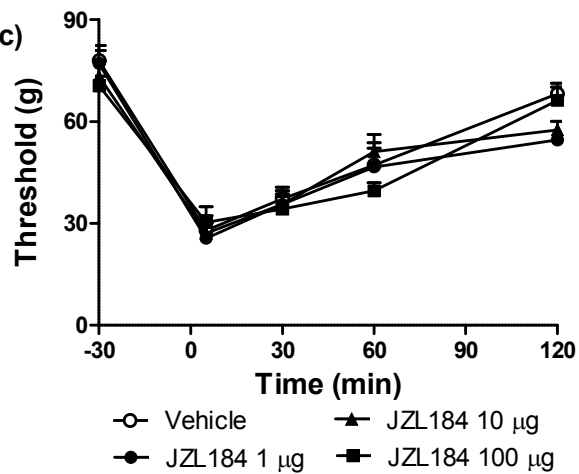


Fig. 3.1. Local injection of the MGL inhibitor JZL184 in the paw suppresses capsaicin-induced nocifensive behavior and thermal hypersensitivity. (a) Only the highest dose of JZL184 (100 μ g i.pl.) suppressed capsaicin-evoked nocifensive behavior compared to vehicle. (b) JZL184 suppressed thermal hyperalgesia in a dose-dependent manner. (c) JZL184 (1, 10, and 100 μ g i.pl.) did not alter capsaicin-induced mechanical hypersensitivity. Data are expressed as mean + SEM. +++ $P < 0.001$, ++ $P < 0.01$, versus vehicle and low dose (1 μ g i.pl.) (ANOVA, Tukey post hoc); X $P < 0.05$ versus all treatments except the highest dose (100 μ g i.pl.) (ANOVA, Tukey post hoc); ^ $P < 0.05$ versus vehicle (ANOVA, Tukey post hoc); * $P \leq 0.05$ JZL184 (100 μ g i.pl.) versus all other groups (ANOVA, Tukey post hoc).

Figure 3.2

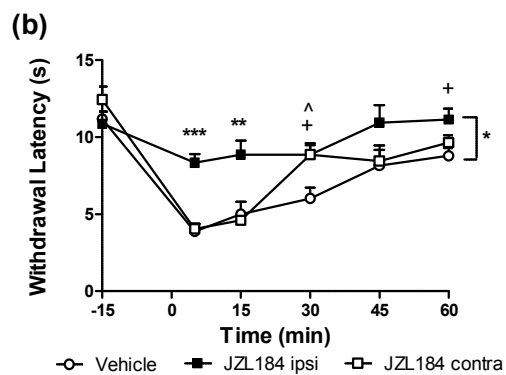
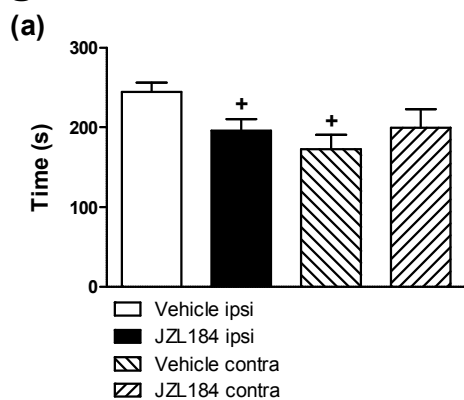


Fig. 3.2. The MGL inhibitor JZL184 suppresses capsaicin-evoked nocifensive behavior and thermal hyperalgesia through a local site of action. A local injection of JZL184 (100 µg i.pl.) ipsilateral but not contralateral to the capsaicin-injected paw suppressed (a) nocifensive behavior and (b) thermal hyperalgesia. Nocifensive behavior was similar following injection of either vehicle or the active dose of JZL184 to the contralateral paw. Data are expressed as mean + SEM. *** $P \leq 0.001$, ** $P < 0.01$, * $P < 0.05$ versus all other groups (ANOVA, Tukey post hoc); + $P < 0.05$ versus vehicle ipsi (t-test, ANOVA, Tukey post hoc); ^ $P < 0.05$ JZL184 contra versus vehicle (ANOVA, Tukey post hoc).

Figure 3.3

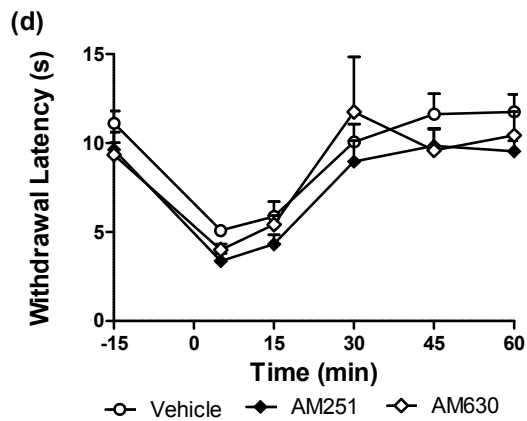
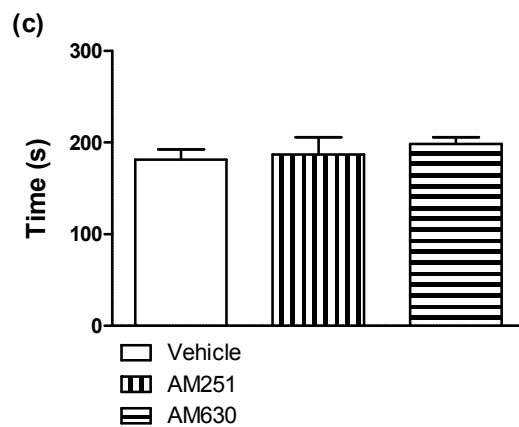
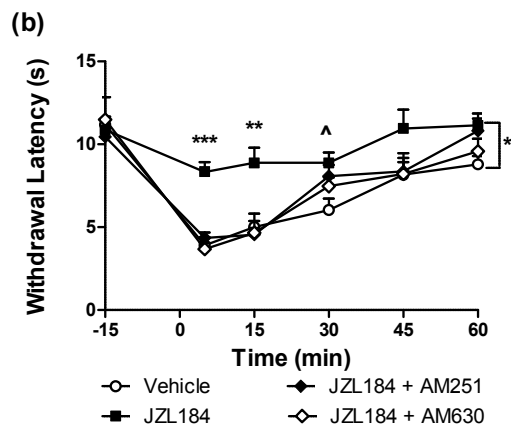
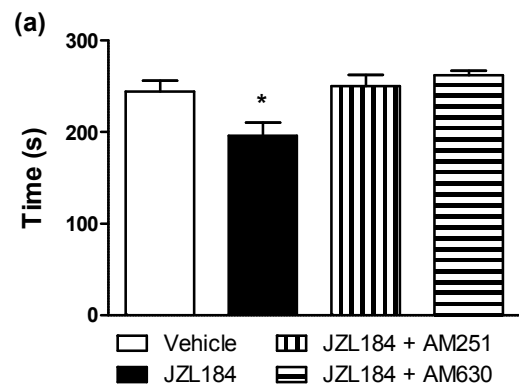
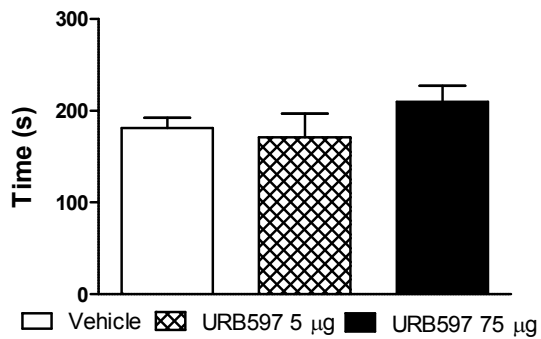


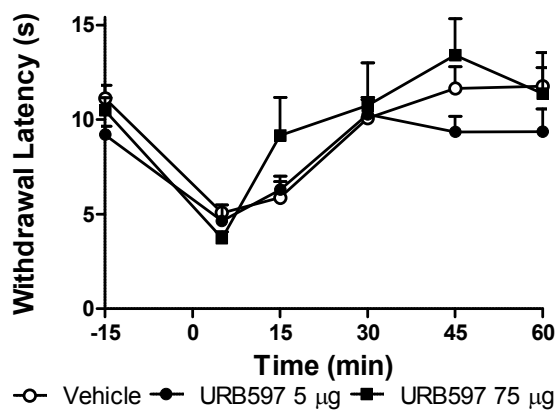
Fig. 3.3. JZL184 suppresses capsaicin-evoked nocifensive behavior and thermal hyperalgesia through CB₁- and CB₂-specific mechanisms. Either the CB₁ antagonist AM251 (80 µg i.pl.) or the CB₂ antagonist AM630 (25 µg i.pl.) decreased the anti-hyperalgesic effect of JZL184 (100 µg i.pl.) in suppressing capsaicin-evoked (a) nocifensive behavior and (b) thermal hyperalgesia. (c, d) Effects of AM251 (80 µg i.pl.) and AM630 (25 µg i.pl.) alone did not differ from vehicle. Data are expressed as mean + SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus all other groups (ANOVA, Tukey post hoc); ^ $P < 0.05$ versus vehicle (ANOVA, Tukey post hoc).

Figure 3.4

(a)



(b)



(c)

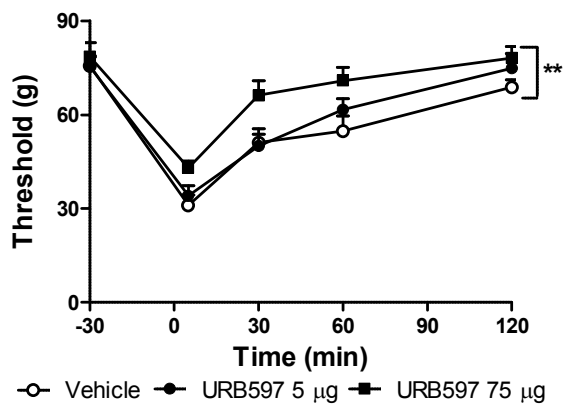
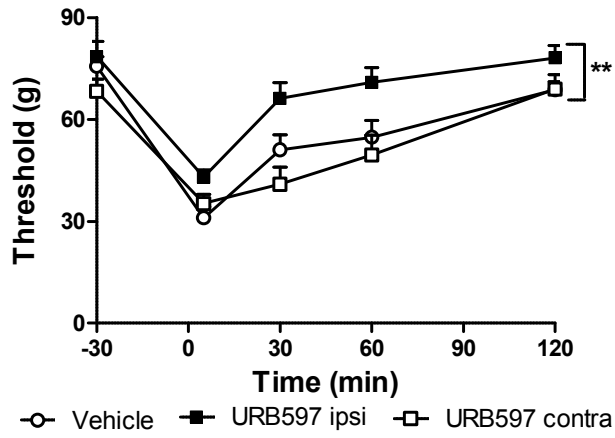


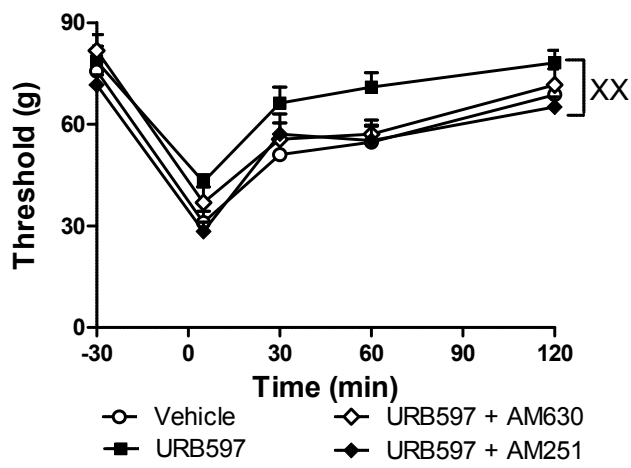
Fig. 3.4. The FAAH inhibitor URB597 attenuates capsaicin-evoked mechanical hypersensitivity. URB597 (5 or 75 μg i.pl.) did not suppress capsaicin-evoked (a) nocifensive behavior or (b) thermal hyperalgesia. The highest dose of URB597 (75 μg i.pl.) suppressed capsaicin-evoked (c) mechanical allodynia. Data are expressed as mean + SEM. ** $P < 0.01$ URB597 (75 μg i.pl.) versus all other groups (ANOVA, Tukey post hoc).

Figure 3.5

(a)



(b)



(c)

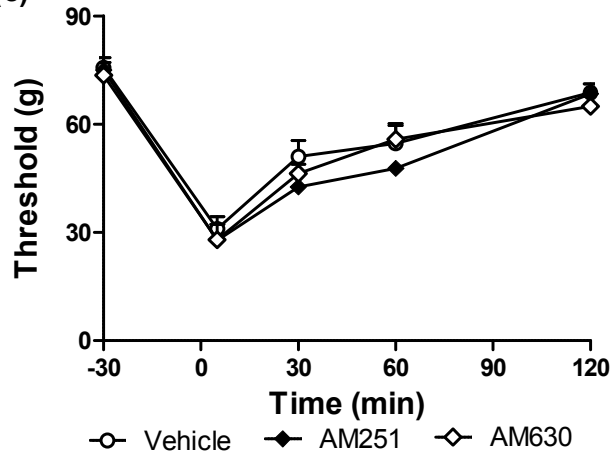


Fig. 3.5. The FAAH inhibitor URB597 suppresses capsaicin-evoked mechanical hypersensitivity through a peripheral site of action. (a) A local injection of URB597 (75 μ g i.pl.) ipsilateral, but not contralateral, to the capsaicin-injected paw attenuated capsaicin-evoked mechanical allodynia. (b) The URB597-induced suppression of capsaicin-induced mechanical hypersensitivity was blocked by the CB₁ antagonist AM251 (80 μ g i.pl.) but not the CB₂ antagonist AM630 (25 μ g i.pl.). (c) Local injection of AM251 (80 μ g i.pl.) or AM630 (25 μ g i.pl.) did not alter capsaicin-evoked mechanical hypersensitivity relative to vehicle. Data are expressed as mean + SEM. ** $P < 0.01$ URB597 ipsi versus all other groups (ANOVA, Tukey post hoc); XX $P < 0.01$ URB597 ipsi versus all groups except URB597 + AM630 (ANOVA, Tukey post hoc).

Figure 3.6

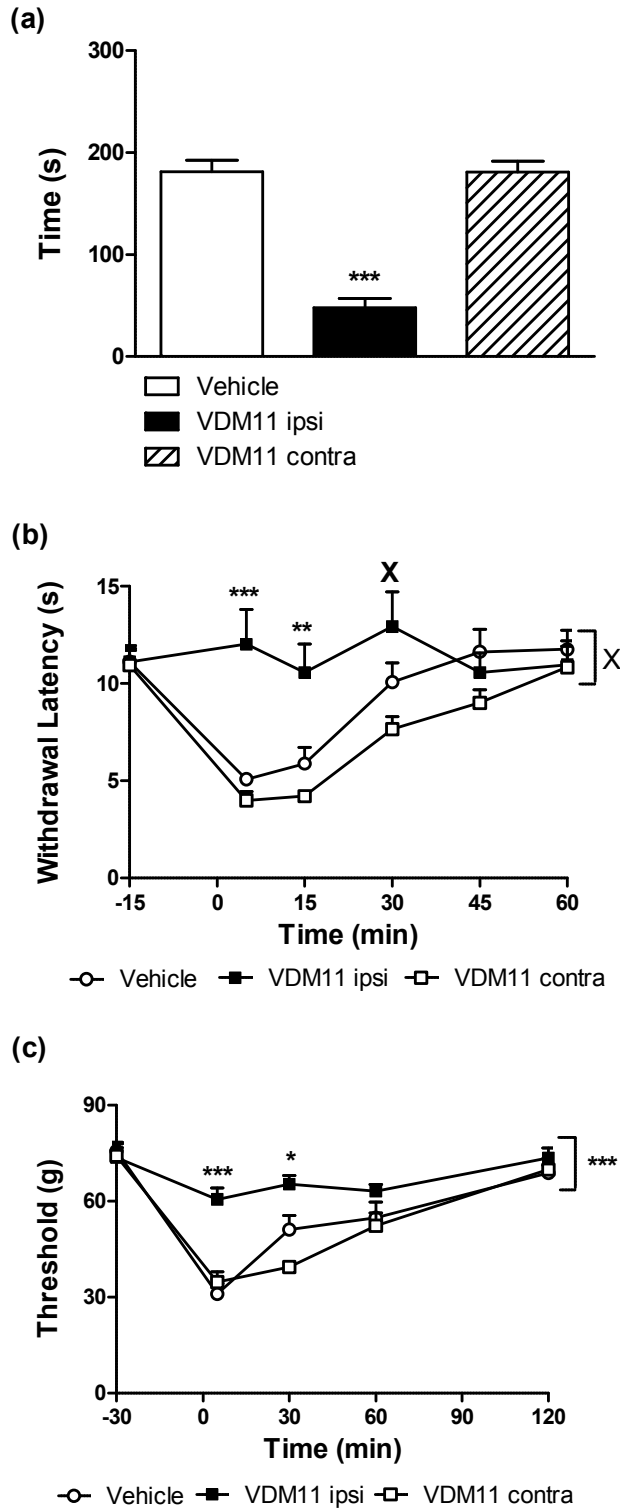
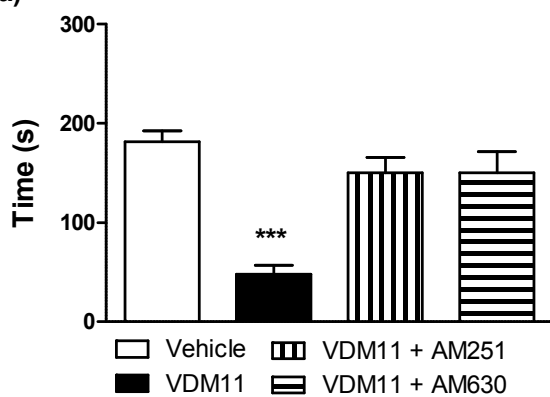


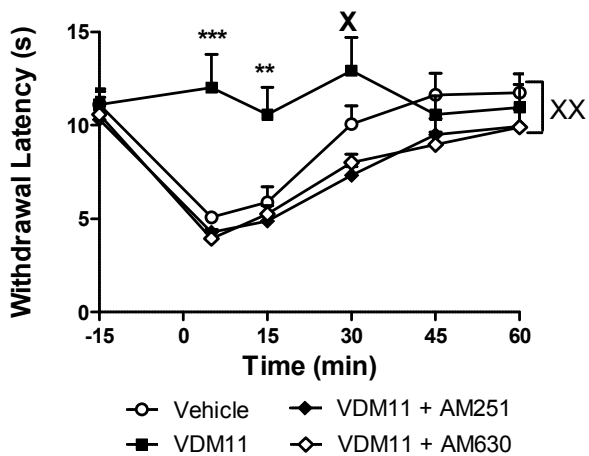
Fig. 3.6. Intraplantar injection of the endocannabinoid uptake inhibitor VDM11 suppresses capsaicin-evoked nocifensive behavior, thermal hyperalgesia, and mechanical allodynia via a peripheral mechanism. Local injection of VDM11 (100 μg i.pl.) in the hindpaw ipsilateral (VMD11 ipsi), but not contralateral (VDM11 contra), to capsaicin injection suppressed (a) nocifensive behavior, (b) thermal hyperalgesia, and (c) mechanical allodynia. Data are expressed as mean + SEM. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P < 0.05$ VDM11 ipsi (100 μg i.pl.) versus all other groups (ANOVA, Tukey post hoc); X $P < 0.05$ VDM11 ipsi (100 μg i.pl.) versus VDM11 contra (100 μg i.pl.) (ANOVA, Tukey post hoc).

Figure 3.7

(a)



(b)



(c)

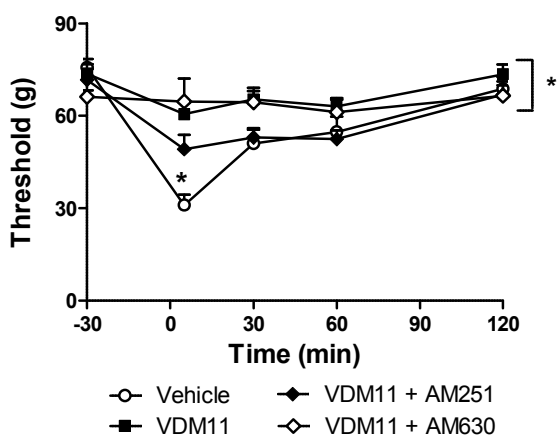


Fig. 3.7. The endocannabinoid uptake inhibitor VDM11 suppresses capsaicin-evoked behavioral sensitization through modality- and cannabinoid receptor subtype-specific mechanisms. The VDM11-induced suppression of capsaicin-evoked (a) nocifensive behavior and (b) thermal hyperalgesia is blocked by either the CB₁ antagonist AM251 (80 µg i.pl.) or the CB₂ antagonist AM630 (25 µg i.pl.). (c) The VDM11 (100 µg i.pl.)-induced suppression of capsaicin-evoked mechanical allodynia and was blocked by AM251 (80 µg i.pl.) but not AM630 (25 µg i.pl.). Data are expressed as mean + SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus all other groups (ANOVA, Tukey post hoc); XX $P < 0.01$, X $P < 0.05$ VDM11 versus all groups except vehicle (ANOVA, Tukey post hoc).

CHAPTER 4: DISCUSSION

Numerous studies have determined the effectiveness of endocannabinoids at relieving pain in a variety of inflammatory and neuropathic pain models. The limited amount of published data regarding the effects endocannabinoids on capsaicin-induced inflammatory pain warranted a closer look at this pain model. In addition, the complete lack of data regarding the effects of pharmacologically inhibiting endocannabinoid degradation led to the present experiments in an attempt to describe for the first time their effects in a capsaicin-induced TRPV1-activated model of inflammatory pain.

The present studies were conducted to evaluate the effectiveness of the pharmacological inhibition of peripheral endocannabinoid degradation at reducing the behavioral hypersensitivities induced by intradermal capsaicin. More specifically, the pharmacological inhibitors of the AEA and 2-AG hydrolyzing enzymes FAAH and MGL (URB597 and JZL184), respectively, and the endocannabinoid uptake inhibitor VDM11 were injected intraplantarly in an attempt to discover the roles of AEA and 2-AG and their therapeutic usefulness in the capsaicin model of inflammatory pain. Behavioral hypersensitivities that were examined include mechanical allodynia, thermal hyperalgesia, and nocifensive behavior.

As summarized in Table 4.1 and illustrated in the schematic of Figure 4.1, capsaicin-induced mechanical allodynia was significantly suppressed by URB597, while thermal hyperalgesia and nocifensive behavior were significantly suppressed by JZL184. VDM11 significantly suppressed all three hypersensitivity modalities, likely through the combined effects

of AEA and 2-AG activity. CB₁ receptor activity was necessary for mechanical allodynia suppression, while CB₁ and CB₂ activity were necessary for thermal hyperalgesia and nocifensive behavior, suggesting endocannabinoid specificity at the cannabinoid receptor subtypes in the periphery: elevated AEA levels activate CB₁, while elevated 2-AG levels are capable of activating both CB₁ and CB₂.

In summary, pharmacologically elevating endocannabinoid levels in the periphery seems to be effective at alleviating capsaicin-induced hypersensitivities, especially when both endocannabinoids are elevated synergistically through an endocannabinoid uptake inhibitor. Given that the capsaicin-induced inflammatory pain model mimics a number of pathological conditions, pharmacologically elevating endocannabinoids in the periphery may prove to be very therapeutically useful for hypersensitivity suppression and pain relief.

TABLE 4.1. Inhibitors of fatty-acid amide hydrolase (URB597), monoacylglycerol lipase (JZL184), and endocannabinoid transport (VDM11) differentially suppress capsaicin-evoked nocifensive behavior, thermal hyperalgesia, and mechanical allodynia.

Compound	Effect on Endocannabinoids	Nocifensive Behavior	Thermal Hyperalgesia	Mechanical allodynia
URB597	↑ AEA	—	—	CB ₁
JZL184	↑ 2-AG	CB ₁ /CB ₂	CB ₁ /CB ₂	—
VDM11	↑ AEA 2-AG	CB ₁ /CB ₂	CB ₁ /CB ₂	CB ₁

FIGURE 4.1

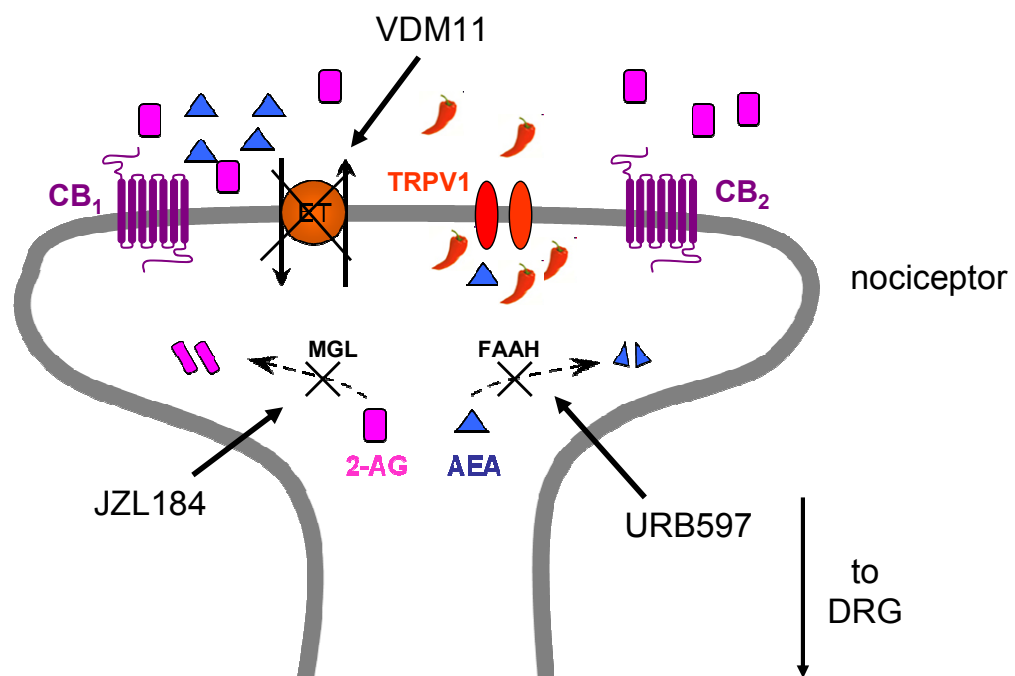


Fig. 4.1. A schematic demonstrating the proposed interaction between endocannabinoids and capsaicin in peripheral nociceptors. The endocannabinoids 2-AG and AEA are degraded by the enzymes MGL and FAAH, respectively. An endocannabinoid uptake transporter (ET) aids in the degradation process by transporting endocannabinoids to their intracellularly-located degrading enzymes. AEA is capable of binding both CB₁ and TRPV1, while 2-AG can bind CB₁ and CB₂ (as suggested by the present data). Note that CB₁, CB₂, and TRPV1 may not all be present on the same nociceptor, but they are all displayed in this schematic for the sake of simplicity.