

Changes in endocannabinoid signaling contribute to the anti-hyperalgesic effect of
URB597 in a murine model of persistent inflammation

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Amy Elizabeth Lindberg

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Dr. Virginia Seybold

August 2011

© Amy Elizabeth Lindberg 2011

Acknowledgements

I am so grateful for the people who have impacted my life during graduate school. Their encouragement, support, and advice as well as the time and energy they poured into my development as a scientist have been foundational to my success. My advisor, Dr. Seybold, deserves special recognition. Ginger, I can never thank you enough for the thoughtful way you guided me through this project, always encouraging me to seek out the answers on my own and never letting me settle for less than my best. You've seen the best in me and cheered me on, and seen the worst in me and helped me become better than I thought I could be. Thank you for giving me the confidence to believe in myself.

To my remaining committee members, Dr. Stan Thayer, Dr. Sabita Roy and Dr. Gibson Wood, thank you for the insightful feedback and constant support throughout my graduate career. Your collective wisdom can't be measured.

To the Seybold lab, especially Lia and Iryna, your support and friendship over the past many years has been invaluable to me. Thank you so much for teaching me how to be a good labmate. Thank you, also, Iryna, for the sharing the data presented in Figure 4 in Chapter 2 of this work.

To my family, you have always encouraged me to do my best and have been pivotal in shaping me into the person I am today. I couldn't have done this without your unconditional love and support. Thank you for providing me with the education and opportunities to succeed. And to the most important person in my life, my husband, Gregg, without you I'd be lost in so many ways. Thank you for inspiring me, for loving me, and for doing life with me. I love you, and Sprout, more than you'll ever know.

Dedication

This thesis is dedicated to my father-in-law, Denny. Although you didn't get to see a copy of the finished draft, I know you saw it in me. I'll always be thankful you believed in me and encouraged me to persevere to the end. I know you are smiling in Heaven. I miss you, always.

Abstract

Modulation of endocannabinoid neurotransmission has a therapeutic benefit in the treatment of inflammatory pain. Studies in this thesis investigated endocannabinoid signaling in a murine model of persistent, peripheral inflammation. Specifically, the ability of URB597, an inhibitor of fatty acid amide hydroxylase (FAAH), which degrades the endogenous cannabinoid anandamide, to reduce mechanical hyperalgesia associated with inflammation was determined.

The first study tested whether local injection of URB597 dose-dependently reduced mechanical hyperalgesia associated with persistent inflammation. Inflammation was induced by injection of Complete Freund's Adjuvant (CFA) in the hind paw of mice and mechanical hyperalgesia was determined using a series of von Frey filaments. The first part of the study resolved that local injection of URB597 dose-dependently reduced mechanical hyperalgesia associated with persistent inflammation and decreased mechanical sensitivity in naïve mice. However, injection of URB597 did not result in increased endocannabinoid content in the plantar skin ipsilateral to the injection as would be expected based on the known mechanism of action of URB597.

The second and third studies investigated the effect of inflammation on levels of FAAH, endocannabinoids and cannabinoid (CB)-1 receptor in naïve and CFA-injected mice to understand the neurochemistry underlying the anti-hyperalgesic effect of URB597. Levels FAAH mRNA decreased and enzyme activity trended toward a decrease in the plantar skin of the inflamed hind paw compared to tissue from naïve mice, but inflammation did not alter level of anandamide in plantar skin ipsilateral to the injections.

In contrast, an increase in FAAH mRNA was accompanied by a decrease in the level of anandamide in dorsal root ganglia (DRGs) ipsilateral to the inflamed hind paw compared to naïve mice. In addition, there is an upregulation of functional CB1 receptors in DRGs ipsilateral to the inflamed hind paw in CFA-injected mice compared to DRGs from naïve mice. Together, these data support a model in which reduced synthesis of AEA in the primary afferent neurons may contribute to the mechanical hyperalgesia associated with peripheral inflammation, and upregulation of CB1 receptors on the primary afferent neurons affected by inflammation may be a compensatory response to decreased basal activation of AEA.

Table of Contents

	Page
Acknowledgements	i
Dedication	ii
Abstract	iii
List of Tables	vii
List of Figures	viii
Chapter One: Introduction	1
I. Cannabinoid effectiveness and limitations.	2
II. Endocannabinoid signaling.	2
III. Nociceptive mechanical transduction.	11
IV. Nociceptive terminology and behavioral assays.	12
V. Peripheral and central mechanisms of sensitization.	14
VI. Experimental models of peripheral inflammation.	17
VII. Treatment of inflammatory pain with cannabinoid receptor agonists.	21
VIII. Inhibition of FAAH as an effective treatment for pain.	25
IX. Current clinical use of cannabinoids.	26
X. Statement of purpose.	27
Chapter Two: The effect of local injection of URB597 on mechanical hyperalgesia associated with persistent peripheral inflammation	31
Introduction	32
Methods	34
Results	38
Discussion	43

Table of Contents Continued

	Page
Chapter Three: Changes in neurochemistry of endocannabinoid signaling associated with persistent peripheral inflammation	59
Introduction	60
Methods	62
Results	69
Discussion	71
Chapter Four: Functional changes in CB1 receptors in neurons isolated from CFA-injected mice	80
Introduction	81
Methods	83
Results	89
Discussion	91
Chapter Five: Concluding statements	104
References	113

List of Tables

	Page
Chapter One	
Chapter Two	
Table 1. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) levels in plantar paw skin from CFA-injected mice treated with local URB597 or vehicle	58
Chapter Three	
Table 1. FAAH enzyme and anandamide content in plantar paw skin from naïve and CFA-injected mice	77
Table 2. FAAH enzyme and anandamide content in nerve tissue from naïve and CFA-injected mice	78
Table 3. AEA level in DRGs from a depolarizing stimulus of 50 mM KCl in naïve and CFA-injected mice	79
Chapter Four	
Chapter Five	

List of Figures

	Page
Chapter One	
Figure 1. Pathways of anandamide biosynthesis	29
Figure 2. Pathways of 2-arachidonoylglycerol synthesis	30
Chapter Two	
Figure 1. URB597 blocked mechanical hyperalgesia associated with peripheral inflammation in a time-dependent manner	52
Figure 2. URB597 dose-dependently reduced mechanical hyperalgesia associated with peripheral inflammation	53
Figure 3. CB1 and CB2 receptor antagonists blocked the anti-hyperalgesic effect of URB597	54
Figure 4. A low dose of URB597 reduced mechanical sensitivity in naïve mice	55
Figure 5. A high dose of URB597 increased mechanical sensitivity in naïve mice	56
Figure 6. The TRPV1 receptor antagonist capsazepine (Capz) blocked the increased mechanical sensitivity in naïve mice induced by a high dose of URB597	57
Chapter Three	
Chapter Four	
Figure 1. Effect of ACPA, a CB1 receptor agonist, on $[Ca^{2+}]_i$	98
Figure 2. Leftward shift in the concentration response to ACPA in large neurons isolated from CFA-injected mice	99
Figure 3. There was no effect of ACPA concentration in small neurons isolated from either naïve or CFA-injected mice	100
Figure 4. Involvement of CB1 and CB2 receptors in the response to ACPA	101

was confirmed by blocking the inhibitory effect by co-application of either AM281 or AM60, CB1 and CB2 receptor antagonists respectively

Figure 5. There was no effect of URB597 in large neurons isolated from either naïve or CFA-injected mice **102**

Figure 6. There was no effect of URB597 in small neurons isolated from either naïve or CFA-injected mice **103**

Chapter Five

Figure 1. Model of endocannabinoid signaling in the periphery. **113**

Chapter 1

Introduction

I. Cannabinoid effectiveness and limitations.

Because pain is a debilitating health problem, clinical management of pain is an area of active research. Although opioid compounds are some of the most effective analgesics, development of tolerance can significantly limit their long term use. Alternative treatments have included cannabinoids, derived from the *cannabis sativa*, which have been used to treat pain for more than four thousand years. However, cannabinoid use is associated with negative psychotropic side effects such as altering sensory perception, causing elation and euphoria, as well as cognitive and memory impairment. In addition to these effects, cannabinoids are also associated with dependence, immobility, and hypothermia. Together, these side effects prevent widespread use of cannabinoids as analgesics.

Despite the limitations of cannabinoids, they are an effective analgesic in nearly every animal model of pain studied including inflammatory, neuropathic, cancer, mechanical, thermal, and chemical pain. As such, medicinal application of cannabinoids has received renewed interest and been effective in humans not only for promoting analgesia (Noyes et al., 1975; Holdcroft et al., 1997), but also in attenuating nausea (Sallan et al., 1975) and stimulating appetite (Nelson et al., 1994). An understanding of the cannabinoid system and its adaptive role in the modulation of pain can give insight into novel therapeutic approaches for pain management.

II. Endocannabinoid signaling.

Cannabinoid Receptors.

Two subtypes of cannabinoid receptors have been identified and characterized (Matsuda et al., 1990; Munro et al., 1993). Both receptor subtypes are G-protein coupled through Gi/o proteins and function to inhibit adenylyl cyclase (Howlett et al., 1986; Felder et al., 1995) and activate mitogen-activated protein kinases including p44/42 MAP kinase (Wartman et al., 1995), p38 kinase (Derkinderen et al., 2001) and JUN-terminal kinase (Rueda et al., 2000). Additionally, CB1 receptors alter neurotransmission by inhibition of N- and P/Q-type calcium channels (Mackie et al., 1993, 1995; Twitchell et al., 1997; Shen and Thayer, 1998; Ross et al., 2001) and activation of potassium channels (Mackie et al., 1995). The overall effect resulting from cannabinoid receptor activation is cellular inhibition.

CB1 receptor expression has been extensively studied in the central nervous system as well as the periphery. CB1 receptors occur predominantly in the nervous system in regions including the neocortex, substantia nigra, periaqueductal gray (PAG), rostral ventrolateral medulla, hippocampus and cerebellum (Herkenham et al., 1991; Jansen et al., 1992; Matsuda et al., 1993; Glass et al., 1997) as well as in the spinal cord (Hohmann and Herkenham 1998), dorsal root ganglia (DRG) (Hohmann and Herkenham 1999; Khasabova et al., 2002) and peripheral nerves (Sañudo-Peña et al., 1999). Frequently, CB1 receptors are localized to nerve endings, suggesting they play a role in modulating presynaptic neurotransmitter release.

CB1 receptor knockout mice have been developed and characterized (Ledent et al., 1999; Zimmer et al., 1999). Although they are healthy and fertile, they show an increased mortality rate. Consistent with the effects of CB1 receptors, these mice display

reduced locomotor activity, increased catalepsy, and hypoalgesia in the formalin and hotplate tests, confirming the role of endogenous cannabinoids in modulating higher brain motor activity and nociceptive responses. Furthermore, CB1 receptor knockout mice show no hypomobility, no catalepsy, and no hypothermia after administration of cannabinoid agonists.

CB2 receptors share only 44% homology with CB1 receptors at the protein level and 68% in the transmembrane domains (Lutz, 2002). CB2 receptor protein is found primarily in cells of the immune system (Facci et al., 1995; Galiegue et al., 1995). Furthermore, expression levels of CB2 mRNA are 10-100 fold higher than CB1 mRNA in immune tissues including the spleen, thymus and peripheral blood mononuclear cells (Galiegue et al., 1995). CB2 receptor mRNA and protein were not thought to be constitutively present in the brain until recently. Since then, CB2 receptor expression has been found in brain microglial cells (Nunez et al., 2004; Carlisle et al., 2002; Carrier et al., 2004) and on neurons in the brains of naïve mice in regions including the cerebellum (Ashton et al., 2006), brainstem (Van Sickle et al., 2005), PAG, thalamus, striatum, cortex, amygdala and hippocampus (Gong et al., 2006), as well as in the spinal cord (Walczak et al., 2005; Walczak et al., 2006; Beltramo et al., 2006). However, many of these studies measured the protein content by Western blot analysis or RNA content by PCR, making it difficult to conclusively determine the cell type expressing CB2 receptors (Van Sickle et al., 2005; Walczak et al., 2005; Ashton et al., 2006; Beltramo et al., 2006; Walczak et al., 2006) Conversely, some studies provide immunochemical evidence that CB2 are expressed on neurons (Van Sickle et al., 2005; Ashton et al., 2006; Gong et al.,

2006) and included either peptide antigen or used CB2 $-/-$ mice to verify the specificity of the antibodies. Supporting a role for functional CB2 receptors in the spinal cord of rats, CB2 receptor agonists dose-dependently decrease capsaicin-induced CGRP release, and this effect persisted in CB1 $-/-$ mice, further ruling out a CB1 receptor-mediated effect (Beltramo et al., 2006).

In addition to inflammatory cells, regions of interest for our studies include the skin and dorsal root ganglia. In the skin, CB2 receptors have been found in cutaneous nerve fiber bundles, on epidermal keratinocytes, and epithelial cells of hair follicles, sebocytes and eccrine sweat glands (Ibrahim et al., 2005; Stander et al., 2005; Walczak et al., 2005) using immunochemical techniques. Evidence for the presence of CB2 receptors on DRG neurons is less clear as data to support both the presence (Beltramo et al., 2006; Walczak et al., 2005; Wotherspoon et al., 2005) and absence (Hohmann and Herkenham, 1999b; Price et al., 2003) of these receptors has been reported. For studies reporting the presence of CB2 receptors on DRG neurons, either the peptide antigen or CB2 $-/-$ mice to confirm the specificity of the CB2 antibodies. For studies reporting the absence of CB2 receptors in DRG neurons, the antibody specificity was confirmed in control tissue exhibiting high levels of CB2 receptor mRNA such as the spleen. However, differences between these studies may be a result of culture conditions influencing receptor expression or of antibody specificity.

CB2 receptors are involved in the regulation of inflammatory reactions and immune responses. Activation of CB2 inhibits the proliferation of cells that produce a robust immune response including T cells (Patrini et al., 1997), B cells (Valk et al.,

1997), natural killer cells (Parolaro, 1999), mast cells and macrophages (Facci et al., 1995). CB2 receptor knockout mice have been shown to have immune system defects related to reduced macrophage functionality (Buckley et al., 2000). In addition to modulating immune responses, CB2 receptors have been shown to produce antinociception by stimulating peripheral release of an endogenous opioid β -endorphin from keratinocytes (Ibrahim et al., 2005).

Endocannabinoids.

Following the identification of cannabinoid receptors, the occurrence of endogenous ligands for CB1 and CB2 receptors were investigated. Two such ligands, N-arachidonyl ethanolamide (anandamide, AEA) and 2-arachidonylglycerol (2-AG) have since been discovered (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995). Current dogma suggests endogenous cannabinoids are released from post-synaptic neurons and act retrogradely on pre-synaptic neurons to modulate cellular signaling. In the primary afferent nerve terminals in the periphery, however, a variation in endocannabinoid neurotransmission may occur. Anandamide can be produced by DRG neurons and released locally to act in an autocrine manner by stimulating CB1 receptors on the axon terminal from which it was released. This is supported by evidence that CB1 receptor mRNA and protein are present in the primary afferent neurons (Hohmann and Herkenham 1999; Ahluwalia et al., 2000, 2002; Bridges et al., 2003; Agarwal et al., 2007). Moreover, lower thresholds to noxious thermal and mechanical stimuli are exhibited by transgenic mice with selective CB1 receptor expression knocked down in

nociceptive neurons (Agarwal et al., 2007) as well as by local injection of a CB1 receptor antagonist in naïve mice (Khasabova et al., 2008).

Anandamide is a partial agonist at both CB1 and CB2 receptors (Hillard 2000; Pertwee and Ross 2002). It acts with greater affinity at CB1 receptors (K_i (CB1 vs. CB2) = 89 vs 371 nM) (reviewed by Hohmann 2002) to induce a tetrad of cannabimimetic effects: analgesia, sedation, catalepsy and hypothermia (Little et al., 1988; Jerman et al., 2002; reviewed by Pacher et al., 2006). Anandamide has also been shown to have low agonist affinity at the transient vanilloid (TRPV1) receptor (Zygmunt et al., 1999; Smart et al., 2000). Pro-nociceptive TRPV1 receptors are ligand-gated, non-selective cationic channels. Importantly, the CB1 receptor binding site is extracellular (Shire et al., 1996) whereas the TRPV1 agonist binding site is intracellular (Jung et al., 1999). This compartmentalization may play a role in regulating the anti- and pro-nociceptive effects of anandamide. As such, TRPV1 receptor activation and subsequent increase in intracellular calcium can drive anandamide synthesis within cells (Ahluwalia et al., 2003a), which can further activate TRPV1 channels (van der Stelt et al., 2005). Alternatively, generated anandamide can be released from the cell and have an inhibitory effect on the level of intracellular calcium (Millns et al., 2001), presumably through CB1 receptor binding and subsequent calcium channel inhibition (Twitchell et al., 1997; Khasabova et al., 2002). Anandamide is generated in neurons in the brain (Di Marzo et al., 1994; Giuffrida et al., 1999), keratinocytes (Maccarrone et al., 2003) and DRGs (Ahluwalia et al., 2003b, van der Stelt et al., 2005) as well as in macrophages (Varga et al., 1998), and vascular endothelium (Deutsch et al., 2007).

The other endogenous cannabinoid, 2-AG, acts as a full agonist at both CB1 and CB2 receptors and is the most efficacious agonist toward both receptor types (Hillard 2000; Savinainen et al., 2001). Reported K_i values for 2-AG are from the nanomolar to micromolar range (Mechoulam et al., 1995; Sugiura et al., 1995). However, unlike anandamide, 2-AG lacks activity at vanilloid receptor 1 (TRPV1 channels; van der Stelt and Di Marzo, 2004). 2-AG can be generated in immune cells, macrophages and microglia (reviewed by Bisogno 2008) as well as in the brain (Stella et al., 1997). Furthermore, levels of 2-AG are 10-25 fold higher than AEA in both skin and brain (Maione et al., 2007).

Endocannabinoid Metabolism.

Both endocannabinoids are fatty acid derivatives that are not stored in cells, but instead are synthesized in an activity-dependent manner (Di Marzo et al., 1994). The primary pathway for anandamide synthesis requires two enzymatic steps, the first of which is *N*-acylation of phosphatidylethanolamine (PE) by a calcium dependent *N*-acyltransferase (NAT). In this step, an acyl group is transferred from the *sn*-1 position of a glycerophospholipid molecule to the amino group of PE. The second step occurs through hydrolysis of *N*-arachidonoyl PE (NArPE) by an *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) to release anandamide and phosphatidic acid (Figure 1, Di Marzo et al., 1994; Sugiura et al., 2002; Okamoto et al., 2004). Alternatively, direct synthesis of anandamide from arachidonic acid and ethanolamine has also been described (Ueda et al., 1995; Paria et al., 1996). Parallel pathways for the generation of anandamide from phosphatidylethanolamine may

also exist (Figure 1). In support of this, a phospholipase (PL)_{A2} that can catalyze the hydrolysis of *N*-arachidonoyl-PE (NArPE) to *N*-arachidonoyl-lysoPE (NAr-lysoPE), which is then acted upon by a lysoPLD to generate anandamide, has been shown in the stomach (Sun et al., 2004). In RAW246.7 macrophages, *N*-acyl-PE is hydrolyzed to phosphoanandamide by a PLC, followed by a dephosphorylation by a phosphatase to produce anandamide (Liu et al., 2006). In light of evidence that anandamide tissue levels are unchanged in NAPE-PLD knockout mice compared to wild-type animals (Leung et al., 2006), clearly multiple pathways for the biosynthesis of anandamide exist. It is likely that anandamide synthesis occurs through the predominant pathway utilizing NAPE-PLD, *in vivo* (Schmid et al., 1983; Hillard and Campbell, 1997; Di Marzo 1998) because NAPE-PLD is expressed in murine primary afferent neurons (Nagy et al., 2009). However, in addition to calcium evoked anandamide synthesis (Ahluwalia et al., 2003b; van der Stelt et al., 2005) primary afferent neurons also produce anandamide in a calcium-independent manner (Vellani et al., 2008).

Multiple synthetic pathways for the production of 2-AG have been reported (Figure 2, adapted from Sugiura et al., 2004). 2-AG can be generated from inositol phospholipids by phospholipase C (PLC) followed by diacylglycerol lipase (DAG lipase) or through the combined actions of phospholipase A₁ and PLC (Stella et al., 1997; Di Marzo 1998; Piomelli et al., 1998; Sugiura et al., 2002; Bisogno et al., 2003). In addition to these pathways, 2-AG can be formed from phosphatidylcholine (PC) through PLC and DAG lipase or through phosphatidic acid through a phosphatase action (Sugiura et al., 2004).

Endocannabinoid signaling is transient and local due to termination by cellular uptake and subsequent catabolism of endocannabinoids. Anandamide is taken up into cells through a yet-uncloned membrane transporter and hydrolyzed by the membrane-bound fatty acid amide hydrolase (FAAH) to release arachidonic acid and ethanolamine (Cravatt et al., 1996). FAAH is localized primarily in the microsomal and mitochondrial fractions of tissue homogenates including the brain, liver, kidney and lung (Schmid et al., 1985; Deutsch et al., 1993) and functions optimally around a pH of 9 *in vitro* (Ueda et al., 2000; Bisogno et al., 2002). FAAH hydrolyzes anandamide in rat neuronal cells (Di Marzo et al., 1994), as well as in “neuronal” cell lines such as mouse neuroblastoma N₁₈TG₂ (Maurelli et al., 1995) and human neuroblastoma CHP100 (Maccarrone et al., 1998), or “non-neuronal” cell lines such as RBL-2H3 (Bisogno et al., 1997) and human lymphoma U937 cells (Maccarrone et al., 1998).

More recently FAAH has been reported in adult rat DRG, sciatic nerve and spinal cord (Lever et al., 2009). cDNAs of FAAH have been cloned in rat, mouse, porcine and human tissues (Cravatt et al., 1996; Giang and Cravatt 1997; Goparaju et al., 1999) and the protein sequences show 80% homology across species (Giang and Cravatt 1997). In addition to degrading anandamide, FAAH has been shown to degrade other fatty acid amides including the anti-inflammatory compound *N*-palmitoyl ethanolamine (Lo Verme et al., 2005a), the appetite modulating ligand *N*-oleoyl ethanolamine (Lo Verme et al., 2005b), and the sleep-inducing agent oleamide (Basile et al., 1999; Cravatt et al., 1996).

Although there is some evidence that FAAH is also able to metabolize 2-AG into arachidonic acid and glycerol (Sugiura et al., 2002), FAAH knockout mice preserve their

ability to hydrolyze 2-AG (Lichtman et al., 2002), and URB597, a selective FAAH inhibitor, reduced anandamide levels, but had no effect on 2-AG levels in the rat brain (Kathuria et al., 2003). Instead, 2-AG is metabolized primarily by monoacylglycerol lipase (MGL) (Goparaju et al., 1999; Dinh et al. 2002), which is unable to metabolize anandamide. MGL activity is associated with both membrane and cytosolic fractions in adipocytes and cerebellum (Sakurada et al., 1981; Ghafouri et al., 2004) and has an optimum pH of around 8 (Tornqvist et al., 1974). Mouse and human MGL show greater than 80% sequence homology (Karlsson et al., 2001).

In addition to degradation of anandamide and 2-AG by FAAH and MGL respectively, both endocannabinoids can be metabolized by cyclooxygenase-2 (COX-2) and other lipoxygenases (LOX) (Kozak et al., 2004; Lambert and Fowler, 2005). This possibility is supported by the ability of COX-2 to metabolize anandamide in FAAH knockout mice *in vivo* (Weber et al., 2004) and co-injection of anandamide and a non-specific cyclooxygenase inhibitor produces synergistic anti-nociceptive effects in the formalin test (Guindon et al., 2006).

III. Nociceptive mechanical transduction.

Primary afferent neurons have their cell bodies located outside of the spinal cord in the dorsal root ganglion, a collection of cell bodies, associated with each spinal nerve. Signals in the form of action potentials from the periphery pass directly from the distal axon to the spinal cord without passing through the cell body. In the dorsal horn of the

spinal cord, the primary afferent neurons synapse on second order neurons and signals are relayed to the brain for higher order processing.

Primary afferent neurons are categorized by axon conduction velocity and the stimuli they transduce (reviewed by McGlone and Reilly, 2010). Myelinated A β -fibers respond to low-threshold stimuli, whereas A δ -fibers are primarily excited by mechanical stimuli that damage the skin (Hunt and McIntyre, 1960; Burgess and Perl 1967; Perl 1968; Handwerker et al., 1987). However, A δ mechano-heat nociceptors (Iggo and Ogawa, 1971; Beck et al., 1974; LaMotte et al., 1982; Treede et al., 1998), which can also respond to cold stimuli (Simone and Kajander, 1996) have been described. Unmyelinated C-polymodal nociceptors respond to noxious mechanical and thermal stimuli (Bessou and Perl, 1969; Beck et al., 1974; Sorkin et al., 1997), in addition to intense cold (Simone and Kajander, 1996). C-nociceptors are preferentially activated by low rates of skin heating, whereas A δ -fibers are activated by high rates of skin heating. It has been suggested that C-mechano-heat nociceptors may evoke burning pain, whereas A δ -mechano-heat fibers may produce pricking pain (Yeomans and Proudfit, 1996; Yeomans et al., 1996).

IV. Nociceptive terminology and behavioral assays.

Nociception is defined by Loeser and Treede (2008) as "the neural processes of encoding and processing noxious stimuli" and is important for protecting the body from tissue damage. Following peripheral and central sensitization, the responses to stimulation in the periphery are enhanced. As such, *allodynia* is a painful sensation to a

previously innocuous stimulus, and *hyperalgesia* is a greater than normal pain sensation to a previously noxious stimulus and the sensation of spontaneous pain. Hyperalgesia can be further classified into primary and secondary hyperalgesia. *Primary hyperalgesia* occurs in the region of tissue damage itself, and is characterized by increased sensitivity to heat, mechanical and chemical stimuli. Primary hyperalgesia is a result of peripheral sensitization. *Secondary hyperalgesia* occurs in the surrounding undamaged tissue area that is increasingly sensitive to mechanical, but not thermal, stimuli, and results from central sensitization (reviewed by Millan, 1999).

A variety of nociceptive assays have been developed over the years to study cutaneous thermal and mechanical nociception. This report will highlight two among the most commonly used: Hargreaves (1988) method for measuring thermal nociception using a radiant heat source, and assessment of mechanical sensitivity through the use of von Frey hairs (von Frey, 1896). In the present work, we chose to focus on cannabinoid modulation of mechanical hyperalgesia in subsequent chapters using von Frey hairs because our preliminary testing produced more consistent results using this method than our radiant heat source of thermal hyperalgesia in mice. Because other studies reviewed utilize thermal nociception for assessment of cannabinoid effectiveness, an understanding of both nociceptive assays is relevant for the duration of this report.

In the Hargreaves test, rodents are placed on a raised clear surface and allowed to acclimate to their surroundings prior to testing. Following acclimation, a radiant heat source is directed at the hind paw. Stimulation to the paw is terminated upon paw

withdrawal or after a cutoff latency is reached (to prevent tissue damage) and the latency to withdraw the limb is recorded.

For assessment of mechanical nociception, typically the up-down method is used to measure tactile allodynia (Chaplan et al., 1994), or withdrawal frequency to a supra-threshold force is used to measure mechanical hyperalgesia. The up-down method consists of applying a series of filaments with various bending forces to the paw in successive order, either ascending or descending. In the absence of a paw withdrawal response, an incrementally stronger filament is applied. In the event of a paw withdrawal, an incrementally weaker filament is applied. When the initial threshold is crossed, this procedure is repeated a predetermined number of times and the 50% mN threshold is interpolated using the formula by Chaplan and colleagues (1994).

Mechanical hyperalgesia is measured by using a single filament presented to the paw ten times at 1-2 s intervals. The number of withdrawals, characterized by vigorous withdrawal or licking of the paw, is counted and expressed as a percent of the stimuli applied.

V. Peripheral and central mechanisms of sensitization.

A variety of stimuli including irritant chemicals, low pH, noxious heat and inflammatory mediators can result in sensitization of nociceptors. Peripheral sensitization of C- and A δ -fiber terminals occurs at the site of inflammation (Hedo et al., 1998; Reeh 1994). Peripheral sensitization of nociceptors is triggered by compounds including bradykinin, prostaglandins and other arachidonic acid products, serotonin,

catecholamines, ATP, adenosine, histamine, excitatory amino acids, and/or neurokinins binding to surface membrane receptors (reviewed by Walker et al., 1995; Coggeshall and Carlton 1997; Burnstock 2000). During inflammation, nerve growth factor (NGF) also plays an important role in the sensitization of nociceptors (Donnerer et al., 1992; Dmitrieva and McMahon 1996; Koltzenburg et al., 1999).

An important step in the initiation of peripheral sensitization is an increase of the intracellular calcium concentration (Guenther et al., 1999; Kress and Guenther, 1999). Increased intracellular calcium leads to activation of second messenger cascades such as protein kinase C, protein kinase A and protein kinase G pathways, altering the responsiveness of primary afferents through actions on ion channels (England et al., 1996; Fitzgerald et al., 1999) or membrane receptors such as TRPV1 (Lopshire and Nicol, 1998).

Strong, persistent noxious stimuli can trigger changes in gene expression in primary afferent neurons through activation of transcription factors (reviewed by Woolf and Salter, 2000). An array of proteins are increased in primary afferent neurons in response to inflammation: CGRP (Donnerer et al., 1992; Galeazza et al., 1995), COX2, which generates prostanoids (Beiche et al., 1998; Samad et al., 2001; Yaksh et al., 2001), dynorphin (Iadorola et al., 1988), and nNOS, which generates nitric oxide (Lam et al., 1996; Dolan et al., 2003) as well as growth factors such as NGF and brain-derived neurotrophic factor (Djoughri and Lawson, 1999; Kerr et al., 1999; Mannion et al., 1999). Interestingly, some of the proteins increased may play a role in endocannabinoid

signaling. COX-2 and other lipoxygenases can degrade endocannabinoids (Weber et al., 2004; Lambert and Fowler, 2005).

Central sensitization is distinct from peripheral sensitization and is a result of hyperexcitability of dorsal horn neurons following tissue injury. Repeated activation of C-fibers by electrical stimulation of the cutaneous receptive fields results in central sensitization and produces a phenomenon referred to as *windup*. Windup occurs when the number of neuronal responses increases with subsequent stimulation (Mendell 1966). Generally, windup is a result of C-fiber afterdischarge-evoked responses of spinal dorsal horn neurons and is involved in the maintenance of inflammatory pain (Dubner 1986). Relevant to our study, C- and A δ -fibers innervating the glabrous skin are sensitized after injection of CFA *in vivo* (Andrew and Greenspan 1999; Djouhri et al., 2006). Central sensitization is characterized by a decrease in threshold for activation (Neugebauer and Schaible 1990; Simone et al., 1991), an increase in firing rate (Guilbaud et al., 1986; Dougherty et al., 1999), increased responses to suprathreshold stimuli (Bessou and Perl 1969; Treede et al., 1992) and recruitment of low threshold A β -fibers (Neumann et al., 1996; Nakatsuka et al., 1999).

Cannabinoid receptors play a role in modulating peripheral sensitization of primary afferent fibers because they attenuate presynaptic transmitter release and tonically modulate the nociceptive threshold for sensory transduction. By inhibiting presynaptic transmitter release, cannabinoid receptors can also modulate the processes that contribute to central sensitization.

VI. Experimental models of peripheral inflammation.

Inflammation is characterized by edema, erythema and hyperalgesia. Peripheral inflammation is caused by release of a wide range of mediators from both immune cells and neurons: including serotonin, histamine, prostaglandin E2 (PGE2), substance P (SP), calcitonin gene-related peptide (CGRP), noradrenaline and bradykinin. Resident mast cells release histamine and serotonin, which act on endothelial cells to cause vasodilation and increased permeability of blood vessels. Additionally, serotonin, as well as prostaglandin, bradykinin, can excite sensory nerve terminals directly to evoke pain. Persistent stimulation causes sensory neurons to release SP, which acts as a vasodilator on blood vessels and stimulates mast cells, increasing degranulation to further promote inflammation.

Peripheral inflammation has been widely studied using a variety of models including carrageenan, formalin and Complete Freund's Adjuvant (CFA). Although each of these models produce peripheral inflammation following subcutaneous injection, the duration of the effect and mechanism of action is distinct and may help explain differences between the results presented here and those of others in the field.

Carrageenan.

Inflammation induced by carrageenan is acute and highly reproducible (Winter et al., 1962). Carrageenan is a mucopolysaccharide extract derived from a species of red alga *Chondrus crispus* (Morris, 2003) and subcutaneous injection of carrageenan into the

hind paw immediately produces the hallmark signs of inflammation—edema, erythema and hyperalgesia.

The inflammatory effect of carrageenan occurs through action of proinflammatory agents including bradykinin, histamine, tachykinins, complement and reactive oxygen/nitrogen species, and prostaglandins, which are generated at the site of injection of by infiltrating cells and act on the primary afferent neurons to promote sensitization. Among these cells, neutrophils readily migrate to the site of inflammation and are capable of generating proinflammatory reactive oxygen species.

Initial edema development is followed by a period of allodynia. Allodynia peaks around 4 h after inoculation, and latency of paw withdrawal from noxious heat and threshold for paw withdrawal from noxious mechanical stimuli are reduced at this time point (Meller et al., 1994). The inflammation persists for 24-96 h (Fletcher et al., 1996) suggesting carrageenan is a relatively acute model of peripheral inflammation. Based on the duration of action and our desire to study changes in endocannabinoid signaling associated with persistent inflammation, this model is less than ideal for our studies.

Formalin.

A second model used to study peripheral injection is that of formalin. Formalin produces bi-phasic (phase 1 and 2) pain-related behaviors including flinching, licking and favoring of the injected limb (Dubuisson and Dennis, 1977; Dickenson and Sullivan, 1987). Formalin injection produces an even more acute duration of nociception than carrageenan, with the early phase peaking in 5 minutes and then subsiding for 10-15

minutes, followed by the late phase, which persists for 20-60 minutes after injection. Phase 1 is thought to be produced by acute activation of primary afferent fibers (Dickenson and Sullivan, 1987). Phase 2 is a result of both inflammation by local release of neuroactive substances including serotonin, histamine, bradykinin, SP, and prostaglandins (Rosland et al., 1990), which sensitize primary afferent fibers and result in central sensitization (Coderre and Melzack, 1992a).

Formalin, unlike carrageenan, also produces an intense increase in the activity of C-fiber afferents (Heapy et al., 1987). However, formalin use as a model of peripheral inflammation and nociception is limited because of the cumbersome and somewhat subjective pain rating scales based on the posture of the animal that are required to interpret nociception in this model (Wheeler-Aceto et al., 1990). However, the phases associated with formalin-induced inflammation make it unique model and can explain its continued use in the field. For our purposes, formalin does not allow us to study changes in endocannabinoid signaling associated with persistent inflammation. Additionally, studies using formalin-induced inflammation also tend to measure endocannabinoids after the behavioral effects indicative of nociception have completely subsided, making it difficult to correlate changes in endocannabinoid signaling with modulation of inflammatory pain.

Complete Freund's Adjuvant.

A third experimental model of peripheral inflammation is injection of CFA. CFA contains an inactivated mycobacteria, *Mycobacterium butyricum*. Its injection produces

the hallmarks of inflammation in the injected limb, which begin within the first few minutes following injection into the hind paw. As with carrageenan, CFA has been studied following subcutaneous injection (Stein et al., 1988). At moderate doses following injection into the hind paw, hyperalgesia typically peaks within 4-6 hours and persists for 10-14 days (Hylden et al., 1989; Iadarola et al., 1988). Following the acute phase of inflammation, T cells, macrophages and polymorphonuclear leukocytes migrate to the region and release a variety of prostaglandins, leukotrienes, and a cascade of cytokines to sensitize sensory neurons.

Following a single intraplantar injection, the inflammation and nociception is significant at 12 h, yet continues to rise over days and persist for up to 2 weeks (Stein et al., 1988; Nagakura et al., 2003). This provides a prolonged window of robust inflammation and nociception in which to test analgesic agents and their ability to inhibit prolonged hyperalgesia and sensitization of peripheral primary afferent neurons, as well as central processing. This sensitization is a result of changes in gene expression to produce pro-nociceptive proteins such as CGRP (Donnerer et al., 1992; Galeazza et al., 1995), COX-2 (Beiche et al., 1998; Samad et al., 2001; Yaksh et al., 2001), dynorphin (Iadorola et al., 1988), and nNOS (Iadorola et al., 1988) that underlie the prolonged duration of hyperalgesia and is one aspect of chronic pain that is distinct from acute pain. Because we are interested endocannabinoid signaling changes during persistent inflammation, a model of CFA-induced inflammation is the best suited to these studies because the inflammation is still robust at two days post injection, and not subsiding as may be the case with carrageenan-induced inflammation.

VII. Treatment of inflammatory pain with cannabinoid receptor agonists.

The anti-nociceptive effects of cannabinoid agonists at both CB1 and CB2 receptors have been observed in a variety of pain models including inflammatory (Richardson et al., 1998a; Clayton et al., 2002; Quartilho et al., 2003; Nackley et al., 2003a, b; Elmes et al., 2005), neuropathic (Herzberg et al., 1997; Fox et al., 2001), and cancer pain (Kehl et al., 2003; Hamamoto et al., 2007; Guerrero et al., 2008; Khasabova et al., 2008; Maida et al., 2008; Potenziari et al., 2008). Although their analgesic ability is mediated through activation of both central and peripheral cannabinoid receptors (Pertwee 2001; Sagar et al. 2005; Gutierrez et al., 2007), the anti-nociceptive effect of cannabinoids is generally believed to be mediated primarily through a central site of action (Walker et al., 1999a). However, in addition to modulation of pain, there is evidence to suggest endocannabinoids tonically modulate nociceptive signaling in the periphery (Agarwal et al., 2007; Khasabova et al., 2008).

Importance of peripheral nociceptors in antinociception.

Despite the long-held belief that the antinociceptive effect of cannabinoids are predominantly mediated centrally (Walker et al., 1999a), there is increasing evidence that peripheral activation of CB1 receptors plays a more prominent role in modulating nociception than initially suggested. In support of this, CB1 receptors are transported peripherally (Hohmann and Herkenham 1999). When the CB1 receptor gene was selectively deleted from peripheral nociceptors, the threshold for sensory transduction

was substantially reduced (Agarwal et al., 2007). Similarly, injection of AM281 (10 µg, i.pl.) a CB1 receptor antagonist, increased the withdrawal frequency to a range of mechanical forces ipsilateral to the injection (Khasabova et al., 2008). Based on these studies, we hypothesized that URB597, an inhibitor of FAAH, would reduce mechanical hyperalgesia following local administration at the site of inflammation. Furthermore, we predicted that anti-hyperalgesia would occur independent of central mediation, thus limiting the adverse centrally-mediated side effects of systemic cannabinoid administration.

It is noteworthy that not only are peripherally administered cannabinoids effective in the treatment of pain during inflammation, but expression of CB1 receptors increase during CFA-induced peripheral inflammation in DRG neurons as well as in nerve fibers innervating the hind paw and facilitate the effect of a peripheral CB1 receptor agonist (Amaya et al., 2006). Therefore, our studies sought to characterize not only functional CB1 receptor expression, but determine if anandamide synthesis and/or degradation was altered during CFA-induced peripheral inflammation. These data will address whether endogenous cannabinoid signaling components may be altered as a result of persistent inflammation, which may improve the effectiveness of cannabinoid analgesics during inflammation.

Peripheral administration of endogenous and synthetic cannabinoids.

Peripheral modulation of inflammatory pain through endogenous and synthetic cannabinoids has also been characterized and is advantageous due to minimal centrally-

mediated adverse side effects. In this regard, during carrageenan-induced inflammation, anandamide administered locally at the site of inflammation suppresses both the development and maintenance of thermal hyperalgesia via a peripheral CB1 receptor (Richardson et al., 1998a). However, Richardson and colleagues did not directly test the involvement of CB2 receptors in this study, and since inflammation involves recruitment of immune cells expressing CB2 receptors to the region in addition to CB1 receptors present in the periphery, it is relevant to directly determine if this receptor subtype contributes to anti-hyperalgesia during peripheral inflammation using both CB1 and CB2 receptor antagonists. However, in the formalin-induced model of inflammation, local administration of anandamide produces anti-nociception in the early phase through a CB-1 receptor dependent mechanism (Calignano et al., 1998) whereas injection of 2-AG attenuates late phase formalin-evoked nociceptive responses in a CB2 receptor-mediated manner (Guindon et al., 2007). Thus, despite the fact that both receptors have been implicated in the formalin-induced model of inflammation, since the nocifensive effect of formalin only persists for approximately sixty minutes, this is not sufficient to draw conclusions about the receptor subtype(s) contributing to anti-hyperalgesic effect of compounds such as URB597 during persistent inflammation. Therefore, our studies with URB597 will address the contribution of both CB1 and CB2 receptors using co-injection of each receptor antagonist. However, previous studies clearly suggest endocannabinoid-mediated anti-nociception is consistent across various models of inflammation.

Local administration of exogenous cannabinoids can also alter the evoked responses of neurons. Anandamide (50 μ g, i.pl.) inhibited noxious mechanically-evoked

responses of spinal neurons in rats with carrageenan-induced hind paw inflammation, an effect that was blocked by the CB2 receptor antagonist SR144528, but not the CB1 receptor antagonist SR141716A (Sokal et al., 2003). Furthermore, intraplantar injection of the CB2 agonist JWH-133 suppresses mechanically evoked responses of wide dynamic range neurons in carrageenan-treated rats via a CB2-specific mechanism (Elmes et al., 2004).

More relevant to our studies in the periphery, Potenziari and colleagues (2008) measured activity in primary afferent neurons. They reported ACEA and methanandamide not only reduced mechanical hyperalgesia associated with CFA injection, but decreased mechanically evoked responses from cutaneous A δ fibers from inflamed, but not naïve, skin in rats a CB1-, but not CB2-dependent, manner (Potenziari et al., 2008).

In addition to the antinociceptive effects of endocannabinoids, synthetic CB1/CB2 receptor agonists such as WIN 55,212-2 and CB1 receptor agonist ACEA administered intraplantarly have also been shown to attenuate the development of mechanical hyperalgesia, allodynia and Fos protein expression evoked by carrageenan (Nackley et al., 2003b; Gutierrez et al., 2007) and both the early and late phases of formalin-evoked nociception (Calignano et al., 1998). Local injection of the CB2 receptor agonist AM1241 inhibits both mechanical and thermal hyperalgesia induced by carrageenan (Nackley et al., 2003a; Quartilho et al., 2003; Gutierrez et al., 2007) or antinociception following a thermal stimulus (Malan et al., 2002), suggesting that CB2 receptors do play a role in persistent inflammation. Most relevant to our studies, in CFA-induced

inflammation, intraplantar administration of the CB1 receptor agonist ACEA attenuates the thermal hyperalgesia associated with CFA-induced inflammation in rats 2 d post inflammation (Amaya et al., 2006). Although these studies suggest cannabinoid agonists are effective in attenuating hyperalgesia, and in most cases determine which receptor subtype is responsible for the effect, they do not set out to characterize the regional changes in endocannabinoid signaling such as the levels of AEA, FAAH enzyme expression and activity and CB1 receptors at a single time point following persistent peripheral inflammation prior to drug administration. Therefore, the goal of the second and third studies in this dissertation is to assess the neuroplasticity of endocannabinoid tone modulating the threshold for nociception during inflammation.

VIII. Inhibition of FAAH as an effective treatment for pain.

Endocannabinoids are released locally and their effects are terminated by rapid breakdown. Thus, disruption of endocannabinoid catabolism provides a unique mechanism for the management of pain. This idea is supported by evidence that the effect of local injection of metabolically stable methanandamide compared to anandamide in the formalin test since methanandamide inhibits nocifensive behaviors associated with both phases of the formalin test, whereas anandamide only inhibited pain behavior associated with the early phase (Calignano et al., 1998). These data suggest degradation of anandamide may account for the difference in the duration of the anti-nociceptive effect. Secondly, genetic deletion of FAAH in mice results in elevated endogenous anandamide levels and these mice exhibit hypoalgesia as well as increases in anandamide-induced

analgesia (Cravatt et al., 2001; Lictman et al., 2004). Together, these data support the idea that disruption of FAAH-mediated degradation of anandamide may be a therapeutic approach for pain management.

In this regard, disrupting catabolism of anandamide through the use of selective FAAH inhibitors such as URB597 (3'-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate), would be predicted to reduce pain-related behavior. Confirming this, systemic injection of URB597 decreases mechanical and thermal hyperalgesia associated with CFA-induced inflammation in rats (Jayamanne et al., 2006). Furthermore, intraplantar injection of URB597 in carrageenan-treated rats produced significant, dose-related increases in levels of anandamide and 2-AG in the hind paw compared to vehicle-treated carrageenan-injected rats (Jhaveri et al., 2008). Coupled with evidence that anandamide synthesis is activity dependent, this mechanism of action limits the ability of locally injected URB597 to have widespread effects on endocannabinoid signaling. Furthermore, URB597 is selective for FAAH over other targets such as cannabinoid receptors and MGL (Kathuria et al., 2003).

IX. Current clinical use of cannabinoids.

Currently, there are two approved cannabinoid drugs available by prescription: Marinol (Δ^9 -THC) and Nabilone (a Δ^9 -THC analogue) (Walker and Huang 2002) and although the Food and Drug Administration has not approved smoked marijuana, fourteen states have enacted laws approving medicinal marijuana (ProCon.org). Although Marinol is approved for the nausea and vomiting associated with chemotherapy for

cancer patients and to assist with loss of appetite with AIDS patients, its utility is limited by its slow and unpredictable pharmacokinetics and production of dysphoria at high doses (Walker and Huang, 2002). Because of the limitations of current cannabinoids on the market, the therapeutic potential of drugs that enhance the activity of endocannabinoids is currently being investigated (reviewed by Bari et al., 2006). In this regard, currently the US Patent and Trademark Office has issued twenty-nine patents related to “FAAH inhibitors,” with the earliest patent being issued on August 7, 2001 (www.patft.uspto.gov).

X. Statement of purpose.

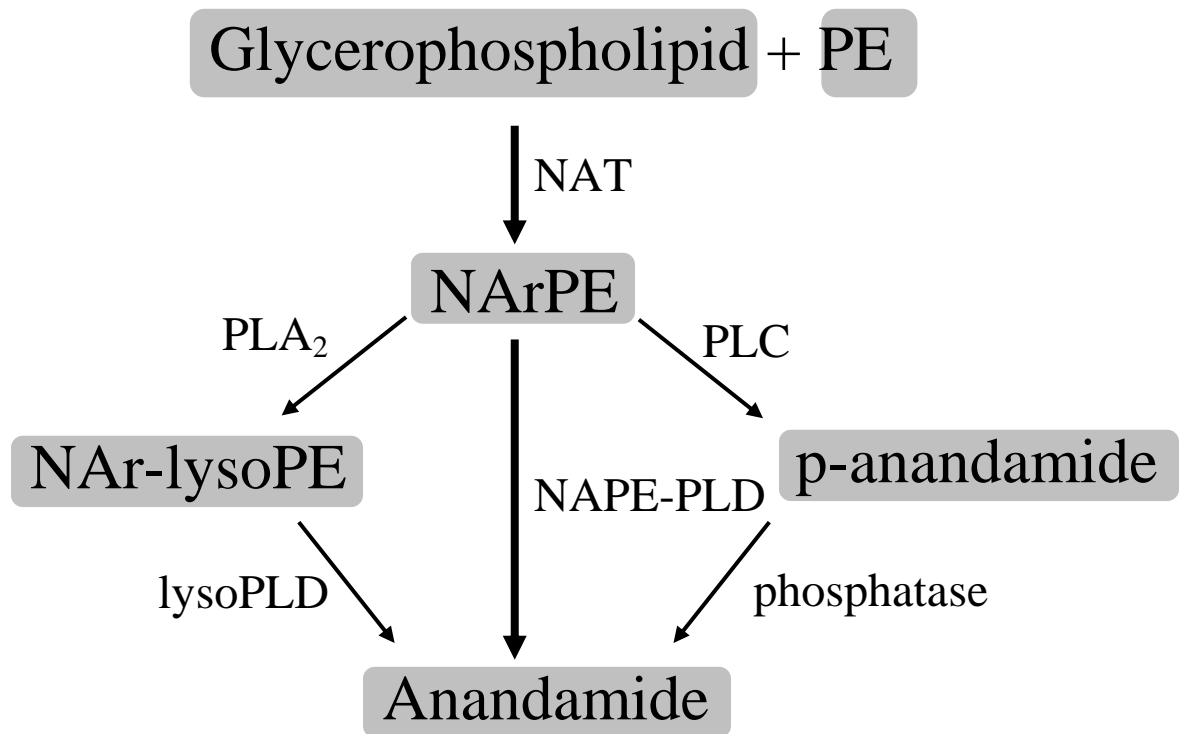
The purpose of my research was to determine whether local injection of URB597, a selective inhibitor of FAAH, is effective in reducing mechanical hyperalgesia associated with CFA-induced inflammation. As part of these studies, I assessed which receptor mediates the effect of URB597 in CFA-injected mice and whether local injection of URB597 results in increased levels of anandamide and/or 2-AG as would be predicted.

The second goal of this research was to assess changes in the neurochemistry of endocannabinoid signaling associated with persistent peripheral inflammation. To do so, I compared endocannabinoids, FAAH, and CB1 receptors between naïve and CFA-injected mice to determine if changes in any of these components of endocannabinoid signaling contribute to, or promote, the mechanical hyperalgesia observed with CFA-induced inflammation. Mechanical hyperalgesia associated with CFA injection may develop, in part, as a result of: 1) increased FAAH mRNA levels and enzyme activity at the site of

inflammation or in the DRG neurons innervating the hind paw, 2) decreased levels of anandamide or 2-AG in these regions, or 3) decreased levels and functional activity of CB1 receptors on peripheral sensory neurons.

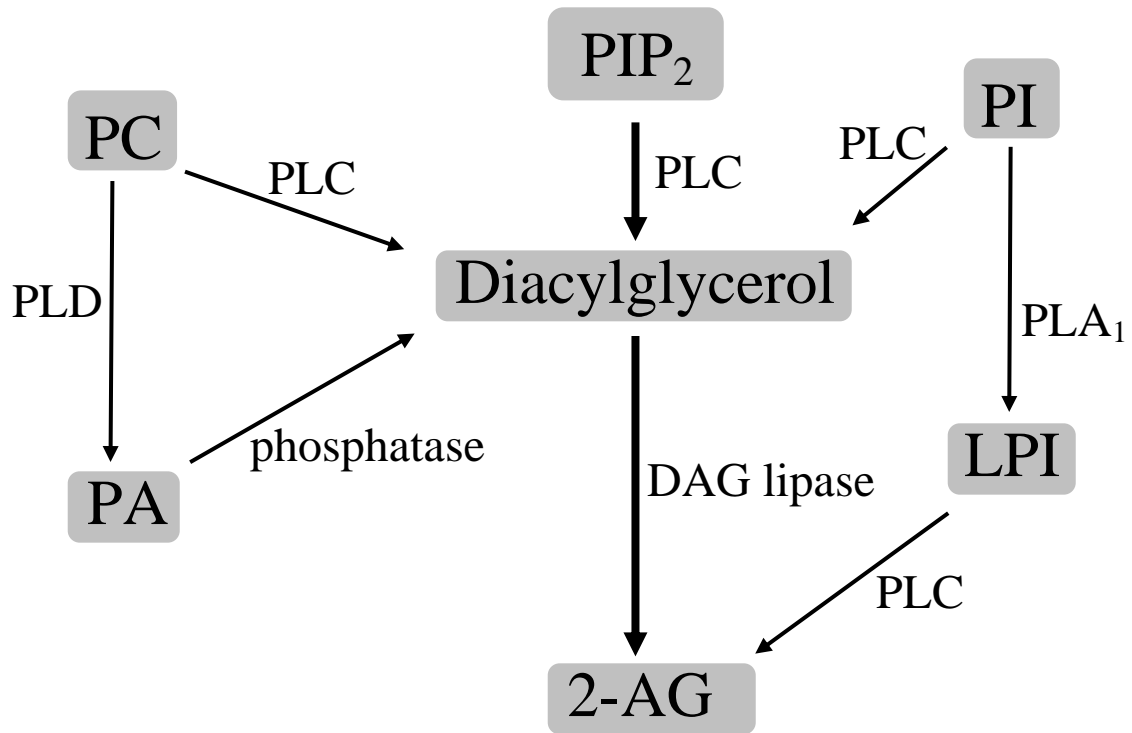
Importantly, the same strain of mice that our lab has characterized cannabinoid signaling in a model of cancer pain was used, allowing us to compare and contrast changes in endocannabinoids signaling in two distinct pain models.

Figure 1. Pathways of anandamide biosynthesis.



PE, phosphatidylethanolamine; NAT, *N*-acyltransferase; NArPE, *N*-arachidonoyl phosphatidylethanolamine; NAPE-PLD, *N*-acylphosphatidylethanolamine-specific phospholipase D; PLA₂, phospholipase A₂; PLC, phospholipase C; NAr-lysoPE, *N*-arachidonoyl-lyso phosphatidylethanolamine; lysoPLD, lyso phospholipase D; p-anandamide, phospho-anandamide.

Figure 2. Pathways of 2-arachidonoylglycerol synthesis.



PIP₂, phosphatidylinositol biphosphate; PI, phosphatidylinositol; PLC, phospholipase C; PLA₁, phospholipase A₁; LPI, lysophosphatidylinositol; 2-AG, 2-arachidonoylglycerol; PC, phosphatidylcholine; PA, phosphatidic acid; PLD, phospholipase D; DAG lipase, diacylglycerol lipase.

Chapter 2

The effect of local injection of URB597 on mechanical hyperalgesia associated with persistent peripheral inflammation

Introduction

Cannabinoid agonists exhibit analgesic effects by acting at both cannabinoid type 1 (CB1) and CB2 receptors (reviewed by Walker and Huang, 2002; Hohmann and Suplita, 2006) and the anti-nociceptive effects of cannabinoid agonists at both CB1 and CB2 receptors have been observed in a model of inflammatory pain (Richardson et al., 1998; Quartilho et al., 2003; Nackley et al., 2003). CB1 and CB2 receptors are also activated by Δ^9 -tetrahydrocannabinol (THC), the psychoactive component in marijuana (Mechoulam, 1986). THC, as well as other direct CB1 agonists, have long been known to possess medicinally beneficial properties, however, widespread distribution of CB1 receptors in the brain leads to side effects such as cognitive and motor impairment, dependence and psychosis (Pacher et al., 2006), limiting systemic administration of cannabinoid agonists as analgesics.

Endogenous ligands for CB1 and CB2 receptors include *N*-arachidonyl ethanolamide (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG). Both anandamide and 2-AG are synthesized on demand in response to an increase in the level of cytoplasmic calcium (Di Marzo et al., 1994). These endocannabinoids are most effective when produced near their target receptor because they undergo rapid catabolism, thereby terminating their signaling. The enzymes primarily responsible for catabolism of anandamide and 2-AG are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL) respectively (Fowler et al., 2005).

Because endocannabinoids undergo rapid breakdown to terminate their signaling, disruption of endocannabinoid catabolism presents a unique opportunity for management

of pain. In support of this, local injection of metabolically stable methanandamide inhibits nocifensive behaviors associated with both early and late phases of the formalin test compared to exogenously injected anandamide, which only inhibited pain behavior associated with the early phase (Calignano et al., 1998). Secondly, when FAAH is genetically deleted in mice, endogenous anandamide levels are elevated and these mice exhibit hypoalgesia as well as increases in anandamide-induced analgesia (Cravatt et al., 2001; Lichtman et al., 2004). Together, these data support the hypothesis that disruption of enzymatic degradation of anandamide may be a therapeutic approach for pain management.

Thus, a third mechanism to disrupt catabolism of anandamide is through inhibition of FAAH. Systemic injection of the FAAH inhibitor, URB597 (3'-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate) decreases thermal and mechanical hyperalgesia associated with Complete Freund's Adjuvant (CFA)-induced inflammation in rats (Jayamanne et al., 2006) and intraplantar injection of URB597 in carrageenan-treated rats produced significant, dose-related increases in levels of anandamide and 2-AG in the hind paw compared to vehicle-treated carrageenan-injected rats (Jhaveri et al., 2008). Unlike injection of methanandamide, URB597 is predicted to elevate endogenous anandamide only where its synthesis is stimulated. Furthermore, URB597 is selective for FAAH over other targets such as cannabinoid receptors and monoacylglycerol lipase (Kathuria et al., 2003).

However, a study of the effect of local injection of URB597 on mechanical hyperalgesia and resulting endocannabinoid levels has not been measured in a model of

persistent, peripheral inflammation. Therefore, the aim of this study is to determine whether local injection of URB597 attenuates mechanical hyperalgesia associated with persistent, peripheral inflammation using a model of CFA-induced inflammation in the hind paw, and if so, which cannabinoid receptor mediates the effect. Additionally, we sought to determine whether injection of URB597 resulted in an increase in the level of anandamide, as would be predicted based on the mechanism of URB597.

Methods

Animals

Adult, male C3H/He mice (National Cancer Institute; 25-30 g) were used in these studies. All procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee. Because Breese and colleagues (2005) found no differences in paw diameter or mechanical sensitivity between naïve mice and saline-injected mice two days post injection, we chose to compare drug treatments in CFA-injected animals to naïve animals in our studies.

Measurement of sensitivity to a mechanical stimulus

A preliminary study determined that the mechanical threshold (Chaplan et al., 1994) on the plantar surface of the hind paw in CFA-injected mice was lower (4.0 ± 0.5 mN, $n=33$) compared to the threshold in naïve mice (18.2 ± 2.4 mN, $n=31$, $p < 0.0001$, Student's *t*-test). The mechanical threshold in naïve mice is consistent with the value reported previously for this strain of mice (Mogil et al., 1999). Based on these data, a von

Frey monofilament with a bending force of 5.7 mN (0.6 g), or just above threshold in CFA-injected mice, was chosen to measure mechanical sensitivity in CFA-injected mice. Naïve mice withdrew their limbs in response to application of this filament approximately 20% of the time, and the increase in the frequency of the withdrawal response to this filament in inflamed hind paws is referred to as mechanical hyperalgesia in this report. The magnitude of the mechanical hyperalgesia measured in CFA-injected mice with this filament allowed reliable assessment of treatments that decreased mechanical hyperalgesia.

Mechanical sensitivity was determined in each mouse by calculating the frequency of withdrawal to a force of 5.7 mN. To conduct this assay, mice were placed on an elevated mesh platform, contained individually under a 70.8 cm² x 4.5 cm deep circular container and allowed to acclimate to the surroundings for 30 minutes prior to testing. The 5.7 mN monofilament was applied to the plantar surface of one hind paw ten times with 1-2 second intervals between stimuli. The number of vigorous responses, characterized by rapid withdrawal or licking of the hind paw, were scored and expressed as a percentage of stimuli applied. All mice were tested before inclusion in the study. Only mice with a baseline response rate of $\leq 40\%$ were assigned to naïve or CFA-injected groups. Baseline values were assessed for each animal for 2 days prior to induction of inflammation.

In separate groups of naïve mice the frequency of withdrawal to mechanical stimuli was conducted, as above, across a range of von Frey monofilaments. Filaments

with bending forces of 3.7, 5.7, 9.8, 13.7 and 19.6 mN were tested following injection of drug or vehicle.

Induction of peripheral inflammation

Inflammation was induced by unilateral, intraplantar (i.pl.) injection of 50 μ l Complete Freund's Adjuvant (CFA; Calbiochem, La Jolla, CA, USA) into the left hind paw under isoflurane anesthesia. Only mice with a $\geq 70\%$ response rate 48 h after injection of CFA were included in the inflamed group in subsequent experiments. In a subset of CFA-injected and naïve animals, paw diameter was measured as an indicator of edema. The measure was made under isoflurane anesthesia using calipers at the widest point in the hind paw ipsilateral to the CFA injection 48 h after the injection or in the left hind paw of naïve mice.

Drug Treatments

At 48 h following induction of inflammation, separate groups of CFA-injected and naïve mice were given an intraplantar injection of URB597 (Cayman Chemical, Ann Arbor, MI, USA) in the left hind paw (ipsilateral to the inflammation in CFA-injected mice). URB597 was injected at doses of 2, 6, 18 and 54 μ g in 10 μ l ethanol under isoflurane anesthesia. The vehicle control group received 10 μ l ethanol. Mechanical sensitivity was scored at 0.5, 2, 6 and 24 h following drug injection.

To determine the effect of dose of URB597, the change in mechanical sensitivity following drug injection (i.e., inhibition of hyperalgesia) in CFA-injected mice was

calculated as a percent of the maximum possible effect on hyperalgesia using the following equation:

$$\% \text{ inhibition of hyperalgesia} = \frac{(\text{predrug score} - \text{postdrug score})}{(\text{predrug score} - \text{pre-CFA score})} \times 100$$

A limited number of values greater than 100 or less than 0 occurred at high and low doses, respectively. Because the study addressed the anti-hyperalgesic effect of URB597, these values were adjusted to 100 and 0 respectively.

To determine pharmacological specificity of the effect of URB597, antagonists AM281 (3 µg) or AM630 (1 µg), for CB1 and CB2 receptors respectively, were co-injected with URB597 (18 µg, i.pl.) into CFA-injected mice. Receptor antagonists were purchased from Tocris (Ellisville, MO USA), dissolved in dimethylsulphoxide (DMSO) and brought to the final desired concentration in saline. Co-injected doses of URB597 and receptor antagonist were diluted to a final dose in 10 µl ethanol:saline (1:1). The vehicle control group was injected with 10 µl ethanol:saline (1:1). Mechanical sensitivity was measured 2 h after drug injection, the most effective time in the time course study.

The ability of URB597 to promote analgesia in naïve mice was assessed at doses of 9 and 18 µg at 2 h. On the basis of results of intraplantar injection of URB597 in naïve mice, two separate groups of naïve mice were tested with either 18 µg URB597 and 100 pmol capsazepine (Capz; Tocris, Ellisville, MO, USA) or capsazepine alone. The dose of 100 pmol capsazepine is consistent with an effective dose that blocks capsaicin-induced licking in mice (Santos and Calixto, 1997; Sakurada et al., 2003). Both URB597 and capsazepine were dissolved in ethanol and brought up to the final injected volume (10 µl) in saline.

In all experiments, experimenters were blinded to the drug treatments during the behavioral testing, and at least two treatments were tested in each behavioral session.

Measurement of endogenous anandamide and 2-AG

Plantar paw skin samples were collected from CFA-injected mice treated with either URB597 (54 µg) or vehicle (10 µl ethanol) 2 h post drug injection. Each sample contained paw skin tissue pooled from two animals. Please see chapter 3 for lipid extraction and targeted isotope-dilution HPLC/atmospheric pressure chemical ionization/mass spectrometry details.

Statistical analyses

All data are presented as the mean \pm S.E.M. for the group. Behavioral data were analyzed using one-way or two-way analysis of variance (ANOVA) or two-way ANOVA for repeated measures with Tukey's *post hoc* test to resolve individual group differences. All other data were analyzed using Student's *t*-test. A value of $p < 0.05$ was considered significant. The ED₅₀ of URB597 was determined by the method of Tallarida and Murray (1987) using a software program developed by M. Ossipov (*Flashcalc*; www.u.arizona.edu/~michaelo/jflashcalc.html).

Results

CFA-induced Inflammation in C3H/He mice

Intraplantar injection of CFA into the hind paw of C3H/He mice produced both visible redness and swelling in the injected paw. After 48 h, paw diameter was greater ipsilateral to the injection in CFA-injected mice compared to naïve mice (3.0 ± 0.01 mm in the CFA group, $n=19$, compared to 2.4 ± 0.01 mm in naïve mice, $n=17$, $p<0.0001$, Student's t -test). The increase in paw diameter was accompanied by a four-fold increase in frequency of withdrawal to the 5.7 mN mechanical stimulus ($80\pm 1.6\%$, in the CFA-injected group, $n=33$, compared to $20\pm 2.3\%$ in naïve mice, $n=31$, $p<0.0001$, Student's t -test).

URB597 attenuated mechanical hyperalgesia during CFA-induced peripheral inflammation

Peripheral injection of URB597 (54 μ g, i.pl.) ipsilateral to the inflamed hind paw reversed the mechanical hyperalgesia associated with inflammation in a time-dependent manner. URB597 significantly inhibited mechanical hyperalgesia associated with peripheral inflammation 2 h after injection and the effect persisted for at least 4 h (Figure 1). By 24 h post-drug injection the effect of URB597 on mechanical hyperalgesia had dissipated and mechanical hyperalgesia was comparable to pre-drug levels. The mean withdrawal frequencies in naïve mice treated with URB597 (54 μ g, i.pl.) were not different from the pre-drug value at any time point. The ethanol vehicle did not alter mechanical sensitivity in CFA-injected or naïve mice at any time point. These data indicate that local injection of URB597 reversed the mechanical hyperalgesia associated with peripheral inflammation between two and six hours post drug injection.

Injection of URB597 (i.pl.) into the inflamed hind paw 48 h after the induction of inflammation dose-dependently reduced the mechanical hyperalgesia associated with peripheral inflammation. URB597 inhibited mechanical hyperalgesia with an ED₅₀ of 4.4 µg (95% CI: 2.7 to 7.4; Figure 2). Doses of 18 and 54 µg URB597 significantly reduced hyperalgesia in CFA-injected mice at 2 h, and mechanical sensitivity following the 54 µg dose of URB597 was not different from the pre-CFA injection value (p=0.39, Student's *t* test). Therefore URB597 is an effective inhibitor of mechanical hyperalgesia in this model of peripheral inflammation.

The anti-hyperalgesic effect of URB597 was mediated locally

To confirm that the anti-hyperalgesic effect of URB597 observed in the time course and dose-response studies was neither mediated centrally nor a result of depressed motor activity, an additional group of CFA-injected mice was given an injection of the highest dose of URB597 (54 µg, i.pl.) in the contralateral paw 48 h after injection of CFA. Mechanical sensitivity was tested in the inflamed hind paw 2 h later. Under these conditions, URB597 did not change the frequency of withdrawal of the inflamed paw to the mechanical stimulus (pre-drug: 73±3%, n=3, post-drug: 81±4%, n=3, p=0.21, Student's *t*-test). Therefore, we conclude that the anti-hyperalgesic effect of intraplantar injection of URB597 into the inflamed paw was locally mediated and did not involve a systemic effect. In addition, these data dismiss the possibility that the reduction in the frequency of withdrawal to the mechanical stimulus following injection of URB597 into the inflamed paw was the result of an impaired motor response.

The effect of URB597 on mechanical sensitivity was mediated by CB1 and CB2 receptors

As a FAAH enzyme inhibitor, intraplantar injection of URB597 is predicted to prevent the degradation of anandamide, thereby increasing the amount of endocannabinoid available for binding to CB1 and CB2 receptors in the periphery. In order to resolve which cannabinoid receptor mediated the anti-hyperalgesic effect of URB597 in this model of peripheral inflammation, the ability of CB1 and CB2 receptor antagonists to block the effect of URB597 on mechanical sensitivity was tested. URB597 (18 µg, i.pl.) and the CB1 receptor antagonist AM281 (3 µg, i.pl.) or the CB2 receptor antagonist AM630 (1 µg, i.pl.) were administered 48 h following induction of inflammation, and mechanical sensitivity was measured 2 h after drug injection. The dose of AM281 (equivalent to 100 µg/kg) is within the range for which structural analogues blocked CB1 receptors (Guindon et al., 2006; Gutierrez et al., 2007) and is comparable to selective blockade of CB1 agonist in a different model in mice (Khasabova et al., 2008). Intraplantar injection of the dose of AM630 (equivalent to 100 µg/kg) blocks CB2 receptors in rats (Quartilho et al., 2003), and a comparable dose blocked the effect of a CB2 receptor agonist in a tumor-related model of mechanical hyperalgesia in mice (I. Khasabova, personal communication). Both the CB1 receptor antagonist, AM281, and the CB2 receptor antagonist, AM630, reversed the anti-hyperalgesic effect of URB597 following co-administration (Figure 3). Compared to vehicle (67±7%), neither AM281 (69±3%, $p=1.0$) nor AM630 (66±5%, $p=1.0$) alone altered mechanical sensitivity in mice with peripheral inflammation at the doses used.

URB597 decreased mechanical sensitivity in naïve mice

Because naïve mice exhibited a low frequency of response to the 5.7 mN monofilament, a separate experiment was designed to determine whether URB597 promoted analgesia in naïve mice. Groups of naïve mice were injected with URB597 (9 or 18 µg, i.pl.) or vehicle and withdrawal frequencies were measured with monofilaments of increasing force 2 h later, the most effective time point in the time course study. The dose of 9 µg was chosen because it produced analgesia in this strain of mice previously in our lab. The dose of 18 µg was chosen because it was the minimally effective dose in CFA-injected mice in the dose-response studies in this report.

The low dose of URB597 (9 µg, i.pl) decreased mechanical sensitivity in response to filaments of increased force (Figure 4). The low dose URB597 significantly attenuated the withdrawal frequency to forces of 9.8, 13.7 and 19.6 mN compared to vehicle-treated animals. The decreased mechanical sensitivity in naïve mice treated with URB597 suggests URB597 promotes analgesia as well as anti-hyperalgesia.

However, the higher dose of URB597 (18 µg, i.pl), which produced anti-hyperalgesia in CFA-injected mice, unexpectedly produced significant hyperalgesia at the 9.8 mN filament in naïve mice (Figure 5). Because anandamide can activate pronociceptive TRPV1 receptors, capsazepine, a TRPV1 receptor antagonist, was co-injected with URB597 (18 µg, i.pl), to determine the contribution of TRPV1 receptors to the hyperalgesia produced by high dose URB597. Co-injection of capsazepine blocked the increase in mechanical sensitivity caused by URB597 (Figure 6). These data indicate

the amount of anandamide following the high dose of URB597 may be sufficiently increased in naïve mice to bind to the TRPV1 receptor, promoting pronociception.

Anandamide and 2-AG levels in plantar paw skin

The prediction was that the anti-hyperalgesic effect of URB597 was mediated by an increase in the local level of anandamide. Therefore we measured levels of anandamide and 2-AG in the hind paw of CFA-injected mice given URB597 (54 µg). Based on the time course studies, the effect of URB597 on mechanical hyperalgesia was most pronounced 2 h after drug injection, therefore samples of plantar paw skin were collected for analysis at 2 h post drug injection. Since the vehicle for injection of URB597 (ethanol) could change endocannabinoid metabolism, we compared endocannabinoid levels in CFA-injected mice given URB597 to CFA-injected mice given ethanol. There was no difference in the level of anandamide between URB597- or ethanol vehicle-treated mice in the plantar paw skin ($p=0.82$, Table 1). Thus, the URB597 compound failed to increase the level of anandamide and 2-AG in the paw skin.

Discussion

The behavioral data from our studies provide clear evidence that locally injected URB597 dose-dependently reduced mechanical hyperalgesia in a model of CFA-induced persistent inflammation. Furthermore, although the FAAH inhibitor URB597 failed to measurably increase levels of anandamide and 2-AG in the hind paws of mice as would have been predicted, the anti-hyperalgesic effect of URB597 was mediated through a

CB1- and CB2-dependent mechanism. Interestingly, in naïve mice, a low dose of URB597 decreased mechanical sensitivity, whereas the higher dose of URB597, that was effective in reducing mechanical hyperalgesia in CFA-injected mice, produced paradoxical pronociception in naïve mice.

URB597 inhibits mechanical hyperalgesia associated with persistent cutaneous inflammation

To our knowledge, we are the first to report that URB597 blocked mechanical hyperalgesia associated with persistent cutaneous inflammation in mice. Previous reports have addressed either systemic injection of URB597 in a model of CFA-induced inflammation in rats (Jayamanne et al., 2006) or local anti-nociceptive effects of URB597 in models of cancer pain in mice (Khasabova et al., 2008) and acute carrageenan-induced inflammatory pain in rats (Jhaveri et al., 2008). The intraplantar dose of URB597 required to reduce mechanical hyperalgesia by 50% in CFA-induced inflammation was 4 µg (134 µg/kg). This dose is comparable to the ED₅₀ to reduce mechanical hyperalgesia in a model of cancer pain in the same strain of mice (Khasabova et al., 2008) and attenuate carrageenan-induced reduction in weight-bearing in rats (100 µg/kg, i.pl.; Jhaveri et al., 2008). Although a high dose of URB597 (400 µg/kg, i.pl.) is less effective than the optimal dose for normalizing weight-bearing in rats following carrageenan injection (Jhaveri et al., 2008), the highest dose of URB597 tested in CFA-injected mice in our study (1.8 mg/kg) continued to promote anti-hyperalgesia through a local

mechanism. Species differences or the model of inflammation (i.e. acute versus persistent) may contribute to these differences.

URB597 did not measurably increase endocannabinoids in plantar paw skin

In vitro evidence suggests that FAAH is able to metabolize both anandamide (Cravatt et al., 1996) and 2-AG (Di Marzo et al., 1998; Goparaju et al., 1998; Lang et al., 1999). Yet studies of anandamide and 2-AG content in the presence of the FAAH inhibitor URB597 or FAAH gene-deletion indicate divergence between FAAH metabolism of these endocannabinoids. For anandamide, both systemic injection of a FAAH inhibitor (Kathuria et al., 2003) and gene-deletion (Cravatt et al., 2001) increase the level of anandamide in the brain. Conversely, URB597 failed to increase 2-AG levels in the rat brain (Kathuria et al., 2003) and 2-AG breakdown is preserved in mutant FAAH *-/-* mice (Lichtman et al., 2002). Based on these studies, it was surprising that intraplantar injection of URB597 did not measurably increase anandamide content in the plantar paw skin of CFA-injected mice. It is noteworthy that these studies were conducted in naïve mice, so the effect of pathophysiological conditions on the drug effect remains to be resolved.

An additional variable includes the effect of drug vehicle on local endocannabinoid levels. In a more recent study, vehicle decreased the level of anandamide in carrageenan-treated mice compared to saline-injected mice, but had no effect on the level of 2-AG (Jhaveri et al., 2008). However, both anandamide and 2-AG were elevated in the hind paw of rats following local injection of URB597 in

carrageenan-induced inflammation (Jhaveri et al. 2008). On the basis that the drug vehicle may alter endocannabinoid levels, we compared URB597-injected to a vehicle control group in our studies. However, we found no increase in either the level of anandamide or 2-AG in the plantar paw skin compared to vehicle-treated mice.

The reasons for these differences are unclear, but may be related in part to both the dosing paradigm of URB597 and the time course of inflammation studied. Jhaveri and colleagues treated the rats with a preventative dose of URB597 thirty minutes prior to injection of carrageenan and measured endocannabinoid content 3 h after carrageenan-induced inflammation, whereas our studies addressed the ability of URB597 to increase endocannabinoids after 2 h in a fully developed model of inflammation that persisted over the course of 48 h.

One possible explanation for the absence of an effect of URB597 on endocannabinoids levels in our study is that under conditions of persistent inflammation, cyclooxygenase-2 (COX-2) is up-regulated (Seibert et al., 1994), and COX-2 and other lipoygenases can degrade endocannabinoids (Lambert and Fowler, 2005) to produce prostaglandin-ethanolamides, also called prostamides (Yu et al., 1997 Burnstein et al., 2000; Guindon and Hohmann, 2008). This possibility is supported by evidence that COX-2 metabolizes anandamide in FAAH knockout mice *in vivo* (Weber et al., 2004) and co-injection of anandamide and a non-specific cyclooxygenase inhibitor produces synergistic anti-nociceptive effects in the formalin test (Guindon et al., 2006). Thus, in our URB597-treated mice with CFA-induced inflammation it is possible that endocannabinoids were diverted to other metabolic pathways during sample preparation,

preventing an observable increase in their tissue levels. Alternatively, the oil-base of CFA may act as a depot for lipophilic endocannabinoids generated in response to inflammation, confounding detection of changes in the paw skin.

Anti-hyperalgesic effect of URB597 is mediated by CB1 and CB2 receptors

Although we were unable to measure increases in anandamide and 2-AG in the present study, the inhibitory effect of URB597 was mediated locally by both CB1 and CB2 receptors during CFA-induced inflammation. The importance of peripheral cannabinoid receptors in nociceptive modulation is exhibited both by genetic disruption of CB1 receptor expression in primary afferent neurons (Agarwal et al., 2007) and by the action of a newly-developed, peripherally restricted inhibitor of FAAH activity, URB937 (Clapper et al., 2010).

Consistent with our data, when URB597 is given systemically in CFA-injected rats, there is an increase in the paw withdrawal threshold that is blocked by either a CB1 or CB2 receptor antagonist (Jayamanne et al., 2006). However, involvement of both CB1 and CB2 receptors may be specific to models of inflammatory pain because in a model of tumor pain in this strain of mice, the anti-hyperalgesic effect of URB597 (i.pl.) was mediated solely by CB1 receptors (Khasabova et al., 2008).

It is not surprising that CB1 receptors, likely through attenuation of presynaptic neurotransmitter release, contribute to the anti-hyperalgesic effect of peripherally administered URB597. Not only are CB1 receptors expressed on primary afferent neurons (Hohmann and Herkenham 1999; Sañudo-Peña et al., 1999; Ahluwalia et al.,

2000, 2002; Khasabova et al., 2002), but their expression is increased in the DRGs and nerve fibers innervating the periphery during CFA-induced peripheral inflammation in mice (Amaya et al., 2006). Combined with the lack of a measurable increase in endocannabinoids in the present work, it is possible that up-regulation of CB1 receptors in the periphery contributes to the anti-hyperalgesic effect of URB597 through this receptor subtype (addressed further in chapters 3 and 4).

On the basis of our data, activation of CB2 receptors also contributed to the effect of URB597 on mechanical hyperalgesia. Anandamide is a partial agonist at both CB1 and CB2 receptors (Hillard 2000; Pertwee and Ross 2002) and although it acts with greater affinity at CB1 receptors ($K_i(\text{CB1 vs. CB2}) = 89 \text{ vs } 371 \text{ nM}$) (reviewed by Hohmann 2002), it is possible that when anandamide breakdown is inhibited by URB597 the local level of anandamide is sufficient to act on CB1 as well as CB2 receptors. CB2 receptors are ideally localized during persistent, cutaneous inflammatory pain to co-mediate the anti-hyperalgesic effect of URB597. CB2 receptors are localized primarily to immune cells (Munro et al., 1993; Galiegue et al., 1995) and their activation inhibits the release of inflammatory mediators from mast cells and macrophages (Facci et al., 1995).

In this case, CB2 receptor activation, through an indirect mechanism, could decrease inflammation-evoked release of sensitizing molecules (such as nerve growth factor, prostanoids, cytokines, histamine, or adenosine triphosphate) that act on peripheral nociceptors to decrease the sensitivity of primary afferent neurons and to reduce nociception (Woolf et al., 1994; Malan et al., 2002; Malan and Porreca 2005). In addition to modulation of immune responses, CB2 receptors have been shown to produce

antinociception by stimulating peripheral release of endogenous opioids from keratinocytes (Ibrahim et al., 2005).

Interestingly, blocking either the CB1 or CB2 receptor subtype alone blocks the anti-hyperalgesic effect of URB597. Recently, our lab has observed synergy of CB1 and CB2 receptor agonists in a model of tumor-evoked cancer pain in the periphery. In this study, the dose-response relationship for co-injection of ACPA and AM1241 (CB1 and CB2 receptor agonists, respectively) shifted nearly 100-fold to the left following local injection into the hind paw (Khasabova et al., under review). Combined with the data in the present study, these data suggest strategies to target multiple cannabinoid receptors in the periphery may enhance the therapeutic potential of cannabinoids.

Although both AM281 and AM630 reversed the effect of URB597, the local doses of the CB1 and CB2 antagonists alone did not enhance mechanical hyperalgesia in our study. These data suggest that a cannabinoid tone mechanism fails to tonically modulate the threshold for nociception in CFA-induced peripheral inflammation. Chapter 3 will address whether inflammation alters endocannabinoid levels, the activity of catabolic enzymes degrading endocannabinoids, or the expression of cannabinoid receptors.

Dose specific effects of local injection of URB597 in naïve mice

It is noteworthy that mechanical analgesia was observed in naïve mice after an intraplantar injection of a low dose of URB597 (9 µg). Importantly, the change occurred in the mid-range of force for mechanical stimuli. Exogenous administration of a dose of

anandamide that is anti-hyperalgesic in tumor-bearing mice does not affect mechanical sensitivity in naïve mice (Khasabova et al., 2008). Rapid catabolism of exogenous anandamide by FAAH may impair the ability of the endocannabinoid to reach the sensory neuron terminal and generate a behavioral effect. Thus, URB597 injection into the paw inhibits FAAH, enabling endogenous anandamide to increasingly bind to receptors and produce analgesia in naïve mice. These data underscore the relevance of understanding the microenvironment of sensory neuron terminals in the paw, and the DRG neurons themselves, in understanding changes in endocannabinoid neurotransmission (to be addressed in chapter 3). Measures of tissue content of endocannabinoids may lack sufficient resolution.

However, the dose of URB597 (18 μ g) that reversed mechanical hyperalgesia in the inflamed hind paw produced unexpected hyperalgesia at a force of 9.8 mN. A higher dose of URB597 would be expected to generate higher levels of anandamide in the tissue and there is evidence to suggest that anandamide binds and activates the TRPV1 receptor at high doses (Zygmunt et al., 1999; Jerman et al., 2002). TRPV1 receptors transduce noxious heat and chemical stimuli, including capsaicin and protons (Caterina et al., 1997; Tominaga et al., 1998; Szallasi and Blumberg 1999). In our studies, co-injection of the TRPV1 receptor antagonist capsazepine with URB597 blocked the increased mechanical sensitivity in naïve mice. Interestingly, capsaicin injection produces hyperalgesia to heat in the immediate vicinity and mechanical hyperalgesia in a larger surrounding area that is inhibited by increased synaptic activity in the spinal cord (Szolcsányi, 1977; Simone et al., 1989; Culp et al., 1989). Thus the mechanical hyperalgesia associated with high dose

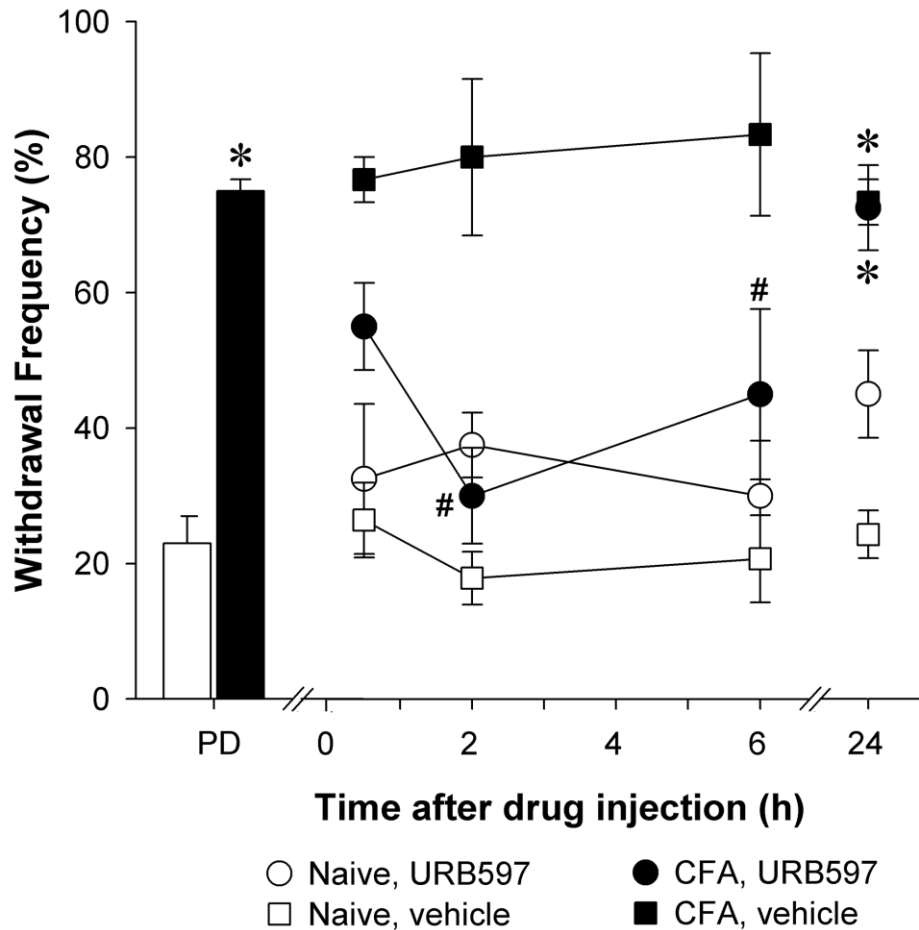
URB597 may be a result of activation of TRPV1 receptors and subsequent central sensitization.

Conclusion

Although we were unable to demonstrate a measurable increase in anandamide content following injection of URB597 in the hind paw, our data verify local treatment with URB597 inhibited mechanical hyperalgesia associated with persistent, peripheral inflammation indirectly through CB1 and CB2 receptor-dependent mechanisms.

Because the same dose in naïve and CFA-injected mice produced opposing behavioral effects, namely pronociception and antihyperalgesia respectively, and the highest dose of URB597 (54 µg) tested failed to produce pronociception in CFA-injected mice, we next sought to characterize any changes in the neurochemistry of FAAH or levels of endocannabinoids and CB1 receptors between naïve and CFA-injected mice that might account for this dose-specific effect of URB597.

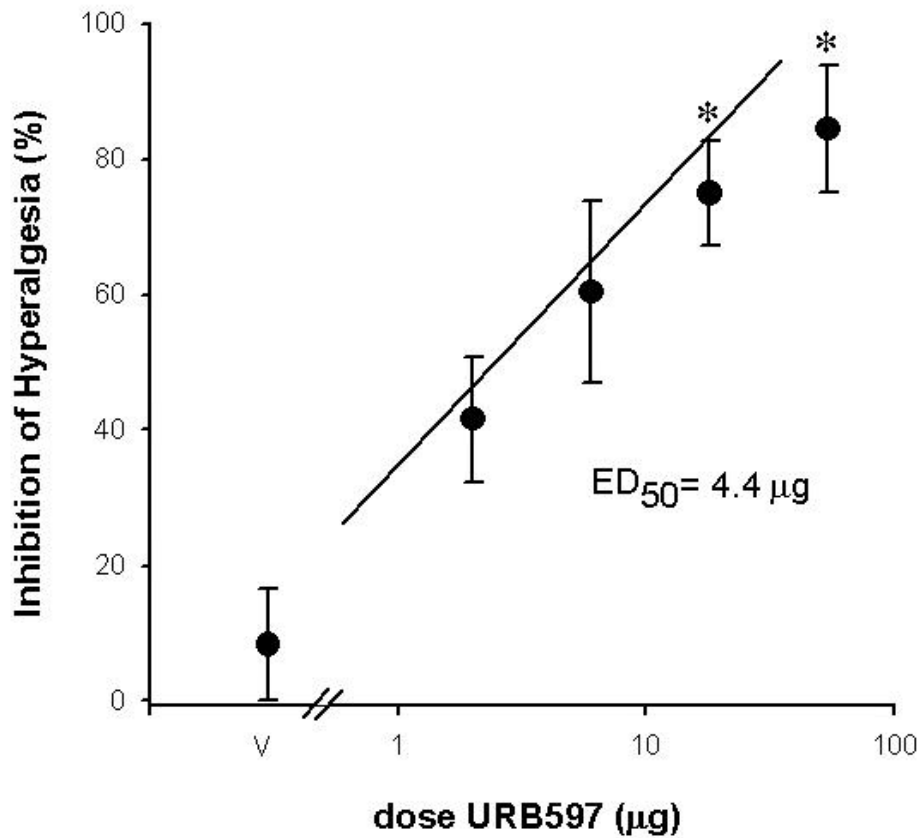
Figure 1. URB597 blocked mechanical hyperalgesia associated with peripheral inflammation in a time-dependent manner.



Mice were injected with CFA two days prior to drug injection. Mechanical sensitivity was measured ipsilateral to CFA injection or in the left hind paw of naïve mice. Compared to naïve mice, the withdrawal frequency in response to a force of 5.7 mN was higher in CFA-injected mice (PD=pre-drug). Mechanical sensitivity was assessed over 24 h following URB597 injection (54 µg, i.pl.). The vehicle was ethanol. For presentation purposes, data for vehicle and drug were pooled for each PD group. For statistical analyses, data for each treatment group were compared to the respective mean PD value within a treatment group. *Significantly different from naïve mice at the respective time

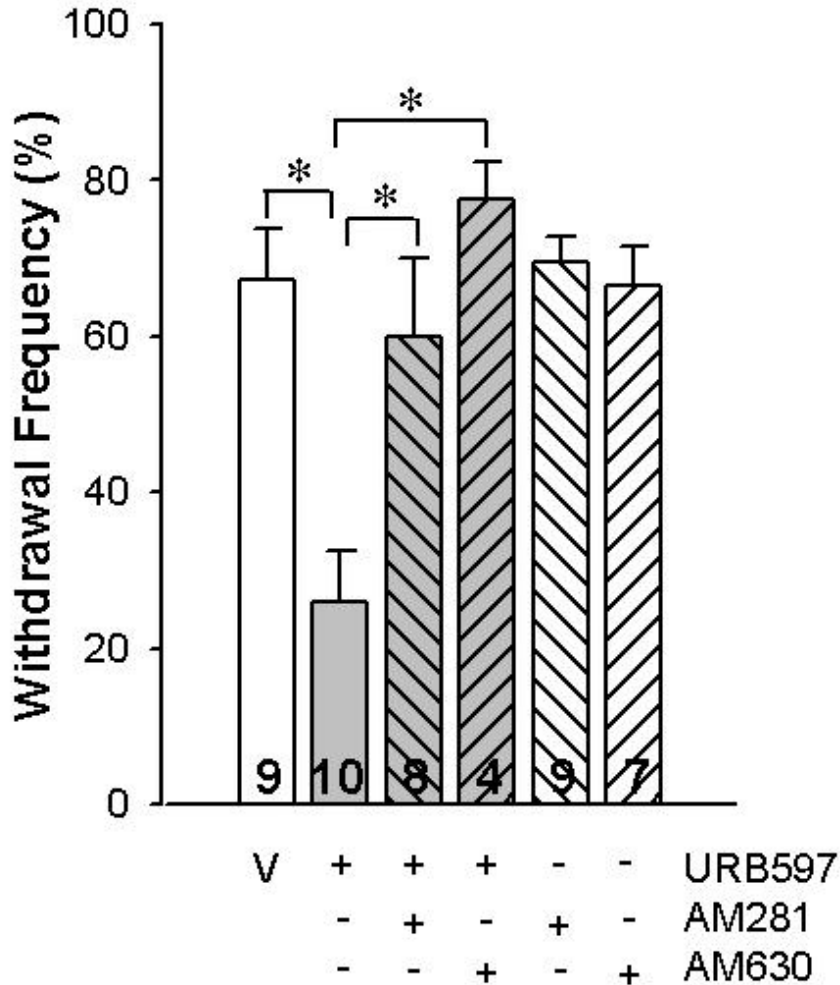
point; # significantly different from PD within the CFA-injected group (two-way repeated measure ANOVA with Tukey's test, $n = 3-7$ mice/treatment group).

Figure 2. URB597 dose-dependently reduced mechanical hyperalgesia associated with peripheral inflammation.



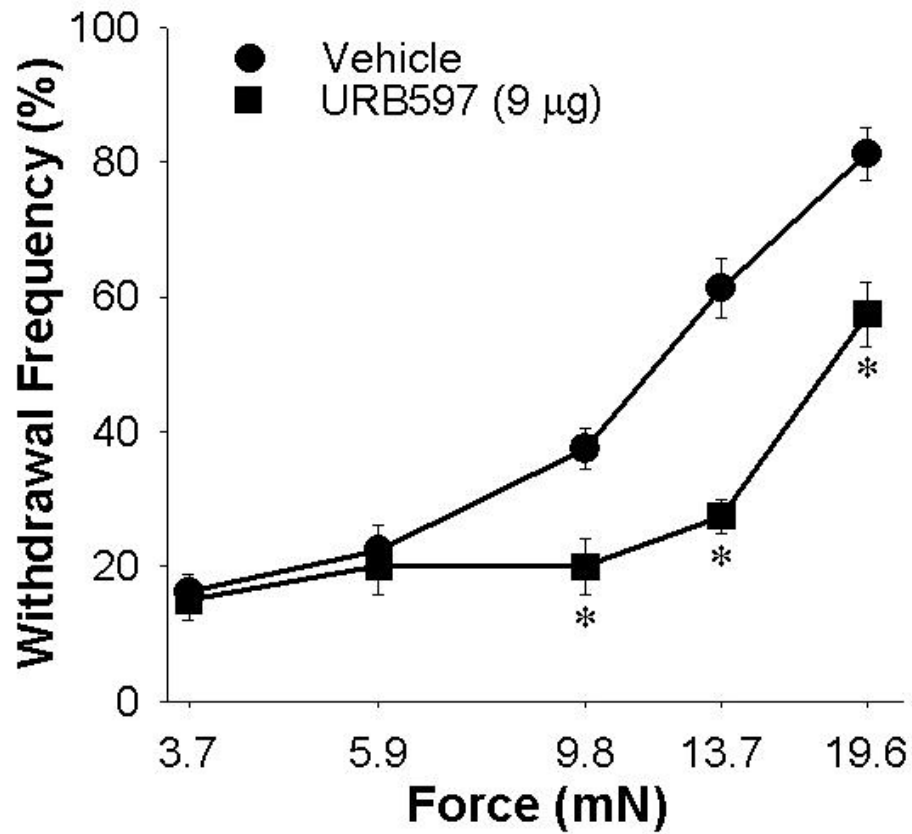
Forty-eight hours following the induction of inflammation the effect of URB597 (2-54 µg, i.pl.) was measured at 2 h after drug injection. The mechanical stimulus was a von Frey monofilament with a force of 5.7 mN. The vehicle (V) was ethanol. A limited number of values greater than 100 or less than 0 occurred at high and low doses, respectively. Because the study addressed the anti-hyperalgesic effect of URB597, these values were adjusted to 100 and 0 respectively. *Significantly different from vehicle (one-way ANOVA with Tukey's test, $n = 3-10$ mice/treatment group).

Figure 3. CB1 and CB2 receptor antagonists blocked the anti-hyperalgesic effect of URB597.



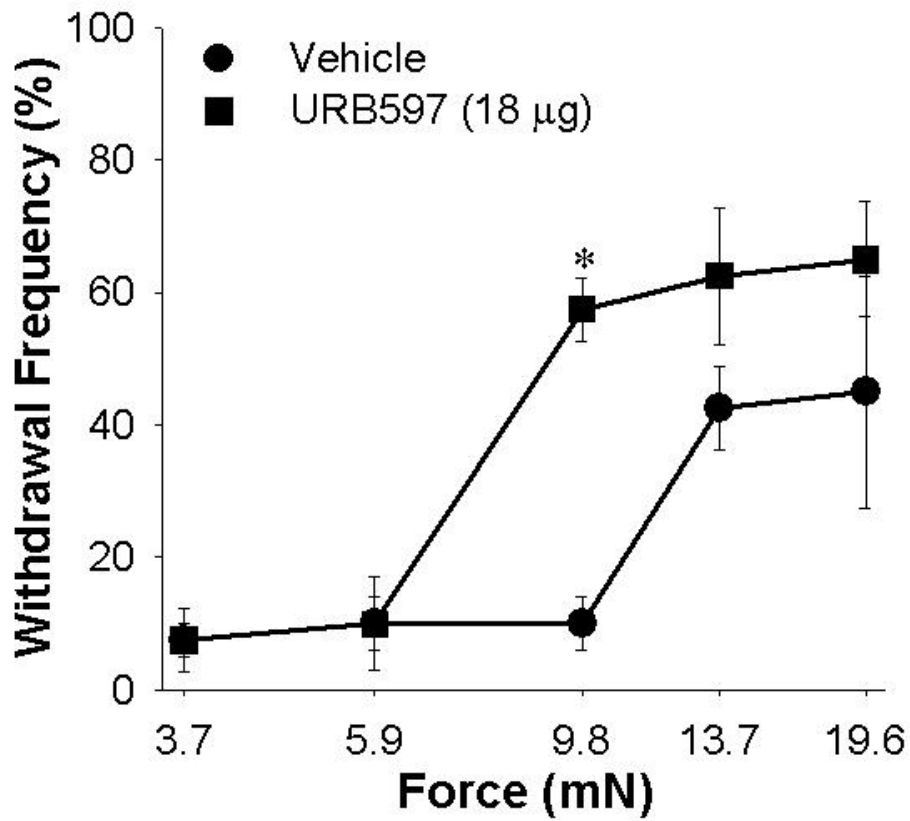
Following induction of inflammation in CFA-injected mice, URB597 (18 μ g in ethanol) and the CB1 receptor antagonist AM281 (3 μ g in saline) or the CB2 receptor antagonist AM630 (1 μ g in saline) were administered (final solution 1:1 ethanol:saline) and mechanical sensitivity was measured at 2 h using the 5.7 mN filament. Data for vehicle (V) were pooled for ethanol ($n = 3$) and 1:1 ethanol:saline ($n = 6$) because there was no statistical difference between them. The vehicle for antagonists alone was ethanol:saline (1:1). The injection volume was 10 μ l. *Significantly different at $p < 0.05$ (one-way ANOVA with Tukey's test). Values inside the bars represent the sample size.

Figure 4. A low dose of URB597 reduced mechanical sensitivity in naïve mice.



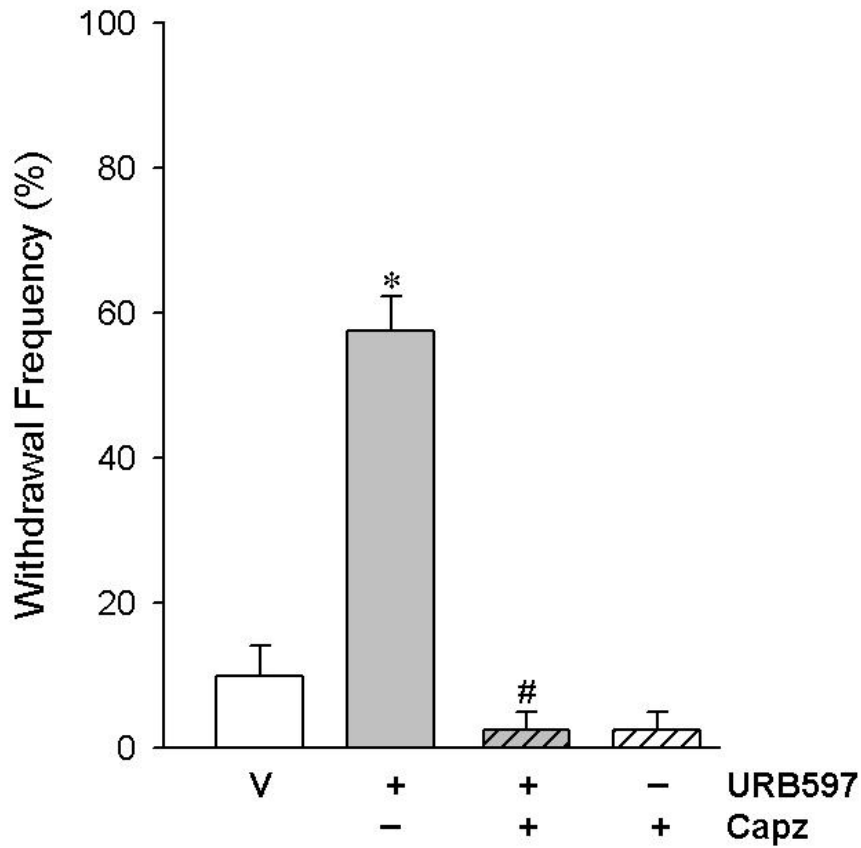
Withdrawal frequency was measured 2 h after injection of URB597 (9 µg) or vehicle. Responses were counted and expressed as a percentage of stimuli applied. *Significantly different from vehicle-injected mice tested with the same monofilament (two-way ANOVA with Tukey's test, $n = 4-5$ mice/group).

Figure 5. A high dose of URB597 increased mechanical sensitivity in naïve mice.



Withdrawal frequency was measured 2 h after injection of URB597 (18 µg) or vehicle. Responses were counted and expressed as a percentage of stimuli applied. *Significantly different from vehicle-injected mice tested with the same monofilament (two-way ANOVA with Tukey's test, $n = 4$ mice/group).

Figure 6. The TRPV1 receptor antagonist capsazepine (Capz) blocked the increased mechanical sensitivity in naïve mice induced by a high dose of URB597.



Naïve mice received an intraplantar injection of drug(s) and mechanical sensitivity was measured at 2 h using the 9.8 mN filament. *Significantly different from vehicle-injected mice tested with the same monofilament; #significantly different from URB597-treated mice (one-way ANOVA with Tukey's test, $n = 4$ mice/group).

Table 1. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) levels in plantar paw skin from CFA-injected mice treated with local URB597 or vehicle.

Condition	Paw	
	(pmol/g tissue)	
	AEA	2-AG
CFA + vehicle	685 ± 75 (4)	27069 ± 1374 (4)
CFA + URB597	657 ± 92 (4)	13970 ± 3223* (4)

Samples from URB597 (54 µg) and vehicle treated mice were collected 2 h post drug injection. Each sample contained tissue pooled from two animals and are reported as pmol/g tissue. *Significantly different from CFA + vehicle (Student's *t*-test). Numbers in parentheses represent the sample size.

Chapter 3

Changes in neurochemistry of endocannabinoid signaling associated with persistent peripheral inflammation

Introduction

The ability of the nervous system to modify its function according to different conditions, is essential to not only the development of the hypersensitivity that underlies inflammatory pain (see reviews by Woolf and Salter, 2000; Kidd and Urban 2001) but the body's response to this hypersensitivity in nociceptive modulation pathways such as in opiate receptor signaling (see reviews by Machelska and Stein 2002; Stein and Lang 2009). In the previous chapter, the same dose of URB597 in naïve mice and mice with intraplantar (i.pl.) injection of Complete Freund's Adjuvant (CFA) produced opposing behavioral effects, namely hyperalgesia and anti-hyperalgesia, respectively. Furthermore, the highest dose of URB597 (54 μg) tested failed to produce hyperalgesia in CFA-injected mice.

Because URB597 blocks the degradation of AEA, we postulate that the behavioral difference resulting from an identical dose of URB597 in naïve mice and those with inflammation was due to the level of AEA relative to that of CB1 and TRPV1 receptors. AEA binds to CB1 receptors with high affinity ($K_i= 89 \text{ nM}$; reviewed by Hohmann, 2002) and to TRPV1 receptors with low affinity ($K_i=5 \mu\text{M}$; reviewed by Ross, 2003). Thus, we hypothesize that if AEA levels decrease in the skin during peripheral inflammation, hyperalgesia results from the loss of CB1 receptor activation. This hypothesis is supported by evidence that deletion of CB1 receptors from nociceptive sensory neurons (Agarwal et al., 2007) and the local injection of a CB1 receptor antagonist (Khasabova et al., 2008) result in hyperalgesia. Therefore, blocking local degradation of AEA in mice with peripheral inflammation would restore CB1 receptor

activation and reverse hyperalgesia. However, in naïve mice, reduced degradation of AEA would result in the accumulation of AEA and activation of TRPV1 receptors, resulting in hyperalgesia.

Measures of AEA in the hind paw skin in models of inflammation are inconsistent. In CFA-injected mice, Agarwal and colleagues (2007) reported an increase in AEA in the hind paw skin of mice at 24 h following a lower dose compared to that used in our study. In contrast, in the formalin model of inflammation, the level of AEA in the paw skin was unchanged in rats (Beaulieu et al., 2000) or decreased in mice (Maione et al., 2007). However, the relationship between AEA content and behavior in the formalin model is difficult to interpret when the AEA level is determined after nocifensive behaviors have subsided. Clearly, in order to understand the relationship of AEA to mechanical hyperalgesia observed 48 h after injection of CFA, AEA has to be measured at the same time point.

A reduction in AEA content in the hind paw may result from an increase in catabolism by fatty acid amide hydrolase (FAAH) or a decrease in synthesis. Similar to reports of AEA levels, reports of FAAH enzyme expression and activity in models of peripheral inflammation are inconsistent. In rats, the amount of FAAH immunoreactivity was unchanged in the lumbar (L)5 DRG 2 d following injection of CFA into the hind paw (Lever et al., 2009). However, FAAH activity decreased 4 h following injection of carrageenan in hind paws of mice (Holt et al. 2005). To our knowledge, FAAH expression and enzyme activity have not been evaluated at the site of inflammation or in

the neurons innervating the hind paw in a single model of persistent, peripheral inflammation in conjunction with AEA content.

Therefore the goal of this study was to compare levels of AEA as well as FAAH expression and enzyme activity in hind paws inflamed by the injection of Complete Freund's Adjuvant to those of naïve mice. Understanding changes in endocannabinoid neurochemistry at the site of inflammation as well as in neurons innervating the region will provide insight into the neuroplasticity of endocannabinoid tone modulating the threshold for nociception in CFA-induced peripheral inflammation.

Methods

Animals

Adult, male C3H/He mice (National Cancer Institute; 25-30 g) were used in these studies. All procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee. Because Breese and colleagues (2005) found no differences in paw diameter or mechanical sensitivity between naïve mice and saline-injected mice two days post injection, we chose to compare drug treatments in CFA-injected animals to naïve animals in our studies.

Measurement of sensitivity to a mechanical stimulus

Mechanical sensitivity was determined in each mouse by calculating the frequency of withdrawal to a force of 5.7 mN (refer to chapter 2 for more detailed assay methods). All mice were tested before inclusion in the study. Only mice with a baseline

response rate of $\leq 40\%$ were assigned to naïve or CFA-injected groups. Baseline values were assessed for each animal for 2 days prior to induction of inflammation.

Induction of peripheral inflammation

Inflammation was induced by unilateral, injection of CFA (50 μ l, i.pl.; Calbiochem, La Jolla, CA, USA) into the left hind paw under isoflurane anesthesia. Only mice with a $\geq 70\%$ response to the 5.7 mN monofilament 48 h after injection of CFA were included in the inflamed group in subsequent experiments.

Measurement of endogenous anandamide

Lipids were extracted from tissue samples as described by Maione and colleagues (2007). Immediately after decapitation under a surgical level of anesthesia, samples of L3-L5 DRGs and plantar paw skin were removed unilaterally from naïve mice and ipsilateral to CFA injection in mice with inflamed hind paws. DRGs were pooled from 2 mice (totaling 6 DRGs/sample) and were homogenized and extracted with 250 μ l of chloroform at 4°C overnight. Paw skin samples were pooled from 2 mice and weighed prior to homogenization and extraction with 500 μ l of chloroform at 4°C overnight. Mixtures were then homogenized with an equal volume of methanol:Tris-HCl (50 mM, 1:1) containing 5-10 pmol of deuterated d_8 -AEA (Cayman Chemical) as an internal standard. Homogenates were centrifuged at 3000 x g for 15 minutes (4°C); the aqueous phase plus debris were collected and extracted again with 1 volume of chloroform. The organic phases were pooled and evaporated with a gentle stream of nitrogen gas. Dried

samples were stored at -80°C until analyzed. Targeted isotope-dilution HPLC/atmospheric pressure chemical ionization/mass spectrometry was conducted on each sample. A Phenomenex Prodigy (ODS-3, 150 x 2 mm) column was used. Mobile phase A was 0.5% formic acid in water and phase B was 0.5% formic acid in acetonitrile. The flow rate was 0.1 ml/min with a gradient that began with 95% A:5% B for 1 min followed by a ramp to 100% B over 10 min and maintenance in buffer B for another 16 min. Analyses were performed at the Analytical Biochemistry Core facility of the Masonic Cancer Center, University of Minnesota. The levels of AEA in tissue samples were estimated from the ratio of the area of the signal of *d*₈-AEA compared to standard curves generated for AEA (0.02 to 20 pmol). Data are expressed as pmoles per gram tissue for paw skin or pmoles per six DRGs.

For studies investigating the capacity of DRGs from naïve or CFA-injected mice to generate AEA, the following modifications to the above protocol were made. Individual samples containing six DRGs were isolated and collected in Puck's (calcium and magnesium free) saline on ice. DRGs were rinsed by removing the Puck's saline and adding 500 µl HEPES buffer containing (in mM) 25 HEPES, 135 NaCl, 2.5 CaCl with 100 nM URB597 and 100 nM JZL184 (4-nitrophenyl-4-(dibenzo[d][1,3] dioxol-5-yl(hydroxy)methyl)piperidine- 1-carboxylate), inhibitors for FAAH and monoglycerol lipase, respectively (Cayman Chemical, Ann Arbor, MI, USA) for 3 x 5 min while shaking. To stimulate the DRGs 50 mM KCl was added for 5 min in 200 µl fresh buffer with inhibitors. Basal treatments were incubated in fresh buffer with inhibitors for 5 min. Samples were then homogenized and processed as above.

Quantification of mRNA by real-time RT-PCR

Lumbar (L)3-L5 dorsal root ganglia (DRGs) and plantar paw skin samples were isolated from mice, immersed in RNAlater (Qiagen, Valencia, CA, USA) and stored at 4°C for determination of FAAH mRNA. Total RNA was isolated from DRG samples using RNeasy Lipid Tissue Mini Kits (Qiagen) and skin samples using RNeasy Fibrous Tissue Mini Kits (Qiagen). RNA was reverse transcribed into cDNA using QuantiTect RT-PCR kits (Qiagen) following the manufacturer's instructions. Real-time PCR was performed using DyNAmo HS SYBR Green Master Mix (Finnzymes, Keilaranta, Finland) with the DNA engine Opticon 2 (MJ Research, Watertown MA, USA) through 45 PCR cycles (94°C for 10 s, 57°C for 20 s, and 72°C for 30 s). Each cDNA sample was run in triplicate for the murine FAAH and the appropriate reference gene: neurofilament protein (200 kD) for DRGs or S15 for skin. Primer pair sequences were as follows: FAAH (GenBank Accession number NM_010173) forward primer 5'-GCT GTG CTC TTT ACC TAC CTG-3' and reverse primer 5'-GAA GCA TTC CTT GAG GCT CAC-3'; neurofilament protein (M35131) forward primer 5'-GAC GGA AGG AGT GAC AGA AG-3' and reverse primer 5'-TCT GGA GAT GCA GCC TCT TC-3'; S15 (BC094409) forward primer 5'-CCG AAG TGG AGC AGA AGA AG-3' and reverse primer 5'-CTC CAC CTG GTT GAA GGT C-3'. Primers were synthesized by Operon Biotechnologies, Inc. (Germantown, MD, USA). Amplicon specificity was confirmed by melting curve analysis, evidence of a single band upon gel electrophoresis, authenticity of the DNA sequence of the band isolated from the gel, and resolution by BLAST analysis that the

sequence of the amplicon was unique to murine FAAH, neurofilament protein or S15 respectively.

The ratio of fold-change in the expression of the mRNA of interest for each sample was calculated by normalization of the cycle threshold values to S15 or neurofilament protein mRNA using the equation derived by Pfaffl (2001) to correct for potential differences in PCR primer efficiencies between the target and reference genes. This approach was validated by the lack of an effect of treatments on the expression of S15 or neurofilament protein and confirmation that data for tissue samples were within the linear range used to determine the efficiency of the primers. The efficiency of each primer set was derived from the slope of its linear graph using the following equation: $\text{efficiency} = 10^{(-1/\text{slope})}$. Samples were collected from normal and CFA-injected mice 3-5 times. In order to control for variability in preparations and assays, data for the target genes (corrected to the reference gene) were normalized to the mean of the control group for each date of sample collection.

Measurement of FAAH enzyme activity

FAAH activity was assayed with modifications as described by Pratt and colleagues (1998). Samples of plantar paw skin, L3-L5 DRGs, and sciatic nerve tissue (2 mm) were assayed. For each sample, two commonly treated tissues (eg. skin from naïve mouse or ligated-sciatic nerve ipsilateral to CFA injection) were pooled and assayed as a single sample. Tissue was removed, frozen in liquid nitrogen, and stored at -80°C until the time of processing.

Paw tissue was weighed and homogenized in 500 µl of assay buffer (50 mM Tris-HCl, 3 mM MgCl₂, 1 mM EDTA, pH 7.4). Samples were sonicated, and the protein concentration of each sample was determined using a commercial Bradford protein assay (Sigma-Aldrich, St. Louis, MO USA). Aliquots of paw skin samples (50 µg of protein per assay tube) were incubated in assay buffer with 1 mg/ml fatty acid-free bovine serum albumin for 30 min at 37°C with [³H]AEA (~100,000 dpm/total assay volume of 500 µl, American Radiolabeled Chemicals, St. Louis, MO, USA).

DRGs and sciatic nerves were weighed and homogenized in 250 µl of assay buffer (50 mM Tris-HCl, 3 mM MgCl₂, 1 mM EDTA, pH 7.4). Aliquots of DRGs and sciatic nerves (90 µl of homogenate per assay tube) were incubated in assay buffer with 1 mg/ml fatty acid-free bovine serum albumin for 1 h at 37°C with [³H]AEA (200,000 dpm/total assay volume of 500 µl, American Radiolabeled Chemicals, St. Louis, MO, USA).

Enzyme reactions for all tissue was stopped by the addition of 2 ml of chloroform:methanol (1:2). After incubating at room temperature for 30 minutes with vortexing every 5 minutes, the phases were separated with the addition of 0.67 ml of chloroform and 0.6 ml of water and centrifugation at 280 x g for 10 minutes. The amount of tritium in 1 ml of each of the aqueous and organic phases was determined using liquid scintillation spectrometry. FAAH activity was expressed as percent hydrolysis of [³H]AEA, taking into account that the radioactivity in the aqueous phase was [³H]ethanolamine (EA), whereas [³H]AEA remained in the organic phase (Pratt et al., 1998) using the following equation:

$$\% \text{ conversion to EA} = \frac{\text{EA dpm in aqueous phase} \times 4.86 \times 100\%}{\text{EA dpm in aq. phase} \times 4.86 + \text{AEA dpm in org. phase} \times 2.66}$$

All samples were run in duplicate for FAAH enzyme activity and the average of the assay tubes minus the % conversion to EA in the blank tubes (no protein) for each experiment was used to report FAAH activity per sample. Values for % conversion for DRGS were multiplied by 2.7 and reported/6 DRGs; for sciatic nerve values were multiplied by 2.7 and reported/2 sciatic nerve segments; for paw skin tissue values are reported per 50 μg protein.

Sciatic nerve ligation

The left sciatic nerve was ligated in naïve mice and CFA-injected mice from 40-44 h following the induction of peripheral inflammation to maximize detection of changes in FAAH enzyme activity in peripheral nerves. The sciatic nerve was isolated under isofluorane anesthesia, and a tight ligature was tied around the nerve (De Repentigny et al., 2003) to block axonal transport of FAAH in the nerve. The sciatic tissue (2 mm) proximal to the ligature was harvested 6 h following ligation. Tissue was removed, frozen in liquid nitrogen, and stored at -80°C until the time of processing.

Statistical analyses

All data are presented as the mean \pm S.E.M. for the group. Data were analyzed using Student's *t*-test. A value of $p < 0.05$ was considered significant.

Results

FAAH enzyme decreased in plantar paw skin during peripheral inflammation without an increase in AEA content

The level of FAAH mRNA decreased by 50% in the plantar paw skin of CFA-injected animals (i.e. the site of inflammation) compared to naïve mice (Table 1) 48 h after induction of hyperalgesia. The change in FAAH enzyme activity in the paw skin ipsilateral to the inflammation in CFA-injected mice showed a similar trend to that of the change in FAAH mRNA, however the results were not statistically significant ($p=0.08$). The level of FAAH activity in plantar skin of naïve mice represented $1.1 \pm 0.2\%$ conversion of [^3H]AEA/50 μg protein in the tissue homogenate ($n=4$). FAAH enzyme activity at the site of inflammation was approximately half of the level in plantar paw skin from naïve mice (Table 1).

In light of a likely decrease in FAAH expression, it was important to determine the effect of CFA-induced inflammation on AEA in the paw skin. The level of AEA in the plantar paw skin ipsilateral to injection of CFA was comparable to that of control mice 48 hr after injection of CFA (Table 1).

FAAH mRNA, but not enzyme activity, increased in L3-L5 DRGs during peripheral inflammation

Although we hypothesized that hyperalgesia associated with peripheral inflammation is due in part to increased AEA catabolism, we did not find evidence to support this in the plantar paw skin. Therefore, we measured FAAH activity in the L3-L5

DRGs during peripheral inflammation as a measure of activity in primary afferent neurons. The level of FAAH mRNA increased in the DRGs of CFA-injected mice compared to naïve mice (Table 2). However, there was no difference in enzyme activity in the L3-5 DRGs innervating the hind paw from CFA-injected mice compared to naïve mice (Table 2). The level of FAAH activity in L3-L5 DRGs of naïve mice represented $3.0 \pm 0.3\%$ conversion of [^3H]AEA/sample ($n=6$).

Since the increase in FAAH mRNA was not associated with an increase in enzyme activity in the DRGs, we investigated whether increased axonal transport of FAAH might occur during peripheral inflammation. To determine this, FAAH enzyme activity was measured in the sciatic nerve following ligation to promote accumulation of enzyme in axons. FAAH activity was measured in samples of sciatic nerve taken proximal to the ligature. Unexpectedly, FAAH enzyme activity in the sciatic nerve from CFA-injected mice decreased by one-third (Table 2, $p=0.03$, Student's t test). The level of FAAH activity in the sciatic nerve of naïve mice represented $2.2 \pm 0.4\%$ conversion of [^3H]AEA/sample ($n=4$).

AEA level in L3-L5 DRGs

In addition to measuring AEA at the site of inflammation, we measured AEA in the DRGs of naïve and CFA-injected mice. In the DRGs, the level of AEA decreased by almost 20% in the CFA-injected group compared to naïve mice (Table 2, $p=0.04$, Student's t test). The reduced level of AEA in the DRGs of CFA-injected mice may be a result of either decreased synthesis or increased degradation. Because the decrease in

AEA content in the DRGs did not occur in conjunction with an increase in FAAH enzyme activity, we tested whether a decrease in synthesis might contribute to the lower level of AEA in DRGs isolated from CFA-injected mice.

The basal level of AEA in DRGs from CFA-treated mice was comparable to that of DRGs from naïve mice (Table 3). AEA is synthesized in a depolarization-evoked manner, and previous studies in our lab confirmed 50 mM KCl is sufficient to stimulate the synthesis of AEA *in vitro*. Treatment with 50 mM KCl stimulated AEA production in DRGs isolated from naïve mice compared to basal conditions ($p=0.004$, Student's *t* test), however, the same stimulus failed to increase the level of AEA in DRGs isolated from CFA-injected mice (Table 3). These data support the hypothesis that a decreased ability to synthesize AEA contributed to the low anandamide content in the DRGs.

Discussion

Through a comprehensive analysis of tissue levels of AEA, FAAH enzyme expression and activity, we extended the scope of knowledge of endocannabinoid signaling to a model of persistent cutaneous inflammation. The data suggest that complex, region-specific changes in endocannabinoid signaling may contribute to the anti-hyperalgesic effect of URB597 during peripheral inflammation.

In the hind paw, the hyperalgesia associated with peripheral inflammation was neither a result of increased FAAH expression and activity nor a reduction in AEA. Rather, our data indicate a decrease in endogenous AEA and the ability to synthesize

AEA in the DRGs contributed to the mechanical hyperalgesia associated with peripheral inflammation (Chapter 2).

Effect of CFA on FAAH activity and AEA in the plantar paw skin

Initially we hypothesized that during inflammation AEA levels decreased in the skin in the hind paw, leading to a loss of CB1 receptor activation and mechanical hyperalgesia. Based on the ability of local injection of URB597 to inhibit mechanical hyperalgesia (Chapter 2), we predicted the hyperalgesia associated with peripheral inflammation resulted from increased FAAH activity in the hind paw. Unexpectedly, FAAH mRNA was reduced and FAAH enzyme activity trended toward a decrease in the plantar skin of the inflamed hind paw compared to naïve mice during peripheral inflammation. Although not consistent with our initial hypothesis, the data are consistent with decreased FAAH activity in carrageenan-inflamed paws in mice (Holt et al., 2005). Decreased FAAH enzyme activity at 4 h (Holt et al., 2005) as well as the trend 2 d after CFA-induced inflammation in the present study suggest decreased FAAH enzyme activity in the plantar skin of the hind paw may be maintained for the duration of peripheral inflammation. Minimizing local degradation of AEA as an adaptive biological response may be employed to moderate mechanical hyperalgesia associated with peripheral inflammation in mice.

Importantly, it is not likely that edema accounts for the lower values in the skin from inflamed hind paws in our study because data for enzyme activity were normalized to protein content and mRNA were normalized to ribosomal mRNA. However, it is

difficult to assess whether the infiltration of immune cells may have altered the amount of protein and mRNA in the skin of the hind paw. Despite this, the change in enzyme activity in the sciatic nerve is consistent with the paw, and therefore not likely related to edema.

In the present study, the endocannabinoid AEA in the plantar skin was unchanged 48 h after CFA injection compared to naïve mice. Despite the lack of a change in AEA in the hind paw between naïve and CFA-injected mice in our study, it is important to note the amount of AEA reported here is within the range described by others (Calignano et al., 1998; Beaulieu et al., 2000) and our lab has detected a decrease in AEA in the plantar paw skin in tumor-bearing mice (Khasabova et al., 2008). Thus, sensitivity in detection would not likely contribute to the lack of a measurable change in this report.

Reports of changes in endocannabinoid levels in the hind paw during inflammation are inconsistent and vary across models of inflammation and species tested. In contrast to the lack of change in endocannabinoids in the hind paw in our report, Agarwal and colleagues (2007) reported an increase in anandamide in the paw skin 24 h after CFA-induced inflammation in mice. The reason for these differences are unclear, but may be related in part to the strain of mice used, the amount of CFA injected, or the time frame in which endocannabinoid measures were performed. Alternatively, it is possible that sampling differences between the studies may contribute to this discrepancy. The skin consists of various layers, and within these layers multiple types of cells including keratinocytes (Maccarrone et al., 2003) and terminals of DRG neurons (Ahluwalia et al., 2003b; van der Stelt et al., 2005) generate anandamide. Studies

examining endocannabinoid content in sublayers of the skin may shed light on this in the future.

It is noteworthy that URB597, an inhibitor of FAAH, blocked mechanical hyperalgesia associated with CFA-induced inflammation following local administration into the hind paw (Chapter 2). The URB597-mediated reduction in mechanical hyperalgesia occurred despite a decrease in the level of FAAH mRNA in the hind paw of CFA-injected mice. Furthermore, in CFA-injected mice, the trend toward a decrease in FAAH enzyme activity did not increase AEA in the hind paw. Although these data do not fit the prediction that a decrease in AEA during inflammation contributed to hyperalgesia, these data are consistent with evidence that FAAH +/- mice do not show an increase in AEA level in the brain (Cravatt et al., 2001). Thus, reduction of FAAH does not inherently increase AEA content.

CFA had no apparent effect on FAAH enzyme activity in the DRGs

In light of unexpected evidence that FAAH mRNA is decreased in the paw skin tissue, we next sought to characterize the contribution of FAAH from the primary afferent neurons responsible for innervating the inflamed hind paw. In the DRGs, although FAAH mRNA was increased in the DRGs associated with peripheral inflammation, FAAH enzyme activity remained unchanged ipsilateral to the inflamed hind paw compared to those from naïve mice. Sensitivity of the assay is unlikely to be an issue in the DRGs because three times the amount of [³H]AEA was converted in the DRGs compared to the paw skin. These results differ from FAAH mRNA and enzyme

activity in this strain of mice in a model of cancer pain in which FAAH mRNA and enzyme activity increased in the DRGs (Khasabova et al., 2008). These differences may result from the duration of pathology, because tumors were present for ten days prior to measurement of FAAH mRNA and enzyme activity, or differences in the chemical interaction of immune and cancer cells with sensory neurons.

Since FAAH mRNA increased in the DRGs, to determine if the lack of an increase in FAAH enzyme activity in the DRGs was due to transport to peripheral terminals, we measured FAAH enzyme activity ipsilateral to CFA injection in ligated sciatic nerves. Interestingly, rather than the predicted increase in FAAH enzyme activity in the sciatic nerve of CFA-injected mice, a significant decrease was observed. An explanation for this may be that the endocannabinoid system, in addition to modulating pain, plays a neuroprotective function in ischemia and trauma (see reviews by Jonsson et al., 2006; Svizenska et al., 2008; Fowler et al., 2010). In this case, inhibition of FAAH enzyme activity would maintain the level of AEA. This may protect the nerve from damage associated with ligation during peripheral inflammation and explain the decrease in FAAH enzyme activity in CFA-injected mice.

Effect of CFA on AEA content in the L3-L5 DRGs

Endocannabinoids are produced on demand (Di Marzo, 1998) and their level is tightly regulated by rapid reuptake and subsequent catabolism. In the absence of evidence supporting increased FAAH enzyme activity in the DRGs associated with inflamed hind paws, it is likely the lower level of AEA in DRGs resulted from decreased synthesis. This

is supported by the decreased capacity of DRGs isolated from CFA-injected mice to generate AEA compared to DRGs isolated from naïve mice. If the DRG is accepted as a mirror of the sensory neuron terminal, the low level of AEA may contribute to hyperalgesia associated with persistent inflammation through reduced basal activation of CB1 receptors on nociceptors (Agarwal et al., 2007).

Conclusion

Taken together, our results underscore the complexity of plasticity in endocannabinoid signaling during persistent inflammation. Resolution at the level of the DRG is important and as such, we report an increase in FAAH mRNA in this region. Although measures of FAAH enzyme activity in the DRG were not as conclusive, the ability to generate AEA is impaired in DRGs isolated from CFA-injected mice compared to naïve animals. Therefore, our data support a model in which decreased ability to synthesize AEA contributed to the mechanical hyperalgesia associated with peripheral inflammation.

Table 1. FAAH enzyme and anandamide content in plantar paw skin from naïve and CFA-injected mice.

Assay	Naïve	CFA-injected
FAAH mRNA	92 ± 10% (14)	45 ± 9%* (15)
FAAH enzyme activity	100 ± 21% (4)	51 ± 9% (4)
AEA content (pmol/g tissue)	565 ± 34 (8)	482 ± 52 (8)

Samples of plantar paw skin ipsilateral to peripheral inflammation were collected 48 h after injection of CFA or from the left side of naïve mice. Quantitative real time RT-PCR was used to measure FAAH mRNA in plantar paw skin isolated from naïve and CFA-injected mice. Abundance of FAAH mRNA was quantified relative to S15 for each sample and then expressed as a percentage of the average value for the naïve group. For the RT-PCR data, one value from the naïve group and two values from the CFA-injected group were omitted because they were greater than two standard deviations from the group mean. FAAH enzyme activity was expressed as percent hydrolysis of [³H]AEA per sample and then expressed as a percentage of the mean for naïve mice. For AEA content, each sample contained tissue pooled from two animals and is reported as pmol/g tissue. *Significantly different from naïve mice in respective assay at $p < 0.01$ (Student's t test). Numbers in parentheses represent the sample size.

Table 2. FAAH enzyme and anandamide content in nerve tissue from naïve and CFA-injected mice.

Assay	Naïve	CFA-injected
FAAH mRNA L3-L5 DRGs	100 ± 13% (14)	141 ± 14%* (15)
FAAH enzyme activity		
L3-L5 DRGs	100 ± 11% (6)	104 ± 13% (4)
Sciatic nerve	100 ± 20% (4)	66 ± 13%* (5)
AEA content (pmol/L3-L5 DRGs)	1.06 ± 0.04 (7)	0.84 ± 0.09* (6)

Samples from CFA-injected mice were collected ipsilateral to peripheral inflammation. Quantitative real time RT-PCR was used to measure FAAH mRNA in L3-5 DRGs isolated from naïve and CFA-injected mice. Abundance of FAAH mRNA was quantified relative to neurofilament protein for each sample. Results were then expressed as a percentage of the average value for the naïve group for each assay. FAAH enzyme activity was expressed as percent hydrolysis of [³H]AEA per sample and then expressed as a percentage of the mean for naïve mice. Samples represent six L3-5 DRGs/sample (from 2 CFA injected mice or both sides of a naïve animal) or two sciatic nerves (from 2 naïve or CFA-injected mice). For AEA content, each sample contained DRGs pooled from two animals and data are reported as pmol/L3-L5 DRGs. *Significantly different from naïve mice ($p < 0.05$, Student's *t* test). Numbers in parentheses represent the sample size.

Table 3. AEA level in DRGs from a depolarizing stimulus of 50 mM KCl in naïve and CFA-injected mice.

Condition	AEA (pmol/six L3-5 DRGs)	
	Basal	KCl (50 mM)
Naïve	0.065 ± 0.004 (6)	0.093 ± 0.006* (5)
CFA	0.086 ± 0.017 (5)	0.086 ± 0.005 (6)

Six L3-5 DRGs/sample (from 2 CFA injected mice or both sides of a naïve animal) were collected and treated with 50 mM KCl or buffer alone for 5 min. *Significantly different from naïve mice under basal conditions ($p < 0.01$, Student's t test). Numbers in parentheses represent the sample size.

Chapter 4

Functional changes in CB1 receptors in neurons isolated from CFA-injected mice

Introduction

The anti-hyperalgesic effect of CB1 receptor agonists is mediated in part by inhibition of primary afferent neurons. Primary afferent neurons are sensory neurons with their cell bodies clustered in the dorsal root ganglia (DRG). Given that DRGs exhibit both the ability to synthesize AEA (Chapter 3) and CB1 receptor-mediated inhibition of depolarization-evoked increases in intracellular free Ca^{2+} concentration (Khasabova et al., 2002; 2004), autocrine signaling in primary afferent neurons is a distinct possibility. Furthermore, there is increasing evidence that peripheral activation of CB1 receptors plays a more prominent role in modulating nociception than initially suggested. This is supported by evidence that selective CB1 receptor gene deletion from peripheral nociceptors reduced the threshold for sensory transduction (Agarwal et al., 2007). Similarly, injection of AM281 (10 μg , i.pl.) a CB1 receptor antagonist, increased the withdrawal frequency to a range of mechanical forces ipsilateral to the injection (Khasabova et al., 2008).

Expression of CB1 receptor mRNA is primarily localized to intermediate- and large-sized neurons (Hohmann and Herkenham, 1999; Bridges et al., 2003) while CB1 receptor immunoreactivity is found in both small (Ahluwalia et al., 2000) and large neurons (Khasabova et al., 2002). Activation of CB1 receptors on DRG neurons produces inhibitory effects through suppression of calcium and sodium channels (Ross et al., 2001; Khasabova et al., 2002; 2004). During CFA-induced inflammation in mice, CB1 receptor mRNA in primary afferent neurons peaks on day two following CFA-induced

inflammation, and CB1 protein expression also increased in DRGs as well as nerve fibers in skin, suggesting transport to the periphery (Amaya et al., 2006). Furthermore, a ten fold lower dose of a CB1 agonist attenuated thermal hyperalgesia at 2 d in CFA-injected mice compared to control mice, suggesting the increase in CB1 receptor expression mediates anti-hyperalgesia (Amaya et al., 2006). In rats, Potenziari and colleagues (2008) reported ACEA and methanandamide not only reduced mechanical hyperalgesia associated with CFA injection, but decreased mechanically evoked responses from cutaneous A δ fibers from inflamed, but not naïve, skin in rats in a CB1-, but not CB2-dependent, manner. Together, these data suggest increased CB1 receptor expression in primary afferent neurons may contribute to modulation of the nociceptive threshold for sensory transduction during inflammation.

In light of evidence that DRGs isolated from CFA-injected mice have a decreased ability to generate AEA compared to DRGs isolated from naïve mice (Chapter 3), we hypothesized that an increase in CB1 receptors in primary afferent neurons may be a compensatory mechanism to promote increased CB1 receptor activation during inflammation. To test this, CB1 receptor mRNA in the DRGs of naïve and CFA-injected mice at the same time point characterized in studies of AEA and FAAH levels were measured. To test the prediction that there would be an increase in functional CB1 receptors in neurons isolated from CFA-injected mice, a bioassay was used to study the inhibitory effect of a CB1 receptor agonist on the depolarization-evoked intracellular free Ca²⁺ level. Since inhibition of FAAH with URB597 blocked CFA-induced mechanical

hyperalgesia *in vivo*, the effect of URB597 on the calcium transient in DRGs from naïve and CFA-injected mice was also compared *in vitro*.

Methods

Animals

Adult, male C3H/He mice (National Cancer Institute; 25-30 g) were used in these studies. All procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Measurement of sensitivity to a mechanical stimulus

Mechanical sensitivity was determined in each mouse by calculating the frequency of withdrawal to a force of 5.7 mN (refer to chapter 2 for more detailed assay methods). All mice were tested before inclusion in the study. Only mice with a baseline response rate of $\leq 40\%$ were assigned to naïve or CFA-injected groups. Baseline values were assessed for each animal for 2 days prior to induction of inflammation.

Induction of peripheral inflammation

Inflammation was induced by unilateral, intraplantar (i.pl.) injection of 50 μ l Complete Freund's Adjuvant (CFA; Calbiochem, La Jolla, CA, USA) into the left hind paw under isoflurane anesthesia. Only mice with a $\geq 70\%$ response rate 48 h after injection of CFA were included in the inflamed group in subsequent experiments.

Quantification of mRNA by real-time RT-PCR

Lumbar (L)3-L5 dorsal root ganglia (DRGs) were isolated from mice, immersed in RNAlater (Qiagen, Valencia, CA, USA) and stored at 4° C for determination of CB1 receptor mRNA. Total RNA was isolated from DRG samples using RNeasy Lipid Tissue Mini Kits (Qiagen). RNA was reverse transcribed into cDNA using QuantiTect RT-PCR kits (Qiagen) following manufacturer's instructions. Real-time PCR was performed using DyNAmo HS SYBR Green Master Mix (Finnzymes, Keilaranta, Finland) with the DNA engine Opticon 2 (MJ Research, Watertown MA, USA) through 45 PCR cycles (94°C for 10 s, 57°C for 20 s, and 72°C for 30 s). Each cDNA sample was run in triplicate for the murine CB1 receptor and the reference gene neurofilament protein (200 kD). Primer pair sequences were as follows: CB1 receptor (GenBank Accession number NM_007726) forward primer 5'-GTT CTG ATC CTG GTG GTG TTG-3' and reverse primer 5'-GTT CAG CAG GCA GAG CAT AC-3'; neurofilament protein (M35131) forward primer 5'-GAC GGA AGG AGT GAC AGA AG-3' and reverse primer 5'-TCT GGA GAT GCA GCC TCT TC-3'. Primers were synthesized by Operon Biotechnologies, Inc. (Germantown, MD, USA). Amplicon specificity was confirmed by melting curve analysis, evidence of a single band upon gel electrophoresis, authenticity of the DNA sequence of the band isolated from the gel, and resolution by BLAST analysis that the sequence of the amplicon was unique to murine CB1 receptor and neurofilament protein respectively.

The ratio of fold-change in the expression of the mRNA of interest for each sample was calculated by normalization of the cycle threshold values to neurofilament

protein mRNA using the equation derived by Pfaffl (2001) to correct for potential differences in PCR primer efficiencies between the target and reference genes. This approach was validated by the lack of an effect of treatments on the expression of neurofilament protein and confirmation that data for tissue samples were within the linear range used to determine the efficiency of the primers. The efficiency of the primer set was derived from the slope of its linear graph using the following equation: efficiency = $10^{(-1/\text{slope})}$. Samples were collected from normal and CFA-injected mice 3-5 times. In order to control for variability in preparations and assays, data for the target gene (corrected to the reference gene) were normalized to the mean of the control group for each date of sample collection.

Isolation of adult murine dorsal root ganglion neurons

Primary cultures of dissociated adult male C3H mouse dorsal root ganglia neurons were prepared from L3-L5 DRGs isolated ipsilateral to the CFA injection or from L3-L5 DRGs isolated from both sides of naïve mice as described previously (Scott 1977; Khasabova et al., 2002, 2007). Following induction of deep anesthesia with isoflurane, mice were decapitated, and DRGs were dissected and collected in Puck's saline (calcium and magnesium free) on ice. After collection, DRGs were enzymatically digested in two 1 h incubations (37°C) with 1.25 mg/ml collagenase D (Boehringer Mannheim, Mannheim, Germany) in Ham's F12/DMEM medium (1:1). DRGs were dissociated into single cells by trituration through flame-polished Pasteur pipets of decreasing diameter and plated at a density of one DRG/coverlip on laminin- (Sigma

Aldrich, St. Louis, MO, USA) coated glass coverslips (Fisher Scientific, Pittsburgh PA, USA). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in Ham's F12/DMEM medium supplemented with L-glutamine (2 mM), glucose (40 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), 5% horse serum, and DNase I (0.15 mg/ml, Sigma, St. Louis, MO, USA). Cells were maintained *in vitro* for 16-24 h prior to use.

Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$)

Cells were loaded with the Ca²⁺-sensitive fluorescent indicator indo-1 acetoxymethyl ester (3 µM, Molecular Probes, Invitrogen, Eugene, OR, USA) in HEPES buffer containing (in mM: 25 HEPES, 135 NaCl, 2.5 CaCl₂, 3.5 KCl, 1 MgCl₂, and 3.3 glucose, pH 7.4, mOsm 335-340) with 2% BSA for 45-75 min at 37°C before recordings were collected. During the experiment, the coverslip was mounted in a superfusion chamber, placed on an inverted microscope stage, and superfused with HEPES buffer at a rate of 1.5 ml/min at room temperature.

Intracellular free calcium was measured in individual neurons using a dual-emission microfluorimeter (Photoscan, Photon Technology International, Princeton, NJ, USA) to detect fluorescence of indo-1 as previously described in our laboratory (Stucky et al., 1996; Khasabova et al., 2002; 2008). Counts from the photomultiplier tubes were collected and summed over 0.5 s and recorded on a computer. Values for $[Ca^{2+}]_i$ were calculated from the equation $[Ca^{2+}]_i = K_D\beta(R - R_{min})/(R_{max} - R)$, where R= 405 nm/485 nm fluorescence emission ratio corrected for background fluorescence. For indo-1, the

dissociation constant (K_D) was 250 nM (Grynkiewicz et al., 1985) and β was the ratio of fluorescence at 485 nM in the absence and presence of a saturation concentration of Ca^{2+} . Remaining values and β were empirically determined in adult murine DRG neurons where $R_{min}=0.275$, $R_{max}=4.73$ and $\beta=4.85$. For each coverslip, background fluorescence for each wavelength was measured in a field the same diameter as the neuron being recorded lacking neurons, processes, or debris.

Using a rectangular diaphragm to restrict the recording area, only one neuron was recorded from each coverslip, and the size was estimated by obtaining the maximum and minimum diameter using a grid mounted in the eyepiece of the microscope. Using these values, the average radius was determined, and that was used to calculate the somal cross-sectional area using the equation $area = \pi r^2$. Data for individual neurons were separated into two groups based on somal size of the neuron, with small neurons being defined as having a somal area $<500 \mu m^2$, and large neurons having a somal area $>500 \mu m^2$. Because murine DRG neurons with a somal size classified here as “small” typically give rise to C-fibers with characteristic nociceptive properties (Hiura and Sakamoto, 1987; Urban and Dray, 1993; Pearce and Duchon, 1994; Dirajlal et al., 2003) these groups allowed us to infer the type of neuron tested in each population.

The following basic protocol was used to study the effect of cannabinoids on the response of individual DRG neurons. After confirmation of a stable baseline in $[Ca^{2+}]_i$, changes in the functional response of DRG neurons were measured by three applications of KCl (50 mM) separated by superfusion of HEPES buffer for 5 min. Following the first KCl application, drugs were included in the HEPES superfusate (Figure 1) at the

concentrations reported in the results. The reported amplitude of the KCl-evoked change in the calcium transient is determined as the difference between the peak amplitude and the baseline prior to KCl application. Variability between treatment groups was minimized by reporting the relative response, defined as the amplitude of the third KCl-evoked Ca^{2+} transient (in the presence of drug) divided by the amplitude of the first response (in the absence of drug). To control for variation among preparations, the relative response for KCl alone (control group) as well as agonist or enzyme inhibitor plus KCl (drug alone) was determined on each day of an experiment when a third drug (receptor antagonist) was tested. To ensure viability of the cell, only cells with a baseline maintained between 90-110% of the starting baseline throughout the duration of the experiment were included in statistical analyses. The maximum amount of cells omitted from any single treatment group was 18% of the total cells tested.

Statistical analyses

All data are presented as the mean \pm S.E.M. for the group. For measurement of Ca^{2+} transients, statistical differences among groups were identified using Student's *t* test or ANOVA with Tukey's multiple comparisons test to resolve individual group differences. A value of $p < 0.05$ was considered statistically significant. *In vitro* data for each treatment were collected from a minimum of three preparations from different animals, with control and treatment groups determined on each day of an experiment to control for variability between preparations of neurons.

Results

CB1 Receptor mRNA increased in the DRGs during peripheral inflammation

Up-regulation of CB1 receptors in sensory neurons innervating the site of inflammation may contribute to the anti-hyperalgesic effect of URB597 in CFA-injected mice. To address this, CB1 receptor mRNA was measured in L3-L5 DRGs using quantitative real time RT-PCR. The level of CB1 receptor mRNA was 1.5 fold greater 48 h after induction of peripheral inflammation compared to DRGs sampled from the same level in naïve mice ($99.5 \pm 8.3\%$ in naïve mice compared to $145.4 \pm 15.9\%$ in CFA-injected mice, $p=0.02$, Student's t test). However, changes in mRNA do not equate to changes in functional CB1 receptor. Therefore, the effects of both a CB1 receptor agonist and URB597 were studied in small- and large-diameter individual DRG neurons.

Effect of ACPA, a CB1 receptor agonist, in two populations of murine DRG neurons

Because cannabinoids inhibit voltage-dependent Ca^{2+} channels in DRG neurons (Ross et al., 2001; Khasabova et al., 2004) a decrease in the Ca^{2+} transient evoked by 50 mM KCl was used as a bioassay for activation of CB1 receptors by the CB1-selective receptor agonist ACPA. For vehicle control treated neurons, there was no difference in the amplitude of intracellular calcium concentration between the first (p1) and third (p3) KCl application in neurons isolated from naïve or CFA-injected mice in either the small (naïve p1= 363.8 ± 56.1 , naïve p3= 317.7 ± 46.6 , $n=15$; CFA-injected mice p1= 339.1 ± 50.1 , CFA-injected mice p3= 319.2 ± 45.7 , $n=24$; $p=0.76$; one-way ANOVA with repeated measures) or large populations (naïve p1= 199.0 ± 16.4 , naïve p3= 190.5 ± 14.3 , $n=42$; CFA-

injected mice $p1=232.2\pm31.9$, CFA-injected mice $p3=229.6\pm33.0$, $n=18$; $p=0.95$; one-way ANOVA with repeated measures).

Because functional CB1 receptors in dissociated DRG neurons from naïve mice are restricted to large size neurons (Khasabova et al., 2004), we first compared the concentration response curve of ACPA in large neurons isolated from naïve to those from CFA-injected mice. Of the concentrations tested on large neurons isolated from naïve mice, only 100 nM ACPA inhibited the relative response (Figure 2). In comparison, for large neurons isolated from CFA-injected mice, only 10 nM ACPA inhibited the transient change in $[Ca^{2+}]_i$, indicating a leftward-shift in the concentration response for ACPA in neurons isolated from CFA-injected mice (Figure 2). There was no effect of ACPA on the relative response of small neurons isolated from naïve or CFA-injected mice at the concentrations tested (Figure 3).

Inhibitory effect of ACPA is blocked by CB1 and CB2 receptor antagonists in large-sized murine DRG neurons

To determine which cannabinoid receptor mediated the effect of ACPA on reducing the amplitude of the depolarization-evoked Ca^{2+} transient in large neurons isolated from naïve mice, we tested co-treatment of ACPA (100 nM) with the CB1 receptor antagonist AM281 (1 μ M) or the CB2 receptor antagonist AM630 (100 nM). Both receptor antagonists blocked the inhibitory effect of ACPA (Figure 4). Because ACPA was expected to be a selective CB1 receptor agonist, we tested the effect of the CB2 receptor agonist AM1241 (500 nM) on the depolarization-evoked Ca^{2+} transient in

order to confirm the presence of functional CB2 receptors. In CHO-K1 cells stably expressing human CB2 receptors, 200 nM was effective on forskolin-stimulated cAMP levels (Bingham et al., 2007). In large neurons isolated from naïve mice, AM1241 had no effect on the mean relative response (1.01 ± 0.21 ; $n = 4$) compared to control (0.87 ± 0.04 ; $n = 9$; $p = 0.39$, Student's t test). Therefore, despite the ability of the CB2 receptor antagonist AM630 to block the inhibitory effect of ACPA, it is not likely that functional CB2 receptors contributed to the effect of ACPA in this preparation.

Effect of URB597, a FAAH inhibitor, in two populations of murine DRG neurons

URB597 inhibits the enzyme that catabolizes AEA. As such, we predicted it would promote an increase in endogenous AEA resulting in the activation of CB1 receptors and attenuation of the KCl-evoked Ca^{2+} transient. In addition, on basis of the results with ACPA, we predicted that the effective concentrations would be different between neurons isolated from naïve and CFA-injected mice. However, URB597 had no effect on the KCl-evoked Ca^{2+} transient in small (Figure 5) or large (Figure 6) neurons at any concentration tested in neurons isolated from either naïve or CFA-injected mice.

Discussion

Understanding the role of CB1 receptors on the primary afferent neurons has become increasingly important as renewed interest in medicinal application of cannabinoid agonists has been explored (Woodridge, 2005, Cabral and Griffin-Thomas, 2008). Our studies provide evidence that CB1 receptor mRNA is increased in DRGs

isolated from CFA-injected mice, and this increase is associated with a left-ward shift in the concentration response to ACPA in large neurons under inflammatory conditions. Together, our data suggest large-sized neurons from CFA-injected mice are more sensitive to a CB1 receptor agonist than neurons isolated from naïve mice. However, the FAAH inhibitor URB597 had no effect on the KCl-evoked Ca^{2+} transient on either population of neurons isolated from naïve or CFA-injected mice.

CB1 receptor expression is increased in the DRGs

Here we report that CB1 receptor mRNA was present in DRGs innervating the hind paw, and during CFA-induced peripheral inflammation, CB1 receptor mRNA significantly increased in this region. Despite the fact that DRGs are composed of both neuronal and non-neuronal supporting cells, it is likely that the increase in CB1 receptor occurred in neurons because previous studies have demonstrated that cannabinoid receptors are localized to neurons using *in situ* hybridization (Hohmann and Herkenham, 1999b), and CB1 receptor immunoreactivity (Ahluwalia et al., 2000; Khasabova et al., 2002; 2008). Furthermore, CB1 receptor immunoreactivity was notably absent among non-neuronal cells in DRG cultures in our lab (Khasabova et al., 2008).

Our data are consistent with others who have also reported increases in CB1 receptor mRNA associated with inflammation and other pain models. In both CFA-induced inflammation (Amaya et al., 2006) and tumor-evoked bone cancer pain (Khasabova et al., 2008), CB1 receptor mRNA increased in the DRGs, allowing for the possibility that increased CB1 receptor mRNA may result in increased functional

receptors in this region. Although we only determined the amount of CB1 receptor mRNA at a single time point post-inflammation (2 d), others have observed a significant increase at both 24 and 48 h, which had subsided by day 4 (Amaya et al., 2006) providing insight into the time course of the increase in CB1 receptor mRNA.

DRGs synthesize CB1 receptors and they are transported to the peripheral terminals (Hohmann and Herkenham 1999a, 1999b; Ahluwalia et al., 2000; Stander et al., 2005). Under inflammatory conditions, increased CB1 receptor mRNA observed in the DRGs may lead to enhanced anterograde transport of this receptor protein to the periphery. However, measurement of CB1 receptor protein expression was not possible in our study because the antibodies available for immunocytochemistry failed to be neutralized by the blocking peptide in our hands. Yet, during inflammation, a lower dose of the CB1 receptor agonist ACEA injected locally reversed thermal hyperalgesia associated with CFA-induced inflammation, which was accompanied by an increase CB1 receptor gene transcription (Amaya et al., 2006). Furthermore, only in CFA-injected mice did we see an anti-hyperalgesic effect of locally injected URB597 (Chapter 2), possibly suggesting this was a result of increased CB1 receptors in the periphery during inflammation.

CB1 receptor function on the DRG neurons

Our study of KCl-evoked intracellular free Ca^{2+} transients in isolated cell bodies from DRG neurons provided direct evidence of CB1 receptor-mediated function in a population of large diameter neurons. Furthermore, neurons isolated from CFA-injected

mice required a ten-fold lower concentration of ACPA to inhibit the calcium transient compared to neurons isolated from naïve mice. This inhibition coincided with an increase in CB1 receptor mRNA in these animals, suggesting an increase in functional CB1 receptor may contribute to the leftward shift in the concentration-response to ACPA that occurred in neurons isolated from CFA-injected mice. The effective concentration of ACPA in naïve mice is consistent with the concentration of another CB1 agonist that attenuated KCl-evoked increase in intracellular free Ca^{2+} in neurons isolated from adult rat DRG neurons in our lab (Khasabova et al., 2004). This increased receptor function was observed after maintaining neurons *in vitro* for 18-24 h, further suggesting changes in gene expression likely account for the change in function as opposed to a transient event of receptor modulation.

It is noteworthy that in small neurons from either naïve or CFA-injected mice, ACPA had no effect on the calcium transient. These results are consistent with previous reports from our lab that observed no effect on the KCl-evoked intracellular Ca^{2+} in small rat DRG neurons (Khasabova et al., 2004) as well as reports that it is primarily large diameter neurons that express CB1 receptors (Hohman and Herkenham, 1999b; Bridges et al., 2003; Khasabova et al., 2004). However, the lack of an effect of the CB1 receptor agonist in small neurons isolated from CFA-injected mice suggests a distinction between the pathophysiology of inflammatory pain and cancer pain. In small neurons isolated from tumor-bearing mice, CB1 receptors displayed increased receptor function sufficient to inhibit the KCl-evoked intracellular Ca^{2+} (Khasabova et al., 2008). This difference may be the result of the different time course of the models, 2 d for CFA-injected mice

compared to 10 d for tumor-bearing mice, or may be a response specifically to mediators released from the fibrosarcoma cells used to develop the tumors in the cancer mice.

Interestingly, both CB1 and CB2 receptor antagonists blocked the inhibitory effect of ACPA in large neurons. These results were unexpected since CB2 receptors primarily localized to immune cells (Galiegue et al., 1995), despite some evidence that CB2 receptors are present in neurons in the spinal cord (Walczak et al., 2006; Beltramo et al., 2006). Furthermore, CB2 receptors have not been shown to alter the response of calcium or potassium channels. Since individual neurons were isolated to measure the calcium transient under continuous superfusion, it is unlikely that an indirect effect of AM630 on immune cell CB2 receptors contributed to the inhibition of calcium transient in our preparation. It is possible that inflammation causes induction of CB2 receptors on primary afferent neurons that is not observed under naïve conditions, and there is limited evidence suggesting this may occur following peripheral nerve injury (Wotherspoon et al., 2005). Although unexpected, the ability of the CB2 receptor antagonist to inhibit the effect of ACPA is consistent with the *in vivo* studies in chapter 2, where co-injection of AM630 inhibited the anti-hyperalgesic effect of URB597.

Effect of URB597 on the DRG neurons

Although the CB1 receptor agonist inhibited the calcium transient in large neurons, URB597 failed to alter the calcium transient in either population of neurons isolated from both naïve or CFA-injected mice. In large neurons isolated from CFA-injected mice, these results are not entirely surprising in light of evidence that both AEA

content and the ability to generate AEA are significantly decreased (Chapter 3). In this case, it is possible that despite the presence of the FAAH inhibitor, DRG neurons are not able to sufficiently generate AEA to stimulate CB1 receptors during inflammatory conditions.

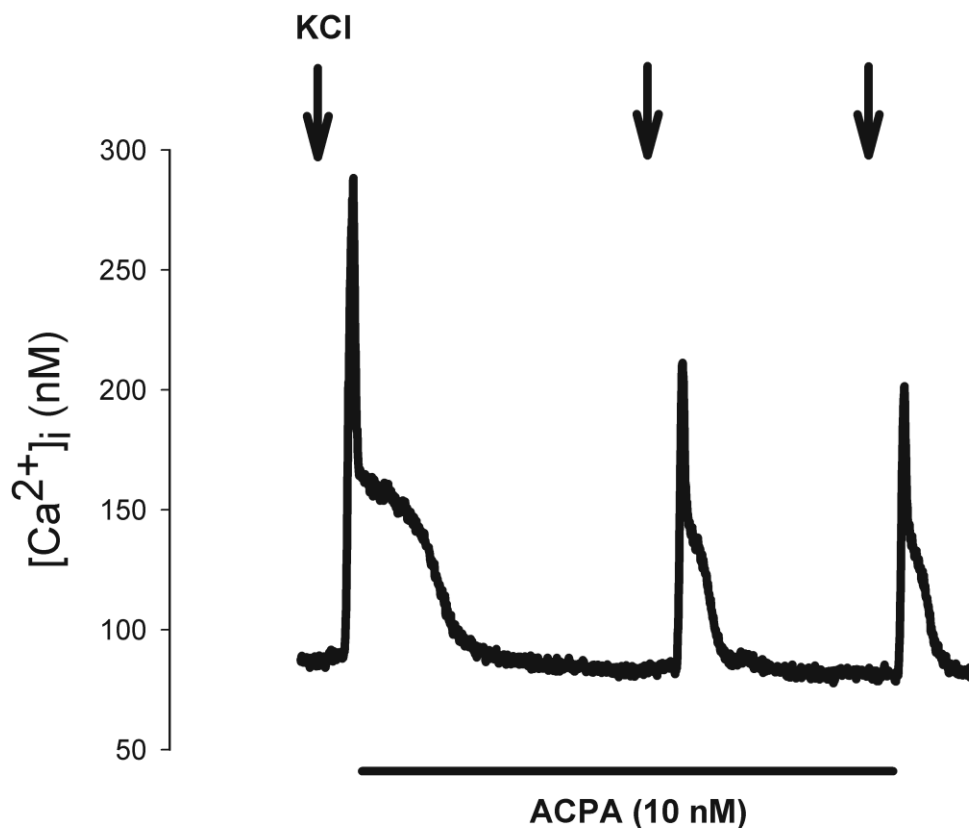
In neurons isolated from both naïve and CFA-injected mice, absence of CB1 receptors on small neurons may contribute to the lack of an effect of URB597 on the free intracellular Ca^{2+} level. These results differ from DRG neurons co-cultured with fibrosarcoma cells in which both the CB1 receptor agonist and URB597 (30 nM) attenuated the KCl-evoked intracellular Ca^{2+} level in a population of small neurons. However, in the cancer model, the reduction of AEA was a result of increased FAAH activity (Khasabova et al., 2008) instead of a decrease in the ability to synthesize AEA as in this model of inflammation. These results demonstrate yet another distinction between models of inflammatory and cancer pain.

Conclusion

In light of evidence that AEA content is significantly reduced in the DRGs of CFA-injected mice and that this decrease is likely the result of a decreased capacity to generate AEA (Chapter 3), our observations support the notion that the increase in CB1 receptor mRNA in the DRGs results in an increase in functional CB1 receptors in large-diameter neurons during inflammation. In this case, up-regulation of CB1 receptors is a compensatory mechanism to decreased basal synthesis of endogenous AEA associated

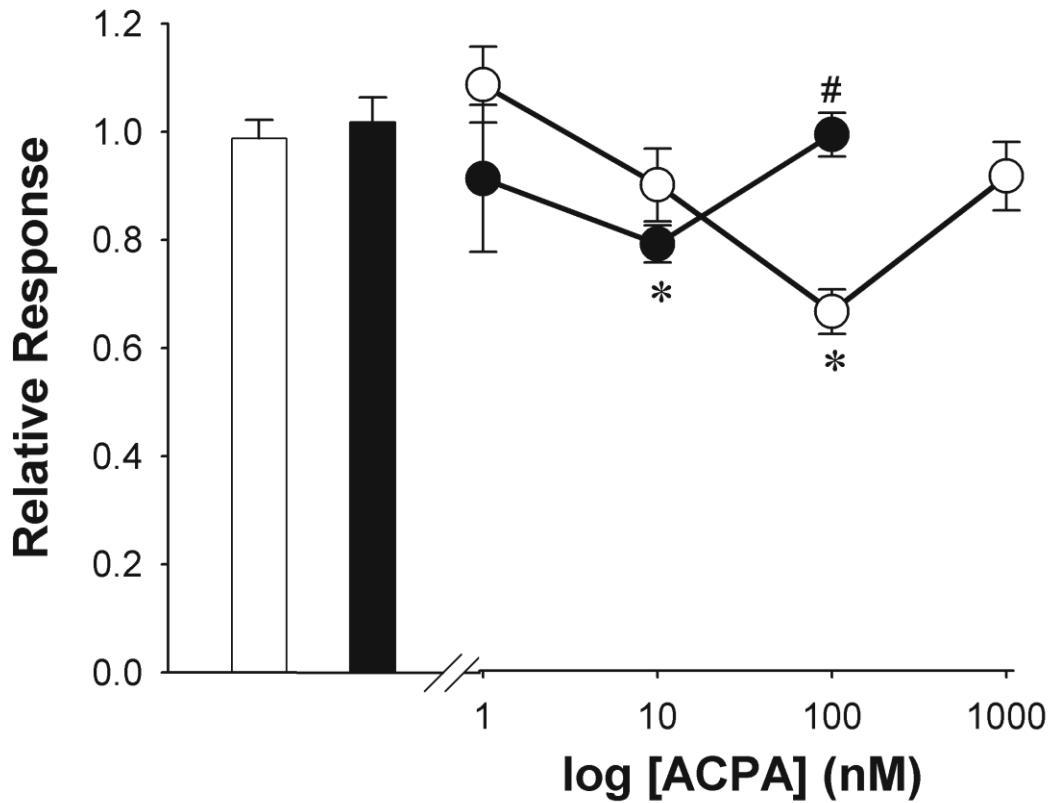
with inflammation. However, since AEA synthesis is impaired, URB597 is unable to promote sufficient CB1 receptor activation to inhibit the KCl-evoked Ca²⁺ transient.

Figure 1. Effect of ACPA, a CB1 receptor agonist, on $[Ca^{2+}]_i$.



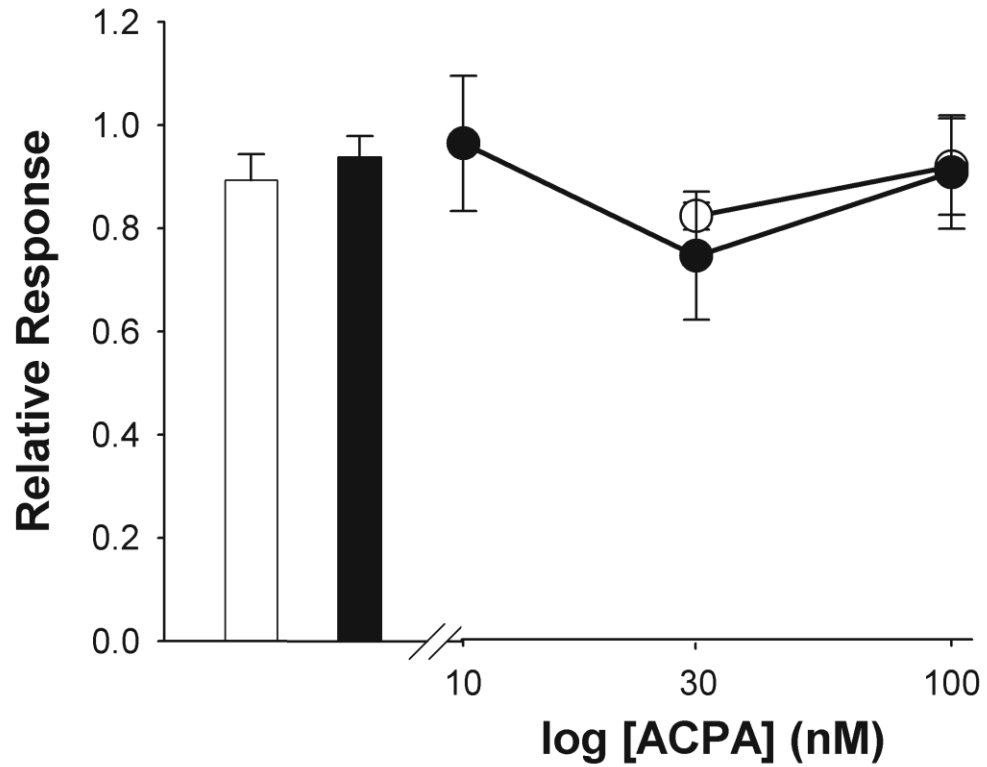
ACPA had no effect on basal $[Ca^{2+}]_i$ but inhibited the Ca^{2+} transient in a representative trace from a large DRG neuron isolated from a CFA-injected mouse. Arrows indicate KCl (50 mM) applications (10 s), and the line below the trace indicates the duration of superfusion with ACPA.

Figure 2. Leftward shift in the concentration response to ACPA in large neurons isolated from CFA-injected mice.



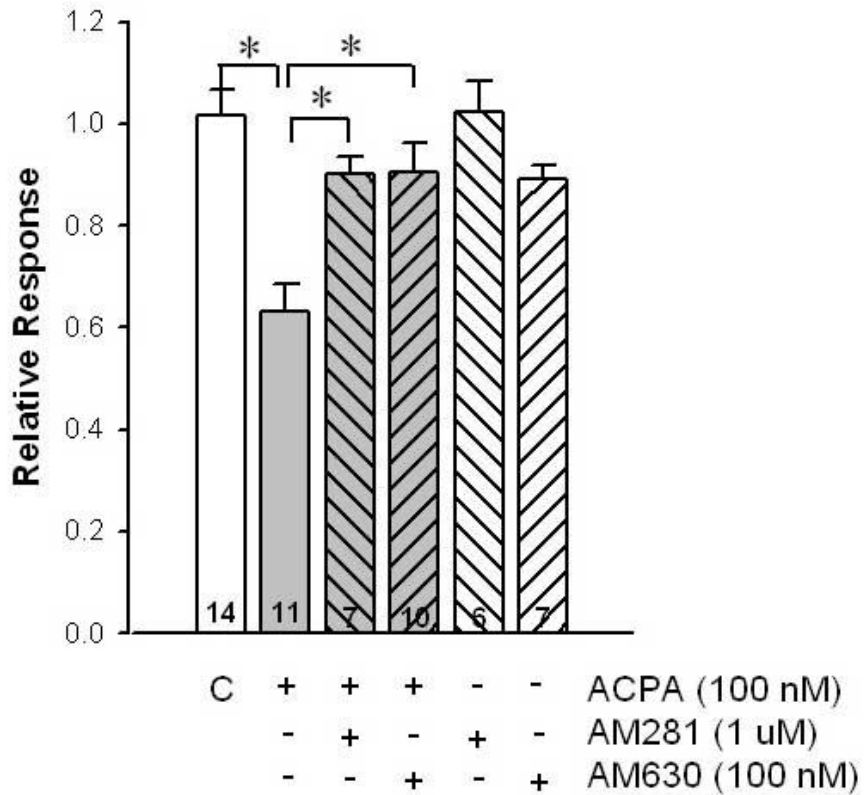
Bars represent vehicle-treated neurons isolated from CFA (filled) or naïve (open) mice. Relative response was defined as the amplitude of the response of a neuron to KCl in the presence of ACPA divided by the amplitude of response in the absence of ACPA. *Significantly different at $p < 0.05$ compared to vehicle (One-way ANOVA w/Tukey test) and #different at $p < 0.05$ compared to neurons isolated from naïve mice at respective time point, two-way RM ANOVA w/Tukey test ($n = 7-25$ /treatment group).

Figure 3. There was no effect of ACPA concentration in small neurons isolated from either naïve or CFA-injected mice.



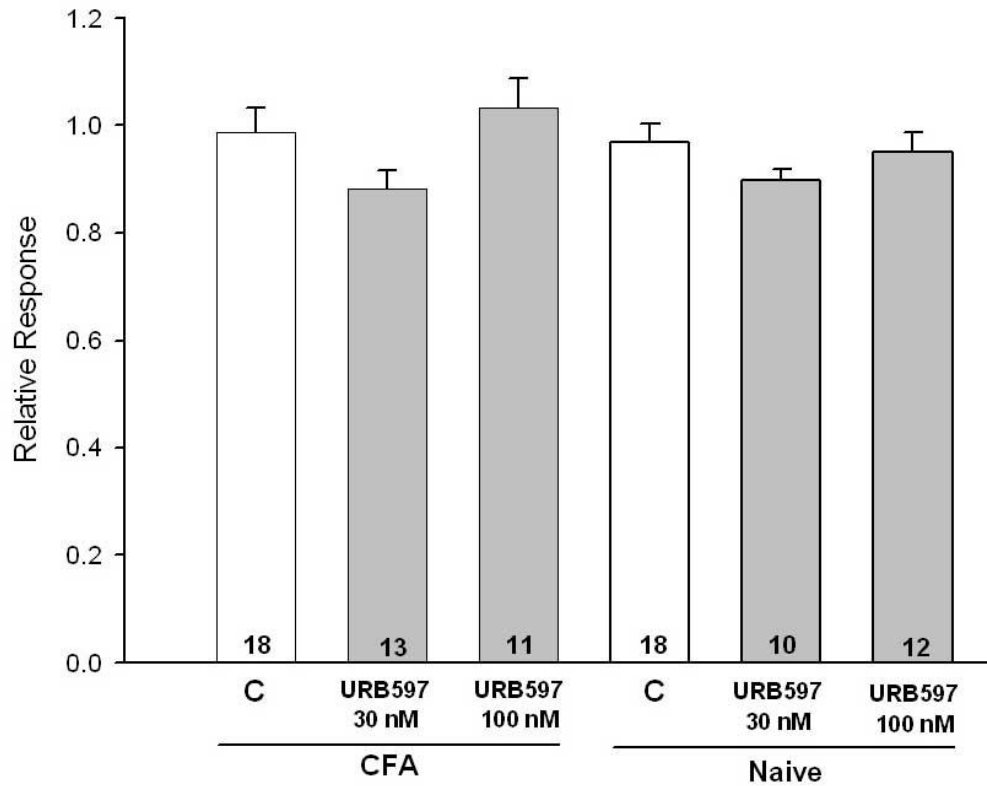
Relative response was defined as the amplitude of the response of a neuron to KCl in the presence of ACPA divided by the amplitude of response in the absence of ACPA. Bars represent vehicle-treated neurons isolated from CFA (filled) or naïve (open) mice. ($n = 6-11$ neurons/treatment group).

Figure 4. Involvement of CB1 and CB2 receptors in the response to ACPA was confirmed by blocking the inhibitory effect by co-application of either AM281 or AM60, CB1 and CB2 receptor angatonists, respectively.



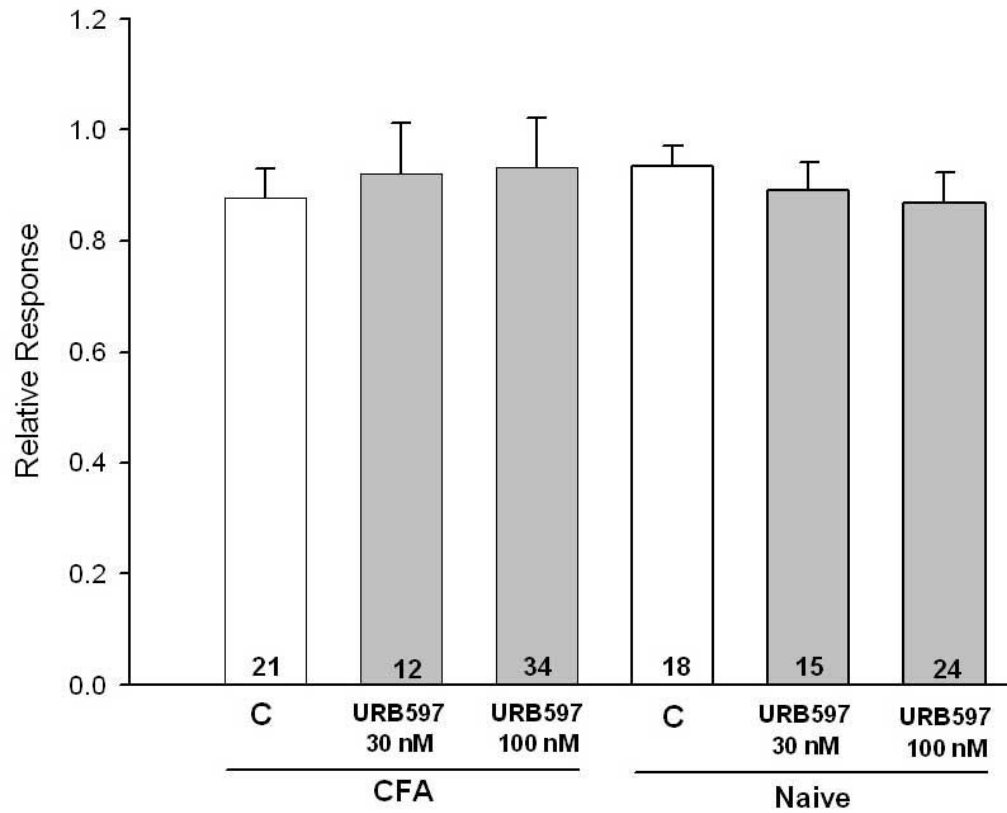
Relative response was defined as the amplitude of the response of a neuron to KCl in the presence of ACPA divided by the amplitude of response in the absence of ACPA.
 *Significantly different at $p < 0.05$ (one-way ANOVA w/Tukey test). Values inside the bars represent sample size.

Figure 5. There was no effect of URB597 in large neurons isolated from either naïve or CFA-injected mice.



Relative response was defined as the amplitude of the response of a neuron to KCl in the presence of URB597 divided by the amplitude of response in the absence of URB597. Values inside the bars represent sample size.

Figure 6. There was no effect of URB597 in small neurons isolated from either naïve or CFA-injected mice.



Relative response was defined as the amplitude of the response of a neuron to KCl in the presence of URB597 divided by the amplitude of response in the absence of URB597. Values inside the bars represent sample size.

Chapter 5

Concluding Statements

The purpose of this thesis was to investigate changes in anandamide signaling that may contribute to mechanical hyperalgesia in a model of persistent, peripheral inflammation and determine whether local injection of URB597 reduced the mechanical hyperalgesia. Specifically, the involvement of AEA, FAAH and CB1 receptors were studied in the hind paw and the primary afferent neurons affected by the peripheral inflammation. The results from these studies indicate 1) local injection of URB597 reduced mechanical hyperalgesia during inflammation, 2) reduced synthesis of AEA in the primary afferent neurons may contribute to the mechanical hyperalgesia associated with peripheral inflammation, 3) up-regulation of CB1 receptors on primary afferent neurons affected by inflammation may be a compensatory response to decreased basal activation of AEA.

Significance of the conclusions and future directions

Anti-hyperalgesic effect of local injection of URB597 during peripheral inflammation

Local injection of URB597, a FAAH inhibitor, promoted anti-hyperalgesia after induction of persistent, cutaneous inflammation in mice through CB1 and CB2 receptors. Whereas most reports in the literature have demonstrated that inhibition of FAAH through systemic injection of a FAAH inhibitor (Kathuria et al., 2003) or gene-deletion (Cravatt et al., 2001) increases AEA in the brain, local injection of URB597 did not measurably increase AEA in the plantar paw skin in mice with peripheral inflammation.

The lack of an increase in AEA following local injection of URB597 during peripheral inflammation is most likely due, at least in part, to decreased synthesis of AEA

in the primary afferent neurons. This conclusion is supported by evidence that DRG isolated from CFA-injected mice have a decreased capacity to synthesize AEA in response to a depolarizing stimulus. However, we cannot rule out the possibility that in addition to reduced synthesis, AEA metabolism is diverted to other pathways and catabolized by COX-2 or other lipoxygenases. Evidence that COX-2 is up-regulated (Seibert et al., 1994) during peripheral inflammation and COX-2-mediated metabolism of AEA in FAAH knockout mice (Weber et al., 2004) both support the possibility that alternative metabolic pathways may contribute to the breakdown of AEA. If this is true then co-injection of URB597 and a non-specific cyclooxygenase inhibitor during inflammation may be expected to measurably increase AEA in the periphery. Another hypothesis that might explain the inability of URB597 to increase AEA may be that measurement of AEA in the paw does not provide sufficient resolution to test for an effect of URB597 on AEA level. Thus, a caveat of the present study design is that the contribution of other relevant cell sources of AEA and FAAH during peripheral inflammation, such as keratinocytes (Maccarrone et al., 2003), macrophages (Varga et al., 1998) and vascular endothelium (Deutsch et al., 2007), were not isolated and measured like the primary afferent neurons (Figure 1).

Alternatively, the anti-hyperalgesic effect of URB597 in the paw may not be a result of increased AEA at all. However, it is unlikely that URB597 is having off target effects that account for the anti-hyperalgesic effect since URB597 is selective for FAAH over other relevant targets such as cannabinoid receptors and monoacylglycerol lipase (Kathuria et al., 2003). Although off target effects cannot be conclusively excluded, the

anti-hyperalgesic effect of URB597 was mediated through a CB1- and CB2-dependent mechanism, suggesting endocannabinoid binding mediated the effect of URB597.

It is noteworthy that both CB1- and CB2-dependent receptor mechanisms contribute to the URB597-mediated reduction in mechanical hyperalgesia during persistent inflammation. These findings contrast with data generated in this strain of mice with tumor-evoked hyperalgesia, in which the effect of URB597 is only mediated by CB1 receptors (Khasabova et al., 2008). In both inflammatory and tumor-evoked pain, it is likely that CB1 receptors contribute to the anti-hyperalgesic effect through attenuation of presynaptic transmitter release and tonic modulation of the nociceptive threshold for sensory transduction. Both selective deletion of the CB1 receptor in nociceptors (Agarwal et al., 2007) or intraplantar injection of a CB1 receptor agonist (Khasabova et al., 2008) have been shown to lower the threshold for sensory transduction in naïve mice. The effect of CB1 receptors in determining the sensitivity to mechanical stimuli persist during peripheral inflammatory conditions when exaggeration of nocifensive behaviors are observed after injection of a CB1 receptor antagonist (Calignano et al., 1998) or selective deletion of CB1 receptors in nociceptors (Agarwal et al., 2007).

The data presented in this dissertation suggest that the role of CB2 receptors may be unique to inflammatory pain. This is supported by evidence that a CB2 receptor antagonist completely blocked the anti-hyperalgesic effect of URB597 in our studies. During persistent, cutaneous inflammation, immune cells expressing CB2 receptors are recruited to the region, and their activation inhibits the release of inflammatory mediators from mast cells and macrophages (Facci et al., 1995). Thus, during inflammation,

activation of CB2 receptors may decrease inflammation-evoked release of sensitizing molecules (such as nerve growth factor, prostanoids, cytokines, histamine, or adenosine triphosphate) that act on peripheral nociceptors to decrease the sensitivity of primary afferent neurons (Woolf et al., 1994; Malan et al., 2002; Malan and Porreca 2005).

Because either a CB1 or CB2 receptor antagonist completely reversed the effect of URB597, we were unable to test the relative contribution of CB1 and CB2 receptors in mediating the effect of URB597. Despite this, in tumor-evoked cancer pain in mice, our lab has observed synergy of CB1 and CB2 receptor agonists. Combined with the data in this dissertation, targeting multiple cannabinoid receptors in the periphery may enhance the therapeutic potential of cannabinoids in the treatment of pain associated with inflammation.

Changes in endocannabinoid signaling associated with peripheral inflammation

The second and third studies in this dissertation characterized regional changes in tissue levels of AEA, FAAH enzyme expression and activity, and CB1 receptors at a single time point following persistent peripheral inflammation. These studies provided insight into the neuroplasticity of endocannabinoid tone modulating the threshold for nociception during inflammation.

The initial hypothesis was that an increase in FAAH activity promoted an increase in the breakdown of AEA and subsequently resulted in mechanical hyperalgesia associated with inflammation. This hypothesis was based on the ability of local injection of URB597 to promote anti-hyperalgesia in the hind paw of CFA-injected mice.

However, data presented in this study do not support this as FAAH mRNA significantly decreased and there was a trend toward decreased FAAH enzyme activity in the hind paw of mice with peripheral inflammation. Consistent with this, FAAH activity is similarly decreased 4 h after carageenan-induced inflammation in mice (Holt et al., 2005), suggesting decreased FAAH enzyme activity in the plantar skin of the hind paw may be maintained for an extended duration during persistent inflammation. If so, this would suggest that inhibition of FAAH to minimize local degradation of AEA may be utilized to moderate mechanical hyperalgesia associated with peripheral inflammation. Although the level of AEA did not increase in the hind paw during peripheral inflammation, in light of evidence that FAAH +/- mice do not show an increase in AEA content in the brain (Cravatt et al., 2001), reduction in FAAH should not be inherently expected to increase AEA content.

To isolate the contribution of FAAH in primary afferent neurons innervating the skin during peripheral inflammation, FAAH mRNA and enzyme activity in the DRG neurons were measured. Distinct differences between mRNA and enzyme activity were characterized for FAAH in the DRGs. In this region, FAAH mRNA increased in DRGs associated with peripheral inflammation, yet there was no change in enzyme activity compared to DRGs isolated from naïve mice. Sensitivity of the assay is unlikely to be an issue because FAAH mRNA and enzyme activity were measurably increased in the DRGs in a model of cancer pain (Khasabova et al., 2008). One hypothesis is that the lack of an increase in FAAH enzyme activity was a result of increased FAAH transport to the primary afferent terminals. If this is true, then FAAH enzyme activity proximal to sciatic

nerve ligation ipsilateral to CFA injection would be expected to increase. However, FAAH enzyme activity was not different in the sciatic nerve between naïve and CFA-injected mice. It is possible that a slow axonal transport rate coupled with FAAH in motor neurons (Tsou et al., 1998b) may have compromised detection of a change in FAAH enzyme activity. Alternatively, since the endocannabinoid system also plays a neuroprotective role in ischemia, oxidative stress, excitotoxicity, neurodegeneration and trauma (reviewed by Jonsson et al., 2006; Svizenska et al., 2008; Fowler et al., 2010) inhibition of FAAH enzyme activity during ligation may be expected to maintain AEA. This would protect the nerve from damage associated with ligation and mask a difference in FAAH enzyme activity during peripheral inflammation.

In persistent peripheral inflammation the lack of an increase in FAAH enzyme activity coupled with the decrease in AEA in the DRGs suggested the decrease in AEA may result from decreased synthesis. In support of this, DRGs isolated from mice with peripheral inflammation display decreased capacity to generate AEA compared to naive conditions. Further experiments could be performed that would elucidate whether decreased synthesis of AEA occurs in the hind paw tissue at the site of inflammation.

Decreases in AEA synthesis associated with inflammation suggested the possibility that CB1 receptors may be up-regulated to counterbalance reduced stimulation. This is supported by an increase in CB1 receptor mRNA as well as increased sensitivity of CB1 receptors to a CB1 receptor agonist in DRG neurons isolated from CFA-injected mice. In other studies, CB1 receptor mRNA in primary afferent neurons peaks on day two following CFA-induced inflammation in DRG neurons, and CB1

protein expression also increased in DRGs as well as nerve fibers in skin, suggesting transport to the periphery (Amaya et al., 2006).

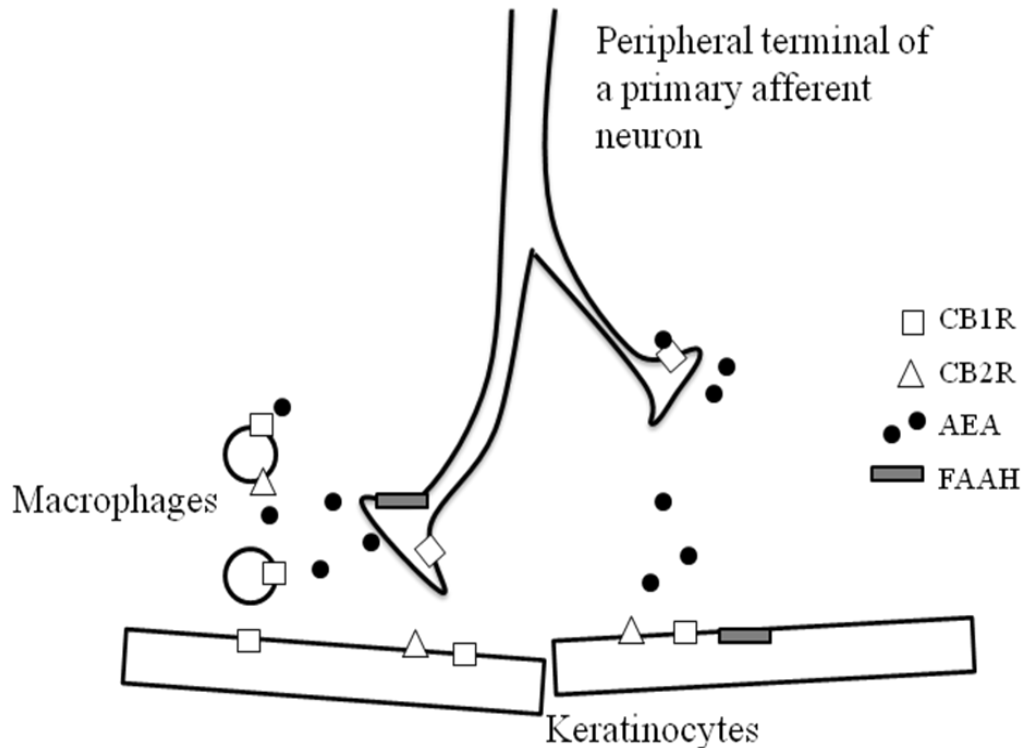
There is consistency between our *in vitro* study that demonstrated a ten fold lower concentration of a CB1 receptor agonist inhibited the increase in KCl-evoked calcium transient in large diameter neurons isolated from CFA-injected mice and other *in vivo* reports. A ten fold lower dose of a CB1 agonist attenuated thermal hyperalgesia at 2 d in CFA-injected mice compared to control mice, suggesting the increase in CB1 receptor expression mediates anti-hyperalgesia (Amaya et al., 2006). However, URB597 failed to increase the KCl-evoked intracellular free Ca^{2+} in the *in vitro* study presented in this dissertation. This was not altogether unexpected in light of evidence that AEA synthesis is reduced in the DRGs.

If the results are to be accepted as physiologically relevant, several assumptions must be made. CFA-induced inflammation is a model for peripheral injury and inflammation. A limitation in using this model is that effects observed could be specific for CFA-induced inflammation and not be representative of inflammatory pathology. Antigens present in CFA produce a cascade of immune responses that may be specific to CFA and not generalize to other forms of inflammation and injury. Thus, one assumption in using this model is that it is representative of inflammation and hyperalgesia produced by other forms of peripheral injury. Secondly, an assumption is made that activity in the sensory nerve terminals can be extrapolated from studies performed on the cell bodies of primary afferent neurons. Sensory nerve terminals are exposed to other tissue types that may differentially modulate their responses in the periphery such as keratinocytes and

immune cells. Third, studies in the periphery do not allow for sufficient resolution of the contribution of CB1 and CB2 receptors and the types of cells responsible for mediating the effect of URB597 on inflammation. In spite of these assumptions, differences in endocannabinoid signaling as well as a locally mediated anti-hyperalgesic effect of URB597, were observed during peripheral inflammation.

These studies provide an important contribution to the literature because they demonstrate that low production of AEA by DRG neurons is not sufficient to mount a URB597-mediated anti-hyperalgesic response during peripheral inflammation. The data presented here suggest that the production of AEA by other tissues in the periphery contribute to the *in vivo* effect of URB597.

Figure 1. Model of endocannabinoid signaling in the periphery.



AEA is released in an activity dependent manner from primary afferent neurons and binds to CB1 receptors on the primary afferent neurons, keratinocytes, and immune cells such as macrophages to produce inhibitory effects. In the periphery, CB1 receptors on neurons suppress neurotransmission by inhibiting calcium channels and activating potassium channels. Activation of cannabinoid receptors on immune cells inhibits the robust immune response produced. In keratinocytes, activation of cannabinoid receptors has been shown to produce antinociception by stimulating peripheral release of the endogenous opioid β -endorphin. AEA signaling is terminated through catabolism by FAAH.

References

- Abbadie C., Brown J.L., Mantyh P.W., and Basbaum A.I. (1996) Spinal cord substance P receptor immunoreactivity increases in both inflammatory and nerve injury models of persistent pain. *Neurosci.* 70, 201-209.
- Agarwal N., Pacher P., Tegeder I., Amaya F., Constantin C.E., Brenner G.J., Rubino T., Michalski C.W., Marsicano G., Monory K., Mackie K., Marian C., Batkai S., Parolaro D., Fischer M.J., Reeh P., Kunos G., Kress M., Lutz B., Woolf C.J., and Kuner R. (2007) Cannabinoids mediate analgesia largely via peripheral type I cannabinoid receptors in nociceptors. *Nat. Neurosci.* 10, 870-879.
- Ahluwalia J., Urban L., Bevan S. and Nagy I. (2003a) Anandamide regulates neuropeptide release from capsaicin-sensitive primary sensory neurons by activating both the cannabinoid 1 receptor and the vanilloid receptor 1 in vitro. *Eur. J. Neurosci.* 17, 2611-2618.
- Ahluwalia J., Yaqoob M., Urban L., Bevan S., and Nagy I. (2003b) Activation of capsaicin-sensitive primary sensory neurones induces anandamide production and release. *J. Neurochem.* 84, 585-591.
- Ahluwalia J., Urban L., Bevan S., Capongna M., and Nagy I. (2002) Cannabinoid 1 receptors are expressed by nerve growth factor- and glial cell-derived neurotrophic factor-responsive primary sensory neurones. *Neurosci.* 110, 747-753.
- Ahluwalia J., Urban L., Capongna M., Bevan S., and Nagy I. (2000) Cannabinoid 1 receptors are expressed in nociceptive primary sensory neurons. *Neurosci.* 100, 685-688.
- Amaya F., Oh-hashii K., Naruse Y., Iijima N., Ueda M., Shimosato G., Tominaga M., Tanaka Y., and Tanaka M. (2003a) Local inflammation increases vanilloid receptor I expression within distinct subgroups of DRG neurons. *Br. Res.* 963, 190-196.
- Amaya F., Shimosato G., Kawasaki Y., Hashimoto S., Tanaka Y., Ji, R. and Tanaka M. (2006) Induction of CB₁ cannabinoid receptor by inflammation in primary afferent neurons facilitates antihyperalgesic effect of peripheral CB₁ agonist. *Pain* 124, 175-183.
- Andrew D., and Greenspan J.D. (1999) Mechanical and heat sensitization of cutaneous nociceptors after peripheral inflammation in the rat. *J. Neurophysiol.* 82, 2649-2656.
- Ashton J.C., Friberg D., Darlington C.L., and Smith P.F. (2006) Expression of the cannabinoid CB₂ receptor in the rat cerebellum: an immunohistochemical study. *Neurosci. Lett.* 396, 113-116.
- Basile A.S., Hanus L., and Mendelson W.B. (1999) Characterization of the hypnotic properties of oleamide. *Neuroreport* 10, 947-951.
- Bari M., Battista N., Fezza F., Gasperi V., and Maccarrone M. (2006) New insights into endocannabinoid degradation and its therapeutic potential. *Mini-Reviews in Med. Chem.* 6, 257-268.

- Beaulieu P., Bisogno T., Punwar S., Farquhar-Smith W.P., Ambrosino G., Di Marzo V., and Rice A.S.C. (2000) Role of the endogenous cannabinoid system in the formalin test of persistent pain in the rat. *Eur. J. Pharmacol.* 396, 85-92.
- Beck P.W., Handwerker H.O., and Zimmermann M. (1974) Nervous outflow from the cat's foot during noxious radiant heat stimulation. *Brain Res.* 67, 373-386.
- Beiche F., Brune K., Geisslinger G., and Goppelt-Struebe M. (1998) Expression of cyclooxygenase isoforms in the rat spinal cord and their regulation during adjuvant-induced arthritis. *Inflamm. Res.* 47, 482-487.
- Beltramo M., Bernardini N., Bertorelli R., Campanella M., Nicolussi E., Fredduzzi S. and Reggiani A. (2006) CB₂ receptor mediated antihyperalgesia: possible direct involvement of neural mechanisms. *Eur. J. Neurosci.* 23, 1530-1538.
- Bessou P. and Perl E.R. (1969) Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. *J. Neurophysiol.* 32, 1025-1043.
- Bessou P. and Perl E.R. (1960)
- Bingham B., Jones P.G., Uveges A.J., Kotnis S., Lu P., Smith V.A., Sun S-C., Resnick, L., Chlenov, M., He Y., Strassle B.W., Cummons T.A., Piesla M.J., Harrison J. E., Whiteside G.T., and Kennedy J.D. (2007) Species-specific in vitro pharmacological effects of the cannabinoid receptor 2 (CB₂) selective ligand AM1241 and its resolved enantiomers. *Br. J. Pharmacol.* 151,1061-1070.
- Bisogno T. (2008) Endogenous cannabinoids: structure and metabolism. *J. Neuroendocrin.* 20, 1-9.
- Bisogno T., De Petrocellis L., and Di Marzo V. (2002) Fatty acid amide hydrolase, an enzyme with many bioactive substrates. Possible therapeutic implications. *Curr. Pharm. Des.* 8, 533-547.
- Bisogno T., Howell F., Williams G., Minassi A., Cascio M.G., Ligresti A., Matias I., Schiano-Moriello A., Paul P., Williams E.J., Gangadharan U., Hobbs C., Di Marzo V., and Doherty P. (2003) Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J. Cell. Bio.* 163, 463-468.
- Bisogno T., Maurelli S., Melck D., De Petrocellis L., and Di Marzo V. (1997) Biosynthesis, uptake and degradation of anandamide and paomitoylethanolamide in leukocytes. *J. Biol. Chem.* 272, 3315-3323.
- Breese N.M., George A.C., Pauers L.E., Stucky C.L. (2005) Peripheral inflammation selectively increases TRPV1 function in IB4-positive sensory neurons from adult mouse. *Pain* 115, 37-49.
- Bridges D., Rice A.S., Egertová M., Elphick M.R., Winter J., and Michael G.J. (2003) Localisation of cannabinoid receptor 1 in rat dorsal root ganglion using in situ hybridisation and immunohistochemistry. *Neurosci.* 119, 803-812.
- Buckley N.E., McCoy K.L., Mezey E., Bonner T., Zimmer A., Felder C.C., Glass M., and Zimmer A. (2000) Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB₂ receptor. *Eur. J. Pharmacol.* 396. 141-149.
- Burgess P.R., and Perl E.R. (1967) Myelinated afferent fibres responding specifically to noxious stimulation of the skin. *J. Physiol.* 190, 541-562.

- Burnstein S.H., Rossetti R.G., Yagen B., and Zurier R.B. (2000) Oxidative metabolism of anandamide. *Prostaglandins & Other Lipid Med.* 61, 29-41.
- Burnstock, G. (2000) P2X receptors in sensory neurons. *Br. J. Anaesth.* 84, 476-488.
- Cabral G.A., and Griffin-Thomas L. (2008) Cannabinoids as thereapeutic agents for ablating neuroinflammator disease. *Endocrine, Metabolic and Immune Disorders – Drug Targets.* 8, 159-172.
- Calignano A., La Rana G., Giuffrida A., and Piomelli D. (1998) Control of pain initiation by endogenous cannabinoids. *Nature* 394, 277-281.
- Carlisle S.J., Marciano-Cabral F., Staab A., Ludwick C., and Cabral G.A. (2002) Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. *Int. Immunopharmacol.* 2, 69-82.
- Carlton S.M., and Coggeshall R.E. (2001) Peripheral capsaicin receptors increase in the inflamed hindpaw: a possible mechanism for peripheral sensitisation. *Neurosci. Lett.* 310, 53–56.
- Carrier E.J., Kearn C.S., Barkmeier A.J., Breese N.M., Yang W., Nithipatikom K., Pfister S.L., Campbell W.B., and Hillard C.J. (2004) Cultured rat microglial cells synthesize the endocannabinoid 2-arachidonylglycerol, which increases proliferation via a CB2 receptor-dependend mechanism. *Mol. Pharmacol.* 65, 999-1007.
- Caterina M.J., Schumacher M.A., Tominaga M., Rosen T.A., Levine J.D., and Julius D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389, 816–824.
- Chaplan S.R., Bach F.W., Pogrel J.W., Chung J.M., Yaksh T.L. (1994) Quantitative assessment of tactile allodynia in the rat paw. *J. Neurosci. Meth.* 53, 55-63.
- Clapper J.R., Moreno-Sanz G., Russo R., Guijarro A., Vacondio F., Duranti A., Tontini A., Sanchini S., Sciolino N.R., Spradley J.M., Hohmann A.G., Calignano A., Mor M., Tarzia G., and Piomelli D. (2010) Anandamide suppresses pain initiation through a peripheral endocannabinoid mechanism. *Nature Neurosci.* 13, 1265-1270.
- Clayton N., Marshall F.H., Bountra C., and O’Shaughnessy C.T. (2002) CB1 and CB2 cannabinoid receptors are implicated in inflammatory pain. *Pain* 96, 253-260.
- Coderre T.J., and Melzack R. (1992a) The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced injury. *J. Neurosci.* 12, 3665-3670.
- Coggeshall R.E., and Carlton S.M. (1997) Receptor localization in the mammalian dorsal horn and primary afferent neurons. *Brain Res. Rev.* 24, 28-66.
- Conti S., Costa B., Colleoni M., Parolaro D., and Giagnoni G. (2002) Antiinflammatory action of endocannabinoids palmitoyl-ethanolamide and the synthetic cannabinoid nabilone in a model of acute inflammation in the rat. *Br. J. Pharmacol.* 135, 181-187.
- Cravatt B.F., Demarest K., Patricelli M.P., Bracey M.H., Giang D.K., Martin B.R., and Lichtman A.H. (2001) Supersensitivity to anandamide and enhanced endogenous

- cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc. Natl. Acad. Sci. U.S.A.* 98, 9371-9376.
- Cravatt B.F., Giang D.K., Mayfield S.P., Boger D.L., Lerner R.A., and Gilula N.B. (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 384, 83-87.
- Coutts A.A., Anavi-Goffer S., Ross R.A., MacEwan D.J., Mackie K., Pertwee R.G., and Irving A.J. (2001) Agonist-induced internalization and trafficking of cannabinoid CB1 receptors in hippocampal neurons. *J. Neurosci.* 21, 2425-2433.
- Culp W.J., Ochoa J., Cline M. and Dotson R. (1989) Heat and mechanical hyperalgesia induced by capsaicin. Cross modality threshold modulation in human C nociceptors, *Brain* 112, 1317-1331.
- De Repentigny Y., Deschenes-Furry J., Jasmin B.J., Kothary R. (2003) Impaired fast axonal transport in neurons of the sciatic nerves from dystonia musculorum mice. *J. Neurochem.* 86, 564-571.
- Derkinderen P., Ledent C., Parmentier M., and Girault J.A. (2001) Cannabinoids activate p38 mitogen-activated protein kinases through CB1 receptors in hippocampus. *J. Neurochem.* 77, 957-960.
- Deutsch D.G., and Chin S.A. (1993) Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem. Pharmacol.* 46, 791-796.
- Deutch D.G., Goligorsky M.S., Schmid P.C., Krebsbach R.J., Schmid H.H., Das S.K., Dey S.K., Arreaza G., Thorup C., Stefano G. and Moore L.C. (1997) Production and physiological actions of anandamide in the vasculature of the rat kidney. *J. Clin. Invest.* 100, 1538-1546.
- Devane W.A., Hanus L., Breuer A., Pertwee R.G., Stevenson L.A., Griffin G., Gibson D., Mandelbaum A., Etinger A., and Mechoulam R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258, 1946-1949.
- Dickenson A.H., and Sullivan A.F. (1987) Subcutaneous formalin-induced activity of dorsal horn neurons in the rat: differential response to an intrathecal opiate administered pre or post formalin. *Pain* 30, 349-360.
- Di Marzo V. (1998) 'Endocannabinoids' and other fatty acid derivatives with cannabimimetic properties: biochemistry and possible physiopathological relevance. *Biochim. Biophys. Acta* 1392, 153-175.
- Di Marzo V., Bisogno T., Sugiura T., Melck D., De Petrocellis L. (1998a) The novel endogenous cannabinoid 2-arachidonoylglycerol is inactivated by neuronal- and basophil-like cells: connections with anandamide. *Biochem J.* 331, 15-19.
- Di Marzo V., Fontana A., Cadas H., Schinelli S., Cimino G., Schwartz J.C., and Piomelli D. (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature.* 372, 686-691.
- Dinh T.P., Carpenter D., Leslie F.M., Freund T.F., Katona I., Sensi S.L., Kathuria S., and Piomelli D. (2002) Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc. Natl. Acad. Sci. USA* 99, 10819-10824.

- Dirajlal S., Pauers L.E., and Stucky C.L. (2003) Differential response properties of IB(4)-positive and -negative unmyelinated sensory neurons to protons and capsaicin. *J. Neurophysiol.* 89, 513-524.
- Djoughri L., Koutsikou S., Fang X., McMullan S., and Lawson S.N. (2006) Spontaneous pain, both neuropathic and inflammatory, is related to frequency of spontaneous firing in intact C fiber nociceptors. *J. Neurosci.* 26, 1281-1292.
- Djoughri L., and Lawson S.N. (1999) Changes in somatic action potential shape in guinea pig nociceptive primary afferent neurons during inflammation in vivo. *J. Physiol.* 520.2, 565-576.
- Dmitrieva N., and McMahon S.B. (1996) Sensitization of visceral afferents by nerve growth factor in the adult rat. *Pain* 66, 87-97.
- Dogrul A., Gul H., Akar A., Yildiz O., Bilgin F., and Guzeldemir E. (2003) Topical cannabinoid antinociception: synergy with spinal sites. *Pain* 105, 11-16.
- Dolan S., Kelly J.G., Huan M., and Nolan A.M. (2003) Transient up-regulation of spinal cyclooxygenase-2 and neuronal nitric oxide synthase following surgical inflammation. *Anesthesiology* 98, 170-180.
- Donaldson L.F., Harmar A.J., McQueen D.S. and Seckl J.R. (1992) Increased expression of preprotachykinin, calcitonin gene-related peptide, but not vasoactive intestinal peptide messenger RNA in dorsal root ganglia during the development of adjuvant monoarthritis in the rat. *Mol. Brain Res.* 16, 143-149.
- Donnerer J., Schuligoi R., and Stein C. (1992) Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: Evidence for a regulatory function of nerve growth factor in vivo. *Neurosci.* 49, 693-698.
- Dougherty P.M., Palecek J., Paleckova V., Sorkin L.S., and Willis W.D. (1992a) The role of NMDA and non-NMDA excitatory amino acid receptors in the excitation of primate spinothalamic tract neurons by mechanical, chemical, thermal and electrical stimuli. *J. Neurosci.* 12, 3025-3041.
- Dougherty P.M., Palecek J., Paleckova V., and Willis W.D. (1994) Neurokinin 1 and 2 antagonists attenuate the responses and NK1 antagonists prevent the sensitization of primate spinothalamic tract neurons after intradermal capsaicin. *J. Neurophysiol.* 72, 1464-1475.
- Dougherty P.M., Palecek J., Paleckova V., and Willis W.D. (1995) Infusion of substance P or neurokinin A by microdialysis alters responses of primate spinothalamic tract neurons to cutaneous stimuli and to iontophoretically released amino acids. *Pain* 61- 411-425.
- Dougherty P.M., Palecek J., Zorn S., and Willis W.D. (1993) Combined application of excitatory amino acids and substance P produces long lasting changes in responses of primate spinothalamic tract neurons. *Brain Res. Rev.* 18, 227-246.
- Dougherty P.M., Sluka K.A., Sorkin L.S., Westlund K.N., and Willis W.D. (1992b) Neural changes in acute arthritis in monkeys. I. Parallel enhancement of responses of spinothalamic tract neurons to mechanical stimulation and excitatory amino acids. *Brain Res. Rev.* 17, 1-13.

- Dougherty P.M., Schwartz A., and Lenz F.A. (1999) Responses of primate spinomesencephalic tract cells to intradermal capsaicin. *Neurosci.* 90, 1377-1392.
- Dougherty P.M and Willis W.D. (1991) Enhancement of spinothalamic neuron responses to chemical and mechanical stimuli following combined micro-iontophoretic application of *N*-methyl-D-aspartic acid and substance P. *Pain* 47, 85-93.
- Dougherty P.M. and Willis W.D. (1992) Enhanced responses of spinothalamic tract neurons to excitatory amino acids accompany capsaicin-induced sensitization in the monkey. *J. Neurosci.* 12, 883-894.
- Drew L.J., Harris J., Millns P.J., Kendall D.A., and Chapman V. (2000) Activation of spinal CB1 receptors inhibits C-fibre driven hyperexcitable neuronal responses and increases [³⁵S]GTPγS binding in the dorsal horn of the spinal cord of noninflamed and inflamed rats. *Eur. J. Neurosci.* 12, 2079-2086.
- Dubner R., (1986) Neuronal plasticity and pain following peripheral tissue inflammation or nerve injury. In: Proceedings of The World Congress on Pain, Pain Research and Clinical Management 5, edited by Bond M., Charlton E., and Woolf C.J. Amsterdam: Elsevier, p/ 263-276.
- Duboisson D. and Dennis S.G. (1977) The formalin test: a quantitative study of the anesthetic effects of morphine, meperidine and brain stem stimulation in rats and cats. *Pain* 4, 161-174.
- Elmes S.J., Jhaveri M.D., Smart D., Kendall D.A., and Chapman V. (2004) Cannabinoid CB2 receptor activation inhibits mechanically evoked responses of wide dynamic range dorsal horn neurons in naïve rats and in rat models of inflammatory and neuropathic pain. *Eur. J. Neurosci.* 20, 2311-2320.
- Elmes S.J., Winyard L.A., Medhurst S.J., Clayton N.M., Wilson A.W., Kendall D.A., and Chapman V. (2005) Activation of CB1 and CB2 receptors attenuates the induction and maintenance of inflammatory pain in the rat. *Pain* 118, 327-335.
- England S., Bevan S., and Docherty R.J. (1996) PBE2 modulates the tetrodotoxin-resistant sodium current in neonatal rat dorsal root ganglion neurons via the cyclic AMP-protein kinase A cascade. *J. Physiol.* 495, 429-440.
- Facci L., Dal Toso R., Romanello S., Buriani A., Skaper S.D., and Leon A. (1995) Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. *Proc. Natl. Acad. Sci. U.S.A.* 92, 3376-3380.
- Fegley D., Gaetani S., Duranti A., Tontini A., Mor M., Tarzia G., and Piomelli D. (2005) Characterization of the fatty acid amide hydrolase inhibitor URB597: effects on anandamide and oleoylethanolamide deactivation. *J. Pharmacol. Exp. Ther.* 313, 352-358.
- Felder C.C., Joyce K.E., Briley E.M., Mansouri J., Mackie K., Blond O., Lai Y., Ma A.L., Mitchell R.L. (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors *Mol. Pharmacol.* 48, 443-450.
- Fitzgerald E.M., Okuse, K., Wood J.N., Dolphin A.C., and Moss S.J. (1999) cAMP-dependent phosphorylation of the tetrodotoxin-resistant voltage-dependent sodium channel SNS. *J. Physiol.* 516, 433-446.

- Fletcher D., Kayser V., and Bilbaud G. (1996) Influence of timing on the analgesic effect of bupivacaine and epinephrine infiltration in carrageenan injected rats. *Anaesthesiology* 84, 1020-1026.
- Fox A., Kesingland A., Gentry C., McNair K., Patel S., Urban L., and James I. (2001) The role of central and peripheral cannabinoid (1) receptors in the antihyperalgesic activity of cannabinoids in a model of neuropathic pain. *Pain* 92, 91-100.
- Fowler C.J., Holt S., Nilsson O., Jonsson K.O., Tiger G., Jacobsson S.O. (2005) The endocannabinoid signaling system: pharmacological and therapeutic aspects. *Pharmacol. Biochem. Behav.* 81:248-262.
- Fowler C. J., Rojo M. L., and Rodriguez-Gaztelumendi A. (2010) Modulation of the endocannabinoid system: neuroprotection or neurotoxicity? *Exp. Neurology* 224, 37-47.
- Galeazza M.T., Garry M.G., Yost H.J, Strait K.A., Hargreaves K.M., and Seybold V.S. (1995) Plasticity in the synthesis and storage of substance P and calcitonin gene-related peptide in primary afferent neurons during peripheral inflammation. *Neurosci.* 66, 443-458.
- Galiegue S., Mary S., Marchand J., Dussosoy D., Carriere D., Carayon P., Bouaboula M., Shire D., LeFur G., and Casellas P. (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* 232, 54-61.
- Gardner D.L. (1960) Production of arthritis in the rabbit by local injection of the mucopolysaccharide carageenin. *Ann. Rheum. Dis.* 19, 369-376.
- Ghafouri N., Tiger G., Razdan R.K., Mahadevan A., Pertwee R.G., Martin B.R., and Fowler C.J. (2004) Inhibition of monoacylglycerol lipase and fatty acid amide hydrolase by analogues of 2-arachidonoylglycerol. *Br. J. Pharmacol.* 143, 774-784.
- Giang D.K., and Cravatt B.F. (1997) Molecular characterization of human and mouse fatty acid amide hydrolases. *Proc. Natl. Acad. Sci. USA* 94, 2238-2242.
- Giuffrida A., Parsons L.H., Kerr T.M., de Fonseca R., Navarro M., and Piomelli D. (1999) Dopamine activation of endogenous cannabinoid signaling in dorsal striatum. *Nature Neurosci.* 2, 358-363.
- Glass M., Dragunow M., and Faull R.L.M. (1997) Cannabinoid receptors in the human brain: a detailed anatomical and quantitative autoradiographic study in the fetal, neonatal and adult human brain. *Neurosci.* 77, 299-318.
- Gold M.S., Levine J.D., and Correa A.M. (1998) Modulation of TTX-R I_{Na} by PKC and PKA and their role in PGE₂-induced sensitization of rat sensory neurons *in vitro*. *J. Neurosci.* 18, 10345-10355.
- Gold M.S., Shuster M.J., and Levine J.D. (1996) Characterization of six voltage-gated K⁺ currents in adult rat sensory neurons. *J. Neurophysiol.* 75, 2629-2646.
- Gong J-P., Onaivi E.S., Ishiguro H., Liu Q-R., Tagliaferro P.A., Brusco A., and Uhl G.R. (2006) Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Res.* 1071, 10-23.

- Goparaju S.K., Kurahashi Y., Suzuki H., Ueda N., and Yamamoto S. (1999) Anandamide amidohydrolase of porcine brain: cDNA cloning, functional expression and site-directed mutagenesis(1). *Biochim. Biophys. Acta.* 1441, 77-84.
- Goparaju S.K., Ueda N., Taniguchi K., and Yamamoto S. (1999) Enzymes of porcine brain hydrolyzing 2-arachidonoylglycerol, an endogenous ligand of cannabinoid receptors. *Biochem. Pharmacol.* 57, 417-423.
- Grynkiewicz G., Poenie M., Tsien R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440-3450.
- Guenther S., Reeh P.W., and Kress M. (1999) Rises in $[Ca^{2+}]_i$ mediate capsaicin- and proton-induced heat sensitization of rat primary nociceptor neurons. *Eur. J. Neurosci.* 11, 3143-3150.
- Guindon J., and Beaulieu P. (2009) The role of the endogenous cannabinoid system in peripheral analgesia. *Curr. Mol. Pharmacol.* 2, 134-139.
- Guindon J., De Lean A., and Beaulieu P. (2006) Local interactions between anandamide, an endocannabinoids, and ibuprofen, a nonsteroidal anti-inflammatory drug, in acute and inflammatory pain. *Pain* 121, 85-93.
- Guindon J., Desroches J., and Beaulieu P. (2007) The antinociceptive effects of intraplantar injections of 2-arachidonoyl glycerol are mediated by cannabinoid CB2 receptors. *Br. J. Pharmacol.* 150, 693-701.
- Guindon J., and Hohmann A.G. (2008) A physiological role for endocannabinoid-derived products of cyclooxygenase-2-mediated oxidative metabolism. *Br. J. Pharmacol.* 153, 1341-1343.
- Guerrero A.V., Quang P., Dekker N., Jordan R.C., and Schmidt B.L. (2008) Peripheral cannabinoids attenuate carcinoma-induced nociception in mice. *Neurosci. Lett.* 433, 77-81.
- Guilbaud G., Kayser V., Benoist J.M., and Gautron M. (1986) Modifications in the responsiveness of rat ventrobasal thalamic neurons at different stages of carrageenin-produced inflammation. *Brain Res.* 385, 86-98.
- Gutierrez T., Farthing J.N., Zvonok A.M., Makriyannis A., and Hohmann A.G. (2007) Activation of peripheral cannabinoid CB1 and CB2 receptors suppresses the maintenance of inflammatory nociception: a comparative analysis. *Br. J. Pharmacol.* 150, 153-163.
- Hamamoto D.T., Giridharagopalan S. and Simone D.A. (2007) Acute and chronic administration of the cannabinoid receptor agonist CP 55,940 attenuates tumor-evoked hyperalgesia. *Eur. J. Pharmacol.* 558, 73-87.
- Handwerker H.O., Anton F., and Reeh P.W. (1987) Discharge pattern of afferent cutaneous nerve fibers from the rat's tail during prolonged noxious mechanical stimulation. *Exp. Brain Res.* 65, 493-504.
- Hanus L., Breuer A., Tchilibon S., Shiloah S., Goldenberg D., Horowitz M., Pertwee R.G., Ross R.A., Mechoulam R., and Fride E. (1999) HU-308: a specific agonist for CB(2), a peripheral cannabinoid receptor. *Proc. Natl. Acad. Sci. U.S.A.* 96, 14228-14233.

- Hargreaves K., Dubner R., Brown F., Flores C., and Joris J. (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 32, 77-88.
- Heapy C.G., Jamieson A., and Russell N.J.W. (1987) Afferent C-fibre and A-delta activity in models of inflammation. *Br. J. Pharmacol.* 90, 164-170.
- Hedo G., Laird J.M.A., and Lopez-Garcia J.A. (1998) Time-course of spinal sensitization following carrageenan-induced inflammation in the young rat: a comparative electrophysiological and behavioural study in vitro and in vivo. *Neurosci.* 92, 309-318.
- Herkenham M., Lynn A.B., Johnson M.R., Melvin L.S., De Costa B.R., and Rice K.C. (1991) Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J. Neurosci.* 11, 563-583.
- Herzberg U., Eliav E., Bennett G.J., and Kopin I.J. (1997) The analgesic effects of R(+)-WIN 55,212-2 mesylate, a high affinity cannabinoid agonist, in a rat model of neuropathic pain. *Neurosci. Lett.* 221, 157-160.
- Hillard C.J. (2000) Biochemistry and pharmacology of the endocannabinoids arachidonylethanolamide and 2-arachidonylglycerol. *Prostaglandins Other Lipid Mediat.* 61, 3-18.
- Hillard C.J., and Campbell W.B. (1997) Biochemistry and pharmacology of arachidonylethanolamide, a putative endogenous cannabinoid, *J. Lipid Res.* 38, 2383-2398.
- Hiura A., Sakamoto Y. (1987) Quantitative estimation of the effects of capsaicin on the mouse primary sensory neurons. *Neurosci. Lett.* 76, 101-106.
- Hohmann A.G. (2002) Spinal and peripheral mechanisms of cannabinoid antinociception: behavioral, neurophysiological and neuroanatomical perspectives. *Chemistry and Physics of Lipids* 121, 173-190.
- Hohmann A.G., and Herkenham, M. (1998) Regulation of cannabinoid and mu opioid receptors in rat lumbar spinal cord following neonatal capsaicin treatment. *Neurosci. Lett.* 252, 13-16.
- Hohmann A.G., and Herkenham M. (1999a) Cannabinoid receptors undergo axonal flow in sensory nerves. *Neurosci.* 92, 1171-1175.
- Hohmann A.G., and Herkenham M. (1999b) Localization of central cannabinoid CB1 receptor messenger RNA in neuronal subpopulations of rat dorsal root ganglia: a double-label in situ hybridization study. *Neurosci.* 90, 923-931.
- Hohmann A.G., and Herkenham M. (2000) Localization of cannabinoid CB(1) receptor mRNA in neuronal subpopulations of rat striatum: a double-label in situ hybridization study. *Synapse* 37, 71-80.
- Hohmann A.G., Martin W.J., Tsou K., and Walker J.M. (1995) Inhibition of noxious stimulus-evoked activity of spinal cord dorsal horn neurons by the cannabinoid WIN 55,212-2. *Life Sci.* 56, 2111-2118.
- Hohmann A.G. and Suplita R.L. 2nd (2006) Endocannabinoid mechanisms of pain modulation. *AAPS J.* 8: E693-E708.

- Hohmann A.G., Tsou K., and Walker J.M. (1998) Cannabinoid modulation of wide dynamic range neurons in the lumbar dorsal horn of the rat by spinally administered WIN 55,212-2. *Neurosci. Lett.* 257, 119-122.
- Hohmann A.G., Tsou K., and Walker J.M. (1999) Intrathecal cannabinoid administration suppresses noxious-stimulus evoked Fos protein-like immunoreactivity in rat spinal cord: comparison with morphine. *Acta. Pharmacol. Sinica.* 20, 1132-1136.
- Holdcroft A., Smith M., Jacklin A., Hodgson H., Smith B., Newton M., and Evans F. (1997) Pain relief with oral cannabinoids in familial Mediterranean fever. *Anaesthesia* 52, 483-486.
- Holt S., Comelli F., Costa B., and Fowler C.J. (2005) Inhibitors of fatty acid amide hydrolase reduce carrageenan-induced hind paw inflammation in pentobarbital-treated mice: comparison with indomethacin and possible involvement of cannabinoid receptors. *Br. J. Pharmacol.* 146, 465-476.
- Honore P., Menning P.M., Rogers S.D., Nichols M.L., Basbaum A.I., Besson J.M., and Mantyh P.W. (1999) Spinal substance P receptor expression and internalization in acute, short-term, and long-term inflammatory pain states. *J. Neurosci.* 19, 7670-7678.
- Howlett A.C., Qualy J.M., and Khachatrian L.L. (1986) Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. *Mol. Pharmacol.* 29, 307-313.
- Hsieh C., Brown S., Derleth C., and Mackie K. (1999) Internalization and recycling of the CB1 cannabinoid receptor. *J. Neurochem.* 73, 493-501.
- Hunt C.C., and McIntyre A.K. (1960) An analysis of fibre diameter and receptor characteristics of myelinated cutaneous afferent fibers in cat. *J. Physiol.* 153, 99-112.
- Hylden J.L.K., Nahin R.L., Traub R.J., and Dubner R. (1989) Expansion of receptive fields of spinal lamina I projection neurons in rats with unilateral adjuvant-induced inflammation: the contribution of dorsal horn mechanisms. *Pain* 37, 229-243.
- Iadarola M.J., Brady L.S., Draisci G., and Dubner R. (1988) Enhancement of dynorphin gene expression in spinal cord following experimental inflammation: stimulus specificity, behavioral parameters and opioid receptor binding. *Pain* 35, 313-326.
- Ibrahim M.M., Porreca F., Lai J., Albrecht P.J., Rice F.L., Khodorova A., Davar G., Makriyannis A., Vanderah T.W., Mata H.P., and Malan T.P. (2005) CB₂ cannabinoid receptor activation produces antinociception by stimulating peripheral release of endogenous opioids. *Proc. Natl. Acad. Sci. USA* 102, 3093-3098.
- Iggo A., and Ogawa H. (1971) Primary cutaneous thermal nociceptors. *J. Physiol.* 216, 77-78P.
- Jansen E.M., Haycock D.A., Ward S.J., and Seybold V.S. (1992) Distribution of cannabinoid receptors in rat brain determined with aminoalkylindoles. *Brain Res.* 575, 93-102.

- Jayamanne A., Greenwood R., Mitchell V.A., Aslan S., Piomelli D., and Vaughan C.W. (2006) Actions of the FAAH inhibitor URB597 in neuropathic and inflammatory chronic pain models. *Br. J. Pharmacol.* 147, 281-288.
- Jhaveri M.D., Richardson D., Robinson I., Garle M.J., Patel A., Sun Y., Sagar D.R., Bennett A.J., Alexander S.P.H., Kendall D.A., Barrett D.A., and Chapman V. (2008) Inhibition of fatty acid amide hydrolase and cyclooxygenase-2 increases levels of endocannabinoids related molecules and produces analgesia via peroxisome proliferator-activated receptor-alpha in a model of inflammatory pain. *Neuropharm.* 55, 85-93.
- Jerman J.C., Gray J., Brough S.J., Ooi L., Owen D., Davis J.B., and Smart D. (2002) Comparison of the effects of anandamide at recombinant and endogenous rat vanilloid receptors. *Br. J. Anaesthesia.* 89, 882-887.
- Jonsson, K.O., Holt S., and Fowler, C.J. (2006) The endocannabinoid system: Current pharmacology research and therapeutic possibilities. *Basic & Clinical Pharmacol. and Toxicol.* 98, 124-134.
- Jung J., Hwany S.W., Kwak J., Lee S., Kang C., Kim W.B., Kim D., and Oh U. (1999) Capsaicin binds to the intracellular domain of the capsaicin-activated ion channel. *J. Neurosci.* 19, 529-538.
- Karlsson M., Reue K., Xia Y.R., Lusis A.J., Langin D., Tornquist H., and Holm C. (2001). Exon-intron organization and chromosomal localization of the mouse monoglyceride lipase gene. *Gene* 272, 11-18.
- Kathuria S., Gaetani S., Fegley D., Valino F., Duranti A., Tontini A., Mor M., Tarzia G., La Rana G., Calignano A., Giustino A., Tattoli M., Palmery M., Cuomo V., and Piomelli D. (2003) Modulation of anxiety through blockade of anandamide hydrolysis. *Nat. Med.* 9, 76-81.
- Kehl L.J., Hamamoto D.T., Wacnik P.W., Croft D.L., Norsted B.D., Wilcox G.L. and Simone D.A. (2003) A cannabinoid agonist differentially attenuates deep tissue hyperalgesia in animal models of cancer and inflammatory muscle pain. *Pain* 103, 175-186.
- Kelly S., and Chapman V. (2001) Selective cannabinoid CB1 receptor activation inhibits spinal nociceptive transmission in vivo. *J. Neurophysiol.* 86, 3061-3064.
- Kelly S. and Donaldson L.F. (2008) Peripheral cannabinoid CB₁ receptors inhibit evoked responses of nociceptive neurons in vivo. *Eur. J. Pharmacol.* 586, 160-163.
- Kelly S., Jhaveri M.D., Sagar D.R., Kendall D.A., and Chapman V. (2003) Activation of peripheral CB₁ receptors inhibits mechanically evoked responses of spinal neurons in noninflamed rats and rats with hindpaw inflammation. *Eur. J. Neurosci.* 18, 2239-2243.
- Kerr B.J., Bradbury E.J., Bennett D.H.L., Trivedi P.M., Dassan P., French J., Shelton D.B., McMahon S.B., and Thompson S.W.N. (1999) Brain-derived neurotrophic factor modulates nociceptive sensory inputs and NMDA-evoked responses in the rat spinal cord. *J. Neurosci.* 19, 5138-5148.
- Khasabova I.A., Khasabov S.G., Harding-Rose C., Coicou L.G., Seybold B.A., Lindberg A.E., Steevens C.D., Simone D.A., and Seybold V.S. (2008) A decrease in

- anandamide signaling contributes to the maintenance of cutaneous mechanical hyperalgesia in a model of bone cancer pain. *J. Neurosci.* 28, 11141-11152.
- Khasabova I.A., Gielissen J., Chandiramani A., Harding-Rose C., Abu Odeh D., Simone D.A., and Seybold V.S. CB1 and CB2 receptor agonists promote analgesia through synergy in a murine model of tumor pain. Under review.
- Khasabova, I.A., Harding-Rose, C., Simone D.A., and Seybold V.S. (2004) Differential effects of CB1 and opioid agonists on two populations of adult rat dorsal root ganglion neurons. *J. Neurosci.* 24, 1744-1753.
- Khasabova I.A., Simone D.A., and Seybold, V.S. (2002) Cannabinoids attenuate depolarization-dependent Ca^{2+} influx in the intermediate-size primary afferent neurons of adult rats. *Neurosci.* 115, 613-625.
- Khasabova I.A., Stucky C.L., Harding-Rose C., Eikmeier L., Beitz A.J., Coicou L.G., Hanson A.E., Simone D.A., and Seybold V.S. (2007) Chemical interactions between fibrosarcoma cancer cells and sensory neurons contribute to cancer pain. *J. Neurosci.* 27, 10289-10298.
- Khasar S.G., Gold M.S. and Levine J.D. (1998) A tetrodotoxin-resistant sodium current mediates inflammatory pain in the rat. *Neurosci. Lett.* 256, 17-20.
- Kidd B.L., and Urban L.A. (2001) Mechanisms of inflammatory pain. *Br. J. Anesth.* 87, 3-11.
- Koltzenburg M., Bennett, D.L.H., Stelton D.L., and McMahon S.B. (1999) Neutralization of endogenous NGF prevents the sensitization of nociceptors supplying inflamed skin. *Eur. J. Neurosci.* 11, 1698-1704.
- Koltzenburg M. (2000) Neuronal mechanisms of cutaneous nociceptive pain. *Clin. J. Pain* 16, S131-138.
- Kozak K.R., Prusakiewicz J.J., and Marnett L.J. (2004) Oxidative metabolism of endocannabinoids by COX-2. *Curr. Pharm. Des.* 10, 659-667.
- Kress M., and Guenther S. (1999) Role of $[Ca^{2+}]_i$ in the TP-induced heat sensitization process of rat nociceptive neurons. *J. Neurophysiol.* 81, 2612-2619.
- Lam H.H., Hanley D.F., Trapp B.D., Saito S., Raja S., Dawson T.M., and Yamaguchi H. (1996) Induction of spinal cord neuronal nitric oxide synthase (NOS) after formalin injection in rat hind paw. *Neurosci. Lett.* 210, 201-204.
- Lambert D.M., and Di Marzo V. (1999) The palmitoylethanolamide and oleamide enigmas: are these two fatty acid amides cannabimimetic. *Curr. Med. Chem.* 6, 757-773.
- Lambert D.M., and Fowler C.J. (2005) The endocannabinoids system: drug targets, lead compounds, and potential therapeutic applications. *J. Med. Chem.* 48, 5059-5087.
- LaMotte R.H., Thalhammer J.G., Torebjörk H.E., and Robinson C.J. (1982) Peripheral neuroal mechanisms of cutaneous hyperalgesia following mild injury by heat. *J. Neurosci.* 2, 765-781.
- Lang W., Qin C., Lin S., Khanolkar A.D., Goutopoulos A. Fan P., Abouzid K., Meng Z., Biegel D., and Makriyannis A. (1999) Substrate specificity and stereoselectivity of rat brain microsomal anandamide amidohydrolase. *J. Med. Chem.* 42, 896-902.
- Ledent C., Valverde O., Cossu G., Petitot F., Aubert J.F., Beslot F., Bohme G.A., Imperato A., Pedrazzini T., Roques B.P., Vassart G., Fratta W., and Parmentier

- M. (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science* 283, 401-404.
- Leterrier C., Laine J., Darmon M., Boudin H., Rossier J., and Lenkei Z. (2006) Constitutive activation drives compartment-selective endocytosis and axonal targeting of type 1 cannabinoid receptors. *J. Neurosci.* 26, 3141-3153.
- Leung D., Saghatelian A., Simon G.M., and Cravatt B.F. (2006) Inactivation of *N*-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. *Biochemistry* 45, 4720-4725.
- Lever I.J., Robinson M., Cibelli M., Paule C., Santha P., Yee L., Hunt S.P., Cravatt B.F., Elphick M.R., Nagy I., and Rice A.S.C. (2009) Localization of the endocannabinoid-degrading enzyme fatty acid amide hydrolase in rat dorsal root ganglion cells and its regulation after peripheral nerve injury. *J. Neurosci.* 29, 3766-3780.
- Lichtman A.H., Hawkins E.G., Griffin G., and Cravatt B.F. (2002) Pharmacological activity of fatty acid amides is regulated, but not mediated, by fatty acid amide hydrolase in vivo. *J. Pharmacol. Exp. Ther.* 302, 73-79.
- Lichtman A.H., Shelton C.C., Advani T., and Cravatt B.F. (2004) Mice lacking fatty acid amide hydrolase exhibit a cannabinoid receptor-mediated phenotypic hypoalgesia. *Pain* 109, 319-327.
- Little P.J., Compton D.R., Johnson M.R., Melvin L.S., and Martin B.R. (1988) Pharmacology and stereoselectivity of structurally novel cannabinoids in mice. *J. Pharm. and Exp. Ther.* 247, 1046-1051.
- Liu J., Wang L., Harey-White J., Osei-Hyiaman D., Razdan R.K., Zhou Z., Chen A., Huang B., Kim H.Y., and Kunos G. (2006) Novel biosynthetic pathway for anandamide. *Proc. Natl. Acad. Sci. USA* 103, 13345-13350.
- Loeser J.D., and Treede R.D. (2008) The Kyoto protocol of IASP basic pain terminology. *Pain* 137, 473-474.
- Lopshire J.C. and Nicol G.D. (1998) The cAMP transduction cascade mediates the prostaglandin E2 enhancement of the capsaicin-elicited current in rat sensory neurons: Whole-cell and single-channel studies. *J. Neurosci.* 18, 6081-6092.
- Lo Verme J., Fu J., Astarita G., La Rana G., Russo R., Calignano A., and Piomelli D. (2005a) The nuclear receptor peroxisome proliferators-activated receptor- α mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol. Pharmacol.* 67, 15-19.
- Lo Verme J., Gaetani S., Fu J., Oveisi F., Burton K., and Piomelli D., (2005b) Regulation of food intake by oleoylethanolamide. *Cell Mol. Life Sci.* 62, 708-716.
- Lo Verme J., Russo R., La Rana G., Fu J., Farthing J., Mattace-Raso G. Meli R., Hohmann A., Calignano A., and Piomelli D. (2006) Rapid broad spectrum analgesia through activation of peroxisome proliferator-activated receptor- α . *J. Pharmacol. Exp. Ther.* 319, 1051-1061.
- Lutz B. (2002) Molecular biology of cannabinoid receptors. *Prostaglandins Leukotrienes Essent. Fatty Acids* 66, 123-142.
- Maccarrone M., Piccirilli S., Battista N., Del Duca C., Nappi G., Corasaniti M.T., Finazzi-Agrò A., and Bagetta G. (2004). Enhanced anandamide degradation is

- associated with neuronal apoptosis induced by the HIV-1 coat glycoprotein gp120 in the rat neocortex. *J. Neurochem.* 89, 1293-1300.
- Maccarrone M., van der Stelt M., Rossi A., Veldink G.A., Vliegthart J.F.G., and Finazzi-Agrò A. (1998) Anandamide hydrolysis by human cells in culture and brain. *J. Bio. Chem.* 273, 32332-32339.
- Mackie K., Devane W.A., and Hille B. (1993) Anandamide an endogenous cannabinoid inhibits calcium currents as a partial agonist in N18 neuroblastoma cells. *Mol Pharmacol.* 44, 498-503.
- Mackie K., Lai Y., Westenbroek R., and Mitchell R. (1995) Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *J. Neurosci.* 15, 6552-6561.
- Machelska H., and Stein C. (2002) Immune mechanisms in pain control. *Anesthes. and Analg.* 95, 1002-1008.
- Maida V., Ennis M., Irani S., Corbo M., and Dolzhkov M. (2008) Adjunctive nabilone in cancer pain and symptom management: a prospective observational study using propensity scoring. *J. Support Oncol.* 6, 119-124.
- Maione S., De Petrocellis L., de Novellis V., Moriello A.S., Petrosino S., Palazzo E., Rossi F.S., Woodward D.F., and Di Marzo V. (2007) Analgesic actions of N-arachidonoyl-serotonin, a fatty acid amide hydrolase inhibitor with antagonistic activity at vanilloid TRPV1 receptors. *Br. J. Pharmacol.* 150, 766-781.
- Malan T.P., Jr., Ibrahim M.M., Deng H., Liu Q., Mata H.P., Vanderah T., Porreca F., and Makriyannis A. (2001) CB2 cannabinoid receptor-mediated peripheral antinociception. *Pain* 93, 239-245.
- Malan T.P., Jr., Ibrahim M.M., Vanderah T.W., Makriyannis A., Porreca F. (2002) Inhibition of pain responses by activation of CB2 cannabinoid receptors. *Chem. Phys. Lipids.* 121, 191-200.
- Malan T.P., Jr., and Porreca F. (2005) Lipid mediators regulating pain sensitivity. *Prostaglandins & Other Lipid Med.* 77, 123-130.
- Mannion R.J., Costigan M., Decosterd I., Amaya F., Ma, O.P., Holstege J.C., Ji R.R., Acheson A., Lindsay R.M., Wilkinson G.A., and Woolf C.J. (1999) Neurotrophins: Peripheral and centrally acting modulators of tactile stimulus-induced inflammatory pain hypersensitivity. *Proc. Natl. Acad. Sci. USA* 96, 9385-9390.
- Martin W.J., Hohmann A.G., and Walker J.M. (1996) Suppression of noxious stimulus-evoked activity in the ventral posterolateral nucleus of the thalamus by a cannabinoid agonist: correlation between electrophysiological and antinociceptive effects. *J. Neurosci.* 16, 6601-6611.
- Martin W.J., Loo C.M., and Basbaum A.I. (1999) Spinal cannabinoids are anti-allodynic in rats with persistent inflammation. *Pain* 82, 199-205.
- Martin W.J., Patrick S.L., Coffin P.O., Tsou K., and Walker J.M. (1995) An examination of the central sites of action of cannabinoid-induced antinociception in the rat. *Life Sci.* 56, 2103-2110.

- Matsuda L.A., Bonner T.I., and Lolait S.J. (1993) Localization of cannabinoid receptor mRNA in rat brain. *J. Comp. Neurol.* 327, 535-550.
- Matsuda L.A., Lolait S.J., Brownstein M.J., Young A.C., and Bonner T.I. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346, 561-564.
- Maurelli S., Bisogno T., De Petrocellis L., De Luccia A., Marino G., and Di Marzo V. (1995) Two novel classes of neuroactive fatty acid amides are substrates for mouse neuroblastoma 'anandamide amidohydrolase'. *FEBS Lett.* 377, 82-86.
- McGlone F., and Reilly D. (2010) The cutaneous sensory system. *Neurosci. And Biobehavioral Rev.* 34, 148-159.
- Mechoulam R. *The pharmacohistory of Cannabis sativa*. Boca Raton, Florida: CRS Press; 1986.
- Mechoulam R., Ben-Shabat S., Hanus L., Ligumsky M., Kaminski N.E., Schatz A.R., Gopher A., Almog S., Martin B.R., Compton D.R., Pertwee R.G., Griffin G., Bayewitch M., Barg J., and Vogel Z. (1995) Identificaiton of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* 50, 83-90.
- Meller S.T., Cummings C.P., Traub R.J., and Gebhart G.F. (1994) The role of nitric oxide in the development and maintenance of the hyperalgesia produced by intraplantar injection of carrageenan in the rat. *Neurosci.* 60, 367-374.
- Mendell L.M. (1966) Physiological properties of unmyelinated fibers projecting to the spinal cord. *Exp. Neurol.* 16, 316-332.
- Meng I.D., Manning B.H., Martin W.J., and Fields H.L. (1996) An analgesia circuit activated by cannabinoids *Nature* 395, 381-383.
- Millan M.J. (1999) The induction of pain, an integrative review. *Prog in Neurobio.* 57, 1-164.
- Millns P.J., Chapman V. and Kendall D.A. (2001) Cannabinoid inhibition of the capsaicin induced calcium response in rat dorsal root ganglion neurons. *Br. J. Pharmacol.* 132,969-971.
- Mogil J.S., Wilson S.G., Bon K., Lee S.E., Chung K., Raber P., Pieper J.O., Hain H.S., Belknap J.K., Hubert L., Elmer G.I., Chung J.M., Devor M. (1999) Heritability of nociception I: responses of 11 inbred mouse strains on 12 measures of nociception. *Pain* 80: 67-82.
- Morris C.J. (2003) Carrageenan-induced paw edema in the rat and mouse. *Methods Mol. Biol.* 225, 115–121.
- Munro S., Thomas K. L., and Abu-Shaar M. (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365, 61-65.
- Nackley A.G., Makriyannis A., and Hohmann A.G. (2003a) Selective activation of cannabinoid CB(2) receptors suppresses spinal fos protein expression and pain behavior in a rat model of inflammation. *Neurosci.* 119, 747-757.
- Nackley A.G., Suplita II R.L., and Hohmann A.G. (2003b) A peripheral cannabinoid mechanism suppresses spinal fos protein expression and pain behavior in a rat model of inflammation. *Neurosci.* 117, 659-670.

- Nackley A.G., Zvonok A.M., Makriyannis A., and Hohmann A.G. (2004) Activation of cannabinoid CB₂ receptors suppresses C-fiber responses and windup in spinal wide dynamic range neurons in the absence and presence of inflammation. *J. Neurophysiol.* 92, 3562-3574.
- Nagakura Y., Okada M., Kohara A., Kiso T., Toya T., Iwai A. Wanibuchi F., and Yamaguchi T. (2003) Allodynia and hyperalgesia in adjuvant-induced arthritic rats: time course of progression and efficacy of analgesia. *J. Pharm. Exp. Ther.* 306, 490-497.
- Nagy B., Fedonidis C., Photious A., Wahba J., Paule C.C., Ma D., Buluwela L., and Nagy I. (2009) Capsaicin-sensitive primary sensory neurons in the mouse express N-Acyl phosphatidylethanolamine phospholipase D. *Neurosci.* 161, 572-577.
- Nakatsuka T., Park J.S., Kumamoto E., Tamaki T., and Yoshimura M. (1999) Plastic changes in sensory inputs to rat substantia gelatinosa neurons following peripheral inflammation. *Pain* 82, 39-47.
- Nelson K., Walsh D., Deeter P. and Sheehan F. (1994) A phase II study of delta-9-tetrahydrocannabinol for appetite stimulation in cancer-associated anorexia. *J. Palliat. Care* 10, 14-18.
- Neugebauer V., Lücke T., and Schaible H.G. (1993) N-Methyl-D-aspartate (NMDA) and non-NMDA receptor antagonists block the hyperexcitability of dorsal horn neurons during development of acute arthritis in rat's knee joint. *J. Neurophysiol.* 70, 1365-1377.
- Neugebauer V. and Schaible H.G., (1990) Evidence for a central component in the sensitization of spinal neurons with joint input during development of acute arthritis in cat's knee. *J. Neurophysiol.* 64, 299-311.
- Neugebauer V., Weiretter F., and Schaible H.G. (1995) Involvement of substance P and neurokinin-1 receptors in the hyperexcitability of dorsal horn neurons during development of acute arthritis in rat's knee joint. *J. Neurophysiol.* 73, 1574-1583.
- Neumann S., Doubell T.P., Leslie T., and Woolf C.J. (1996) Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. *Nature* 384, 360-364.
- Noyes Jr., R., Brunk S.F., Baram D.A., and Canter A. (1975) Analgesic effect of Delta-9-Tetrahydrocannabinol. *J. Clin. Pharm.* 15, 139-143.
- Nunez E., Benito C., Pazos M.R., Barbachano A., Fajardo O., Gonzalez S., Tolon R.M., and Romero J. (2004) Cannabinoid CB₂ receptors are expressed by perivascular microglial cells in the human brain: an immunohistochemical study. *Synapse* 53, 208-213.
- Okamoto Y., Morishita J., Tsuboi K., Tonai T., and Ueda N. (2004) Molecular Characterization of a Phospholipase D Generating Anandamide and Its Congeners. *J. Biol. Chem.* 279, 5298-5305.
- Pacher P., Bátkai S., and Kunos G. (2006) The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol Rev.* 58, 389-462.
- Paria B.C., Deutsch D.D., and Dey S.K. (1996) The uterus is a potential site for anandamide synthesis and hydrolysis: differential profiles of anandamide synthase

- and hydrolase activities in the mouse uterus during the periimplantation period. *Mol. Reprod. Dev.* 45, 183-192.
- Parolaro D. (1999) Presence and functional regulation of cannabinoid receptors in immune cells. *Life Sci.* 65, 637-644.
- Patrini G., Sacerdote P., Fuzio D., Manfredi B, and Parolaro D. (1997) Regulation of immune functions in rat splenocytes after acute and chronic in vivo treatment with CP-55, 940, a synthetic cannabinoid compound. *J. Neuroimmunol.* 80, 143-148.
- Pearce R.J., and Duchen M.R. (1994) Differential expression of membrane currents in dissociated mouse primary sensory neurons. *Neurosci.* 63, 1041-1056.
- Perl E.R. (1968) Myelinated afferent fibres innervating the primare skin and their response to noxious stimuli. *J. Physiol.* 197, 593-615.
- Pertwee R.G. (2001) Cannabinoid receptors and pain. *Prog. Neurobiol.* 63, 569-611.
- Pertwee R.G., and Ross R.A. (2002) Cannabinoid receptors and their ligands. *Prostaglandins Leukot. Essent. Fatty Acids* 66, 101-121.
- Pfaffl M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, e45.
- Piomelli D., Beltramo M., Giuffrida A., and Stella N. (1998) Endogenous cannabinoid signaling. *Neurobiol. Dis.* 5, 462-473.
- Potenzieri C., Harding-Rose C., and Simone D.A. (2008) The cannabinoid receptor agonist, WIN 55,212-2, attenuates tumor-evoked hyperalgesia through peripheral mechanisms. *Brain Res.* 1215, 69-75.
- Pratt P.F., Hillard C.J., Edgemond W.S., Campbell W.B. (1998) N-arachidonylethanolamide relaxation of bovine coronary artery is not mediated by CB1 cannabinoid receptor. *Am. J. Physiol.* 274, H375-H381.
- Price T.J., Helesic G., Parghi D., Hargreaves K.M., and Flores C.M. (2003) The neuronal distribution of cannabinoid receptor type 1 in the trigeminal ganglion of the rat. *Neuroscience* 120, 155-162.
- ProCon.org “Fourteen Legal Medical Marijuana States and DC: Laws, Fees, and Possession Limits”
<http://medicalmarijuana.procon.org/view.resource.php?resourceID=000881>
 (accessed August 12, 2010)
- Quartilho A., Mata H.P., Ibrahim M.M., Vanderah T.W., Porreca F., Makriyannis A., and Malan T.P. (2003) Inhibition of inflammaoty hyperalgesia by activation of peripheral CB2 cannabinoid receptors. *Anesthesiology* 99, 955-960.
- Randic M., Hecimovic H., and Ryu P.D. (1990) Sustance P modulates glutamate-induced currents in acutely isolated rat spinal dorsal horn neurons. *Neurosci. Lett.* 117, 74-80.
- Reeh P.W. (1994) Chemical excitation and sensitization of nociceptors. In : Cellular Mechanisms of Sensory Processing, edited by Urban I. Heidelberg, Germany: Springer-Verlag, p. 119-131.
- Richardson J.D., Aanonsen L., and Hargreaves K.M. (1998b) Antihyperalgesic effects of spinal cannabinoids. *Eur. J. Pharmacol.* 345, 145-153.
- Richardson J.D., Kilo S. and Hargreaves K.M. (1998a) Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB₁ receptors. *Pain* 75, 111-119.

- Rosland J.H., Tjølsen A., Maehle B., and Hole K. (1990) The formalin test in mice: effect of formalin concentration. *Pain* 42, 235-242.
- Ross R.A. (2003) Anandamide and vanilloid TRPV1 receptors. *Br. J. Pharm.* 140, 790-801.
- Ross R.A., Coutts A.A., McFarlane S.M., Anavi-Goffer S., Irving A.J., Pertwee R.G., MacEwan D.J., and Scott R.H. (2001) Actions of cannabinoid receptor ligands on rat cultured sensory neurones: implications for antinociception. *Neuropharmacology* 40, 221-232.
- Rueda D., Galve-Roperh I., Haro A., and Guzman M. (2000) The CB(1) cannabinoid receptor is coupled to the activation of c-Jun N-terminal kinase. *Mol. Pharmacol.* 58, 814-820.
- Sagar D.R., Kelly S., Millns P.J., O'Shaughnessey C.T., Kendall D.A., and Chapman V. (2005) Inhibitory effects of CB1 and CB2 receptor agonists on responses of DRG neurons and dorsal horn neurons in neuropathic rats. *Eur. J. Neurosci.* 22, 371-379.
- Sakurada T., Matsumura T., Moriyama T., Sakurada C., Ueno S., and Sakurada S. (2003) Differential effects of intraplantar capsaizepine and ruthenium red on capsaicin-induced desensitization in mice. *Pharm. Biochem. and Beh.* 75, 115-121.
- Sakurada T., and Noma A. (1981) Subcellular localization and some properties of monoacylglycerol lipase in rat adipocytes. *J. Biochem.* 90, 1413-1419.
- Sallan S.E., Zinberg N.E., and Frei III, E. (1975) Antiemetic effect of delta-9-tetrahydrocannabinol in patients receiving cancer chemotherapy. *N. Engl. J. Med.* 293, 795-797.
- Samad T.A., Moore K.A., Sapirstein A., Billet S., Allchorne A., Poole S., Bonventre J.V. and Woolf C.J. (2001) Interleukin-1 beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* 410, 471-475.
- Santos A.R.S., and Calixto J.B. (1997) Ruthenium red and capsaizepine antinociceptive effect in formalin and capsaicin models of pain in mice. *Neurosci. Lett.* 235, 73-76.
- Sañudo-Peña M.C., Strangman, N.M., Mackie K., Walker J.M., and Tsou K. (1999) CB₁ receptor localization in the spinal cord and roots, dorsal root ganglion, and peripheral nerve. *Acta. Pharmacol. Sin.* 12, 1115-1120.
- Savinainen J.R., Järvinen T., Laine K., and Laitinen J.T. (2001) Despite substantial degradation, 2-arachidonoylglycerol is a potent full efficacy agonist mediating CB(1) receptor-dependent G-protein activation in rat cerebellar membranes. *Br. J. Pharmacol.* 134, 664-672.
- Schmid P.C., Reddy P.V., Natarajan V., and H.H.O. Schmid H.H.O., Metabolism of N-acylethanolamine phospholipids by mammalian phosphodiesterase of the phospholipase D type. *J. Biol. Chem.* 258, 9302-9306.
- Schmid P.C., Zuzarte-Augustin M.L., and Schmid H.H. (1985) Properties of rat liver N-acylethanolamine amidohydrolase. *J. Biol. Chem.* 260, 14145-14149.
- Scott B.S. (1977) Adult mouse dorsal root ganglia neurons in cell culture. *J. Neurobiol.* 8, 417-427.

- Seibert K., Zhang Y., Leahy K., Hauser S., Masferrer J., Perkins W., Lee L., and Isakson P. (1994) Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc Natl Acad Sci U.S.A.* 91, 12013-12017.
- Seybold V.S., Jia Y-P., Abrahams L.G. (2003) Cyclooxygenase -2 contributes to central sensitization in rats with peripheral inflammation. *Pain* 105, 47-55.
- Shen M., and Thayer S.A. (1998) The cannabinoid agonist WIN55,212-2 inhibits calcium channels by receptor-mediated and direct pathways in cultured rat hippocampal neurons. *Brain Research* 783, 77-84.
- Shire D., Calandra B., Delpuch M., Dumont X., Kaghad M., Le Fur G., Caput D., and Ferrara P. (1996) Structural features of the central cannabinoid CB1 receptor involved in the binding of the specific CB1 antagonist SR 141716A. *J. Biol. Chem.* 271, 6941-6946.
- Showalter V.M., Compton D.R., Martin B.R., and Abood M.E. (1996) Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): identification of cannabinoid receptor subtype selective ligands. *J. Pharmacol. Exp. Ther.* 278, 989-999.
- Simone D.A., Baumann T.K., and LaMotte R.H. (1989) Dose-dependent pain and mechanical hyperalgesia in humans after intradermal injection of capsaicin, *Pain* 38, 99-107.
- Simone D.A., and Kajander K.C. (1996) Excitation of rat cutaneous nociceptors by noxious cold. *Neurosci. Lett.* 213, 53-56.
- Simone D.A., Sorkin L.S., Oh, U., Chung J.M., Owens C., LaMotte R.H., and Willis W.D. (1991) Neurogenic hyperalgesia: central neural correlates in responses of spinothalamic tract neurons. *J. Neurophysiol.* 66, 228-246.
- Smart D., Gunthorpe M.J., Jerman J.C., Nasir S., Gray J., Muir A.I., Chambers J.K., Randall A.D., and Davis J.B. (2000) The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br. J. Pharmacol.* 129, 227-230.
- Sokal D.M., Elmes S.J., Kendall D.A., and Chapman V. (2003) Intraplantar injection of anandamide inhibits mechanically-evoked responses of spinal neurones via activation of CB2 receptors in anaesthetised rats. *Neuropharmacology* 45, 404-411.
- Sorkin L.S., Xiao W.H., Wagner R., and Myers R.R. (1997) Tumour necrosis factor- α induces ectopic activity in nociceptive primary afferent fibres. *Neurosci.* 81, 255-262.
- Stander S., Schmelz M., Metze D., Luger T., and Rukwied R. (2005) Distribution of cannabinoid receptor 1 (CB1) and 2 (CB2) on sensory nerve fibers and adnexal structures in human skin. *J. Dermatol. Sci.* 38, 177-188.
- Stein C., and Lang L.J. (2009) Peripheral mechanisms of opioid analgesia. *Curr. Opinion in Pharmacol.* 9, 3-8.
- Stein C., Millan M.J., and Herz A. (1988) Unilateral inflammation of the hindpaw in rats as a model of prolonged noxious stimulation: alterations in behavior and nociceptive thresholds. *Pharmacol. Biochem and Beh.* 31, 445-451.

- Stella N., Schweitzer P., and Piomelli D. (1997) A second endogenous cannabinoid that modulates long-term potentiation. *Nature* 388, 773-778.
- Stucky C.L., Thayer S.A., and Seybold V.S. (1996) Prostaglandin E₂ increases the proportion of neonatal rat dorsal root ganglion neurons that respond to bradykinin. *Neurosci.* 74, 1111-1123.
- Sugiura T., Kondo S., Sukagawa A., Nakane S., Shinoda A., Itoh K., Yamashita A., and Waku K. (1995) 2-Arachidonylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun.* 215, 89-97.
- Sugiura T., Kobayashi Y., Oka S., and Waku K. (2002) Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. *Prostaglandins Leukotrienes Essent. Fatty Acids* 66, 173-192.
- Sugiura T., Oka S., Gokoh M., Kishimoto S., and Waku K. (2004) New perspectives in the studies on endocannabinoid and cannabis: 2-arachidonoylglycerol as a possible novel mediator of inflammation. *J. Pharmacol. Sci.* 96, 367-375.
- Sun Y-X., Tsuboi K., Okamoto Y., Tonai T., Murakami M., Kudo I., and Ueda N. (2004) Biosynthesis of anandamide and *N*-palmitoylethanolamine by sequential actions of phospholipase A₂ and lysophospholipase D. *Biochem. J.* 380, 749-756.
- Svizenska I., Dubovy P., and Sulcova A. (2008) Cannabinoid receptors 1 and 2 (CB1 and CB2), their distribution, ligands and functional involvement in nervous system structures—a short review. *Pharmacol. Biochem. & Beh.* 90, 501-511.
- Szallasi A., and Blumberg P.M. (1999) Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol. Rev.* 51, 159-212.
- Szolcsányi J. (1977) A pharmacological approach to elucidation of the role of different nerve fibres and receptor endings in mediation of pain. *J. Physiol. (Paris)* 73, 251-259.
- Tallarida R.J. and Murray R.B. (1987) *Manual of Pharmacological Calculations with Computer Programs* pp 26-31. Springer Verlag, New York.
- Thompson S.W.N., Dray A., and Urban L. (1994) Injury-induced plasticity of spinal reflex activity: NK1 neurokinin 1 receptor activation and enhanced A- and C-fiber mediated responses in the rat spinal cord *in vitro*. *J. Neurosci.* 14, 3672-3687.
- Tominaga M., Caterina M.J., Malmberg A.B., Rosen T.A., Gilbert H., Skinner K., Raumann B.E., Basbaum A.I., and Julius D. (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* 21, 531-543.
- Tornqvist H., Krabisch L., and Belfrage P. (1974) Simple assay for monoacylglycerol hydrolase activity of rat adipose tissue. *J. Lipid Res.* 15, 291-294.
- Treede R.D., Meyer R.A., and Campbell J.N. (1998) Myelinated mechanically insensitive afferents from monkey hairy skin: Heat-response properties. *J. Neurophysiol.* 80, 1082-1093.
- Treede R.D., Meyer R.A., Raja S.N., and Campbell J.N. (1992) Peripheral and central mechanisms of cutaneous hyperalgesia *J. Neurophysiol.* 91, 2770-2781.
- Tsou K., Lowitz K.A., Hohmann A.G., Martin W.J., Hathaway C.B., Bereiter D.A., and Walker J.M. (1996) Suppression of noxious stimulus-evoked expression of FOS protein-like immunoreactivity in rat spinal cord by a selective cannabinoid agonist. *Neurosci.* 70, 791-798.

- Tsou K., Nogueron M.I., Muthian S., Sañudo-Pena M.C., Hillard C.J., Deutsch D.G., and Walker J.M. (1998) Fatty acid amide hydrolase is located preferentially in large neurons in the rat central nervous system as revealed by immunohistochemistry. *Neurosci. Lett.* 254, 137-140.
- Twitchell W., Brown S., and Mackie K. (1997) Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. *J Neurophysiol.* 78, 43-50.
- Ueda N., Kurahashi Y., Yamamoto S., and Tokunaga T. (1995) Partial purification and characterization of the porcine brain enzyme hydrolyzing and synthesizing anandamide. *J. Biol. Chem.* 270, 23823-23827.
- Ueda N., Puffenbarger R.A., Yamamoto S., and Deutsch D.G. (2000) The fatty acid amide hydrolase (FAAH). *Chem. Phys. Lipids.* 108, 107-121.
- United States Patent and Trademark Office, www.patft.uspto.gov
- Urban L., and Dray A. (1993) Actions of capsaicin on mouse dorsal root ganglion cells in vitro. *Neurosci. Lett.* 157, 187-190.
- Urban L., Naeem S., Patel I.A., and Dray A. (1994) Tachykinin induced regulation of excitatory amino acid responses in the rat spinal cord in vitro. *Neurosci. Lett.* 168, 185-188.
- Valk P., Verbakel S., Vankan Y., Hol S., Mancham S., Ploemacher R., Mayen A., Lowenberg B., and Delwel R. (1997) Anandamide, a natural ligand for the peripheral cannabinoid receptor is a novel synergistic growth factor for hematopoietic cells *Blood* 90, 1448-1457.
- van der Stelt M., and Di Marzo V. (2004) Endovanilloids. Putative endogenous ligands of transient receptor potential vanilloid 1 channels. *Eur. J. Biochem.* 271, 1827–1834.
- van der Stelt M., Trevisani M., Vellani V., De Petrocellis L., Schiano Moriello A., Campi B., McNaughton P., Geppetti P., and Di Marzo V. (2005) Anandamide acts as an intracellular messenger amplifying Ca²⁺ influx via TRPV1 channels. *EMBO J.* 24, 3026-3037.
- Van Sickle M.D., Duncan M., Kingsley P.J., Mouihate A., Urbani P., Mackie K., Stella N., Makriyannis A., Piomelli D., Davison J.S., Marnett L.J., Di Marzo V., Pittman Q.J., Patel K.D., and Sharkey K.A. (2005) Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science* 310, 329-332.
- Varga K., Wagner J.A., Bridgen D.T., and Kunos G. (1998) Platelet- and macrophage derived endogenous cannabinoids are involved in endotoxin-induced hypotension. *FASEB. J.* 12, 1035-1044.
- Vellani V., Petrosino S., De Petrocellis L., Valenti M., Prandini M., Magherini P.C., McNaughton P.A., and Di Marzo V. (2008) Functional lipidomics: Calcium-independent activation of endocannabinoid/endovanilloid lipid signaling in sensory neurons by protein kinase C and A and thrombin. *Neuropharmacol.* 55, 1274-1279.
- von Frey M. (1896) Treatise on the sensory functions of the human skin. Trans. From German by Biederman-Thorson in H.O. Handwerker and K. Brune, K (eds.), 1987. Classical German Contributions to Pain Research, (pp. 69-131) Vth World Congress on Pain, Hamburg. Original German: Untersuchungen über die

- Sinnesfunktionen der Menschlichen Haut. Vol. 23, Koenigl. Saech. Ges. Wissensch., Leipzig, S. Hirzel.
- Walczak J.S., Pichette V., Leblond F., Desbiens K., and Beaulieu P. (2005) Behavioral, pharmacological and molecular characterization of the saphenous nerve partial ligation: a new model of neuropathic pain. *Neuroscience* 132, 1093-1102.
- Walczak J.S., Pichette V., Leblond F., Desibens K., and Beaulieu P. (2006) Characterization of chronic constriction of the saphenous nerve, a model of neuropathic pain in mice showing rapid molecular and electrophysiological changes. *J. Neurosci. Res.* 83, 1310-1322.
- Walker J.M., Hohmann A.G., Martin W.J., Strangman N.M., Huang S.M., and Tsou K. (1999) The neurobiology of cannabinoid analgesia. *Life Sci.* 65, 665-673.
- Walker J.M., and Huang S.M. (2002) Cannabinoid analgesia. *Pharmacol. and Ther.* 95, 127-135.
- Walker J.M., Huang S.M., Strangman N.M., Tsou K., and Sañudo-Peña M.C. (1999) Pain modulation by release of the endogenous cannabinoid anandamide. *Proc. Natl. Acad. Sci. U.S.A.* 96, 12198-12203.
- Walker K., Perkins M., and Dray A. (1995) Kinins and kinin receptors in the nervous system. *Neurochem. Int.* 26, 1-16.
- Wartmann M., Campbell D., Subramanian A., Burstein S.H., and Davis R.J. (1995) The MAP kinase signal transduction pathway is activated by the endogenous cannabinoid anandamide. *FEBS Lett.* 359, 133-136.
- Weber A., Ni J., Ling K.H., Acheampong A., Tang-Liu D.D., Burk R., Cravatt B.F., and Woodward D. (2004) Formation of prostamides from anandamide in FAAH knockout mice analyzed by HPLC with tandem mass spectrometry. *J. Lipid Res.* 45, 757-763.
- Wheeler-Aceto H., Porreca F., and Cowan A. (1990) The rat paw formalin test: comparison of noxious agents. *Pain* 40, 229-238.
- Winter C.A., Risley E.A., and Nuss G.W. (1962) Carrageenan-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. *Proc. Soc. Exp. Biol.* 111, 544-547.
- Wise L.E., Cannavacciuolo R., Cravatt B.F., Martin B.F., Lichtman A.H. (2008) Evaluation of fatty acid amides in the carrageenan-induced paw edema model. *Neuropharmacol.* 54, 181-188.
- Woolf C.J., Safieh-Garabedian B., Ma Q.P., Crilly P., Winter J. (1994) Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. *Neurosci.* 62, 327-331.
- Woolf C.J., and Salter M.W. (2000) Neuronal Plasticity: Increasing the gain in pain. *Science* 288, 1765-1768.
- Woodridge E., Barton S., Samuel J., Osorio J., Dougherty A., and Holdcroft A. (2005) Cannabis use in HIV for pain and other medical symptoms. *J. of Pain and Symptom Management.* 29, 358-367.
- Wotherspoon G., Fox Z., McIntyre P., Colley S., Bevan S., and Winter J. (2005) Peripheral nerve injury induces cannabinoid receptor 2 protein expression in rat sensory neurons. *Neuroscience* 135, 235-245.

- Yaksh T.L., Dirig D.M., Conway C.M., Svensson C., Luo Z.D., and Isakson P.C. (2001) The acute antihyperalgesic action of nonsteroidal, anti-inflammatory drugs and release of spinal prostaglandin E₂ is mediated by the inhibition of constitutive spinal cyclooxygenase-2 (COX-2) but not COX-1. *J. Neurosci.* 21, 5847-5853.
- Yeomans D.C., Pirec V., and Proudfit H.K. (1996) Nociceptive responses to high and low rates of noxious cutaneous heating are mediated by different nociceptors in the rat: Behavioral evidence. *Pain* 68, 133-140.
- Yeomans D.C., and Proudfit H.K. (1996) Nociceptive responses to high and low rates of noxious cutaneous heating are mediated by different nociceptors in the rat: Electrophysiological evidence. *Pain* 68, 141-150.
- Yu M., Ives D., and Ramesha C.S. (1997) Synthesis of prostaglandin E₂ ethanolamide from anandamide by cyclooxygenase-2. *J. Biol. Chem.* 272, 21181-21186.
- Zimmer A., Zimmer A.M., Hohman A.G., Herkenham M., and Bonner T.I. (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB₁ receptor knockout mice. *Proc Natl Acad Sci U.S.A.* 96, 5780-5785.
- Zygmunt P.M., Petersson J., Andersson D.A., Chuang H., Sorgard M., Di Marzo V., Julius D., and Hogestatt E.D. (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 400, 452-457.