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Effect of cannabinoids on pain-stimulated and pain-depressed behavior in rats

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Effects of Cannabinoids on Pain-Stimulated
and Pain-Depressed Behavior in Rats

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

by

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Table of Contents

List of Abbreviations

%MCR: %Maximum control rate

2-AG: 2-Arachidonoylglycerol

AA: Arachidonic acid

ACEA: Arachidonyl-2'-chloroethylamide

Amg: Amygdala

ASIC: Acid-sensing ion channel

cAMP: Cyclic adenosine monophosphate

CBR: Cannabinoid receptor

DA: Dopamine

GABA: Gamma-Aminobutryric acid

GPR55: G-protein coupled receptor 55

IP: Intraperitoneal

LH: Lateral hypothalamus

LPS: Lipopolysaccharide

LTD: Long-Term Depression

MAGL: Monoacylglycerol lipase

mg/kg: Milligrams per kilogram

NAc: Nucleus accumbens

NAPE: N-arachidonoylphoshphatidylethanolamine

NAPE-PLD: N-arachidonoylphoshphatidylethanolamine phospholipase D

NSAID: Nonsteroidal anti-inflammatory drug

OEA: Oleoylethanolamide

PAG: Periaqueductal gral

PBN: Parabrachial nucleus

PEA: Palmitoylethanolamide

PIM: Pro-inflammatory molecules

PLC: Phospholipase C

PPAR-α: Peroxisome proliferator-activated receptor-α

PPQ: p-phenylquinone

RVM: Rostral ventromedial medulla

Sep: Septum

THC: Δ9-tetrahydrocannabinol

TRPV1: Transient receptor potential vanilloid-1

VTA: Ventral tegmental area

List of Drugs

CP55940-mixed CB1R/CB2R high efficacy agonist

GW405833-selective CB2R agonist

Ketoprofen-nonsteroidal anti-inflammatory drug

Lipopolysaccharide (LPS)-endotoxin, toll-like 4 receptor (TL4R) agonist

MK886-Peroxisome proliferator-activated receptor-α (PPAR-α) antagonist

PF3845-irreversible fatty acid amide hydrolase (FAAH) inhibitor (increases endogenous endocannabinoid anandamide and other fatty acid ethanolamines such as PEA and OEA that are agonists for PPAR-α)

Rimonabant-selective CB1R antagonist

SR144528-selective CB2R antagonist

∆9-Tetrahydrocannabinol (THC)-mixed CB1R/CB2R low efficacy agonist

URB597-irreversible FAAH inhibitor (increases endogenous endocannabinoid anandamide and other fatty acid ethanolamines such as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) that are agonists for PPAR-α)

Abstract

EFFECTS OF CANNABINOIDS ON PAIN-STIMULATED
AND PAIN-DEPRESSED BEHAVIOR IN RATS

By Andrew J. Kwilasz, BA, Psychology

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

Virginia Commonwealth University, 2013.

Major Director: Dr. S. Stevens Negus, PhD, Professor,
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Cannabinoids produce antinociception in many preclinical models of acute and chronic pain. In contrast, cannabinoids produce inconsistent analgesia in humans, showing little or no efficacy in treating acute pain, with modest efficacy in treating chronic inflammatory pain. This discrepancy may reflect an overreliance on preclinical assays of pain-stimulated behaviors, defined as behaviors that increase in rate or intensity following delivery of a noxious stimulus. In these assays, antinociception is indicated by a reduction in pain-stimulated behaviors, and antinociception is produced either by a reduction in sensory sensitivity to the noxious stimulus (i.e. true analgesia) or by false positive motor impairment. This dissertation addresses this weakness by complementing cannabinoid effects in conventional assays of pain-stimulated behavior with their effects in novel assays of pain-depressed behavior. Pain-depressed behaviors are defined as behaviors that decrease in rate or intensity following presentation of a noxious stimulus. Motor impairment does not produce false positive antinociception in assays of pain-depressed behavior, because antinociception is indicated by a blockade

or reversal of pain-induced behavioral depression. In this dissertation, an intraperitoneal (IP) injection of lactic acid served as an acute noxious stimulus to stimulate stretching (pain-stimulated behavior) or depress intracranial self-stimulation (ICSS) (pain-depressed behavior), whereas, IP injection(s) of lipopolysaccharide (LPS) served as a chronic/acute inflammatory-related noxious stimulus to stimulate mechanical allodynia (pain-stimulated behavior) or depress ICSS (pain-depressed behavior). Cannabinoids tested in the assays of acid-stimulated stretching and acid-depressed ICSS included: mixed CB1R/CB2R agonists THC and CP55940, drugs that modulate levels of the endogenous cannabinoid agonist anandamide (URB597 and PF3845), and a selective CB2R agonist, GW405833. THC was also tested in assays of LPS-stimulated mechanical allodynia and LPS-depressed ICSS. In general, mixed CB1R/CB2R agonists were ineffective or exacerbated pain-depressed behavior regardless of noxious stimulus. Contrastingly, URB597 and GW405833 produced antinociception in the assay of acid-depressed ICSS; however their effects were not mediated by CBRs. All compounds produced antinociception in the assay of pain-stimulated behavior, except for PF3845. These results suggest that assays of pain-depressed behavior may be useful for development of cannabinoid analgesic medications, but that further research is needed to determine mechanisms underlying cannabinoid-mediated antinociception in these assays.

CHAPTER ONE

Introduction and Background

1.1 Use of cannabinoids for the treatment of pain

The marijuana plant (*Cannabis sativa*) is a natural source of cannabinoids used for centuries to treat pain (Mechoulam and Ben-Shabat, 1999). Δ9-tetrahydrocannabinol (THC), the primary active constituent of marijuana, has been studied extensively with the intent of characterizing its therapeutic properties. Many cannabinoids originate from the marijuana plant and are classified by their structural relationship to THC, whereas synthetically developed cannabinoids are classified by their activity at cannabinoid receptors (Weissman, 1981). Moreover, endogenous ligands for cannabinoid receptors have been identified and are also classified as cannabinoids. Most of THC's behavioral effects are exerted through its action as a low-efficacy agonist at the cannabinoid-1 receptor (CB1R) (Devane et al., 1988; Melvin et al., 1993; Onaivi et al., 1995); however, in addition to CB1Rs, THC also activates the cannabinoid-2 receptor (CB2R) (Felder et al. 1995). As a result of discovery of THC's pharmacological actions, several mixed synthetic cannabinoid agonists for CB1Rs and CB2Rs have been developed (Howlett 1995). Moreover, selective agonists have been developed for CB1Rs and CB2Rs, such as Arachidonyl-2'-chloroethylamide (ACEA) (Hillard et al., 1999) and GW405833 (Marriot and Huffman, 2008), respectively.

THC and other mixed CB1R/CB2R agonists are highly efficacious in nearly all preclinical assays of pain. For example, studies modeling acute nociception have shown

that THC and other CBR agonists such as CP55940, WIN55212-2, and HU210 are all effective at increasing the latency to paw withdrawal on a hot plate in rats (De Vry et al., 2004; Hama and Sagen, 2009). Other studies using tail-flick assays to measure spinal reflexes elicited by noxious thermal stimuli have found similar results (Rubino et al., 1994; Patrini et al., 1997; Wiley et al., 2007), as have studies using models of acute inflammatory pain such as stimulation of stretching by intraperitoneal (IP) injection of dilute acid (Sofia et al., 1975; Booker et al., 2009) or stimulation of paw flinching by intraplantar administration of dilute formalin (Finn et al., 2004; Khodayar et al., 2006). THC and CBR agonists have also been shown to produce antinociception in preclinical models of chronic pain, such as those that model inflammatory or neuropathic pain (Lim et al., 2003; Hsieh et al., 2011). For example, an intraplantar injection of Complete Freund's Adjuvant (CFA) into the rat hindpaw produces a transient inflammatory reaction accompanied by paw swelling and hypersensitive paw-withdrawal from mechanical and thermal stimuli occurring as early as 24 h after injection and lasting up to two weeks (Schepers et al. 2008a; Schepers et al. 2008b; Yang and Gao 2010). Several studies have demonstrated the effectiveness of cannabinoids in attenuating CFA-induced inflammation and associated nociceptive responses (Amaya et al. 2006; Jayamanne et al. 2006). Furthermore, chronic pain has been associated with neuropathy in humans, and neuropathic pain has been modeled in rodents using procedures (e.g. nerve injury or chemotherapeutic drug treatment like paclitaxel) that measure hypersensitive withdrawal responses from mechanical or thermal stimuli. Cannabinoids often produce antinociception in these assays manifested as a blockade of neuropathy-induced hypersensitivity (Costa et al., 2004; Pascual et al., 2005).

Selective CB2R agonists also decrease pain-related behaviors in many preclinical studies (Wilkerson and Milligan, 2011). For example, selective CB2R agonists are effective at increasing the latency of time for a rat to withdraw its paw from an innocuous mechanical stimulus or noxious thermal stimulus after acute tissue damage (i.e. paw incision) as well as after other inflammatory (i.e. intraplantar CFA) or nerve-related injuries (Malan et al., 2001; Valenzano et al., 2005; Whiteside et al., 2005; Hsieh et al., 2011). Moreover, CB2R agonists have been shown to be more potent against inflammatory-related stimuli such an intraplantar injection of CFA versus an acute tissue injury such as intraplantar paw incision (Valenzano et al., 2005). One potential advantage of CB2R agonists is that they do not produce CB1R-mediated side effects such as behavioral depression/sedation and abuse-related effects, and this property makes the CB2R an attractive target for candidate cannabinoid analgesics (Marriott and Huffman, 2008).

The endogenous cannabinoid system, or *endocannabinoid system*, has also gained considerable attention as a target to treat pain and inflammation (Pacher et al., 2006; Schlosburg et al., 2009; Alvarez-Jaimes and Palmer, 2011). The endocannabinoid system is comprised primarily of two endogenous cannabinoid ligands for CB1Rs and CB2Rs, anandamide (AEA) and 2-arachidonylglycerol (2-AG), and the respective enzymes that degrade them, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL). In particular, the inhibition of FAAH, which has the effect of increasing endogenous levels of AEA in synapses, has been demonstrated as a promising alternative to CB1R agonist-based cannabinoid medications (Pacher et al., 2006; Schlosburg et al., 2009; Alvarez-Jaimes and Palmer, 2011). FAAH inhibitors such

as URB597 and PF3845 produce antinociception in many assays of preclinical pain (Jayamanne et al., 2006; Ahn et al., 2009; Clapper et al., 2010; Kinsey et al., 2010; Booker et al., 2012; Ghosh et al., 2012). Furthermore, FAAH inhibitors are a useful strategy to reduce side effects associated with the CB1R, as FAAH inhibitors increase CBR activity only when and where endogenous AEA is actively being synthesized and released from cells. This property of FAAH inhibitors imbues them with temporal and spatial selectivity for activation of CBRs not obtainable with direct CBR agonists (Pacher et al., 2006; Schlosburg et al., 2009; Alvarez-Jaimes and Palmer, 2011). FAAH inhibitors have also been shown to increase other anti-inflammatory fatty acid ethanolamines, such as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), which activate peroxisome proliferator-activated receptor-alpha (PPAR-α) (Pacher et al., 2006; Schlosburg et al., 2009; Alvarez-Jaimes and Palmer, 2011). PPAR-α activation alone produces antinociception in several preclinical models of pain (Russo et al., 2007; Costa et al., 2008; Clapper et al., 2010), and has also been shown to reduce nociceptor field size and paw oedema in rat and mouse models of inflammatory pain (Clapper et al. 2010; Sagar et al. 2008). Overall, the preclinical literature provides abundant support for the antinociceptive effects of FAAH inhibitiors mediated by both CBRs and PPAR-α.

Despite the overwhelming evidence of the antinociceptive capabilities of CBR agonists in preclinical studies, CBR agonists have not been as successful in well-controlled clinical studies (Rice, 2006; Karst et al., 2010). Most of the studies on CBR agonists in humans have investigated either smoked marijuana and/or THC, including its synthetic forms marinol or dronabinol. Smoked marijuana and oral THC have

displayed some weak efficacy at treating various forms of acute nociception (Greenwald and Stitzer, 2000; Wallace et al., 2007; Cooper et al., 2013); however other studies investigating effects of THC and other CBR agonists on acute inflammatory conditions such as post-operative pain and experimentally-induced sunburn have displayed virtually no efficacy, with pain exacerbated by higher doses in some studies (Raft et al., 1977; Buggy et al., 2003; Naef et al., 2003; Beaulieu, 2006; Kraft et al., 2008; Klooker et al., 2011). A single clinical study has been conducted demonstrating the ability of Sativex (a formulation of THC and a non-psychoactive cannabinoid cannabidiol in a sublingual spray) to relieve morning pain at rest and during movement in patients with chronic inflammatory pain associated with rheumatoid arthritis (Blake et al., 2006), and Sativex is also approved in several countries to treat muscle spasticity and related pain in another inflammatory disorder, multiple sclerosis (Leussink et al., 2012). Additionally, some clinical trials exist that support weak efficacy of CBR agonists for the treatment of various types of neuropathic pain, although other studies fail to show improvement in symptoms (Berman et al., 2004; Svendsen et al., 2004; Abrams et al., 2007; Nurmikko et al., 2007; Rog et al., 2007; Ellis et al., 2009). Recently, clinical trials have been conducted to test the efficacy of the FAAH inhibitor PF7845 for the treatment of osteoarthritis-related pain (Huggins et al., 2012) and the efficacy of the selective CB2R agonist GW842166 (Ostenfeld et al., 2011) for the treatment of dental pain; however neither of these compounds displayed analgesic efficacy in these studies. Several other CB2R agonists are also in various phases of clinical development (see Wilkerson and Milligan, 2011). Taken together, these studies demonstrate that although CBR agonists may have some efficacy to relieve certain forms of chronic pain, further studies are

needed to determine the specific pathologies/circumstances in which CBR agonists as analgesic medications are most useful.

In summary, there is a discordance between the robust and reliable antinociceptive effects of CBs in preclinical studies and the lack of consistent analgesic effects in the clinic, especially under conditions of acute pain. This discordance may reflect an overreliance of preclinical research on assays that measure pain-stimulated behavior, as explained in section 1.3.

1.2. Cannabinoid receptor pharmacology

The CBR system consists of at least two known receptors: CB1R and CB2R. CBRs are G-protein-coupled receptors coupled to $G_{i/o}$ (Howlett, 2002; Demuth and Molleman, 2006; Lovinger, 2008). Activation of CBRs causes dissociation of G_α and $G_{\beta\gamma}$ subunits from the G-protein coupled receptor. The G_α subunit decreases activity of adenylyl cyclase activity, which subsequently decreases production of cyclic adenosine monophosphate (cAMP) (Howlett et al., 1990). Decreased cAMP has been shown to lead to decreased protein kinase A activity, which subsequently leads to increased activity of G-protein coupled inwardly-rectifying and A-type potassium channels and decreased probability of the cell firing (Mackie et al., 1995; McAllister et al., 1999). Moreover, the $G_{\beta\gamma}$ subunit causes decreased activity of N- and P/Q-type calcium channels and subsequently decreases probability of neurotransmitter release, regardless of whether an action potential occurs (Mackie et al., 1995; De Waard et al., 2005).

CB1Rs are the most abundant receptors in the mammalian central nervous system; however they are also present in peripheral tissues at much lower levels (Pacher et al., 2006). CB1Rs are typically located on the presynaptic axon terminals of glutamatergic and gamma-aminobutyric acid (GABA)-ergic neurons. When glutamate or GABA are released from these neurons, "on demand" endogenous biosynthesis and release of endocannabinoids occurs in the post-synaptic cell. Once released, endocannabinoid agonists such as AEA and 2-AG travel retrogradely back across the synapse to bind and activate presynaptically-located CB1Rs, which serves as a negative feedback mechanism of glutamate and GABA release (Figure 1.1) (Ahn et al., 2008). Moreover, CB1R activation has been shown to lead to short-term depression (STD) and long-term depression (LTD) of glutamatergic synapses and GABAergic synapses (Kano et al., 2009; Peterfi et al., 2012). Endocannabinoid-mediated LTD is a phenomenon by which signaling at the CB1R decreases future probability of glutamate or GABA release, and is a form of synaptic plasticity, which serves to weaken synaptic connections. CB1R agonists and more commonly endocannabinoids such as AEA and 2-AG have been shown to elicit LTD (Mackie, 2006). In addition to regulating LTD via CB1Rs, AEA has also been shown to regulate LTD via activation of transient receptor potential vanilloid-1 (TRPV1) ion channels (Chavez et al., 2010; Di Marzo, 2010; Grueter et al., 2010). CB1Rs have been found on primary afferent nociceptors and are distributed on their cell bodies, their peripheral terminals in various tissues, and their central terminals in the spinal cord (Veress et al., 2012). Furthermore, CB1Rs are distributed in brain regions associated with pain processing such as the prefrontal cortex (PFC), periaqueductal gray (PAG), and amygdala (Amg) (Hohmann et al., 1999;

Hohmann and Herkenham, 1999). CB1Rs are also present in brain regions related to reward and motivated behavior, such as the nucleus accumbens (NAc) and ventral tegmental area (VTA) (Herkenham et al., 1991), and these brain regions may also play a role in the experience of pain and expression of pain-related behaviors (Jarcho et al., 2012). Given the wide distribution of CB1Rs in the central nervous system, CB1R-mediated effects of CBR agonists on pain processing are vast, having the potential ability to modulate afferent pain signals and inflammation as well as other affective signs of pain (Figure 1.2) (Smith et al., 2001; Pacher et al., 2006). Cannabinoid agonists and/or endocannabinoids have also been shown to induce LTD at synapses between primary nociceptors and secondary nociceptors or between primary nociceptors and motor neurons (Yuan and Burrell, 2010; Kato et al., 2012; Yuan and Burrell, 2012; Yuan and Burrell, 2013), as well as in brain regions such as the VTA, NAc, and hippocampus (Chavez et al., 2010; Di Marzo, 2010; Grueter et al., 2010; Kortleven et al., 2011; Peterfi et al., 2012; Labouebe et al., 2013). LTD occurring in physiological regions such as these may also contribute to long-term modulation of afferent pain signals. The abundance of CB1Rs in the central nervous system are also responsible for the large number of side effects caused by CB1R agonists, including sedation, memory- and cognitive- deficits, as well as the potential for abuse (Kaufmann et al., 2010). Overall, these side effects as well as others have dampened enthusiasm for direct CB1R agonists as candidate analgesic drugs (Karst and Wippermann, 2009).

In contrast to CB1Rs, CB2Rs have been found primarily on immune-related cells, and were traditionally thought to only be expressed in peripheral tissues (Massi et al., 2006; Schlosburg et al., 2009; Alvarez-Jaimes and Palmer, 2011). More recently,

studies have found CB2Rs expressed in the central nervous system (Svizenska et al., 2008; Alvarez-Jaimes and Palmer, 2011). Although some studies have shown the presence of CB2Rs on neurons (Svizenska et al., 2008), most CB2Rs receptors expressed in the central nervous system are thought to be on immune system cells, primarily microglia (Cabral et al., 2008; Wilkerson and Milligan, 2011). Microglia are cells that become primed and then activated by pro-inflammatory molecules, such as cytokines, which are released as a result of ongoing inflammation. During the primed phase, microglia have been shown to express a high number of CB2Rs, and activation of these CB2Rs can inhibit migration and activation of the microglial cells (Cabral et al., 2008). Once activated, however, microglia release pro-inflammatory molecules, participating in a positive feedback mechanism of inflammation that is insensitive to CB2R agonists (Cabral et al., 2008; Milligan and Watkins, 2009; Wilkerson and Milligan, 2011). The effects of CB2R agonists are not as well-characterized as effects of CB1R agonists; however, CB2R agonists are thought to exert their antinociceptive properties at least in part through inhibition of the microglial response to inflammation (Figure 1.3) (Wilkerson and Milligan, 2011).
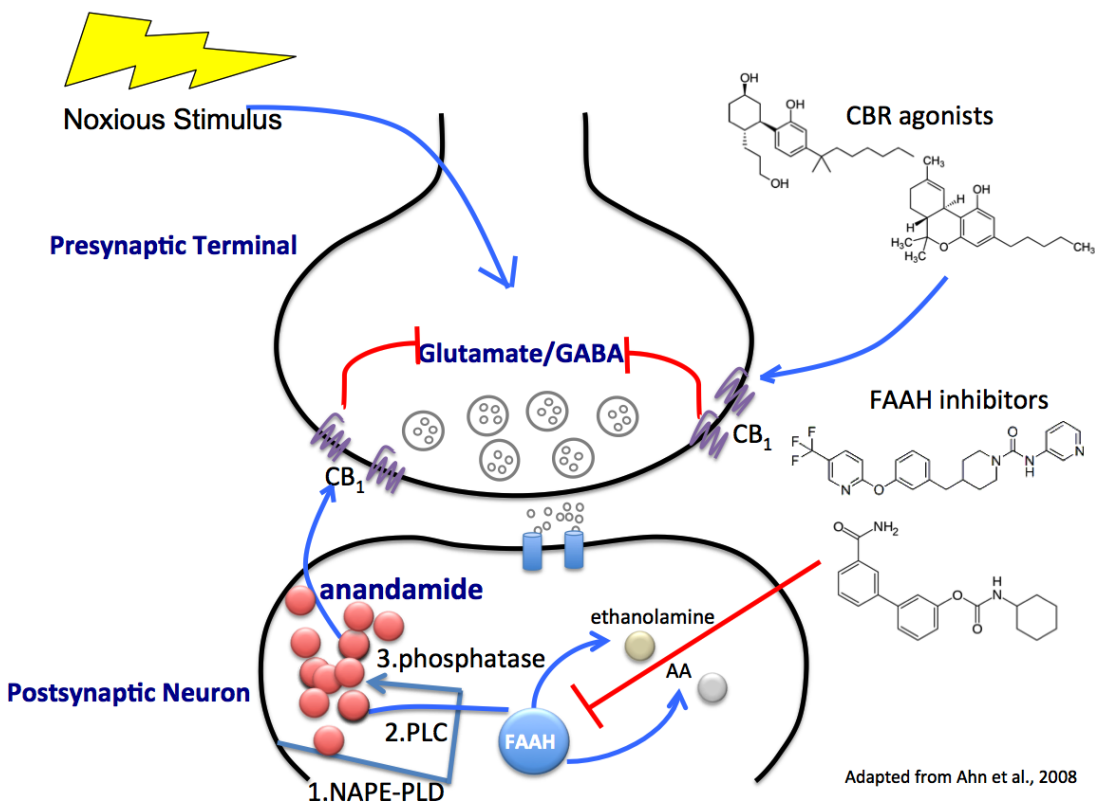
Figure 1.1

Figure 1.1. Depiction of a synapse with presynaptically-located CB1Rs. Displays biosynthetic and degradation pathways for AEA, and the effects of CB1R agonists or FAAH inhibition on glutamate or GABA release from a synapse due to noxious stimulation. AEA's biosynthetic pathway is not fully understood but can involve cleavage of the phospholipid precursor N-arachidonoylphosphatidylethanolamine (NAPE) from the cell membrane by the enzyme NAPE-phospholipase D (NAPE-PLD), whereas other biosynthetic pathways have been shown to involve cleavage via phospholipase C (PLC) and phosphatase (Liu et al., 2006). CBR agonists such as THC and CP55940 act directly at the CB1R to inhibit neurotransmitter release, whereas FAAH inhibitors such as URB597 and PF3845 act indirectly by inhibiting the degradation of AEA into ethanolamine and arachidonic acid (AA) by FAAH, causing increased AEA levels and subsequent CB1R activation (Ahn et al., 2008).
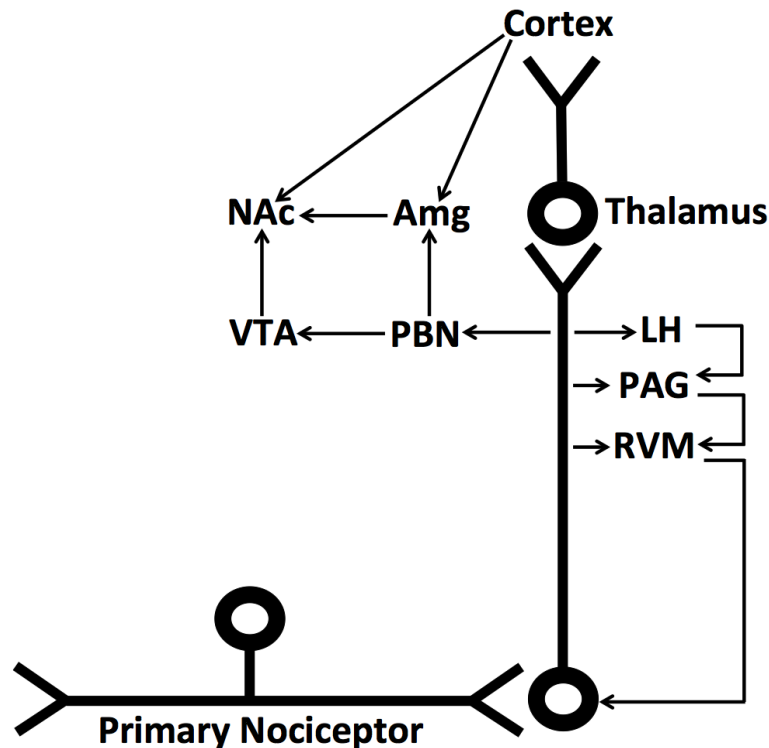
Figure 1.2

Figure 1.2. Depicts the primary pain pathway and its interaction with CB1Rs, as well as the ability of pain signals to influence brain areas regulating reward and emotion. Primary afferents synapse with secondary afferents in the dorsal horn of the spinal cord. Secondary afferents then synapse with tertiary afferents in the thalamus, and also send collateral projections to lower brain areas such as the rostral ventromedial medulla (RVM), the periaqueductal gray (PAG), and the lateral hypothalamus (LH). Tertiary synapses finally terminate in cortical areas. Furthermore, secondary nociceptors also send projections to the parabrachial nucleus (PBN), which then project to areas involved in reward and emotion such as the ventral tegmental area (VTA) and amygdala (Amg), both of which also synapse with the nucleus accumbens (NAc) (Rice, 2006). CB1Rs have been found in every brain region depicted in this figure (Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992; Hohmann et al., 1999; Hohmann and Herkenham, 1999) and thus have the ability to partake in modulation of pain signals at many different stages of pain processing.
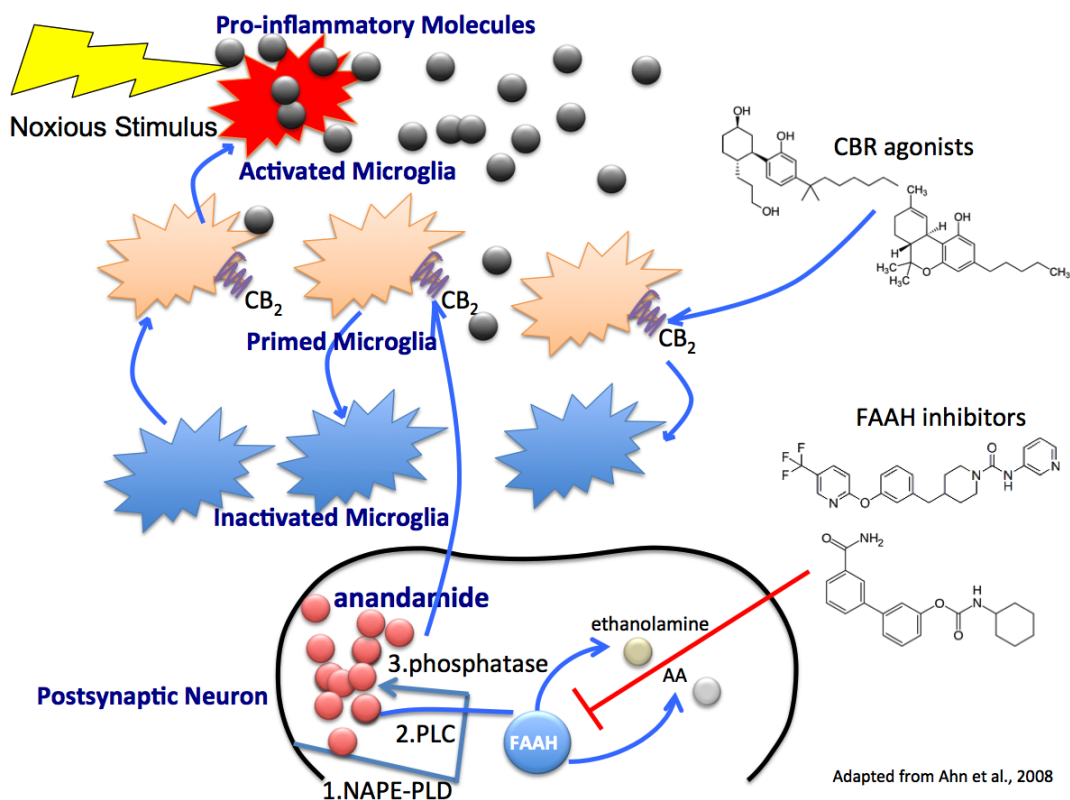
Figure 1.3

Figure 1.3. Depiction of microglial cells expressing CB2Rs. A noxious stimulus may produce tissue damage and subsequent release of pro-inflammatory molecules such as cytokines. These pro-inflammatory molecules serve to "prime" the microglial cells, causing them to express a large number of CB2Rs and migrate toward areas of inflammation. CB2R agonists can bind directly to CB2Rs on microglial cells and inhibit this migration as well as subsequent activation of microglial cells (Cabral et al., 2008), whereas FAAH inhibitors such as URB597 and PF3845 act indirectly by inhibiting the degradation of AEA into ethanolamine and arachidonic acid (AA) by FAAH, causing increased AEA levels and subsequent CB2R activation (Ahn et al., 2008). 2-AG may also be released by microglia via autocrine and paracrine mechanisms (not shown in figure) (Cabral et al., 2008).

1.3. Preclinical assays of pain-stimulated and pain-depressed behavior

Traditional preclinical assays of pain typically measure a category of pain-related behaviors that our lab has called "pain-stimulated behaviors." Pain-stimulated behaviors are defined as behaviors (e.g. withdrawal responses) that increase in rate or intensity following delivery of a noxious stimulus. In assays of pain-stimulated behavior, antinociception is indicated by decreases in the target behavior. However, decreases in pain-stimulated behavior can be produced either by a reduction in sensory sensitivity to the noxious stimulus (i.e. true analgesia) or by nonselective behavioral depressant effects (e.g. sedation, motor impairment) that limit the subject's ability to respond. Sedative drugs such as cannabinoid agonists are thus especially prone to produce false-positive antinociception in assays of pain-stimulated behavior (De Vry et al., 2004; Finn et al., 2004; Kwilasz and Negus, 2012). In this dissertation, two assays of pain-stimulated were employed: lactic acid-stimulated stretching and lipopolysaccharide (LPS)-stimulated mechanical allodynia. In the assay of lactic acid-stimulated stretching, animals received an IP injection of dilute lactic acid and were immediately observed for stretching behavior for a 30 min period. A stretch is defined as a contraction of the abdomen that occurs concurrently with extension of at least one hind limb, and this behavior increases (pain-*stimulated* behavior) following delivery of an IP acid injection, indicative of nociception. In the assay of LPS-stimulated mechanical allodynia, LPS, a pro-inflammatory constituent of gram-negative bacterial cell walls, is administered as an IP injection, which has been shown in previous studies to produce mechanical allodynia (Cahill et al., 1998; Hains et al., 2010). In these studies, mechanical allodynia was defined as an increased paw-withdrawal response (pain-*stimulated* behavior) to a series

of Von Frey filaments, of graded stiffness, applied to the paw. Clinically effective analgesics such as ketoprofen and morphine (Flecknell, 2009; Sarzi-Puttini et al., 2010) have been shown to be effective in assays of pain-stimulated behavior (Pereira Do Carmo et al., 2009; Kwilasz and Negus, 2012); however, clinically ineffective analgesics that produce sedation, such as kappa opioid agonists and dopamine receptor antagonists, also produce false-positive antinociception in assays of pain-stimulated behavior (Stevenson et al., 2006; Negus et al., 2010b). These data suggest that preclinical assays of pain-stimulated behavior alone are not sufficient to predict the efficacy of candidate analgesics.

In contrast to assays of pain-stimulated behavior, "pain-depressed behaviors" are defined as behaviors such as feeding, locomotion, or operant behavior that decrease in rate or intensity following delivery of a noxious stimulus. Assays of pain-depressed behavior have two attributes important to the assessment of candidate analgesics. First, antinociception is indicated by increases in the target behavior, and as a result, assays of pain-depressed behavior are not vulnerable to false-positive effects caused by nonselective behavioral depression (Negus et al., 2010a; Negus et al., 2010b; Kwilasz and Negus, 2012). Second, assays of pain-depressed behavior may model pain-related functional impairment and/or depressed mood used to assess pain in both human and veterinary medicine (Cleeland and Ryan, 1994; Dworkin et al., 2005; National Research Council, 2011), and thus may provide insight into effects of candidate analgesics on these clinically relevant components of pain (Negus et al., 2006; Negus et al., 2010a). In view of these attributes, we have argued that assays of pain-depressed behavior may complement conventional assays of pain-stimulated behavior and increase the

predictive validity of preclinical candidate analgesic assessment (Negus et al., 2006; Negus et al., 2010a). In this dissertation, three assays of pain-depressed behavior were employed: lactic acid-depressed intracranial self-stimulation (ICSS), lactic acid-depressed feeding, and LPS-depressed ICSS. ICSS is an operant assay in which subjects respond on a lever to receive pulses of electrical stimulation delivered via electrodes implanted in the brain's "reward pathway" (Pereira Do Carmo et al., 2009a; Negus et al., 2010a; Negus et al., 2010b; Negus et al., 2011). In the assays of acid depressed ICSS or acid-depressed feeding, an acute IP injection of dilute lactic acid served as the noxious stimulus to depress both ICSS and feeding behaviors. Similarly, in the assay of LPS-depressed ICSS, either chronic or acute IP injections of LPS were administered, which produced depression of ICSS behavior. LPS or pro-inflammatory cytokine administration has previously been shown to produce inflammation-related decreases in behavior such as ICSS (Anisman et al., 1996; Anisman et al., 1998; Borowski et al., 1998; Barr et al., 2003; van Heesch et al., 2013), feeding (Kubera et al., 2013), and social interaction (Konsman et al., 2008). It was hypothesized that all cannabinoid drugs tested would produce antinociception in assays of pain-stimulated behavior, whereas they would lack or show reduced efficacy in assays of pain-depressed behavior, especially under conditions in which an acute noxious stimulus was used (see section 1.5 below).

1.4. Neurobiology for convergence of ICSS, pain, and cannabinoids

ICSS increases mesolimbic DA. Lateral hypothalamic ICSS (i.e. the type of ICSS used in the studies of this dissertation) has been shown to stimulate increases in mesolimbic dopamine (DA) (Neill et al., 2002; Wise, 2005; Cheer et al., 2007). These ICSS-stimulated increases in mesolimbic DA, most commonly observed in striatal areas such as the NAc, have been shown to be dependent on release of glutamate from brain areas such as the LH and septum to the VTA. The VTA is one of the main brain regions in which cell bodies of dopaminergic afferents are found. Glutamate activates glutamate receptors on the cell bodies of these dopaminergic neurons, and stimulates the dopaminergic neurons in the VTA to release DA into the NAc (Shizgal, 1989). DA in the NAc then stimulates D1 and D2 DA receptors on GABAergic medium spiny neurons in the NAc, which has been correlated with reward-related events and behaviors, such as operant drug self-administration, increases in locomotor activity, and ICSS (Neill et al., 2002; Wise, 2005; Cheer et al., 2007). Similarly, decreases in mesolimbic DA have been correlated with depressed activity and/or mood as well increased pain (Neugebauer et al., 2009; Dellagioia et al., 2012; Jarcho et al., 2012).

IP Lactic acid decreases mesolimbic DA. The studies in chapters 2-4 of this dissertation employ IP lactic acid as a noxious stimulus. Acids such as lactic acid are a source of protons (i.e. in the form of hydronium ions), which when placed in an aqueous solution will increase the concentration of protons in that solution. When lactic acid is administered to the peritoneal cavity of a rat, the concentration of proton ions in the peritoneal cavity will thus increase. Primary afferent nociceptors present in the

peritoneal cavity possess receptors for protons such as TRPV1 and acid-sensing ion channels (ASICs), and are stimulated by activation of these receptors (Holzer, 2011). Moreover, lactic acid may cause tissue damage, which causes release of pro-inflammatory molecules such as cytokines, and these molecules can further depolarize primary afferent nociceptors to transmit pain signals (Rice, 2006). Pro-inflammatory molecules have also been shown to increase expression of proton-sensitive ion channels such as TRPV1 on primary afferent nociceptors (Rice, 2006; Holzer and Holzer-Petsche, 2009), and this phenomenon could also contribute to an enhanced pain signal following acid administration. The primary pain pathway and its connections to reward-related regions of the brain are shown in Figure 1.2. In Figure 1.4, the intersection between the effects of noxious stimulation, ICSS, and cannabinoids on these reward-related brain regions is illustrated in greater detail. ICSS indirectly stimulates dopaminergic neurons that project to the NAc via glutamatergic afferents in the LH and septum; however these dopaminergic neurons are also inhibited by noxious stimulation in at least two ways. First, peripheral noxious stimulation such as after IP lactic acid administration activates primary nociceptors and ultimately glutamatergic projections from the PBN and Amg to GABAergic interneurons in the VTA (Jhou et al., 2009; Neugebauer et al., 2009; Coizet et al., 2010). These GABAergic interneurons can then inhibit mesolimbic dopaminergic neurons in the VTA. Second, tissue damage following IP lactic acid administration can cause release of pro-inflammatory molecules such as cytokines, and these molecules may further sensitize primary afferents to produce increased stimulation of pain pathways (Rice, 2006; Holzer and Holzer-Petsche, 2009). Pro-inflammatory molecules may also signal at the blood brain barrier

to cause release of pro-inflammatory molecules and subsequent inflammatory

processes in the brain (Erickson et al., 2012). Brain inflammation, pain, and depression

of mood have also been correlated with decreased mesolimbic DA (Neugebauer et al.,

2009; Dellagioia et al., 2012; Jarcho et al., 2012), although mechanisms underlying

these processes are not fully understood.

CB1/2R agonists increase mesolimbic DA. CB1/2R agonists have been shown to

increase mesolimbic DA (Tanda et al., 1997; Lecca et al., 2006), and thus may be able

to reverse the depression of mesolimbic DA by noxious stimulation. Cannabinoid

receptor agonists have also been shown to activate CB1Rs in the VTA (Fitzgerald et al.,

2012), which are thought to disinhibit dopaminergic cell activity by presynaptically

inhibiting GABAergic interneurons in this region. Moreover, CB1R agonists have been

shown to stimulate VTA dopaminergic cell firing activity (French et al., 1997). CB1Rs

would thus be in a position to inhibit/reverse the excitatory pain signals from the PBN

and Amg to the GABAergic interneurons of the VTA. In addition to the effects of CB1R

agonists on pain signals, CB2R agonists have also been shown to inhibit immune cell-

related functions, such as macrophage- and microglia-activation and migration

(Wilkerson and Milligan, 2011). Inhibition of macrophage activation in the peritoneal

cavity following IP acid administration could decrease further release of pro-

inflammatory molecules that desensitize the primary afferent nociceptor, whereas

inhibition of microglial activation in the spinal cord and brain may also contribute to

decreased pain pathway activation and subsequent decreases in mesolimbic DA (Rice,

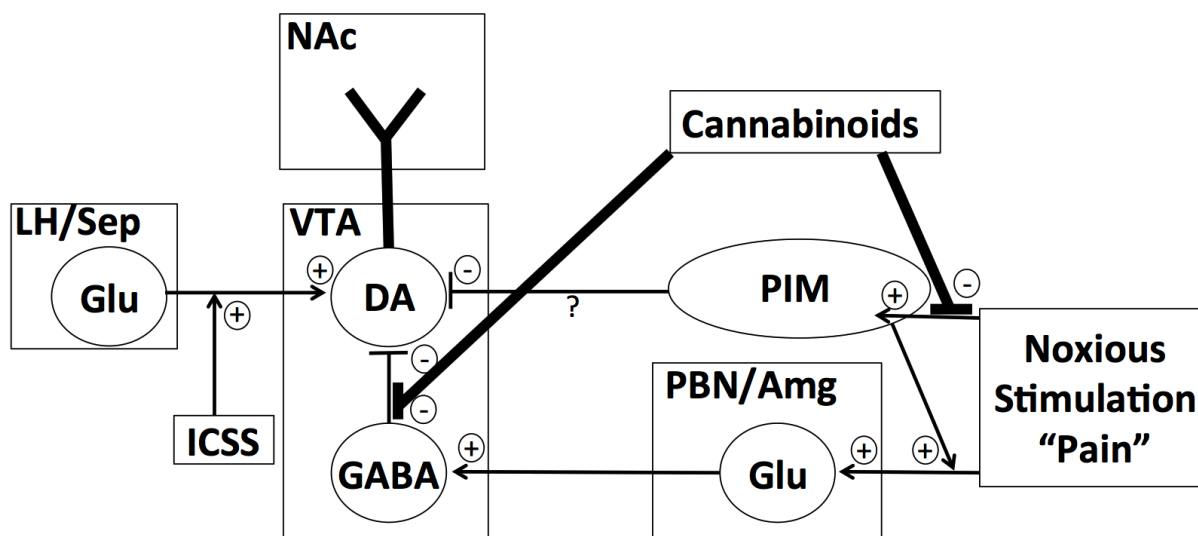2006; Holzer and Holzer-Petsche, 2009).

Figure 1.4

Figure 1.4. Depiction of the intersection between ICSS, pain, and cannabinoids on reward-related circuitry. ICSS behavior is dependent on mesolimbic DA and glutamate. Glutamatergic afferents from regions that include LH and septum (Sep) are stimulated directly by ICSS and subsequently stimulate dopaminergic cell bodies in the VTA. Noxious stimulation increases glutamatergic signals at primary afferent nociceptors, which ultimately project signals to brain regions such as the PBN and Amg. The PBN and Amg subsequently can stimulate GABAergic interneurons in the VTA to inhibit DA release in the NAc. Noxious stimulation also stimulates production of pro-inflammatory molecules (PIM), which can further enhance the sensitivity of nociceptors to noxious stimulation. CB1R agonists can disinhibit VTA DA neurons by presynaptically inhibiting GABA release from GABAergic interneurons in the VTA. This property also imbues CB1R agonists with the potential to block/reverse the inhibition of mesolimbic DA neurons via noxious stimulation signals from the PBN and Amg. Moreover, CB2R agonists can inhibit macrophage and microglial cell activation, which reduces release of pro-inflammatory cytokines in both peripheral and central regions, respectively. Ultimately, this may lead to a blockade/reversal of pain-induced decreases in mesolimbic DA.

1.5. Introduction to data chapters

Chapter 2. Effects of the mixed CB1R/CB2R agonists THC and CP55940 on acute pain-stimulated and pain-depressed behavior. In these studies, intraperitoneal (IP) injection of dilute acid served as an acute noxious stimulus to stimulate stretching (a pain-stimulated behavior) and to depress ICSS (a pain-depressed behavior). Initial experiments indicated that the cannabinoid agonists THC and CP55940 failed to produce antinociception in the assay of acid-depressed ICSS. Two follow-up studies were conducted to further evaluate conditions under which THC and/or CP55940 might be effective. First, previous studies with another drug class (delta opioid agonists) showed that expression of antinociception in the assay of acid-depressed ICSS could be obscured by rate-decreasing effects, but that repeated drug treatment could produce selective tolerance to rate-decreasing effects and unmask antinociception (Negus et al., 2012). Accordingly, THC effects in assays of acid-stimulated stretching and acid-depressed ICSS were evaluated during chronic THC administration to test the hypothesis that repeated THC might produce selective tolerance to rate-decreasing effects and unmask antinociception in the assay of acid-depressed ICSS. Second, effects of THC and CP55940 were evaluated in an assay of acid-induced depression of feeding (Stevenson et al., 2006; Kwilasz and Negus, 2012). Feeding is reliably stimulated by THC and other cannabinoid agonists in the absence of pain (Williams et al., 1998; Miller et al., 2004; Jarbe and DiPatrizio, 2005; Farrimond et al., 2011), suggesting that cannabinoids might be more effective in blocking acid-induced depression of feeding than acid-induced depression of ICSS. The nonsteroidal anti-inflammatory drug (NSAID) and clinically effective analgesic ketoprofen (Flecknell,

2009; Sarzi-Puttini et al., 2010) was tested as a positive control in assays of acid-stimulated and acid-depressed behavior. In accordance with the lack of efficacy of cannabinoid agonists to treat acute pain in clinical studies (Rice, 2006; Karst et al., 2010; Kraft, 2012), we predicted that THC and CP55940 would be ineffective whereas ketoprofen would be effective to produce antinociception in assays of pain-depressed behavior.

Chapter 3. Effects of the FAAH inhibitors URB597 and PF3845 on acute pain-stimulated and pain-depressed behavior. In these studies, we evaluated the effects of two structurally-unrelated FAAH inhibitors, URB597 and PF3845, in assays of acute pain-stimulated and pain-depressed behavior. Plasma and brain levels of the fatty acid ethanolamines AEA, PEA, and OEA were also quantified as biomarkers for FAAH inhibitor activity. As in the previous chapter, an IP injection of dilute lactic acid served as the noxious stimulus to stimulate stretching and depress ICSS. Both URB597 and PF3845 have been shown to produce antinociception in several preclinical assays of pain-stimulated behavior (Ahn et al., 2009; Clapper et al., 2010; Booker et al., 2012; Ghosh et al., 2012), and furthermore to produce a significantly reduced profile of side effects compared to direct CB1R agonists (Karst and Wippermann, 2009; Schlosburg et al., 2009; Alvarez-Jaimes and Palmer, 2011). Due to the reduced side effect profile of FAAH inhibitors, including reduced sedation, we predicted that FAAH inhibitors might be more effective than cannabinoid receptor agonists in assays of pain-depressed behavior.

Chapter 4. Effects of the CB2R agonist GW405833 on acute pain-stimulated and pain-depressed behavior. In these studies, we assessed the effects of the CB2R

agonist GW405833 in assays of acute pain-stimulated and pain-depressed behavior. As in the previous chapters, the noxious stimulus employed in these assays was an acute IP injection of lactic acid, which either stimulated stretching or depressed ICSS. Due to their antinociceptive effects in many models of acute and chronic pain and their reduced side-effect profile versus mixed CB1R/CB2R agonists including reduced sedation (Malan et al., 2001; Valenzano et al., 2005; Whiteside et al., 2005; Hsieh et al., 2011), we predicted that a CB2R agonist might also be more effective than mixed CB1R/CB2R agonists at producing antinociception in an assay of pain-depressed behavior.

Chapter 5. Effects of THC on acute and repeated LPS-induced stimulation of mechanical allodynia and depression of ICSS. In these studies, repeated or acute injections of LPS, a pro-inflammatory constituent of gram-negative bacterial cell walls, were used to model inflammatory-related pain as described previously (Watkins et al., 1994; Cahill et al., 1998; Hains et al., 2010). Hypersensitivity of withdrawal responses from mechanical stimuli applied to the hindpaw (mechanical allodynia) served as a measure of pain-stimulated behavior, and decreases in ICSS served as a measure of pain-depressed behavior. Both mechanical sensitivity and ICSS were evaluated daily in each rat during chronic IP LPS treatment. LPS was also administered IP acutely in a separate experiment. In this study, change in body temperature after acute LPS administration was also measured as a physiological indicator of LPS effects. THC was evaluated for its ability to block LPS-induced stimulation of mechanical allodynia and depression of ICSS. We predicted that both chronic and acute LPS would stimulate mechanical allodynia and depress ICSS and that acute LPS would increase body temperature. Given that cannabinoids produce robust anti-inflammatory in preclinical

studies (Puffenbarger et al., 2000; Croxford and Yamamura, 2005; Pascual et al., 2005; Valenzano et al., 2005; Whiteside et al., 2005; Cabral et al., 2008; Burstein and Zurier, 2009; Wilkerson and Milligan, 2011), have displayed some efficacy in a clinical trial for the treatment of the inflammatory disorder rheumatoid arthritis (Blake et al., 2006), and are clinically indicated in several countries to treat muscle spasticity and related pain associated with another inflammatory disorder, multiple sclerosis (Leussink et al., 2012), we predicted that THC would be effective to block LPS-stimulated mechanical allodynia and LPS-depressed ICSS.

CHAPTER TWO

Effects of the mixed CB1R/CB2R agonists THC and CP55940

on acute pain-stimulated and pain-depressed behavior.

Published:

2.1. Introduction

Marijuana has been used for centuries to treat pain, and THC, the primary active constituent of marijuana, as well as other cannabinoid agonists such as CP55940 produce antinociception in nearly all preclinical assays of pain (Rice, 2006; Karst et al., 2010). Despite the robust antinociceptive effects of cannabinoid agonists in preclinical assays of pain in animals, in well-controlled clinical trials in humans, cannabinoid agonists do not produce analgesia against acute pain and have weak efficacy with a narrow therapeutic window against chronic pain (Raft et al., 1977; Rice, 2006; Karst et al., 2010; Kraft, 2012). This disparity between preclinical and clinical findings suggest that traditional preclinical assays of pain used to study cannabinoids in animals may not be sufficient to predict clinical analgesic effects in humans.

Traditional preclinical assays used to measure pain can be classified as assays of pain-stimulated behavior. In assays of pain-stimulated behavior, delivery of a noxious

stimulus increases the rate, frequency, or intensity of the target behavior (e.g. withdrawal response), and antinociception is indicated by drug-induced decreases in the target behavior. However, drug-induced decreases may be the result of either nonspecific behavioral depression (i.e. sedation/motor suppression) or a decreased sensitivity to the noxious stimulus (i.e. analgesia). Cannabinoid agonists such as THC and CP55940 and other drugs that produce behavioral depression are thus prone to producing false-positive antinociception in assays of pain-stimulated behavior (De Vry et al., 2004; Finn et al., 2004; Kwilasz and Negus, 2012). In contrast, in assays of pain-depressed behavior, delivery of a noxious stimulus decreases the rate, frequency, or intensity of the target behavior, and antinociception is indicated by drug-induced increases in the target behavior. Behavioral depressants thus do not produce false-positive antinociception in assays of pain-depressed behavior (Negus et al., 2010a; Negus et al., 2010b; Kwilasz and Negus, 2012). Furthermore, pain-depressed behavior may model clinically relevant dimensions of pain, such as functional impairment and/or depressed mood, that are often used to diagnose pain in both human and veterinary medicine (Cleeland and Ryan, 1994; Dworkin et al., 2005; National_Research_Council, 2011).

The goal of the present study was to assess the effects of THC and CP55940 in assays of acute pain-stimulated and pain-depressed behavior. THC and CP55940 were tested in assays of lactic acid-stimulated stretching, lactic acid-depressed ICSS, and lactic acid-depressed feeding. The nonsteroidal anti-inflammatory drug and clinically effective analgesic ketoprofen (Flecknell, 2009; Sarzi-Puttini et al., 2010) was also included as a positive control.

2.2. Methods

Subjects

Seventy-nine male Sprague-Dawley rats (Harlan, Frederick, MD, USA) weighing approximately 300-320 g (age 10-11 weeks) at the time of surgery and/or delivery were individually housed and maintained on a 12 h light/dark cycle with lights on from 6:00 a.m. to 6:00 p.m. Rats had free access to food and water except during testing. Animal maintenance and research were in compliance with National Institutes of Health guidelines on care and use of animal subjects in research and adhered to guidelines of the Committee for Research (National Research Council, 2011). All animal use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Assay of lactic acid-stimulated stretching

Behavioral procedure. Twenty-six rats that failed to meet the criteria for ICSS within 4 weeks (see below) were used for studies of lactic acid-stimulated stretching as described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013). During test sessions, rats were placed into an acrylic test chamber (31.0 x 20.1 x 20.0 cm) for a 30 min observation period that began immediately after injection of dilute lactic acid (1.8% in a volume of 1 ml/kg). A stretch was operationally defined as a contraction of the abdomen followed by a stretching of at least one hind limb, and the number of stretches during the observation period was counted.

Studies with acute THC were conducted in four phases. First, a THC dose-effect curve was determined by administering THC (0.32-10 mg/kg or vehicle) 30 min prior to

acid. Doses were delivered in a Latin-square dose order across rats and separated by at least one week. Second, the time course of effects produced by 3.2 mg/kg THC was determined by varying the interval between administration of THC and acid (10, 30, 100, 300 min, and 24 h). A dose of 3.2 mg/kg THC was chosen for time course studies because it was the lowest dose to significantly decrease acid-stimulated stretching during dose-effect testing. Each pretreatment time was tested in a different test session in randomized order, and test sessions were separated by at least one week. Third, to assess the role of cannabinoid 1 receptors in mediating THC effects, THC-induced antinociception was evaluated for its sensitivity to antagonism by the cannabinoid 1 receptor antagonist rimonabant. For these studies, rimonabant (0.01-1.0 mg/kg or vehicle) was administered 20 min prior to THC (3.2 mg/kg), and acid was administered 30 min after THC. All THC and rimonabant doses were delivered in a Latin-square dose order across rats and separated by at least one week.

Finally, to assess the potential for antinociceptive tolerance to repeated THC, acid-stimulated stretching was evaluated following chronic treatment with THC (3.2 mg/kg/day). Initially a vehicle test was conducted in which rats were administered THC vehicle prior to treatment with acid. Beginning one week later, THC (3.2 mg/kg) was administered once daily for 22 days. On days 1, 8, 15 and 22, acid (1.8% in 1 ml/kg) was administered 30 min after THC, and the stretching response was evaluated. Effects of 3.2 mg/kg THC on acid-stimulated stretching were redetermined one additional time two weeks after termination of chronic THC.

To provide a comparison for results with THC, two additional groups of rats were used to evaluate the high-efficacy cannabinoid 1 receptor agonist CP55940 (0.0032-0.1

mg/kg or vehicle) and the NSAID ketoprofen (1 mg/kg or saline). In both cases, the test drug was administered 30 min prior to acid, and tests were separated by one week.

Data Analysis. Drug effects on acid-stimulated stretching were evaluated by repeated measures one-way analysis of variance (ANOVA) or *t* test as appropriate. A significant ANOVA was followed by Newman Keul's or Dunnett's post hoc test, and the criterion for significance was set at $p < 0.05$.

Assay of intracranial self-stimulation (ICSS)

Surgery. All rats were anesthetized with isoflurane (2.5-3% in oxygen; Webster Veterinary, Phoenix, AZ, USA) for implantation of stainless steel electrodes (Plastics One, Roanoke, VA, USA). One pole (the cathode) of each bipolar electrode was 0.25 mm in diameter and covered with polyamide insulation except at the flattened tip, whereas the other pole (the anode) was 0.125 mm in diameter and uninsulated. The cathode was implanted in the left medial forebrain bundle at the level of the lateral hypothalamus (2.8 mm posterior to bregma, 1.7 mm lateral from the midsagittal suture, and 8.8 mm below the skull). The anode was wrapped around one of the three skull screws to serve as the ground, and the skull screws and electrode assembly were secured to the skull with orthodontic resin. The animals were allowed to recover for at least 7 days prior to commencing ICSS training.

Apparatus. Experiments were conducted in sound-attenuating boxes that contained modular acrylic test chambers (29.2 x 30.5 x 24.1 cm) equipped with a response lever (4.5 cm wide, extended 2 cm through the center of one wall, 3 cm off the floor), stimulation lights (three lights colored red, yellow, and green, positioned 7.6 cm

directly above the response lever), a 2 W house light, and an ICSS stimulator (Med Associates, St. Albans, VT, USA). Electrodes were connected to the stimulator via a swivel connector (Model SL2C, Plastics One, Roanoke, VA, USA). The stimulator was controlled by computer software that also controlled programming parameters and data collection (Med Associates, St. Albans, VT, USA).

Behavioral procedure. After initial shaping of lever press responding, rats were trained under a continuous reinforcement schedule of brain stimulation using procedures similar to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013). During sessions lasting 30-60 min, each lever press resulted in the delivery of a 0.5 s train of square wave cathodal pulses (0.1 ms pulse duration), and stimulation was accompanied by illumination of the stimulus lights over the lever. Responses during the 0.5 s stimulation period did not earn an additional stimulation. Initially, the frequency of stimulation was held constant at 158 Hz, and the stimulation intensity for each rat was adjusted gradually to the lowest value that would sustain a high rate of reinforcement (> 30 stimulations/min). This intensity (100-280 µA across rats) was then held constant for the remainder of the study, and frequency manipulations were introduced. Sessions involving frequency manipulations consisted of sequential 10 min components. During each component, a descending series of 10 current frequencies (158 to 56 Hz in 0.05 log increments) was presented, with each frequency available during sequential 1 min frequency trials. Each frequency trial began with a 10 s time out, during which responding had no scheduled consequences. During the last 5 s of this time out, five noncontingent "priming" stimulations were delivered at the frequency available during that trial, and the lever lights were illuminated during

each stimulation. Noncontingent stimulations were separated by intervals of 0.5 s. This noncontingent stimulation was then followed by a 50 s "response" phase, during which responding produced electrical stimulation under the continuous reinforcement schedule. Training continued with presentation of three to six sequential components per day until rats reliably responded for only the first four to six frequency trials of all components for at least three consecutive days. In general, rats were implanted with electrodes and trained on ICSS procedures in groups of 10-12. The first six rats in each group to meet training criteria were then advanced to testing, while the remaining rats were assigned to assays of acid-stimulated stretching as described above.

Once training was completed, testing was initiated. The first component of each test session was considered to be an acclimation component, and data from this component were discarded. Data from the second and third "baseline" components were used to calculate baseline parameters of the frequency-rate curves for that session (see "Data Analysis"). Drugs were administered immediately after removing the subjects from the operant chamber after the third baseline component. Studies of THC effects on ICSS were conducted in four phases. In the first phase, the effects of acute THC on ICSS were studied in two separate groups of rats. In the first group of rats, THC effects on ICSS were studied in the absence of the noxious stimulus (control ICSS). Subjects were placed in their home cages after administration of THC (0.32-10 mg/kg or vehicle) and then transferred back to the operant chambers at designated times (30, 100, 180, 300 min) for two consecutive "test" components, totaling 20 min at each time point. In the second group of rats, THC effects on ICSS were studied in the presence of the noxious stimulus (acid-depressed ICSS). Subjects were administered THC (0.32-3.2

mg/kg or vehicle) 30 min prior to lactic acid (1.8% in a volume of 1 ml/kg), which was administered immediately before two consecutive "test" components. THC and acid doses were administered in Latin-square order and were separated by at least one week. The second phase examined the effects of 3.2 mg/kg THC administered 180 and 300 min before acid treatment. These times were selected because initial results indicated that treatment with 3.2 mg/kg THC significantly decreased acid-stimulated stretching after 180 and 300 min but did not significantly decrease ICSS at these pretreatment times in the absence of a noxious stimulus. For these experiments, subjects were placed in their home cages after THC administration and then injected with acid and transferred back to the operant chamber at the designated time (180 or 300 min) for two consecutive "test" components. Each pretreatment time/dose combination was tested in a different test session in randomized order, and test sessions were separated by at least one week. In the third phase, the ability of rimonabant to block THC effects on ICSS was investigated. In these experiments, rimonabant (1 mg/kg or vehicle) was administered 50 min prior to testing and 20 min before THC (3.2 mg/kg or vehicle). THC and rimonabant doses were administered in Latin-square order and were separated by at least one week. In phases 1-3, training and test sessions were conducted Monday-Friday for the duration of the experiment, with test sessions conducted on Thursdays or Fridays.

The final phase of studies with THC examined effects of chronic THC in two separate groups of rats. In the first group, the effects of chronic THC were studied in the absence of the acid noxious stimulus (control ICSS). For these experiments, subjects initially received chronic treatment of THC vehicle (1 ml/kg/day) for three weeks while

being tested with THC (1-10 mg/kg or vehicle) once/week in a Latin-Square dose order. Following this treatment regimen, subjects were treated for 11 days with 1 mg/kg/day THC, 11 days with 3.2 mg/kg/day THC, and lastly 11 days with 10 mg/kg/day THC (i.e. 33 days of total THC treatment). On the last four days of treatment with each dose of chronic THC, subjects were tested with THC (1-10 mg/kg or vehicle) one dose/day in a Latin-Square dose order. Subjects that received test THC doses lower than the chronic THC dose for that day were administered the difference of the test and chronic doses at the end of the test session. In the second group of rats, the effects of chronic THC were studied in the presence of the noxious stimulus (acid-depressed ICSS). For these experiments, THC vehicle was initially administered prior to acid vehicle. One week later, THC vehicle was administered prior to acid. Beginning one week later, THC (3.2 mg/kg) was administered once daily for 22 days. On days 1, 8, 15, and 22, acid (1.8% in 1 ml/kg) was administered 30 min after THC, and ICSS was evaluated as described above. Effects of 3.2 mg/kg THC on acid-depressed ICSS were redetermined one additional time two weeks after termination of chronic THC. In both groups of rats, training and test sessions were conducted seven days/week.

In addition to these studies with THC, two additional groups of rats were tested with either CP55940 or ketoprofen. For these studies, one group was treated with CP55940 (0.01-0.32 mg/kg or vehicle) and the other with ketoprofen (1 mg/kg or saline) 30 min before treatment with 1.8% lactic acid or lactic acid vehicle (sterile water). Training and test sessions were conducted Monday-Friday for the duration of the experiment, with test sessions conducted on Thursdays or Fridays.

Data Analysis. The primary dependent variable in this ICSS procedure was the reinforcement rate in stimulations per minute during each frequency trial. To normalize these data, raw reinforcement rates from each trial in each rat were converted to percent maximum control rate (%MCR), with the MCR defined as the mean of the maximal rates observed during the second and third "baseline" components for that session in that rat. Thus, %MCR values for each trial were calculated as (Reinforcement Rate During a Frequency Trial ÷ Maximum Control Rate) x 100. For each test session, data from the second and third components were averaged to yield a baseline frequency-rate curve. Data from each test (two consecutive "test" components) were averaged for each test for each rat. Baseline and test curves were then averaged across rats to yield mean baseline and test curves for each manipulation. For statistical analysis, results were compared by repeated measures two-way ANOVA, with treatment and ICSS frequency as the two factors. A significant ANOVA was followed by Holm-Sidak post hoc test, and the criterion for significance was set at $p < 0.05$.

To provide an additional summary of ICSS performance, the total number of stimulations per component was calculated as the sum of stimulations delivered across all 10 frequency-trials of each component. Test data were then normalized to individual baseline data using the equation Percent Baseline Total Stimulations per Component = (Mean Total Stimulations per Test Component ÷ Mean Total Stimulations per Baseline Component) x 100. Data were then averaged across rats in each experimental condition and compared by repeated measures one-way ANOVA or two-way ANOVA where appropriate. A significant one-way ANOVA was followed by Newman Keul's or

Dunnett's post hoc test, a significant two-way ANOVA was followed by Holm-Sidak post hoc test, and the criterion for significance was set at $p < 0.05$.

Assay of lactic acid- and prefeeding-depressed feeding

Behavioral procedure. Sixteen rats were used for feeding studies. During test sessions, rats were placed into an acrylic test chamber (31 x 20.1 x 20 cm) within a sound- and light-attenuating cabinet for a 30 min feeding session. Rodent 45 mg purified food pellets (Product #F0021, Bio-Serv, Frenchtown, NJ) were delivered in pre-weighed glass petri dishes (60 x 15 mm) (Corning Life Sciences, Pittston, PA) securely taped to the bottom left corner of the test chamber. Spilled pellets/dust were collected at the end of the session and added back to the petri dish, which was then re-weighed. Percent Body Weight Food Consumed after each session was determined with the following equation: [(Pre-Session Dish Weight – Post-Session Dish Weight) ÷ [Daily Subject's Weight (g)] x 100]. Initial baseline feeding sessions were conducted for two weeks weeks until stable feeding baselines were achieved. Studies were conducted in four phases. First, pain-related depression of feeding was established by administering dilute lactic acid (0.56-1.8% or vehicle in a volume of 1 ml/kg) immediately before the test session. For these studies, drug vehicle was also administered 30 min before acid. Second, THC and ketoprofen effects on pain-related depression of feeding were determined by administering THC (0.32-3.2 mg/kg or vehicle) or ketoprofen (1 mg/kg or vehicle) 30 min prior to injection of lactic acid (1.8% or vehicle in a volume of 1 ml/kg), which was administered immediately before the test session. Third, satiation-related depression of feeding was established by exposing rats to a 60 min prefeeding session

30 min prior to the test session. Prefeeding sessions were similar to test sessions, except that they were conducted in separate chambers not housed in sound- and light-attenuated cabinets. Finally, THC and ketoprofen effects on satiation-related depression of feeding were determined by administering THC (0.1-1 mg/kg or vehicle) or ketoprofen (1 mg/kg or vehicle) immediately after a 60 min prefeeding session and 30 min prior to the test session. THC doses were delivered in a Latin-square dose order across rats and separated by at least one week. Ketoprofen was tested after THC. Rats were housed in their homecages with free access to food and water at all times except during feeding sessions.

In addition to these studies with THC and ketoprofen, an additional group of rats was tested with CP55940 under conditions of acid- and satiation-related depression of feeding. In this study, animals were treated with CP55940 (0.0032-0.032 mg/kg or vehicle) 30 min before the feeding session using procedures identical to those with THC and ketoprofen. In all feeding studies, rats were housed in their homecages with free access to food and water at all times except during feeding sessions. Training and test sessions were conducted Monday-Friday, with test sessions conducted on Wednesdays or Fridays.

Data Analysis. Drug effects on acid- and satiation-related depression of feeding were evaluated by repeated measures one-way ANOVA. A significant ANOVA was followed by Newman Keul's or Dunnett's post hoc test, and the criterion for significance was set at $p < 0.05$.

Drugs

Lactic acid was purchased from Sigma Chemical Co. (St. Louis, MO). THC, CP55940, and rimonabant were provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). Ketoprofen was purchased from Spectrum Chemical Co. (New Brunswick, NJ). Lactic acid was prepared in sterile water. THC, CP55940, and rimonabant were prepared in a vehicle consisting of ethanol, emulphor EL-620 (Rhone-Poulenc; Princeton, NJ), and sterile saline in a ratio of 1:1:18, respectively. Ketoprofen was prepared in sterile saline except in feeding tests, in which it was prepared in the same vehicle as THC. All solutions were injected intraperitoneally in a volume of 1 ml/kg.

2.3. Results

Effects of THC on acid-stimulated stretching. Figure 2.1 shows that THC produced dose-dependent, time-dependent, and rimonabant-reversible antinociception in the assay of acid-stimulated stretching. IP administration of acid (1.8% lactic acid in a volume of 1 ml/kg) stimulated approximately 30 stretches after administration of THC vehicle (*gray bars* in all panels). Figure 2.1a shows that stretching was significantly lower 30 min after administration of 3.2 and 10 mg/kg THC than after THC vehicle. Figure 2.1b shows that 3.2 mg/kg THC produced a significant reduction in acid-stimulated stretching from 10-300 min with recovery after 24 h. Figure 2.1c shows that rimonabant dose-dependently blocked the antinociceptive effect of 3.2 mg/kg THC, and significant antagonism was achieved at a dose of 1.0 mg/kg rimonabant.

Effects of THC on ICSS in the absence of a noxious stimulus. Figure 2.2 shows that THC produced a dose-dependent, time-dependent, and rimonabant-reversible decrease in ICSS in the absence a noxious stimulus. During each test session, a "baseline" frequency-rate curve was determined before testing to permit determination of the Maximum Control Rate (MCR) for that session. Over the course of the entire study this group of rats, the average MCR was 59.7 ± 2.6 stimulations/trial and the average baseline total stimulations was 266.6 ± 56.8. Reinforcement rates during each frequency trial of a session were then expressed as a percentage of that session's MCR, and the average baseline frequency-rate curve for studies with THC is shown in Figure 2.2a as a *gray line*. Rats generally did not respond at frequencies of 56-89 Hz, and reinforcement rates increased across a frequency range of 89-158 Hz. Maximum reinforcement rates were usually observed at the highest stimulation frequencies. When administered 30 min prior to an ICSS session, THC produced a dose-dependent rightward and downward shift in the ICSS frequency-rate curve (Fig. 2.2a). Low doses of 0.32 and 1 mg/kg THC had no effect on control ICSS in the absence of the acid noxious stimulus; however, treatment with 3.2 mg/kg THC significantly decreased reinforcement rates at a single frequency of 89 Hz, and treatment with 10 mg/kg THC significantly decreased reinforcement rates at frequencies of 89-158 Hz compared to treatment with THC vehicle. THC also produced a dose-dependent and time-dependent decrease in total stimulations (Fig. 2.2b). Low doses of 0.32 and 1 mg/kg THC had no effect on total stimulations at any time point, but 3.2 mg/kg THC significantly decreased total stimulations at a pretreatment time of 30 min, and 10 mg/kg THC significantly decreased total stimulations at pretreatment times of

30-180 min. THC-induced decreases in ICSS were also blocked by pretreatment with rimonabant (Fig. 2.2c). Rimonabant (1 mg/kg) administered 50 min before testing had no effect on ICSS alone, but significantly blocked decreases in ICSS induced by 30 min pretreatment with 3.2 mg/kg THC.

Effects of THC on acid-induced depression of ICSS. Figure 2.3 shows that the same noxious stimulus used in the stretching assay (IP injection of 1.8% lactic acid in 1 ml/kg) depressed ICSS. Treatment with acid vehicle had little effect on the frequency-rate curve; however, treatment with 1.8% lactic acid depressed ICSS, producing a significant rightward shift in the frequency-rate curve and a decrease in total stimulations delivered across all frequencies. Figure 2.4 shows that THC failed to produce antinociception in the assay of acid-depressed ICSS. Rather, when administered 30 min before acid treatment, THC produced a further, dose-dependent depression of ICSS. Low doses of 0.32 and 1 mg/kg THC significantly decreased ICSS at a single frequency of 141 Hz, and treatment with 3.2 mg/kg THC significantly decreased ICSS at frequencies of 126, 141, and 158 Hz compared to treatment with THC vehicle (Fig. 2.4a). Additionally, treatment with THC (0.32-3.2 mg/kg) or its vehicle 30 min before acid administration significantly decreased total stimulations delivered across all frequencies, and there was a trend for treatment with THC to exacerbate acid-induced decreases in total stimulations, but this trend did not achieve statistical significance (Fig. 2.4b). A dose of 3.2 mg/kg THC also failed to block acid-induced depression of ICSS when it was administered 180 or 300 min before acid treatment (Fig. 2.4c). These were times at which this dose of THC significantly decreased acid-

stimulated stretching (Fig. 2.1b) but did not significantly decrease ICSS in the absence of a noxious stimulus (Fig. 2.2b).

Effects of CP55940 and ketoprofen on acid-stimulated stretching and on ICSS in the absence or presence of acid. Figure 2.5a-b shows that CP55940, like THC, produced antinociception in the assay of acid-stimulated stretching but not in the assay of acid-depressed ICSS. However, unlike THC, CP55940 was approximately 10-fold more potent to produce antinociception in the assay of acid-stimulated stretching than to decrease control ICSS in the absence of the acid stimulus. In particular, doses of 0.01 and 0.032 mg/kg CP55940 produced significant antinociception in the assay of acid-stimulated stretching (Fig. 2.5a) while 10-fold higher doses of 0.1-0.32 mg/kg were required to decrease control ICSS (Fig. 2.5b, *open bars*). Despite this evidence for selective antinociception, CP55940 still failed to block acid-induced depression of ICSS. Rather, CP55940 only exacerbated acid-induced depression of ICSS (Fig. 2.5b, *closed bars*) at the same doses of 0.1 and 0.32 mg/kg that decreased control ICSS (Fig. 2.5a, *open bars*). Figure 2.5c-d shows that, in contrast to THC and CP55940, the NSAID ketoprofen produced antinociception in assays of both acid-stimulated stretching and acid-depressed ICSS. Thus, a dose of 1.0 mg/kg ketoprofen significantly reduced acid-stimulated stretching (Fig. 2.5c). The same dose of 1.0 mg/kg ketoprofen had no effect on control ICSS, but significantly blocked acid-induced depression of ICSS (Fig. 2.5d).

Effects of chronic THC treatment on acid-stimulated stretching and on ICSS in the absence or presence of acid. Chronic THC treatment produced a dose-dependent tolerance to THC-induced rate-decreasing effects on control ICSS. Specifically, complete tolerance was observed to the rate-decreasing effects of 3.2

mg/kg THC following chronic treatment with 3.2 and 10 mg/kg/day THC but not following 1 mg/kg/day THC (Fig. 2.6). In the assay of acid-stimulated stretching, chronic THC treatment (3.2 mg/kg/day) produced duration-dependent partial tolerance to THC-induced antinociceptive effects. Partial tolerance to THC-induced antinociceptive effects was observed on day 15 of chronic THC treatment (Fig. 2.7a). No greater tolerance was produced by an additional 7 days of treatment, and full THC antinociception recovered 2 weeks after termination of chronic THC. In contrast, chronic THC treatment (3.2 mg/kg/day) did not produce antinociception in the assay of acid-depressed ICSS (Fig. 2.7b) at treatment durations that produced significant antinociception in the assay of acid-stimulated stretching (Fig. 2.7a) and complete tolerance to THC-induced rate-decreasing effects on control ICSS (Fig. 2.6). Analysis of frequency-rate curves (*data not shown*) indicated that THC initially exacerbated acid-induced depression of ICSS on days 1 and 8 of chronic THC treatment, and that tolerance to this effect developed by days 15 and 22 of chronic THC treatment. No greater tolerance was produced by an additional 7 days of treatment, and THC exacerbation of acid-induced depression of ICSS recovered 2 weeks after termination of chronic THC.

Effects of THC, ketoprofen, and CP55940 on feeding depressed by acid or prefeeding. Lactic acid produced a concentration-dependent decrease in food consumption, and exposing rats to a 60 min prefeeding session before testing also significantly decreased food consumption by approximately the same extent as 1.8% lactic acid (Fig. 2.8a). Neither THC nor ketoprofen significantly altered food consumption in the absence of acid or prefeeding (Fig. 2.8b). Ketoprofen but not THC significantly blocked acid-induced depression of feeding (Fig. 2.8c), and in contrast,

THC but not ketoprofen significantly attenuated prefeeding-induced depression of feeding (Fig. 2.8d). CP55940 did not produce significant effects on acid- (Fig. 2.8e) or prefeeding- (Fig. 2.8f) induced depression of feeding, although an intermediate dose of 0.01 mg/kg CP55940 did more than double mean food consumption after prefeeding. Higher CP55940 doses were not tested in the feeding assays because they significantly decreased both control and acid-depressed ICSS (Fig. 2.5b).
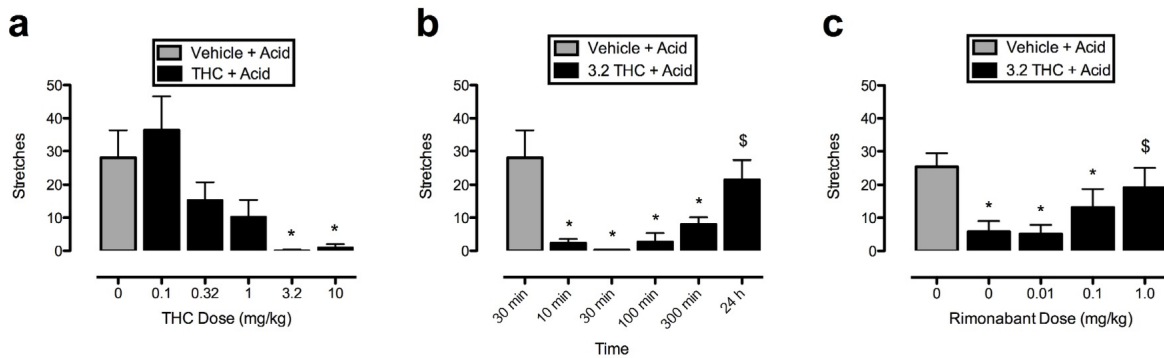
Figure 2.1

Figure 2.1. Δ9-tetrahydrocannabinol (THC) produced dose-dependent, time-dependent, and rimonabant-reversible blockade of lactic acid-stimulated stretching. The left panel *(a)* shows effects of THC (0.1-10 mg/kg) or its vehicle administered 30 min before acid treatment. Abscissa: dose THC in milligrams per kilogram. Ordinates (all panels): number of stretches observed during a 30 min observation period. The center panel *(b)* shows effects of THC (3.2 mg/kg) administered 10 min-24 h before acid treatment. Effects of vehicle administered 30 min before acid treatment are included for comparison. Abscissa: time following THC or vehicle administration. The right panel *(c)* shows the effects of 50 min pretreatment with rimonabant (0.01-1.0 mg/kg) or its vehicle and 30 min pretreatment with THC (3.2 mg/kg) before acid treatment. Effects of rimonabant vehicle + THC vehicle + acid are included for comparison. Abscissa: dose rimonabant in milligrams per kilogram. One-way ANOVA indicated significant main effects of THC treatment in panel *a* [$F(5,25)=6.63$; $p<0.001$], time in panel *b* [$F(5,25)=7.65$; $p<0.001$], and rimonabant dose in panel *c* [$F(4,20)=11.21$; $p<0.001$]. *Asterisks* (*) indicate significantly different from vehicle + acid in all panels or *dollar signs* ($) indicate significantly different from 30 min pretreatment with THC (3.2 mg/kg) in panel *b* and from THC (3.2 mg/kg) + rimonabant vehicle in panel *c* as determined by Newman-Keuls post hoc test, $p < 0.05$. All *bars* show mean ± SEM in six rats.

Figure 2.2

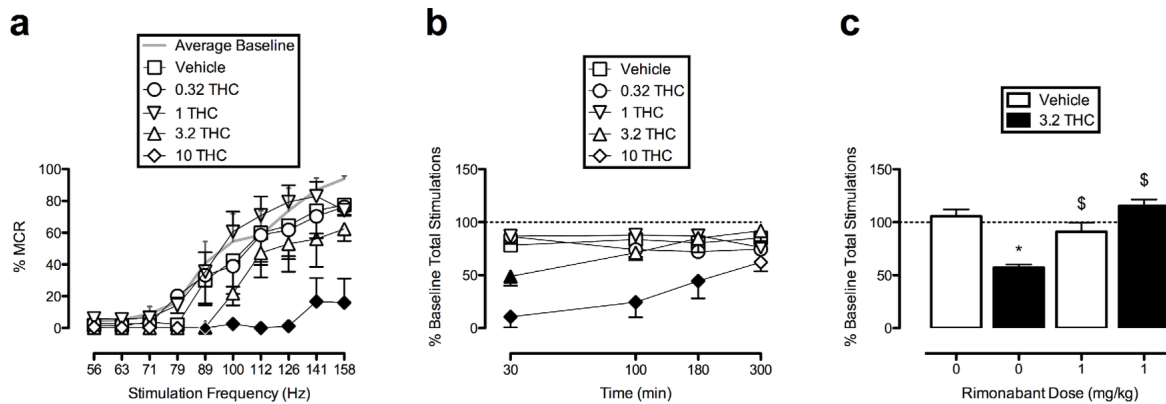**Figure 2.2.** Δ9-tetrahydrocannabinol (THC) produced dose-dependent, time-dependent, and rimonabant-reversible depression of intracranial self-stimulation (ICSS) in the absence of a noxious stimulus. The left panel *(a)* shows ICSS frequency-rate curves determined 30 min after treatment with THC (0.32-10 mg/kg) or its vehicle. Abscissa: frequency of electrical brain stimulation in hertz (log scale). Ordinate: percent maximum control response rate (%MCR). The average baseline ICSS frequency-rate curve for the entire study in this group of rats is shown by the *gray line* for comparison, but these data were not included in statistical analysis. Two-way ANOVA indicated a significant main effect of THC treatment [$F_{(4,20)}=11.78$; $p<0.001$], a significant main effect of frequency [$F_{(9,45)}=19.31$; $p<0.001$], and a significant frequency × treatment interaction [$F_{(36,180)}=4.77$; $p<0.001$]. *Filled symbols* indicate frequencies at which reinforcement rates after THC treatment were significantly lower than rates after THC vehicle treatment as determined by Holm-Sidak post hoc test, $p < 0.05$. All data show mean ± SEM in six rats. The center panel *(b)* shows the total number of stimulations per component expressed as a percent of baseline stimulations per component following treatment with THC (0.32-10.0 mg/kg) or its vehicle at various pretreatment times. Abscissa: time following THC or vehicle administration. Ordinate: percent baseline total number of stimulations per component. Two-way ANOVA indicated a significant main effect of THC treatment [$F_{(4,20)}=12.35$; $p<0.001$] and a significant treatment × time interaction [$F_{(12,60)}=5.93$; $p<0.001$]. *Filled symbols* indicate significantly lower than vehicle treatment at indicated time as determined by Holm-Sidak post hoc test, $p < 0.05$.

All data show mean ± SEM in six rats. The right panel *(c)* shows the total number of stimulations per component expressed as a percent of baseline stimulations per component following 50 min pretreatment with rimonabant (1 mg/kg) or its vehicle and 30 min pretreatment with THC (3.2 mg/kg) or its vehicle. Abscissa: dose rimonabant in milligrams per kilogram. Ordinate: percent baseline total number of stimulations per component. One-way ANOVA indicated a significant main effect of treatment [$F_{(3,12)}=17.04$; $p<0.001$]. The a*sterisk* (*) indicates significantly different from rimonabant vehicle + THC vehicle and *dollar signs* ($) indicate significantly different from rimonabant vehicle + THC (3.2 mg/kg) as determined by Newman-Keuls post hoc test, $p < 0.05$. All *bars* show mean ± SEM in five rats.
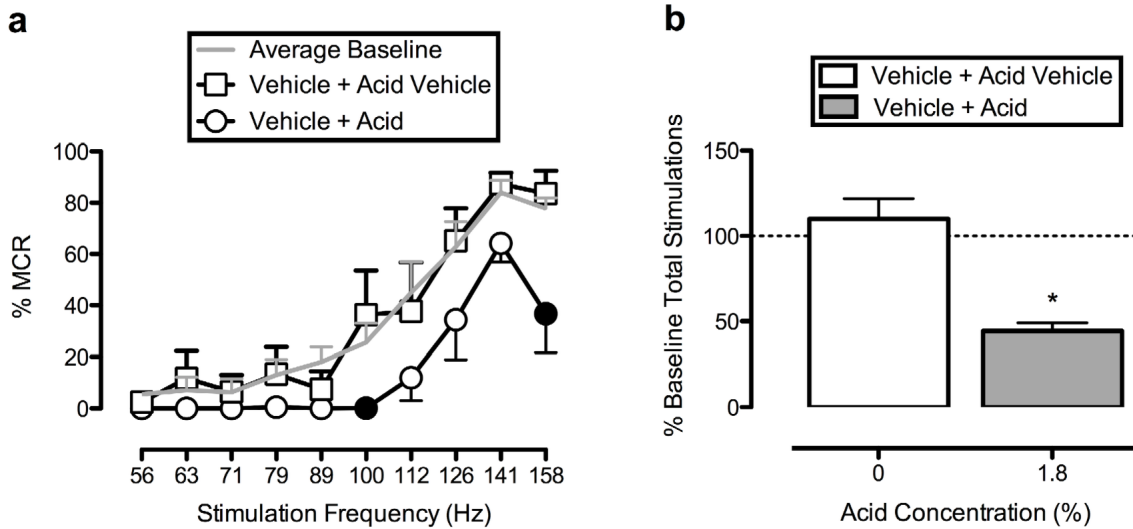
Figure 2.3

Figure 2.3. Lactic acid depresses intracranial self-stimulation (ICSS). The left panel *(a)* shows ICSS frequency-rate curves determined after treatment with THC vehicle 30 min before lactic acid vehicle or 1.8% lactic acid administration. Abscissa: frequency of electrical brain stimulation in hertz (log scale). Ordinate: percent maximum control response rate (%MCR). The average baseline ICSS frequency-rate curve for the entire study in this group of rats is shown by the *gray line* for comparison, but these data were not included in statistical analysis. Two-way ANOVA indicated a significant main effect of frequency [$F_{(9,36)}=23.92$; $p<0.001$] and a significant frequency × treatment interaction [$F_{(9,36)}=2.40$; $p=0.030$]. *Filled symbols* indicate frequencies at which reinforcement rates after acid treatment were significantly lower than rates after acid vehicle treatment as determined by Holm-Sidak post hoc test, $p < 0.05$. The right panel *(b)* shows the total number of stimulations per component expressed as a percent of baseline stimulations per component determined after treatment with THC vehicle 30 min before lactic acid vehicle or 1.8% lactic acid administration. Abscissa: Lactic acid concentration. Ordinate: percent baseline total number of stimulations per component. The *asterisk* (*) indicates 1.8% lactic acid significantly depressed ICSS compared to 0% lactic acid (i.e. lactic acid vehicle) as determined by paired *t* test [$t_{(4)}=6.95$; $p=0.002$]. All *bars* show mean ± SEM in five rats.
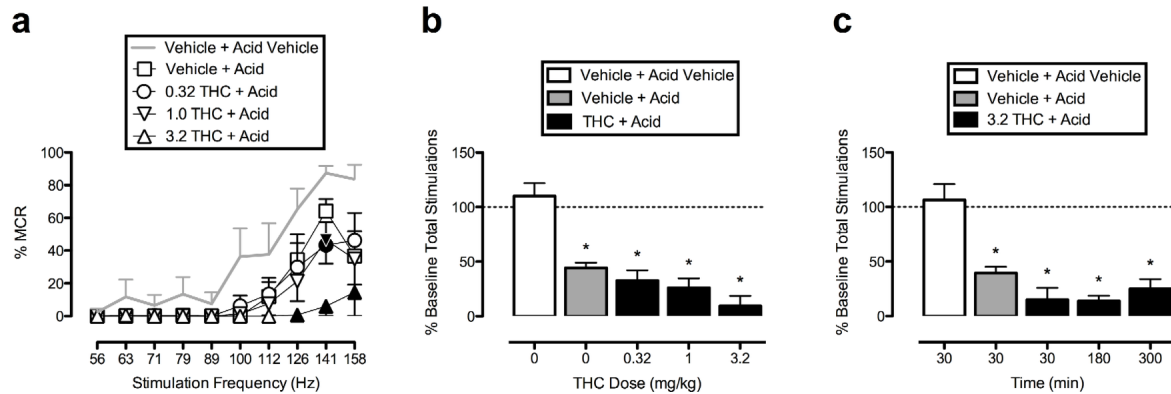
Figure 2.4

Figure 2.4. Δ9-tetrahydrocannabinol (THC) exacerbates lactic acid-induced depression of intracranial self-stimulation (ICSS). The left panel *(a)* shows ICSS frequency-rate curves determined after treatment with THC (0.32-3.2 mg/kg) or its vehicle 30 min before acid administration. Abscissa: frequency of electrical brain stimulation in hertz (log scale). Ordinate: percent maximum control response rate (%MCR). The THC vehicle + acid vehicle frequency-rate curve is shown by the *gray line* for comparison, but these data were not included in statistical analysis. Two-way ANOVA indicated a significant main effect of THC treatment [$F_{(3,12)}=5.16$; p=0.016], a significant main effect of frequency [$F_{(9,36)}=6.68$; $p<0.001$], and a significant frequency × treatment interaction [$F_{(27,108)}=4.62$; $p<0.001$]. Filled symbols indicate frequencies at which reinforcement rates after THC + acid treatment were significantly lower than after vehicle + acid treatment as determined by Holm-Sidak post hoc test, $p < 0.05$. All data show mean ± SEM in five rats. The center panel *(b)* shows the total number of stimulations per component expressed as a percent of baseline stimulations per component after treatment with THC (0.32-3.2 mg/kg) or its vehicle 30 min before acid administration. Abscissa: dose THC in milligrams per kilogram. Ordinate: percent baseline total number of stimulations per component. One-way ANOVA indicated a significant main effect of treatment [$F_{(4,16)}=19.26$; $p<0.001$]. The *asterisks* (*) indicate treatment with THC vehicle + acid or THC + acid significantly depressed ICSS compared to treatment with THC vehicle + acid vehicle as determined by Newman Keul's post hoc test, $p < 0.05$. All bars show mean ± SEM in five rats. The right panel *(c)*

shows the total number of stimulations per component expressed as a percent of baseline stimulations per component following treatment with THC vehicle 30 min before acid vehicle or acid administration, or THC (3.2 mg/kg) 30-300 min before acid administration. Abscissa: time following THC or vehicle administration. Ordinate: percent baseline total number of stimulations per component. One-way ANOVA indicated a significant main effect of treatment [$F(4,12)=14.43$; $p<0.001$]. The *asterisks* (*) indicate treatment with THC vehicle + acid or THC (3.2 mg/kg) + acid significantly depressed ICSS compared to treatment with THC vehicle + acid vehicle as determined by Newman Keul's post hoc test, $p < 0.05$. All bars show mean ± SEM in four rats.
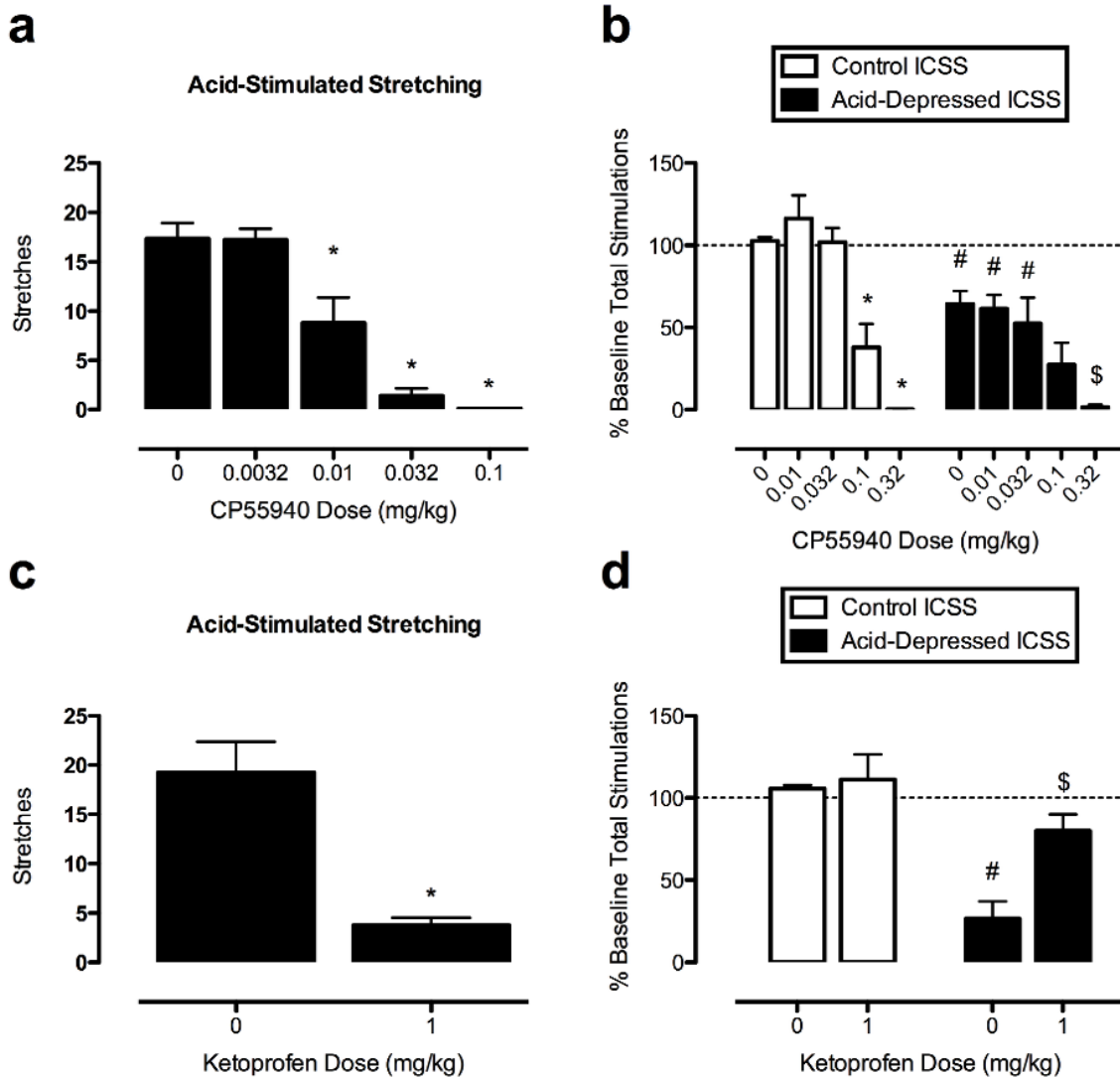
Figure 2.5

Figure 2.5. Effects of CP55940 (top panels) and ketoprofen (bottom panels) on lactic acid-stimulated stretching and lactic acid-induced depression of intracranial self-stimulation (ICSS). Abscissae (all panels): drug dose in mg/kg. Left ordinates: number of stretches observed during 30 min observation periods. Right ordinates: percent baseline total number of stimulations per component. CP55940 dose-dependently blocked acid-stimulated stretching (panel a, [$F_{(4,16)}=38.80$; $p<0.001$]) as indicated by one-way ANOVA. Two-way ANOVA on ICSS data in the presence and absence of acid treatment indicated a significant main effect of CP55940 dose [$F_{(4,16)}=34.44$; $p<0.01$],

a significant main effect of acid treatment ([F(1,4)=15.69; *p*=0.017], but no significant interaction (panel b). Ketoprofen blocked acid-induced stimulation of stretching (panel *c*, [t(3)=4.43; *p*=0.021]) as indicated by t-test. Two-way ANOVA on ICSS data in the presence and absence of acid treatment indicated a significant main effect of ketoprofen dose ([F(1,3)=16.95 *p*=0.026], a significant main effect of acid treatment ([F(1,3)=13.71 *p*=0.034], but no significant interaction (panel d). In panels *a* and *c*, *asterisks* (*) indicate significant difference from a "0" drug dose (i.e. vehicle) + lactic acid as determined by one-way ANOVA followed by Dunnett's post hoc test (CP55940) or by t-test (ketoprofen), *p* < 0.05. In panels *b* and *d*, a*sterisks* (*) indicate significant difference from a "0" drug dose (i.e. vehicle) + lactic acid vehicle, *dollar signs* (*$*) indicate significant difference from a "0" drug dose + lactic acid, and *number signs* (#) indicate significant depression of ICSS by lactic acid as determined by two-way ANOVA followed by Holm-Sidak post hoc test, *p* < 0.05. All *bars* show mean ± SEM in five rats (CP55940) or four rats (ketoprofen).
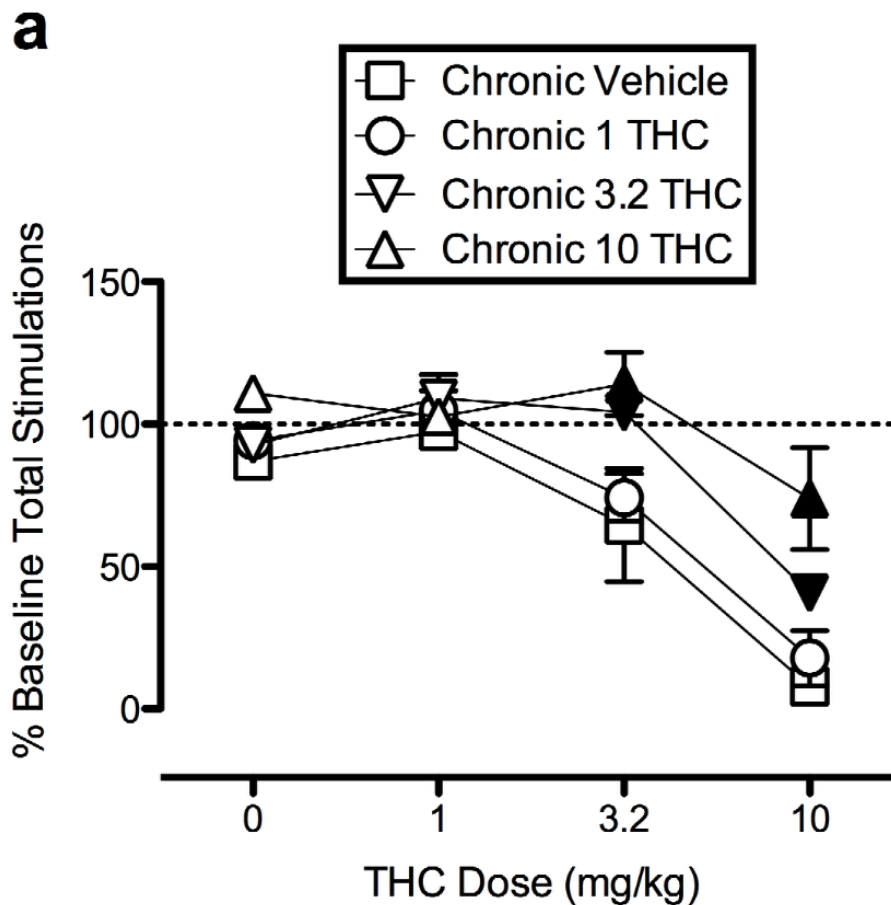
Figure 2.6

Figure 2.6. Chronic administration of THC produces tolerance to its rate-decreasing effects on intracranial self-stimulation (ICSS) in the absence of a noxious stimulus. Abscissa: THC challenge dose (mg/kg). Ordinate: percent baseline total number of stimulations per component. Two-way ANOVA indicated a significant main effect of chronic THC dose [$F_{(3,12)}=16.27$; $p<0.001$], a significant main effect of THC challenge dose [$F_{(3,12)}=55.33$; $p<0.001$], and a significant interaction [$F_{(9,36)}=3.58$; $p=0.003$]. *Filled symbols* indicate chronic THC + THC challenge dose combinations after which reinforcement rates were significantly higher than rates after the same THC challenge administered during chronic vehicle, as determined by Holm-Sidak post hoc test, *p* < 0.05. All *bars* show mean ± SEM in five rats.
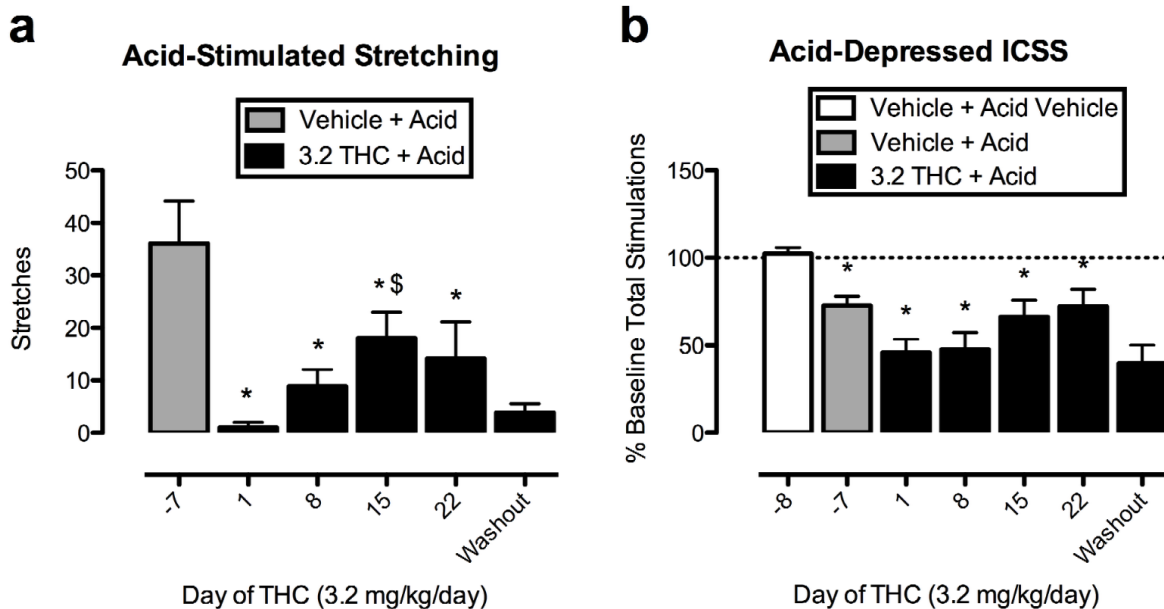
Figure 2.7

Figure 2.7. Chronic administration of THC produces partial tolerance to its antinociceptive effects in the assay of acid-stimulated stretching but does not unmask antinociceptive effects in the assay of acid-depressed intracranial self-stimulation (ICSS). Abscissae (all panels): Day of THC (3.2 mg/kg/day) administration. Left ordinate: number of stretches observed during 30 min observation periods. Right ordinate: percent baseline total number of stimulations per component. Effects of a 2-week washout period following chronic THC administration are also shown for comparison but were not included in the statistical analysis. The left panel (*a*) shows the effects of chronic administration of THC in the assay of acid-stimulated stretching. One-way ANOVA indicated a significant main of THC treatment duration (panel *a*, [$F_{(4,20)}=9.41$; $p<0.001$]). *Asterisks* (*) indicate treatment with THC produced significant antinociception compared to treatment with THC vehicle on days 1, 8, 15, and 22 of chronic THC administration, and *dollar signs* ($) indicate significant tolerance to this antinociceptive effect on day 15 compared to day 1 of chronic THC administration as determined by Newman-Keul's post hoc test, $p < 0.05$. The right panel (*b*) shows the effects of chronic administration of THC in the assay of acid-depressed ICSS. One-way

ANOVA indicated a significant main effect of treatment (panel *b*, [$F_{(5,20)}=10.08$; *p*<0.001]). *Asterisks* (*) indicate treatment with acid significantly depressed ICSS compared to treatment with lactic acid vehicle as determined by Newman-Keul's post hoc test, *p* < 0.05. Chronic THC administration failed to alter THC effects on acid-induced depression of ICSS. All *bars* show mean ± SEM in six rats (stretching) or five rats (ICSS).
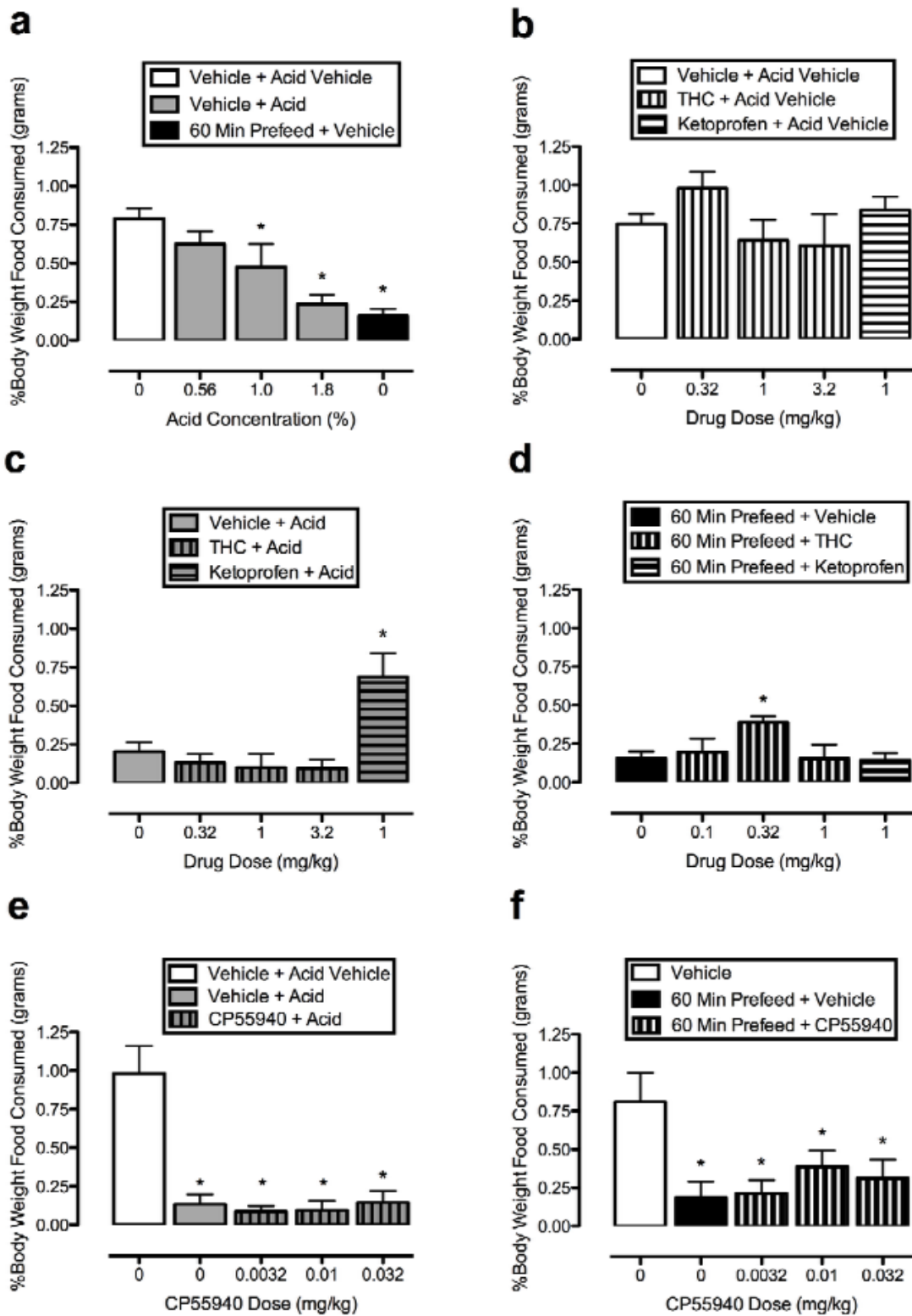
Figure 2.8

Figure 2.8. THC and ketoprofen effects on feeding depressed by acid or prefeeding. The upper left panel (*a*) shows the effects of lactic acid vehicle, lactic acid (0.56-1.8%), or a 60 min prefeeding session on feeding. Abscissa: percent acid concentration. Ordinates (all panels): percent body weight food consumed in grams during a 30 min feeding session. One-way ANOVA indicated a significant main effect of treatment [$F_{(4,20)}=16.30$; $p<0.001$]. *Asterisks* (*) indicate lactic acid (1-1.8%) or a 60 min prefeeding session significantly decreased feeding as determined by Dunnett's post hoc test, $p < 0.05$. All *bars* show mean ± SEM in six rats. The upper right panel (*b*) shows effects of THC (0.32-3.2 mg/kg), ketoprofen (1 mg/kg), or vehicle administered 30 min before acid vehicle treatment on control feeding. Abscissa: drug dose in milligrams per kilogram. THC and ketoprofen did not significantly alter feeding in the absence of lactic acid or a 60 min prefeeding session. All *bars* show mean ± SEM in seven rats. The middle left panel (*c*) shows the effects of THC (0.32-1 mg/kg), ketoprofen (1 mg/kg), or vehicle administered 30 min before 1.8% lactic acid. Abscissa: drug dose in milligrams per kilogram. One-way ANOVA indicated a significant main effect of treatment [$F_{(4,24)}=10.46$; $p<0.001$]. The *asterisk* (*) indicates ketoprofen significantly blocked acid-induced depression of feeding as determined by Dunnett's post hoc test, $p < 0.05$. All *bars* show mean ± SEM in seven rats. The middle right panel (*d*) shows the effects of THC (0.1-1 mg/kg), ketoprofen (1 mg/kg), or vehicle administered immediately after a 60 min prefeeding session and 30 min before the test session. Abscissa: drug dose in milligrams per kilogram. One-way ANOVA indicated a significant main effect of treatment [$F_{(4,28)}=2.88$; $p=0.041$]. The *asterisk* (*) indicates THC significantly blocked prefeeding-induced depression of feeding as determined by Dunnett's post hoc test, $p < 0.05$. All *bars* show mean ± SEM in eight rats. The bottom left panel (*e*) shows the effects of CP55940 (0.0032-0.032 mg/kg or vehicle) administered 30 min before 1.8% lactic acid. One-way ANOVA indicated a significant main effect of acid treatment [$F_{(4,28)}=19.64$; $p<0.001$]. *Asterisks* (*) indicate lactic acid (1.8%) significantly decreased feeding as determined by Newman-Keuls post hoc test, $p < 0.05$. All *means ± SEM* represent eight rats. The bottom right panel (*f*) shows the effects of CP55940 (0.0032-0.032 mg/kg or vehicle) administered immediately after a 60 min prefeeding

session and 30 min before the test session. One-way ANOVA indicated a significant main effect of prefeeding treatment [$F_{(4,28)}=4.25$; $p=0.008$]. *Asterisks* (*) indicate a 60 min prefeeding session significantly decreased feeding vs. CP55940 vehicle alone as determined by Newman-Keuls post hoc test, $p < 0.05$. CP55940 did not alter feeding under either condition tested. All means ± SEM represent eight rats.

2.4. Discussion

The purpose of this study was to assess effects of the cannabinoid receptor agonists THC and CP55940 in assays of pain-stimulated and pain-depressed behavior in rats. There were four main findings. First, in agreement with the large literature on antinociceptive effects of cannabinoid agonists in assays of pain-stimulated behavior (Rice, 2006; Karst et al., 2010), THC and CP55940 dose-dependently decreased acid-stimulated stretching. Second, THC and CP55940 also decreased control ICSS in the absence of the acid noxious stimulus; however, depression of control ICSS was shorter in duration (THC) or occurred at lower doses (CP55940) than depression of acid-stimulated stretching. Furthermore, chronic administration of THC produced complete tolerance to THC-induced depression of control ICSS and only produced partial tolerance to THC-induced antinociception in the assay of acid-stimulated stretching. These findings suggest that nonselective behavioral depression may have contributed to, but could not account entirely for, cannabinoid antinociception in the assay of acid-stimulated stretching. Third, despite evidence for antinociception in the assay of acid-stimulated stretching, both acute and chronic THC and acute CP55940 failed to produce antinociception in the assay of acid-depressed ICSS. Lastly, THC and CP55940 also failed to produce antinociception in the assay of acid-depressed feeding. Collectively, these findings demonstrate that although cannabinoid agonists are effective to produce antinociception in assays of pain-stimulated behavior, they are ineffective in assays of acute pain-depressed behavior. The effects of THC and CP55940 contrast with the antinociceptive efficacy of clinically effective analgesics such as ketoprofen (Kwilasz and Negus, 2012, present study) and morphine (Negus et al., 2006; Pereira Do Carmo

et al., 2009) in these assays of pain-depressed behavior. Moreover, the lack of cannabinoid efficacy in these assays of acute pain-depressed behavior in rats agrees with the general lack of efficacy of cannabinoids in treating acute pain in humans (Raft et al., 1977; Rice, 2006; Karst et al., 2010; Kraft, 2012). Taken together, these results do not support the use of cannabinoid agonists to treat the behavioral depressant effects of acute pain and further suggest that preclinical assays of pain-depressed behavior may be useful during cannabinoid drug development for predicting clinical drug effects on pain in humans.

CHAPTER THREE

Effects of the FAAH inhibitors URB597 and PF3845

on acute pain-stimulated and pain-depressed behavior.

Submitted to the Journal of Pain in March 2013

3.1. Introduction

FAAH inhibitors increase physiological levels of the endocannabinoid AEA (which targets CB1Rs and CB2Rs) as well as other fatty acid ethanolamines such as PEA and OEA (which target PPAR-α). FAAH inhibitors such as URB597 and PF3845, like mixed cannabinoid agonists, produce antinociception in many traditional preclinical assays of pain but, unlike cannabinoid agonists, have been shown to produce fewer side effects than traditional cannabinoid agonists such a sedation/motor suppression (Schlosburg et al., 2009; Alvarez-Jaimes and Palmer, 2011). Recently, a clinical trial for the treatment of osteoarthritis-related pain failed with the FAAH inhibitor PF7845, which is structurally-related to PF3845. This disparity between preclinical and clinical findings suggests that traditional preclinical assays of pain used to study FAAH inhibitors in animals may not be sufficient alone to predict clinical analgesic effects in humans.

The goal of the present study was to compare effects of the two well-characterized FAAH inhibitors URB597 and PF3845 in preclinical assays of acute pain-stimulated and pain-depressed behavior in rats. Assays of pain-stimulated and pain-depressed behavior have been used previous to examine the effects of opioids, nonsteroidal anti-inflammatory drugs, and other drug classes (Pereira Do Carmo et al.,

2009; Negus et al., 2010b; Kwilasz and Negus, 2012; Negus et al., 2012; Rosenberg et al., 2013). In particular, we have previously shown that the CBR agonists THC and CP55940 failed to produce antinociception in assays of pain-depressed behavior (Kwilasz and Negus, 2012, Chapter 2), a finding concordant with the poor clinical efficacy of CBR agonists as analgesics in humans (Raft et al., 1977; Rice, 2006; Karst et al., 2010; Kraft, 2012). Insofar as FAAH inhibitors display weaker efficacy than direct CBR agonists to produce motor impairment, we predicted that URB597 and PF3845 might be more likely than CBR agonists to produce antinociception in assays of pain-depressed behavior.

3.2. Methods

Subjects

One hundred and two male Sprague-Dawley rats (Harlan, Frederick, MD, USA) weighing approximately 300 g at the time of delivery were used in these studies. All housing, maintenance, and research conditions in this chapter are identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Assay of acid-stimulated stretching

Behavioral procedure. Thirty-four rats that failed to meet the criteria for ICSS within 4 weeks (see below) were used for studies of lactic acid-stimulated stretching as described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

In four separate groups of rats, FAAH inhibitor dose-effect curves were determined for URB597 (1-10 mg/kg or vehicle) or PF3845 (1-10 mg/kg or vehicle) administered 60 min or 240 min prior to acid (i.e. 1 group/drug/time point). These pretreatment times were selected based on previous studies that reported peak brain levels of AEA, PEA, and OEA approximately 60 min post-treatment with URB597 and approximately 240 min post-treatment with PF3845 (Fegley et al., 2005; Ahn et al., 2009). To determine whether URB597-induced antinociception was mediated by CB1Rs or CB2Rs, URB597 (10 mg/kg) was administered 240 min before acid in combination with the CB1R-selective antagonist rimonabant (1 mg/kg) or the CB2R-selective antagonist SR144528 (1 mg/kg) administered 30 min before acid. These antagonist doses and pretreatment times were based on previous studies that have demonstrated antagonism of cannabinoid agonist-induced antinociception with rimonabant (Kwilasz and Negus, 2012) and SR144528 (Hohmann et al., 2004). Rimonabant and SR144528 were administered after URB597 because of their ability to act as competitive antagonists at CB1Rs (Thomas et al., 1998; Jarbe et al., 2010) and CB2Rs (Griffin et al., 1999), respectively. To determine whether URB597-induced antinociception was mediated by PPAR-α, the PPAR-α antagonist MK886 (1-3.2 mg/kg) was administered 30 min before URB597 (10 mg/kg), which was administered 240 min before acid. The doses and treatment time for MK886 were based on previous studies that demonstrated antagonism of URB597 and PPAR-α agonist effects with MK886 (Mazzola et al., 2009; Mascia et al., 2011). MK886 was administered prior to URB597 to ensure blockade of the transcription-enhancing effects of PEA, OEA, which have been implicated in their therapeutic effects (Schoonjans et al., 1996; Gervois and Mansouri, 2012). PF3845-

induced pronociception was also assessed for mediation by CB1/2Rs and PPAR-α using identical procedures to those used for URB597. Doses/treatments for all phases were delivered in a randomized dose order across rats and separated by at least one week.

Data Analysis. All methods of data analysis in this chapter are identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Assay of intracranial self-stimulation (ICSS)

Surgery. All surgical procedures in this chapter are identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Apparatus. All apparatus and materials used in this chapter are identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Behavioral procedure. After initial shaping of lever press responding, rats were trained under a continuous reinforcement schedule of brain stimulation using procedures identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Studies of FAAH inhibitor effects on ICSS were conducted in three phases, and the experimental design of each phase is shown in Figure 3.1. In the first phase, subjects were treated with URB597 (1-10 mg/kg or vehicle) or PF3845 (1-10 mg/kg or vehicle) at time "0" and with the intraperitoneal acid noxious stimulus (1.8% lactic acid in

a volume of 1 ml/kg) administered 60 min later. ICSS was evaluated for 20 min before and 20 min after acid injection to assess FAAH inhibitor effects in the absence and presence of the noxious stimulus. Specifically, ICSS was evaluated from 40-60 min to assess effects of the FAAH inhibitor in the absence of the noxious stimulus and again from 60-80 min to assess effects of the FAAH inhibitor on acid-induced depression of ICSS. The second phase was identical except that the acid noxious stimulus was administered 260 min after the FAAH inhibitor, and ICSS was assessed from 240-260 min (FAAH inhibitor effects on ICSS in the absence of the noxious stimulus) and from 260-280 min (FAAH inhibitor effects on acid-induced depression of ICSS). In the third phase, to determine whether URB597-induced antinociception in the assay of acid-depressed ICSS was mediated by CB1Rs or CB2Rs, URB597 (10 mg/kg) was administered at time "0", either rimonabant (1 mg/kg) or SR144528 (1 mg/kg) was administered after 210 min, and the acid noxious stimulus was administered after 260 min. Lastly, in the fourth phase, to determine whether URB597-induced antinociception in the assay of acid-depressed ICSS was mediated by PPAR-α, MK886 (1-3.2 mg/kg) was administered 30 min prior to URB597 (10 mg/kg), which was administered at time "0," and the acid noxious stimulus was administered after 260 min. ICSS was evaluated for 20 min before and 20 min after acid injection in phases three and four as in the other phases. FAAH inhibitor doses and cannabinoid/PPAR-α antagonist + URB597 combinations were administered in randomized order and were separated by at least one week. Training and test sessions were conducted Monday-Friday, with test sessions conducted on Wednesdays, Thursdays, or Fridays.

Data Analysis. All methods of data analysis in this chapter are identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Measurement of brain and plasma fatty acid ethanolamines

Experimental procedure. Forty-three rats were used for the measurement of brain and plasma fatty acid ethanolamines following FAAH inhibitor administration. The fatty acid ethanolamines evaluated were anandamide (AEA), palmitoylethanolamide (PEA), and oleoylethanolamide (OEA). Rats were dosed in their home cages with URB597 (10 mg/kg, vehicle) or PF3845 (10 mg/kg, vehicle) and sacrificed via decapitation with guillotine at 60 or 240 min after drug administration. Naive rats that did not receive any injection prior to sacrifice were also included as controls. Immediately after decapitation, brains were harvested, quickly frozen in isopentane on dry ice, and stored at -80°C until assay. For plasma samples, trunk blood was collected immediately after decapitation and centrifuged at 1250 x g to separate plasma from blood cells. Plasma samples were then stored at -80°C until assay.

Procedure for tissue extraction. On the day of assay, the pre-weighed rat brains were homogenized with 5 ml chloroform:methanol (2:1 v/v containing 0.0348 g PMSF/ml). One quarter of the homogenate (1.25 ml) was taken and diluted to 1.4 ml with the chloroform:methanol used for homogenization. Internal standards (50 µl of each of 2 pmol AEA-d8, 1 nmol 2-AG-d8, 3.3 nmol PEA-d4 and 3 nmol OEA-d4) were added to each sample. Homogenates were vortexed and mixed with 0.3 ml of 0.73% w/v NaCl, vortexed again, and then centrifuged for 10 min at 3200 x g and 4°C. The aqueous

phase plus debris were collected and extracted again twice with 0.8 ml chloroform. The organic phases from the three extractions were pooled, and organic solvents were evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml chloroform and mixed with 1 ml cold acetone. The mixtures were then centrifuged for 5 min at 1800 x g and 4°C to precipitate proteins. The upper layer of each sample was collected and evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml methanol and placed in auto-sample vials for analysis.

Rat plasma (200 µl) was mixed with 50 µl of each of the internal standards mentioned above and then mixed with 2.8 ml chloroform:methanol (2:1 containing 0.0348 g PMSF/ml). Samples were vortexed and 0.6 ml of 0.73% w/v NaCl was added to each sample, vortexed, and then centrifuged for 10 min at 3200 x g and 4°C. The aqueous phase plus debris were collected and extracted again twice with 1.6 ml chloroform. The organic phases from the three extractions were pooled and the organic solvents were evaporated under nitrogen gas. The dried samples were reconstituted with 0.1 ml chloroform and mixed with 1 ml cold acetone. The upper layer of each sample was then collected and processed as described above.

High performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) quantification. An HPLC/MS/MS system was used to identify and quantify AEA, 2-AG, PEA, and OEA in brain and plasma samples. The system used was a 3200 Q trap with a turbo V source for TurboIonSpray (Applied Biosystems, Carlsbad, CA, USA) attached to a SCL HPLC system (Shimadzu, Kyoto, Japan) controlled by Analyst 1.4.2 software (AB Sciex, Framingham, MA, USA). The chromatographic separation was performed using a Discovery® HS C18, 4.6 x 15 cm, 3

micron (Supelco, Bellefonte, PA, USA). The mobile phase consisted of (10:90) water:methanol with 0.1% ammonium acetate and 0.1% formic acid and was delivered at a flow rate of 0.3 ml/min. The source temperature was set at 600°C and had a curtain gas at a flow rate of 30 ml/min. The ionspray voltage was 5000 V with ion source gases 1 and 2 flow rates of 60 and 50 ml/min, respectively. The mass spectrometer was run in positive ionization mode, and the acquisition mode used was multiple reaction monitoring. The following transitions were monitored: (348>62) and (348>91) for AEA; (356>62) for AEA-d8; (379>287) and (279>269) for 2-AG; (387>96) for 2-AG-d8; (300>62) and (300>283) for PEA; (304>62) for PEA-d4; (326>62) and (326>309) for OEA; and (330>66) for OEA-d4. Calibration curves were constructed with each analytical batch for each analyte. Curves were constructed using linear regression based on the peak area ratios of each analyte and its deuterated internal standard. The extracted calibration curves ranged from 0.039 pmol to 40 pmol for AEA, from 0.0625 nmol to 64 nmol for 2-AG, and from 0.156 nmol to 0.5 nmol for PEA and OEA. The total run time for the analytical method was 8 minutes.

Data Analysis. Prior to data analysis, AEA brain level data were transformed to pmol/g of brain tissue, 2-AG, PEA, and OEA brain level data were transformed to nmol/g of brain tissue, and AEA, PEA, and OEA plasma level data were transformed to pmol/ml of plasma. 2-AG plasma levels were below the threshold of detection. Statistical analysis of brain and plasma levels of AEA, PEA, and OEA did not reveal significant differences between naïve and vehicle-treated animals; thus drug-treatment levels were normalized to mean vehicle-treatment levels for each condition using the equation: (Drug-Treatment Level ÷ Mean Vehicle-Treatment Level) x 100. Drug effects

on brain and plasma fatty acid amides were evaluated by two-way ANOVA, with treatment and treatment time as the two factors. A significant ANOVA was followed by Holm-Sidak post hoc test, and the criterion for significance was set at $p < 0.05$.

Drugs

Lactic acid was purchased from Sigma Chemical Co. (St. Louis, MO, USA). AEA, AEA-d8, 2-AG, 2-AG-d8, PEA, PEA-d4, OEA, OEA-d4, and MK886 were purchased from Cayman Chemical (Ann Arbor, MI, USA). URB597, rimonabant, and SR144528 were provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD, USA). PF3845 was provided by Dr. Ben Cravatt at The Scripps Research Institute (San Diego, CA, USA) and by Anu Mahadevan at Organix Laboratories (Woburn, MA, USA). Lactic acid was prepared in sterile water. MK886 was prepared in a vehicle consisting of 20% DMSO and 80% sterile water. URB597 was prepared in a vehicle consisting of 1% carboxymethylcellulose (Sigma), 1% Tween 80 (Sigma), 2% dimethyl sulfoxide (Sigma), and 96% sterile saline. PF3845 was prepared in a vehicle consisting of 10% ethanol, 10% emulphor EL-620 (Rhone-Poulenc; Princeton, NJ, USA), and 80% sterile saline. Rimonabant and SR144528 were prepared in a vehicle consisting of 5% ethanol, 5% cremophor (Sigma), and 90% sterile saline. All solutions were injected intraperitoneally in a volume of 1 ml/kg except for URB597, which was injected intraperitoneally in a volume of 2 ml/kg.

3.3. Results

Effects of FAAH inhibitors on acid-stimulated stretching. Figure 3.2 shows that IP administration of lactic acid (1.8% in a volume of 1 ml/kg) stimulated approximately 15 stretches after administration of vehicle (gray bars in all panels). URB597 (1-10 mg/kg) dose-dependently and significantly decreased stretching after both 60 and 240 min (panels 3.2a and 3.2c, respectively). Conversely, PF3845 (1-10 mg/kg) had no effect on stretching after 60 min (panel 3.2b), and it dose-dependently increased stretching after 240 min (panel 3.2d; an effect that was replicated in a separate group of rats and found to be insensitive to rimonabant, data not shown). Figure 3.3 shows that the antinociceptive effect of URB597 in the assay of acid-stimulated stretching was antagonized by administration of the CB1R antagonist rimonabant (1 mg/kg, panel 3.3a), but not by the CB2R antagonist SR144528 (1 mg/kg, panel 3.3b) or the PPAR-α antagonist MK886 (1-3.2 mg/kg, panel 3.3c). Rimonabant, SR144528, and MK886 did not alter acid-stimulated stretching when administered alone. Figure 3.4a shows that the pronociceptive effect of PF3845 in the assay of acid-stimulated stretching was not antagonized by administration of the CB1R antagonist rimonabant (1 mg/kg) or CB2R antagonist SR144528 (1 mg/kg). Figure 3.4b also shows that the pronociceptive effect of PF3845 in the assay of acid-stimulated stretching was not able to be repeated enough times to properly determine the effect of MK886 on PF3845-induced pronociception. Nonetheless, stretching is highest after MK886 + PF3845 versus PF3485 alone and vehicle, suggesting PPAR-α is not likely to play a role in PF3845-induced pronociception.

Effects of FAAH inhibitors on ICSS in the absence of a noxious stimulus.

Over the course of the entire ICSS study, the average baseline number of stimulations per component was 188.31 ± 45.91, and data in Figures 3.5, 3.7, and 3.9 show drug effects expressed as a percent of the baseline number of stimulations per component in each group of rats. Figure 3.5 shows that both URB597 (1-10 mg/kg) and PF3845 (1-10 mg/kg) produced dose-dependent decreases in ICSS from 40-60 min after treatment, but not from 240-260 min after treatment, in the absence of the noxious stimulus.

During each test session, a "baseline" frequency-rate curve was determined before testing to permit determination of the Maximum Control Rate (MCR) for that session. Over the course the entire study in all groups of rats, the average MCR was 51.56 ± 4.95 stimulations/trial. Reinforcement rates during each frequency trial of a session were then expressed as a percentage of that session's MCR, and the average baseline frequency-rate curves for studies with URB597 and PF3845 are shown in Figure 3.6 as gray lines. Maximum reinforcement rates were usually observed at the highest stimulation frequencies and decreased with lower stimulation frequencies. In general, when administered 40 min prior to an ICSS session, both URB597 and PF3845 produced rightward and downward shifts in the ICSS frequency-rate curves (Figs. 3.6a and b). Forty min after administration of URB597, a low dose of 1 mg/kg URB597 increased ICSS at a single frequency of 158 Hz, whereas higher doses of 3.2 and 10 mg/kg URB597 significantly decreased ICSS at frequencies of 126, 141, and 158 Hz (Fig. 3.6a). Forty minutes after administration of PF3845, low doses of 1 and 3.2 mg/kg PF3845 produced no effect on ICSS, whereas a higher dose of 10 mg/kg PF3845 significantly decreased ICSS at a single frequency of 141 Hz (Fig. 3.6b). Both URB597

and PF3845 produced less dramatic rate-decreasing effects when administered 240 min prior to an ICSS session (Figs. 3.6c and d). Following 240 min administration with URB597, a low dose of 1 mg/kg URB597 significantly decreased ICSS at a frequency of 141 Hz, a higher dose of 3.2 mg/kg URB597 significantly decreased and increased ICSS at frequencies of 158 Hz and 89 Hz, respectively, and the highest dose of 10 mg/kg URB597 significantly increased ICSS at a frequency of 89 Hz (Fig. 3.6c). Following 240 min administration with PF3845, low doses of 1 and 3.2 mg/kg PF3845 produced no effect on ICSS, whereas a higher dose of 10 mg/kg PF3845 significantly decreased ICSS at frequencies of 112 and 126 Hz (Fig. 3.6d).

Effects of FAAH inhibitors on acid-induced depression of ICSS. Figure 3.7 shows that the lactic acid noxious stimulus significantly decreased ICSS when it was administered 60 min or 260 min after vehicle. This acid-induced depression of ICSS served as a manifestation of pain-related behavioral depression, and drugs were evaluated for their ability to block acid-induced depression of ICSS. Figure 3.7 also shows that URB597 but not PF3845 produced dose- and time-dependent antinociception in the assay of acid-depressed ICSS. Specifically, URB597 (1-10 mg/kg) did not significantly alter acid-induced depression of ICSS after 60 min (panel 3.7a), but it partially, dose-dependently and significantly attenuated acid-induced depression of ICSS after 260 min (panel 3.7c). Conversely, PF3845 (1-10 mg/kg) did not significantly alter acid-induced depression of ICSS after either 60 (panel 3.7b) or 260 min (panel 3.7d).

Both URB597 and PF3845 displayed small but significant antinociceptive effects in the assay of acid-induced depression of ICSS based on the frequency-rate curve

analysis (Fig. 3.8). URB597 (10 mg/kg) administered 260 min before acid treatment significantly attenuated acid-induced depression of ICSS at a single frequency of 126 Hz, however this point was also significantly different than URB597 vehicle + acid vehicle treatment (Fig. 3.8c). PF3845 (3.2-10 mg/kg) administered 60 min before acid treatment also significantly attenuated acid-induced depression of ICSS (Fig. 3.8b). A dose of 3.2 mg/kg PF3845 significantly attenuated acid-induced depression of ICSS at frequencies of 141 and 158 Hz, and a dose of 10 mg/kg PF3845 significantly attenuated acid-induced depression of ICSS at a single frequency of 158 Hz. URB597 (1-10 mg/kg) administered 60 min before acid treatment and PF3845 (1-10 mg/kg) administered 260 min before acid treatment produced no effects on acid-induced depression of ICSS.

Figure 3.9 (panels a and b) show that the CB1R antagonist rimonabant and the CB2R antagonist SR144528 failed to reverse URB597-induced antinociception in the assay of acid-depressed ICSS. Administration of 1 mg/kg rimonabant (Fig. 3.9a) or 1 mg/kg SR144528 (Fig. 3.9b) 50 min before the ICSS session had no effect on the antinociceptive effects of 10 mg/kg URB597 administered 260 min before the ICSS session. Furthermore, these doses of rimonabant (Fig. 3.9a) and SR144528 (Fig. 3.9b) produced no effects on acid-induced depression of ICSS alone. Experiments with the PPAR-α antagonist MK886 were less straightforward (Figure 3.9c). In this group of rats, URB597 (10 mg/kg) produced statistically ambiguous evidence for antinociception, such that ICSS after URB597 + acid was not statistically different from ICSS after either acid vehicle alone or acid alone, and MK886 did not significantly alter these weak effects of URB597.

Effects of FAAH inhibitors on plasma and brain fatty acid ethanolamines.

Figure 3.10 shows that both URB597 (10 mg/kg) and PF3845 (10 mg/kg) produced time-dependent increases in plasma and brain fatty acid ethanolamines. URB597 increased plasma PEA and OEA after 60 and 240 min; however it did not significantly increase plasma AEA at either of these times. In brain, URB597 increased AEA and OEA after 60 min and AEA, OEA and PEA after 240 min. PF3845 increased plasma AEA and OEA after 60 min and AEA, OEA, and PEA after 240 min. In brain, PF3845 increased AEA, OEA, and PEA after 240 min but not 60 min. No significant differences were found in brain levels of 2-AG after administration of either FAAH inhibitor at any time point (*data not shown*). Levels of endocannabinoids/fatty acid ethanolamines in naïve animals were as follows: in plasma in pmol/ml*,* AEA (0.90 ± 0.18), PEA (16.81 ± 3.11), and OEA (9.16 ± 1.47), and in brain in pmol/g, AEA (5.26 ± 1.41), and in nmol/g, PEA (0.21 ± 0.06), OEA (0.08 ± 0.01), and 2-arachidonoylglycerol (9.49 ± 1.04). 2-AG levels in plasma were below the limits of detection for all treatments.
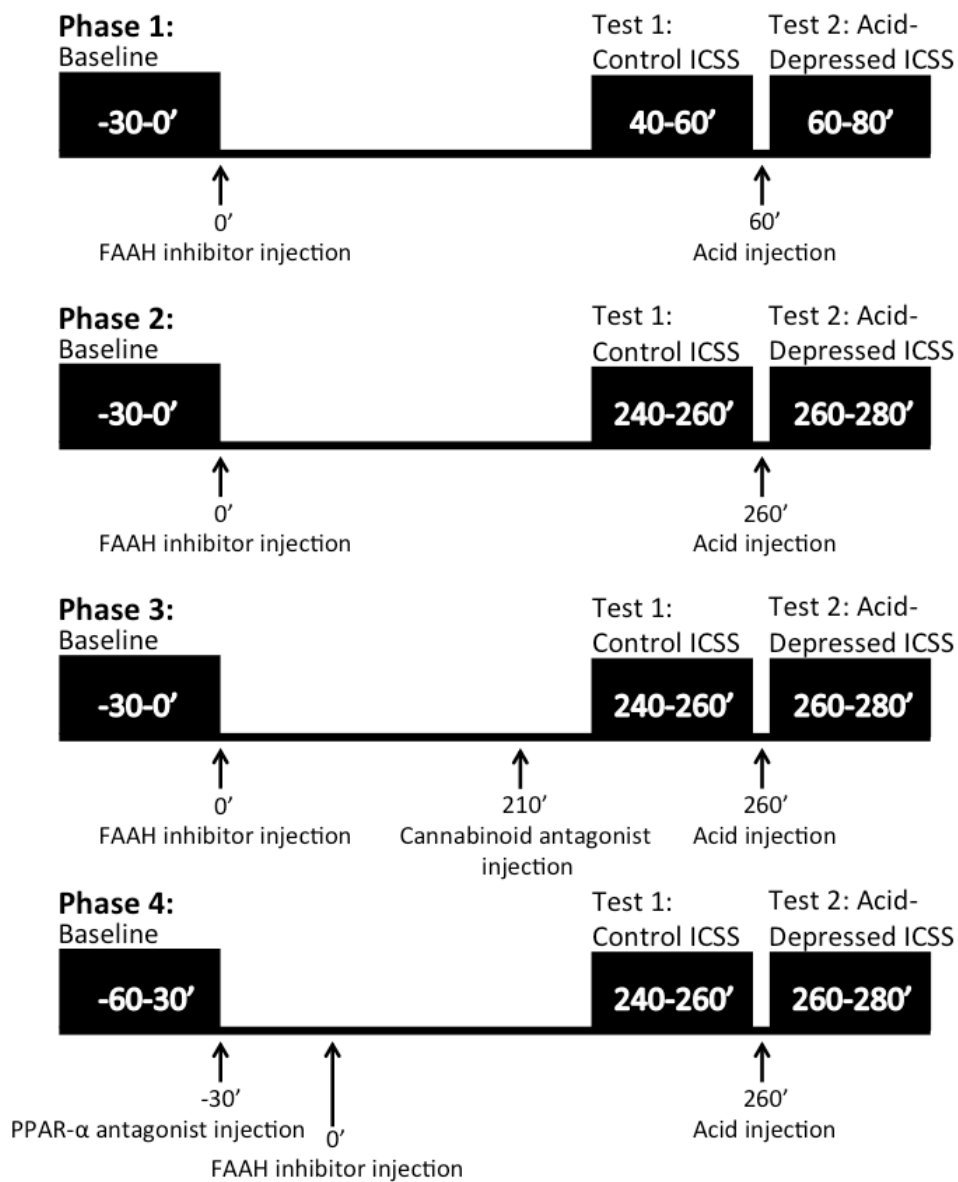
Figure 3.1

Figure 3.1. Diagram of experimental design used for drug treatments and behavioral testing in the assay of acid-depressed intracranial self-stimulation (ICSS).
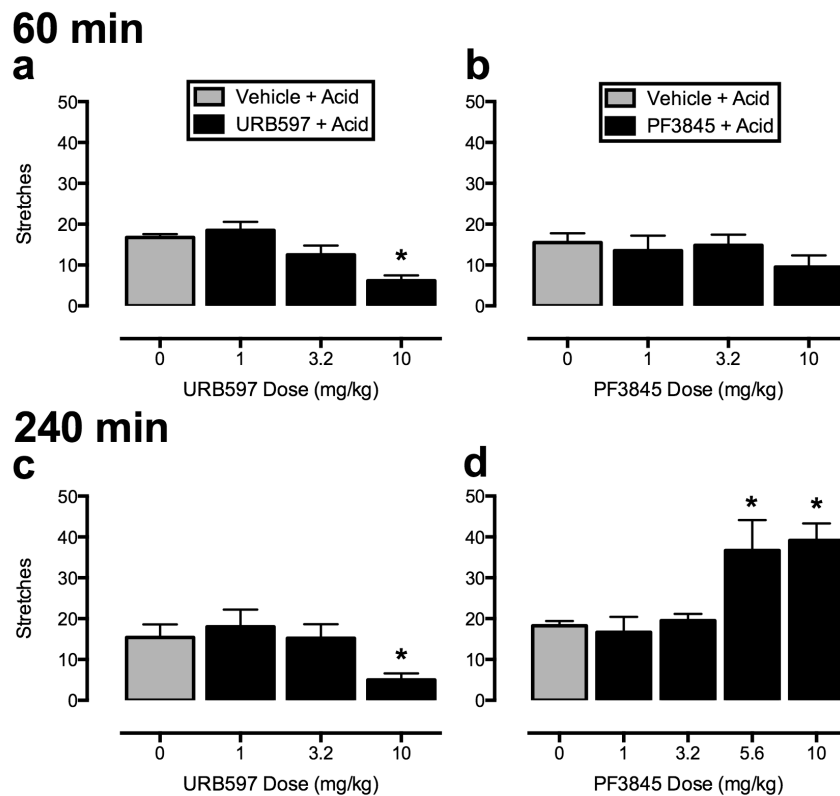
Figure 3.2

Figure 3.2. Effects of FAAH inhibitors on acid-stimulated stretching. Abscissae: dose FAAH inhibitor in milligrams per kilogram. Ordinates: number of stretches observed during a 30 min observation period. The left panels (a and c) show the effects of URB597 (1-10 mg/kg or vehicle) administered 60 min (panel a) or 240 min (panel c) before acid treatment. One-way repeated measures ANOVA indicated significant main effects of URB597 after 60 min (panel a) $[F_{(3,15)}=9.59; p<0.001]$ and 240 min (panel c) $[F_{(3,12)}=14.63; p<0.001]$. All bars show mean ± SEM in six (60 min) or five (240 min) rats. The right panels (b and d) show the effects of PF3845 (1-10 mg/kg or vehicle) administered 60 min (panel b) or 240 min (panel d) before acid treatment. One-way repeated measures ANOVA indicated a significant main effect of PF3845 treatment 240 min post-treatment (panel d) $[F_{(4,20)}=11.71; p<0.001]$. All bars show mean ± SEM in six rats. *Asterisks* (*) indicate significantly different from vehicle + acid in all panels as determined by Dunnett's post hoc test, $p < 0.05$.
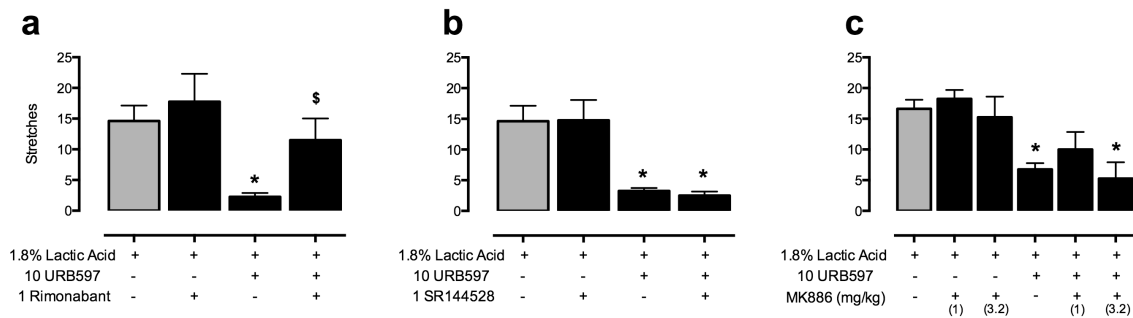
Figure 3.3

Figure 3.3. URB597-induced antinociception in the assay of acid-stimulated stretching is antagonized by rimonabant but not by SR144528 or MK886. Abscissae: Treatment conditions. Ordinates: number of stretches observed during a 30 min observation period. The left panel (a) shows the effect of URB597 (10 mg/kg or vehicle) administered 240 min before acid treatment in combination with rimonabant (1 mg/kg or vehicle) administered 30 min before acid treatment. One-way repeated measures ANOVA indicated a significant main effect of treatment in panel a [$F_{(3,9)}$=9.47; p=0.004]. The center panel (b) shows the effect of URB597 (10 mg/kg or vehicle) administered 240 min before acid treatment in combination with SR144528 (1 mg/kg or vehicle) administered 30 min before acid treatment. One-way repeated measures ANOVA indicated a significant main effect of treatment in panel b [$F_{(3,9)}$=17.03; p<0.001]. The right panel (c) shows the effect of MK886 (1-3.2 mg/kg or vehicle) administered 30 min before URB597 (10 mg/kg or vehicle), which was administered 240 min before acid treatment. One-way repeated measures ANOVA indicated a significant main effect of treatment in panel c [$F_{(5,15)}$=7.27; p=0.001]. *Asterisks* (*) in all panels indicate significantly different from vehicle + acid, and the d*ollar sign* ($) in panel a indicates significantly different from URB597 (10 mg/kg) + acid as determined by Newman-Keuls post hoc test, p < 0.05. All bars show mean ± SEM in four rats.
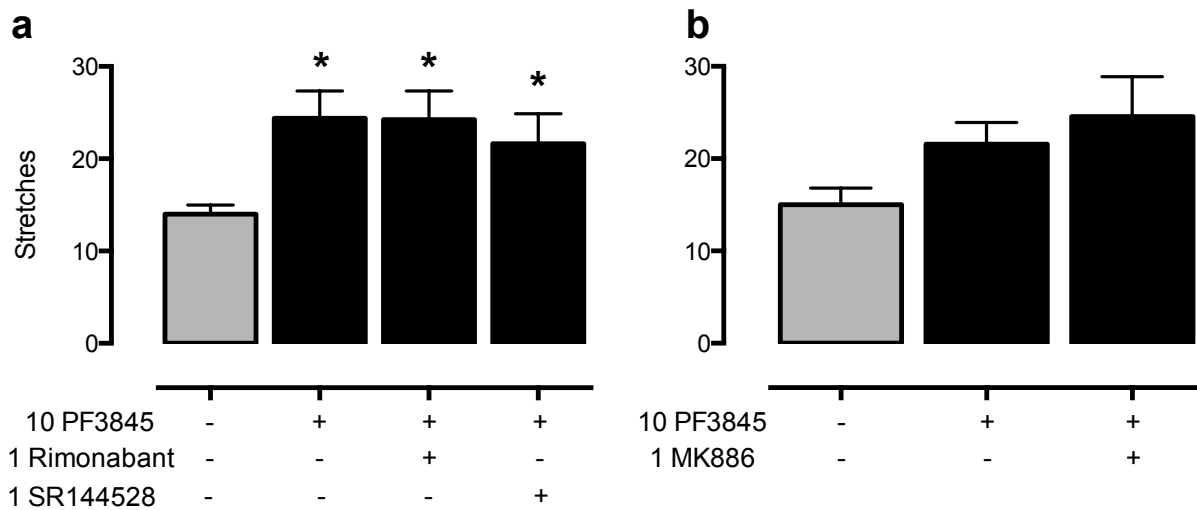
Figure 3.4

Figure 3.4. PF3845-induced pronociception in the assay of acid depressed ICSS is not antagonized by rimonabant, SR144528, or MK886. Abscissae: Treatment conditions. Ordinates: percent baseline total number of stimulations per component. The left panel (a) shows the effects of PF3845 (10 mg/kg or vehicle) administered 240 min before acid treatment in combination with rimonabant (1 mg/kg) or SR144528 (1 mg/kg) administered 30 min before acid treatment. One-way repeated measures ANOVA indicated a significant main effect of PF3845 treatment in panel a [$F_{(3,21)}=5.56$; p=0.006]. Asterisks (*) in panel a indicate significantly different from vehicle + acid treatment as indicated by Newman-Keuls post hoc test, $p < 0.05$. All bars show mean ± SEM in eight rats. The right panel (b) shows the effects of PF3845 (10 mg/kg or vehicle) administered 240 min before acid treatment in combination with MK886 (1 mg/kg) administered 30 min before PF3845 treatment. One-way repeated measures ANOVA did not indicate an effect of treatment. All bars show mean ± SEM in seven rats.

Figure 3.5
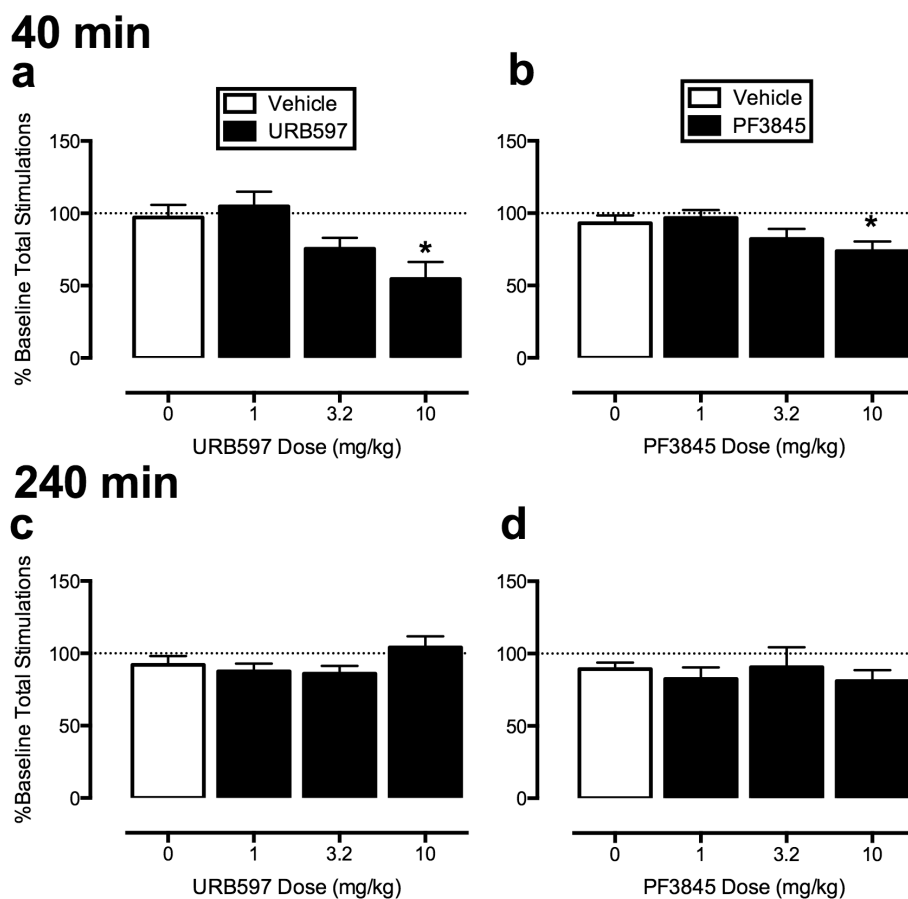
Figure 3.5. Effects of URB597 and PF3845 on control ICSS in the absence of a noxious stimulus. Abscissae: dose FAAH inhibitor in milligrams per kilogram. Ordinates: percent baseline total number of stimulations per component. The left panels (a and c) show the effects of URB597 (1-10 mg/kg or vehicle) administered 40 min (panel a) or 240 min (panel c) before ICSS. One-way repeated measures ANOVA indicated a significant main effect of URB597 treatment 40 min post-treatment (panel a) [$F(3,12)=13.47$; $p<0.001$]. The right panels (b and d) show the effects of PF3845 (1-10 mg/kg or vehicle) administered 40 min (b) or 240 min (d) before ICSS. One-way repeated measures ANOVA indicated a significant main effect of PF3845 treatment 40 min post-treatment (panel b) [$F(3,12)=5.51$; $p=0.013$]. Asterisks (*) in all panels indicate significantly different from vehicle treatment as indicated by Dunnett's post hoc test, $p < 0.05$. All bars show mean ± SEM in five rats.

Figure 3.6
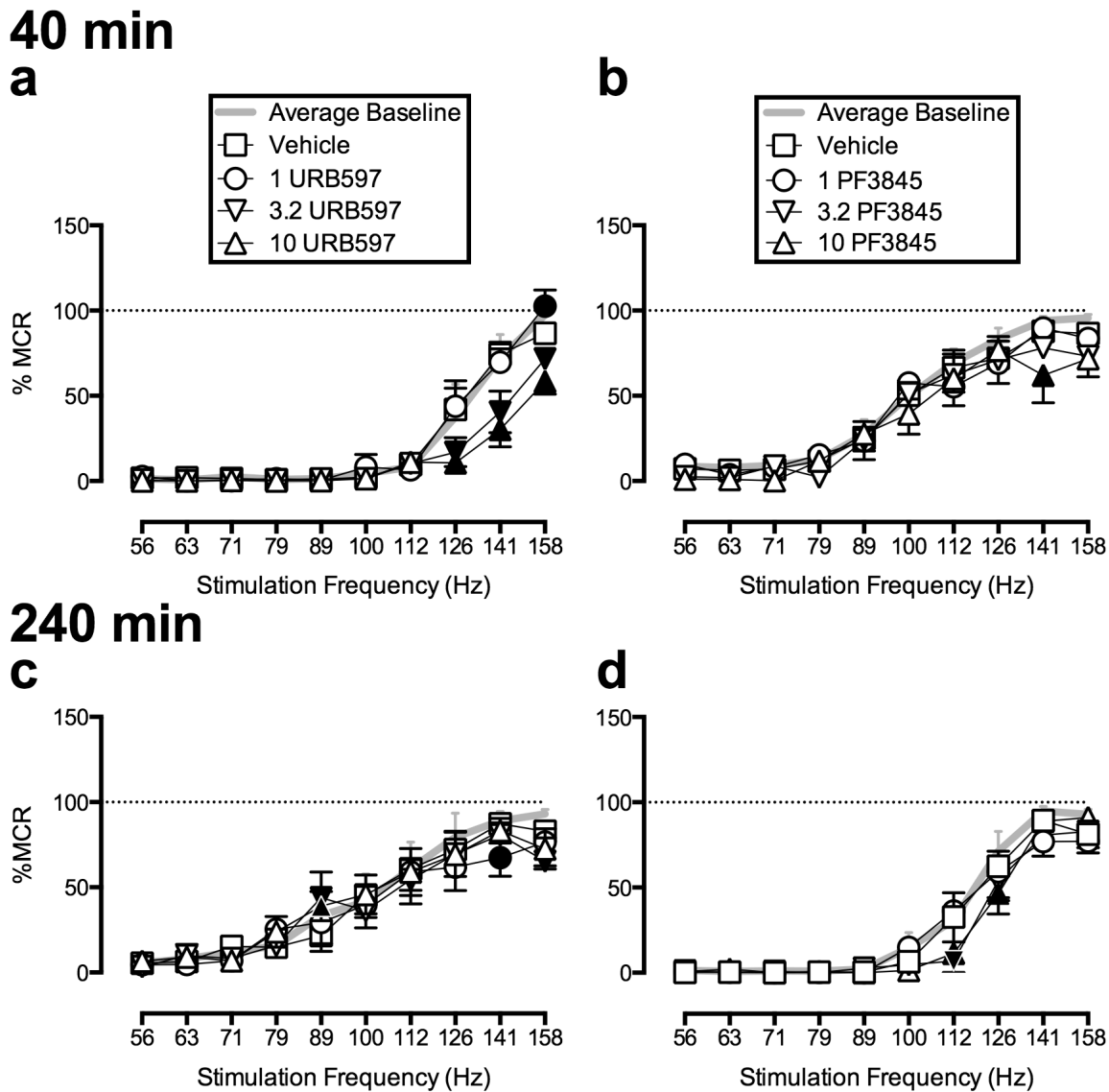
Figure 3.6. Full ICSS frequency-rate curves for Figure 3.5. Abscissae: frequency of electrical brain stimulation in hertz (log scale). Ordinates: percent maximum control response rate (%MCR). The left panels (a and c) show ICSS frequency-rate curves determined 40 min (panel a) or 240 min (panel c) after treatment with URB597 (1-10 mg/kg or vehicle). The average baseline ICSS frequency-rate curve for each study is shown by the gray line for comparison, but these data were not included in statistical analysis. Two-way repeated measures ANOVA indicated a significant main effect of

URB597 treatment in panel a [F(3,12)=15.89, $p<0.001$], a significant main effect of frequency in panels a [F(9,36)=41.79; $p<0.001$]) and c [F(9,36)=14.61; $p<0.001$]), and a significant treatment × frequency interaction panels a [F(27,108)=7.38; $p<0.001$]) and c [F(27,108)=1.61; $p=0.045$]). The right panels (b and d) show ICSS frequency-rate curves determined 40 min (panel b) or 240 min (panel d) after treatment with PF3845 (1-10 mg/kg or vehicle). Two-way repeated measures ANOVA indicated a significant main effect of PF3845 treatment in panel b [F(3,12)=5.00; $p=0.018$], a significant main effect of frequency in panels b [F(9,36)=47.94; $p<0.001$]) and d [F(9,36)=44.66; $p<0.001$]), and a significant treatment × frequency interaction in panel d [F(27,108)=2.81; $p<0.001$]). *Filled symbols* indicate frequencies at which reinforcement rates after URB597 or PF3845 treatment were significantly different than rates after vehicle treatment as determined by Holm-Sidak post hoc test, $p < 0.05$. All data show mean ± SEM in five rats.

Figure 3.7
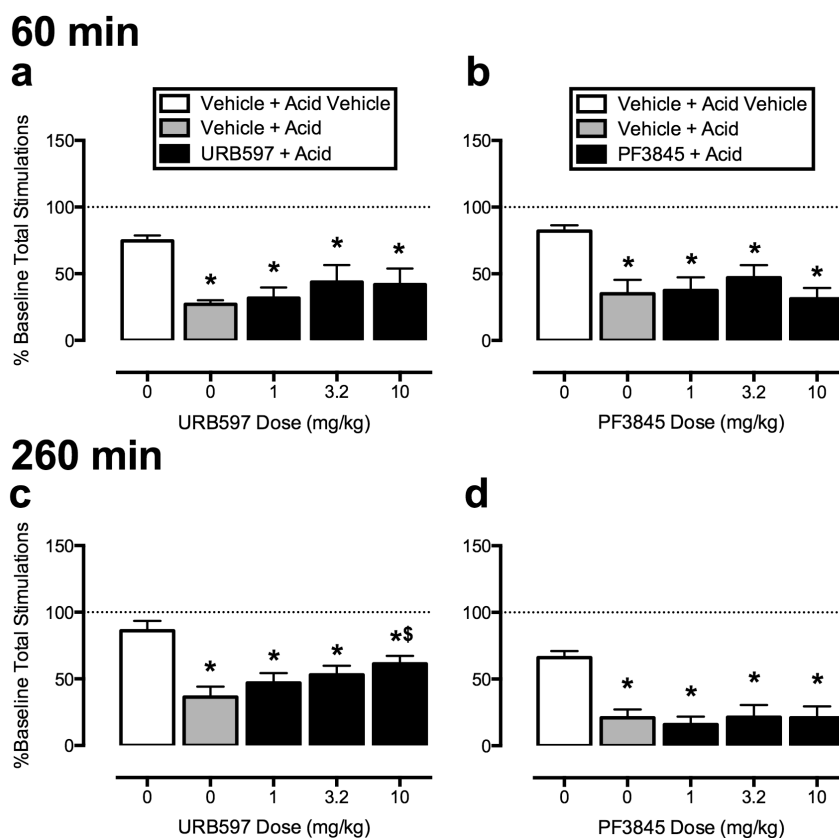
Figure 3.7. Effects of URB597 and PF3845 on acid-induced depression of ICSS. Abscissae: dose FAAH inhibitor in milligrams per kilogram. Ordinates: percent baseline total number of stimulations per component. The left panels (a and c) show the effects of URB597 (1-10 mg/kg or vehicle) administered 60 min (panel a) or 260 min (panel c) before acid treatment. One-way repeated measures ANOVA indicated a significant main effect of treatment in panels a [$F_{(4,16)}$=4.59; p=0.012] and c [$F_{(4,16)}$=9.83; p<0.001]. The right panels (b and d) show the effects of PF3845 (1-10 mg/kg or vehicle) administered 60 min (panel b) or 260 min (panel d) before acid treatment. One-way repeated measures ANOVA indicated a significant main effect of treatment in panels b [$F_{(4,16)}$=17.73; p<0.001] and d [$F_{(4,16)}$=10.96; p<0.001]. *Asterisks* (*) in all panels indicate significantly different from vehicle + acid vehicle treatment, and *dollar signs* ($) in panel c indicate significantly different from vehicle + acid treatment as indicated by Newman-Keuls post hoc test, p < 0.05. All bars show mean ± SEM in five rats.

Figure 3.8

60 min
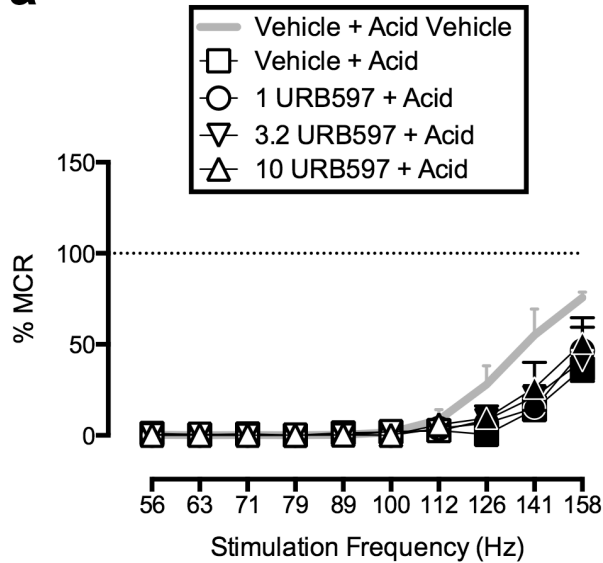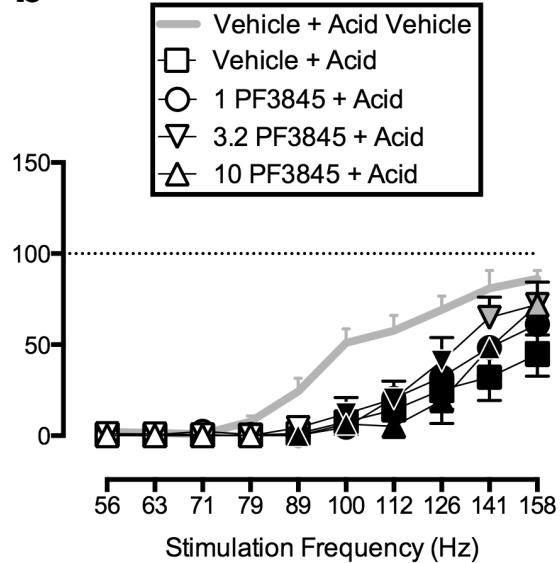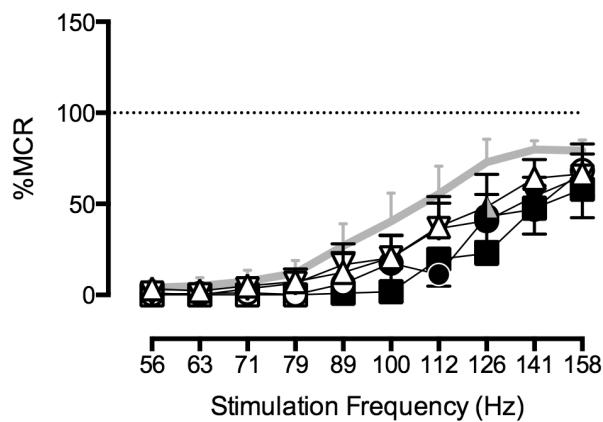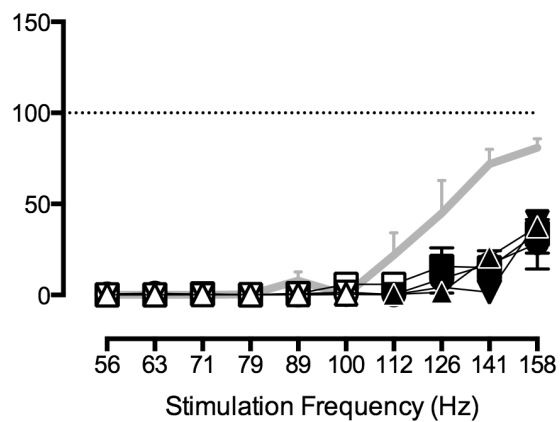
Figure 3.8. Full ICSS frequency-rate curves for Figure 3.7. Abscissae: frequency of electrical brain stimulation in hertz (log scale). Ordinates: percent maximum control response rate (%MCR). The left panels (a and c) show ICSS frequency-rate curves determined in the presence of the acid noxious stimulus 60 min (panel a) or 260 min (panel c) after treatment with URB597 (1-10 mg/kg or vehicle). Two-way repeated measures ANOVA indicated a significant main effect of treatment in panels a [F(4,16)=6.68; p=0.002] and c [F(4,16)=5.42 p=0.006]), a significant main effect of

frequency in panels a [$F_{(9,36)}$=24.42; $p<0.001$]) and c [$F_{(9,36)}$=16.13; $p<0.001$]), and a significant treatment × frequency interaction in panels a [$F_{(36,144)}$=3.41; $p<0.001$]) and c [$F_{(36,144)}$=1.76; p=0.010]). The right panels (b and d) show ICSS frequency-rate curves determined in the presence of the acid noxious stimulus 60 min (panel b) or 260 min (panel d) after treatment with PF3845 (1-10 mg/kg or vehicle). Two-way repeated measures ANOVA indicated a significant main effect of PF3845 treatment in panels b [$F_{(4,16)}$=22.00; $p<0.001$] and d [$F_{(4,16)}$=8.61; $p<0.001$]), a significant main effect of frequency in panels b [$F_{(9,36)}$=24.55; $p<0.001$]) and d [$F_{(9,36)}$=21.97; $p<0.001$]), and a significant treatment × frequency interaction in panels b [$F_{(36,144)}$=4.05; $p<0.001$]) and d [$F_{(36,144)}$=4.46; $p<0.001$]). *Black filled symbols* indicate frequencies at which reinforcement rates after vehicle + acid or drug + acid treatment were significantly lower than rates after vehicle + acid vehicle treatment, *gray filled symbols* indicate frequencies at which reinforcement rates after PF3845 + acid treatment were significantly higher than rates after vehicle + acid treatment, and *half-filled gray and black symbols* indicate frequencies at which reinforcement rates after URB597 + acid treatment were both significantly lower than rates after vehicle + acid vehicle treatment and significantly higher than rates after vehicle + acid treatment as determined by Holm-Sidak post hoc test, $p < 0.05$. All data show mean ± SEM in five rats.

Figure 3.9
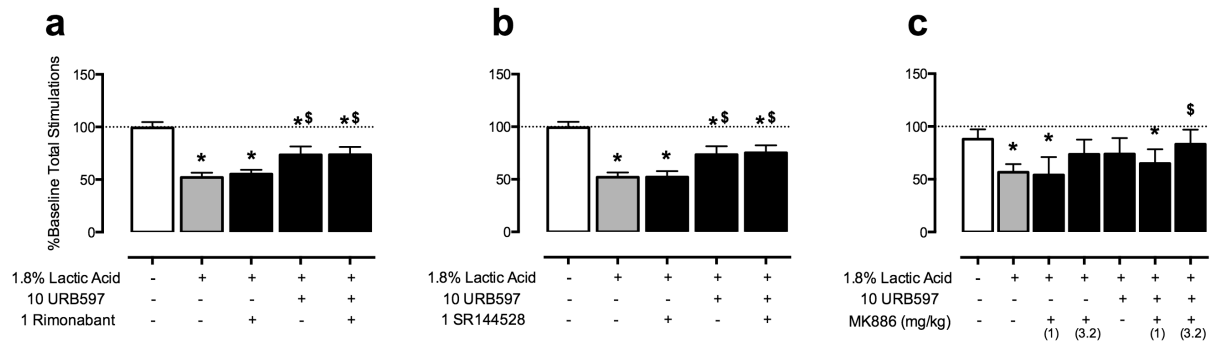
Figure 3.9. URB597-induced antinociception in the assay of acid depressed ICSS is not antagonized by rimonabant, SR144528, or MK886. Abscissae: Treatment conditions. Ordinates: percent baseline total number of stimulations per component. The left panel (a) shows the effects of URB597 (10 mg/kg or vehicle) administered 260 min before acid treatment in combination with rimonabant (1 mg/kg or vehicle) administered 50 min before acid treatment. One-way repeated measures ANOVA indicated a significant main effect of treatment in panel a [$F(4,16)=13.68$; $p<0.001$]. The center panel (b) shows the effects of URB597 (10 mg/kg or vehicle) administered 260 min before acid treatment in combination with SR144528 (1 mg/kg or vehicle) administered 50 min before acid treatment. One-way repeated measures ANOVA indicated a significant main effect of treatment in panel b [$F(4,16)=14.53$; $p<0.001$]. The right panel (c) shows the effects of MK886 (1-3.2 mg/kg or vehicle) administered 30 min before URB597 (10 mg/kg or vehicle), which was administered 260 min acid treatment. One-way repeated measures ANOVA indicated a significant main effect of treatment in panel c [$F(6,24)=5.46$; $p<0.001$]. Asterisks (*) in all panels indicate significantly different from vehicle + acid vehicle treatment, and *dollar signs* ($) in all panels indicate significantly different from vehicle + acid treatment as indicated by Newman-Keuls post hoc test, $p < 0.05$. All bars show mean ± SEM in five rats.

Figure 3.10
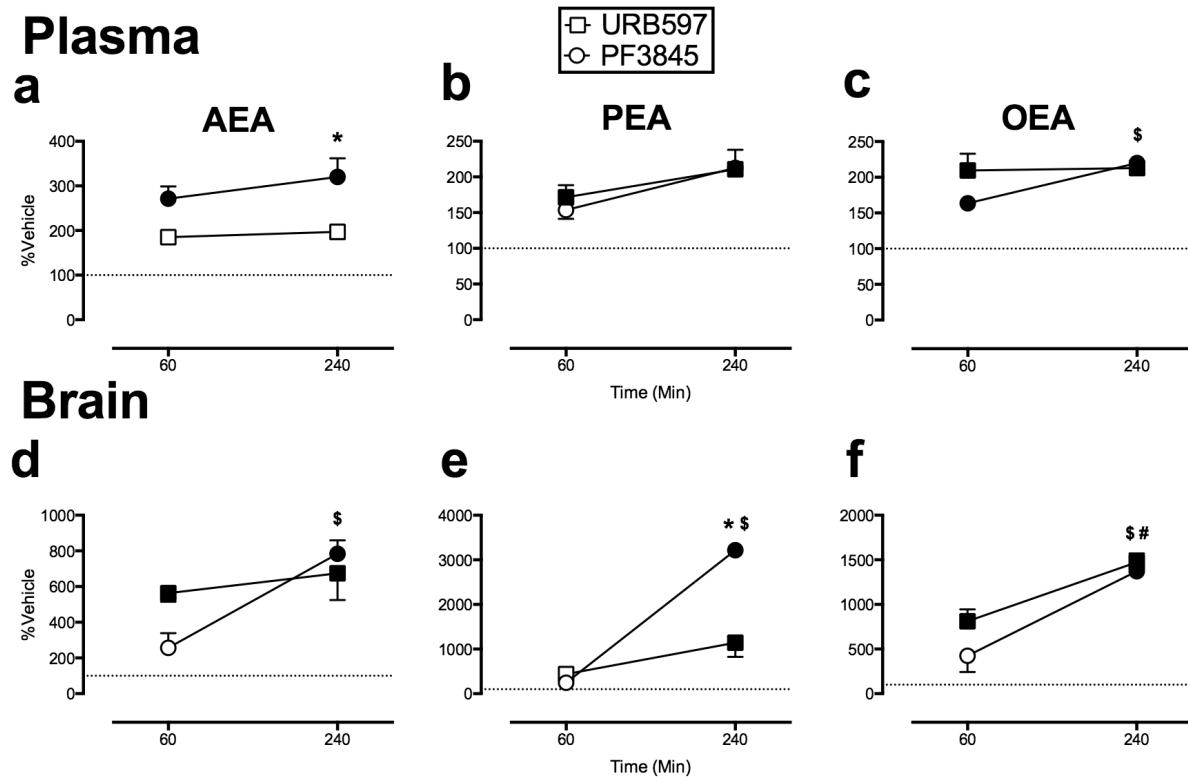
Figure 3.10. Both URB597 and PF3845 produced time-dependent increases in plasma and brain fatty acid ethanolamines. Abscissae: time post-drug administration in minutes. Ordinates: percent of vehicle-treatment. All panels show the effects of URB597 (10 mg/kg or vehicle) and PF3845 (10 mg/kg or vehicle) on plasma (upper panels) and brain (lower panels) levels of anandamide (AEA) (panels a and d), palmitoylethanolamide (PEA) (panels b and e), and oleoylethanolamide (OEA) (panels c and f). In plasma, two-way ANOVA indicated a significant main effect of treatment (panel a [F(2,17)=37.17; p<0.001], panel b [F(2,17)=26.15; p<0.001], and panel c [F(2,17)=71.77; p<0.0001]), a significant main effect of time (panel b [F(1,17)=8.06; p=0.011] and panel c [F(1,17)=5.93; p=0.026]), and a significant treatment × time interaction (panel c [F(2,17)=5.15; p<0.018]). In brain samples, two-way ANOVA indicated a significant main effect of treatment (panel d [F(2,17)=22.92; p<0.001], panel e [F(2,17)=69.14; p<0.001], and panel f [F(2,17)=59.62; p<0.001]), a significant main effect of time (panel d [F(1,17)=10.11; p=0.006], panel e [F(1,17)=110.2; p<0.001], and

panel f [F(1,17)=43.01; *p*<0.001]), and a significant treatment × time interaction (panel d [F(2,17)=6.01; *p*=0.011], panel e [F(2,17)=61.68; *p*<0.001], and panel f [F(2,17)=12.36; *p*<0.001]). *Filled symbols* indicate significantly different from vehicle treatment (i.e. 100%), *asterisks* (*) indicate a significant difference between URB597 and PF3845 treatment, *number* signs (#) indicate a significant difference in URB597 treatment 60 min versus 240 min post-treatment, and *dollar signs* ($) indicate a significant difference in PF3845 treatment 60 min versus 240 min post-treatment as indicated by Holm-Sidak post hoc test, *p* < 0.05. All points show mean ± SEM in four rats except for URB597 60 min post-treatment which shows mean ± SEM in three rats.

3.4. Discussion

This study assessed effects of the FAAH inhibitors URB597 and PF3845 in assays of pain-stimulated and pain-depressed behavior in rats. There were four main findings. First, URB597 produced partial but dose-dependent antinociception in both assays, and these results provide some support for further consideration of URB597 as a candidate analgesic drug. Second, PF3845 failed to produce antinociception in either assay, and the poor preclinical efficacy of PF3845 agrees with the poor clinical efficacy of the structurally-related FAAH inhibitor PF7845 to treat osteoarthritis-related pain (Huggins et al., 2012). Together, these findings highlight the potential for differential effects by drugs with nominally similar mechanisms of action. Third, URB597-induced antinociception in the assay of acid-stimulated stretching was mediated by CB1Rs, but not CB2Rs or PPAR-α, whereas in the assay of acid-depressed ICSS, URB597-induced antinociception was not mediated by CBRs or PPAR-α. Lastly, both URB597 and PF3845 similarly increased biomarkers AEA, PEA, and OEA that have previously been associated with antinociceptive effects of FAAH inhibitors (Ahn et al., 2009; Ahn et al., 2011). These results challenge prevailing notions regarding the importance of FAAH inhibition, AEA/fatty acid ethanolamine generation, and cannabinoid receptor/PPAR-α activation in mediating the antinociceptive effects of FAAH inhibitors and suggest differences in the potential of FAAH inhibitors as candidate analgesics for treatment of acute pain.

CHAPTER FOUR

Effects of the CB2R agonist GW405833

on acute pain-stimulated and pain-depressed behavior

Manuscript in preparation for submission for publication

4.1. Introduction

CBR agonists are classified by their ability to bind to and activate CB1Rs and/or CB2Rs (Howlett, 1995; Pertwee, 2010). CB1Rs are located primarily on nerve cells (Devane et al., 1988; Matsuda et al., 1990; Herkenham et al., 1991), whereas CB2Rs have been found primarily on immune-related cells, such as macrophages and microglia (Munro et al., 1993; Van Sickle et al., 2005; Wilkerson and Milligan, 2011). Both specific CB1R agonists and CB2R agonists have been shown to produce antinociception in many traditional preclinical assays of pain, however CB1R agonists have also been associated with cannabimimetic-related side effects such as behavioral depression/sedation, which has dampened overall clinical enthusiasm for these compounds (Rice, 2006; Karst et al., 2010). In contrast, CB2R agonists do not produce cannabimimetic-related side effects until they reach doses that produce nonspecific CB1R-mediated effects (Anikwue et al., 2002; Valenzano et al., 2005; Whiteside et al., 2005; Karst and Wippermann, 2009). Several CB2R agonists are in various stages of clinical development (see Wilkerson and Milligan, 2011 for review); however, recently a clinical trial for the treatment of dental-related pain failed with the CB2R agonist GW842166 (Ostenfeld et al., 2011). This disparity between preclinical and clinical

findings suggests that traditional preclinical assays of pain used to study CB2R agonists in animals may not be sufficient alone to predict clinical analgesic effects in humans.

The goal of the present study was to assess the effects of the CB2R agonist GW405833 in preclinical assays of acute pain-stimulated and pain-depressed behavior in rats. Assays of pain-stimulated and pain-depressed behavior have been used previous to examine the effects of opioids, nonsteroidal anti-inflammatory drugs, and other drug classes (Pereira Do Carmo et al., 2009; Negus et al., 2010b; Kwilasz and Negus, 2012; Negus et al., 2012; Rosenberg et al., 2013). We have previously shown that the CBR agonists THC and CP55940 failed to produce antinociception in assays of pain-depressed behavior (Kwilasz and Negus, 2012), a finding concordant with the poor clinical efficacy of CBR agonists as analgesics in humans (Raft et al., 1977; Rice, 2006; Karst et al., 2010; Kraft, 2012). We have also shown in Chapter 3 of this dissertation that the FAAH inhibitor URB597 produces non-CBR-mediated antinociception in the assay of pain-depressed behavior. Given that CB2R agonists display weaker efficacy than CB1R agonists to produce motor impairment similar to FAAH inhibitors, we predicted that GW405833 might be more likely than CB1R agonists to produce antinociception in assays of pain-depressed behavior.

4.2. Methods

Subjects

Fifteen male Sprague-Dawley rats (Harlan, Frederick, MD, USA) weighing approximately 300-320 g (age 10-11 weeks) were used for these studies. All housing, maintenance, and research conditions in this chapter are identical to those described

previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Assay of lactic acid-stimulated stretching

 Behavioral procedure. Six rats that failed to meet the criteria for ICSS within 4 weeks (see below) were used for studies of lactic acid-stimulated stretching as described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

 Studies with GW405833 were conducted in two phases. First, a GW405833 dose-effect curve was determined by administering GW405833 (3.2-32 mg/kg or vehicle) 60 min prior to acid. Second, to assess the role of CB1Rs and CB2Rs in mediating GW405833 effects, GW405833-induced antinociception in the assay of acid-stimulated stretching was evaluated for its sensitivity to antagonism by the CB1R antagonist rimonabant and the CB2R antagonist SR144528, respectively. For these studies, rimonabant (1 mg/kg or vehicle) or SR144528 (1 mg/kg or vehicle) was administered 20 min prior to GW405833 (32 mg/kg), and acid was administered 60 min after GW405833. These antagonist doses and pretreatment times were based on previous studies that have demonstrated antagonism of cannabinoid agonist-induced antinociception with rimonabant (Kwilasz and Negus, 2012) and SR144528 (Hohmann et al., 2004). All treatments were delivered in randomized order across rats and separated by at least one week.

Data Analysis. All methods of data analysis in this chapter are identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Assay of intracranial self-stimulation (ICSS)

Surgery. All surgical procedures in this chapter are identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Apparatus. All apparatus and materials used in this chapter are identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Behavioral procedure. After initial shaping of lever press responding, rats were trained under a continuous reinforcement schedule of brain stimulation using procedures identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Studies with GW405833 were conducted in three phases. In the first phase, the effects of GW405833 on ICSS were studied in the absence of the noxious stimulus (control ICSS). Subjects were placed in their home cages after administration of GW405833 (1-32 mg/kg or vehicle) and then transferred back to the operant chambers at the designated times (60, 120, and 240 min) for two consecutive "test" components, totaling 20 min at each time point. In the second phase, GW405833 effects on ICSS were studied in the presence of the noxious stimulus (acid-depressed ICSS). Subjects were administered GW405833 (3.2-32 mg/kg or vehicle) 60 min prior to lactic acid

(1.8% in a volume of 1 ml/kg), which was administered immediately before two consecutive "test" components. Lastly, in the third phase, to assess the role of CB1Rs and CB2Rs in mediating GW405833 effects, GW405833-induced antinociception in the assay of acid-depressed ICSS was evaluated for its sensitivity to antagonism by the CB1R antagonist rimonabant and the CB2R antagonist SR144528, respectively. For these studies, rimonabant (1 mg/kg or vehicle) or SR144528 (1 mg/kg or vehicle) was administered 20 min prior to GW405833 (32 mg/kg or vehicle), and acid was administered 60 min after GW405833. All treatments were delivered in randomized order across rats and separated by at least on week.

Data Analysis. All methods of data analysis in this chapter are identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Drugs

Lactic acid was purchased from Sigma Chemical Co. (St. Louis, MO, USA). GW405833 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rimonabant and SR144528 were provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD, USA). Lactic acid was prepared in sterile water. GW405833 was prepared in a vehicle consisting of 10% ethanol, 10% cremophor (Sigma), and 90% sterile saline. Rimonabant and SR144528 were prepared in a vehicle consisting of 5% ethanol, 5% cremophor, and 90% sterile saline. All solutions were injected intraperitoneally in a volume of 1 ml/kg.

4.3. Results

Effects of GW405833 on acid-stimulated stretching. Figure 4.1 shows that IP administration of lactic acid (1.8% in a volume of 1 ml/kg) stimulated approximately 15 stretches after administration of vehicle (*gray bar*). GW405833 (3.2-32 mg/kg) dose-dependently and significantly decreased stretching 60 min after administration. Figure 4.2 shows that the antinociceptive effect of GW405833 in the assay of acid-stimulated stretching was significantly but only partially antagonized by administration of the CB1R antagonist rimonabant (1 mg/kg, Fig. 4.2a), but not by the CB2R antagonist SR144528 (1 mg/kg, Fig. 4.2b).

Effects of GW405833 on ICSS in the absence of the noxious stimulus. During each test session, a "baseline" frequency-rate curve was determined before testing to permit determination of the Maximum Control Rate (MCR) for that session. Over the course of the entire study in this group of rats, the average MCR was 51.6 ± 5.1 stimulations/trial and the average baseline total stimulations was 215.2 ± 34.1. Reinforcement rates during each frequency trial of a session were then expressed as a percentage of that session's MCR, and the average baseline frequency-rate curve for all tests in the control ICSS phase is shown in Figure 4.3a as a *gray line*. Rats generally did not respond at frequencies of 56-89 Hz, and reinforcement rates increased across a frequency range of 89-158 Hz. Maximum reinforcement rates were usually observed at the highest stimulation frequencies.

When administered 60 min prior to an ICSS session, GW405833 produced minimal effects on the ICSS frequency-rate curve (Fig. 4.3a). A low dose of 3.2 mg/kg GW405833 increased ICSS at a single frequency of 100 Hz, whereas an intermediate

dose of 10 mg/kg GW405833 decreased ICSS at a single frequency of 112 Hz. GW405833 did not produce any effect on total stimulations at either 60, 120, or 240 min post-administration (Fig. 4.3b).

Effects of GW405833 on acid-induced depression of ICSS. Figure 4.4 shows that the same noxious stimulus used in the stretching assay (IP injection of 1.8% lactic acid in 1 ml/kg) depressed ICSS and that GW405833 (3.2-32 mg/kg) dose-dependently produced antinociception in the assay of acid-depressed ICSS. Treatment with acid vehicle had little effect on the frequency-rate curve; however, treatment with 1.8% lactic acid depressed ICSS, producing a significant rightward shift in the frequency-rate curve (Fig. 4.4a) and a decrease in total stimulations delivered across all frequencies (*gray bar*, Fig. 4.4c). When administered 60 min prior to acid, GW405833 (3.2-32 mg/kg) produced significant but partial antinociception in the assay of acid-depressed ICSS (32 mg/kg, Figs. 4.4b and 4.4c). A dose of 32 mg/kg GW405833 significantly attenuated acid-induced depression of ICSS at a single frequency of 112 Hz (Fig. 4.4b). Additionally, total stimulations delivered across all frequencies after treatment with 32 mg/kg GW405833 were not significantly different from treatment with acid vehicle, however they were also not different than treatment with acid alone (Fig. 4.4c).

Figure 4.5 shows that the CB1R antagonist rimonabant and the CB2R antagonist SR144528 failed to reverse GW405833-induced antinociception in the assay of acid-depressed ICSS. Pretreatment with 1 mg/kg rimonabant (Fig. 4.5a) or 1 mg/kg SR144528 (Fig. 4.5b) had no effect on the antinociceptive effects of 32 mg/kg GW405833. Furthermore, these doses of rimonabant (Fig. 4.5a) and SR144528 (Fig. 4.5b) produced no effects on acid-induced depression of ICSS alone. Statistical

analysis did provide some support for a partial attenuation of GW405833-induced antinociception with rimonabant, insofar as GW405833 + acid was not different than control, whereas ICSS after rimonabant + GW05833 + acid was depressed relative to control but also significantly different than acid alone.

Figure 4.1
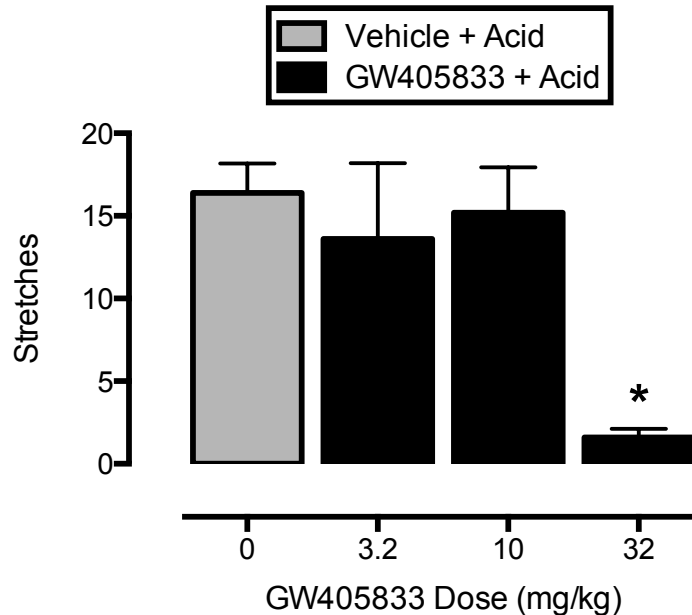
Figure 4.1. GW405833 produced dose-dependent blockade of lactic acid-stimulated stretching. Figure 4.1 shows the effects of GW405833 (3.2-32 mg/kg) or its vehicle administered 60 min before acid treatment. Abscissa: dose GW405833 in milligrams per kilogram. Ordinate: number of stretches observed during a 30 min observation period. One-way ANOVA indicated a significant main effect of GW405833 treatment [$F_{(3,12)}=7.81$; $p=0.004$]. The *asterisk* (*) indicates significantly different from vehicle + acid as determined by Dunnett's post hoc test, $p < 0.05$. All *bars* show mean ± SEM in five rats.

Figure 4.2
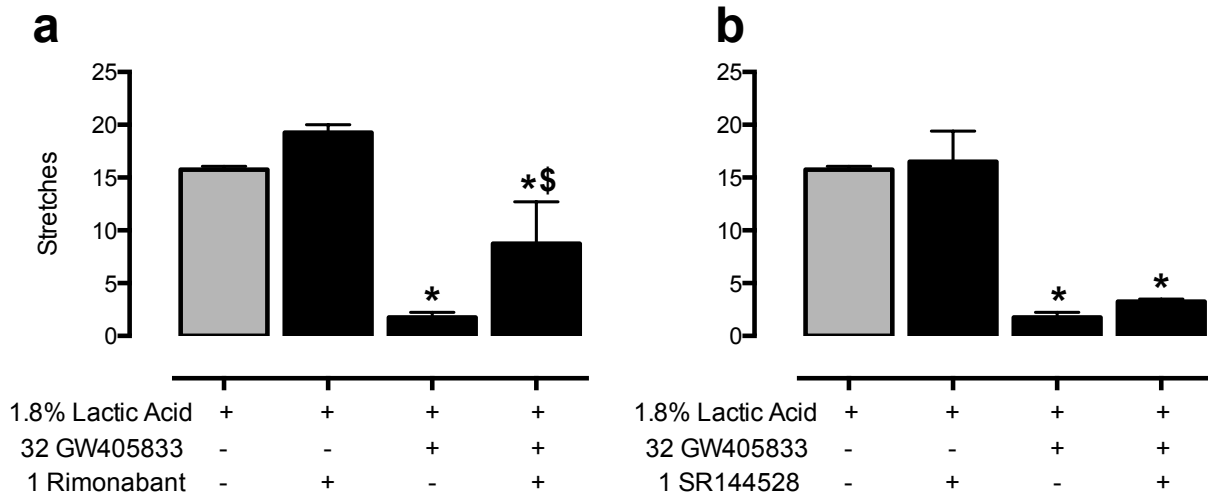
Figure 4.2. GW405833-induced antinociception in the assay of acid-stimulated stretching is attenuated by rimonabant but not by SR144528. Abscissae: Treatment conditions. Ordinates: number of stretches observed during a 30 min observation period. The left panel (*a*) shows the effect of rimonabant (1 mg/kg or vehicle) administered 80 min before acid treatment in combination with GW405833 (32 mg/kg or vehicle) administered 60 min before acid treatment. One-way repeated measures ANOVA indicated a significant main effect of treatment in panel *a* [F(3,9)=18.75; p<0.001]. The right panel (*b*) shows the effect of SR144528 (1 mg/kg or vehicle) administered 80 min before acid treatment in combination with GW405833 (32 mg/kg or vehicle) administered 60 min before acid treatment. One-way repeated measures ANOVA indicated a significant main effect of treatment in panel *b* [F(3,9)=32.68; p<0.001]. *Asterisks* (*) in all panels indicates significantly different from vehicle + acid, and the *dollar sign* ($) in panel *a* indicates significantly different from GW405833 (32 mg/kg) + acid as determined by Newman-Keuls post hoc test, p < 0.05. All *bars* show mean ± SEM in four rats.

Figure 4.3
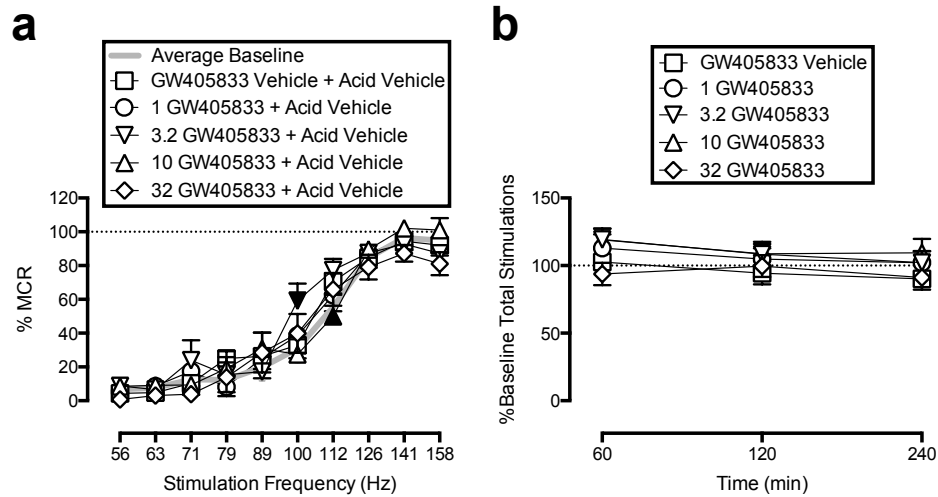
Figure 4.3. GW405833 produced dose-dependent and time-dependent alterations of intracranial self-stimulation (ICSS) in the absence of a noxious stimulus. The left panel *(a)* shows ICSS frequency-rate curves determined 60 min after treatment with GW405833 (1-32 mg/kg) or its vehicle. Abscissa: frequency of electrical brain stimulation in hertz (log scale). Ordinate: ICSS rate expressed as percent maximum control response rate (%MCR). The average baseline ICSS frequency-rate curve for the entire study in this group of rats is shown by the *gray line* for comparison, but these data were not included in statistical analysis. Two-way ANOVA indicated a significant main effect of frequency [$F(9,45)=55.90$; *p*<0.001] and a significant frequency × treatment interaction [$F(27,135)=2.22$; *p*=0.002]. *Filled symbols* indicate frequencies at which reinforcement rates after GW405833 treatment were different than rates after GW405833 vehicle treatment as determined by Holm-Sidak post hoc test, *p* < 0.05. All *symbols* show mean ± SEM in six rats. The right panel *(b)* shows the total number of stimulations per component expressed as a percent of baseline stimulations per component following treatment with GW405833 (1-32 mg/kg) or its vehicle at various pretreatment times. Abscissa: time following GW405833 or vehicle administration. Ordinate: percent baseline total number of stimulations per component. Two-way ANOVA indicated a significant main effect of time [$F(2,10)=8.23$; *p*=0.008]. All *symbols* show mean ± SEM in six rats.

Figure 4.4
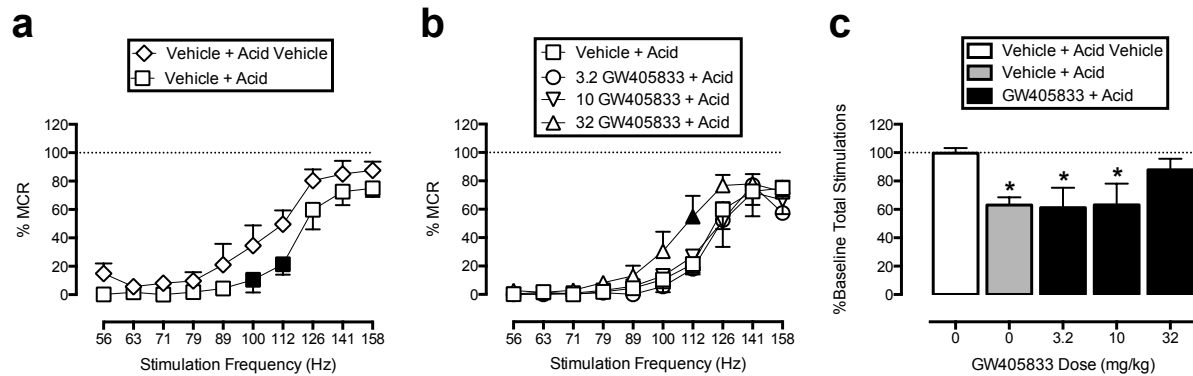
Figure 4.4. Lactic acid depresses intracranial self-stimulation (ICSS) and GW405833 produces antinociception in the assay of acid-depressed ICSS. The left panel *(a)* shows ICSS frequency-rate curves determined after treatment with GW405833 vehicle 60 min before lactic acid vehicle or 1.8% lactic acid administration. Abscissa: frequency of electrical brain stimulation in hertz (log scale). Ordinate: ICSS rate expressed as percent maximum control response rate (%MCR). Two-way ANOVA indicated a significant main effect of acid treatment [$F_{(1,4)}=24.69$; $p=0.008$] and a significant main effect of frequency [$F_{(9,36)}=23.87$; $p<0.001$]. *Filled symbols* indicate frequencies at which reinforcement rates after acid treatment were significantly lower than rates after acid vehicle treatment as determined by Holm-Sidak post hoc test, *p* < 0.05. All *symbols* show mean ± SEM in five rats. The center panel *(b)* shows ICSS frequency-rate curves determined after treatment with GW405833 (3.2-32 mg/kg) or its vehicle 60 min before acid administration. Abscissa: frequency of electrical brain stimulation in hertz (log scale). Ordinate: ICSS rate expressed as percent maximum control response rate (%MCR). Two-way ANOVA indicated a significant main effect of GW405833 treatment [$F_{(3,12)}=3.52$; $p=0.049$], a significant main effect of frequency [$F_{(9,36)}=27.47$; *p*<0.001], and a significant frequency × treatment interaction [$F_{(27,108)}=1.59$; *p*=0.049]. Filled symbols indicate frequencies at which reinforcement rates after GW405833 + acid treatment were significantly higher than after vehicle + acid treatment as determined by Holm-Sidak post hoc test, *p* < 0.05. All *symbols* show mean ± SEM in five rats. The right

panel *(c)* shows the total number of stimulations per component expressed as a percent of baseline stimulations per component after treatment with GW405833 (3.2-32 mg/kg) or its vehicle 60 min before administration of acid or its vehicle. Abscissa: dose GW405833 in milligrams per kilogram. Ordinate: percent baseline total number of stimulations per component. One-way ANOVA indicated a significant main effect of treatment [$F_{(4,16)}=4.25$; *p*=0.016]. The *asterisks* (*) indicate significantly different from GW405833 vehicle + acid vehicle as determined by Newman Keul's post hoc test, *p* < 0.05. ICSS after 32 mg/kg GW405833 + acid was not different from either the vehicle + acid vehicle or the vehicle + acid conditions. All *bars* show mean ± SEM in five rats.

Figure 4.5
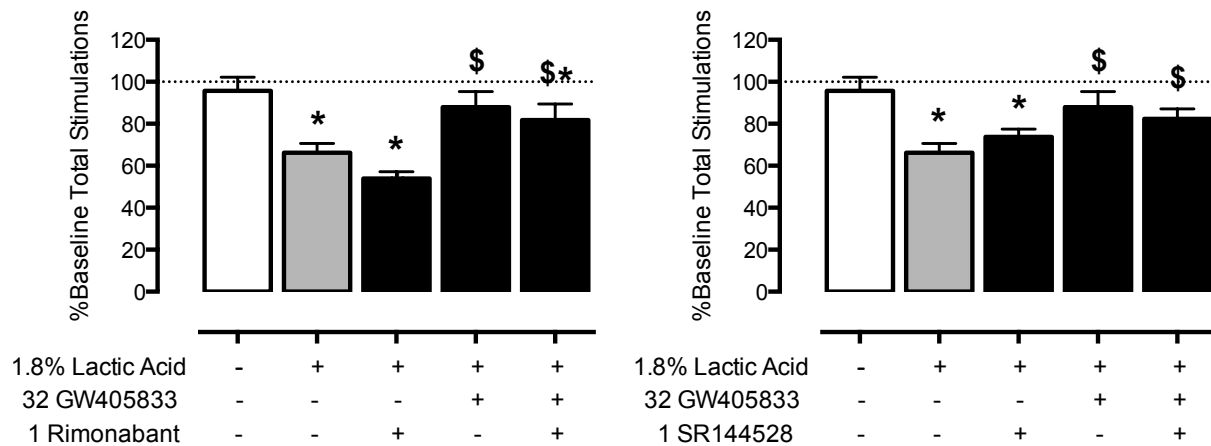
Figure 4.5. GW405833-induced antinociception in the assay of acid depressed ICSS is not antagonized by rimonabant or SR144528. Abscissae: Treatment conditions. Ordinates: percent baseline total number of stimulations per component. The left panel (*a*) shows the effect of rimonabant (1 mg/kg or vehicle) administered 80 min before acid treatment in combination with GW405833 (32 mg/kg or vehicle) administered 60 min before acid treatment. One-way repeated measures ANOVA indicated a significant main effect of treatment in panel a [$F_{(4,20)}$=19.54; p<0.001]. The right panel (*b*) shows the effect of SR144528 (1 mg/kg or vehicle) administered 80 min before acid treatment in combination with GW405833 (32 mg/kg or vehicle) administered 60 min before acid treatment. One-way repeated measures ANOVA indicated a significant main effect of treatment in panel b [$F_{(4,20)}$=6.90; p=0.001]. *Asterisks* (*) in all panels indicate significantly different from vehicle + acid vehicle treatment, and *dollar signs* ($) in all panels indicate significantly different from vehicle + acid treatment as determined by Newman-Keuls post hoc test, p < 0.05. All *bars* show mean ± SEM in six rats.

4.4. Discussion

The goal of these studies was to assess the effects of the CB2R agonist GW405833 in assays of acute pain-stimulated and pain-depressed behavior in rats. There were three main findings. First, GW405833 produced dose-dependent and CB1R-mediated but not CB2R-mediated antinociception in the assay of acid-stimulated stretching. These results demonstrate the potential for GW405833 to produce CB1R-mediated antinociception at higher doses (i.e. 32 mg/kg and higher), and are in agreement with other studies that have reported GW405833 produces CB1R-like effects such as behavioral depression and antinociception at high doses (Valenzano et al., 2005; Whiteside et al., 2005). Second, GW405833 produced no effect on ICSS in the absence of the noxious stimulus. These results are the first to report on the effects of a CB2R agonist in an assay of ICSS and show that GW405833 does not produce abuse-related facilitation of ICSS that is characteristic of many other drugs of abuse (Carlezon and Chartoff, 2007; Bauer et al., 2013). Lastly, GW405833 also produced dose-dependent and non CBR-mediated antinociception in the assay of acid-depressed ICSS, although there was also weak evidence this effect was partially mediated by CB1Rs. Collectively, these results show that GW405833 produces antinociception in both assays of pain-stimulated and pain-depressed behavior, but through non CB2R-mediated mechanisms. Further research is necessary to determine the other mechanisms mediating GW405833-induced antinociception in assays of acute pain-stimulated and pain-depressed. Lastly, these results question the importance of CB2R activation for the treatment of acute pain.

CHAPTER FIVE

Effects of THC on acute and repeated LPS-induced stimulation

of mechanical allodynia and depression of ICSS.

5.1. Introduction

Marijuana has been used for centuries to treat pain, and CBR agonists produce antinociception in many preclinical assays of pain (Rice, 2006; Karst et al., 2010). CBR agonists have also been shown to produce anti-inflammatory effects in many preclinical assays, and this effect is thought to be at least partially responsible for the antinociceptive effects of CBR agonists in these studies (Croxford and Yamamura, 2005; Burstein and Zurier, 2009; Stein and Machelska, 2011). Specifically, CBR agonists have been shown to decrease physiological increases in pro-inflammatory-related molecules, such as cytokines, after treatment with an inflammatory challenge, such as LPS (Puffenbarger et al., 2000; Roche et al., 2006). In clinical studies, CBR agonists have been shown to have some efficacy to treat inflammatory-related pain (Blake et al., 2006), and have also been approved to treat multiple sclerosis-related muscle spasticity and associated pain and in several countries worldwide (Leussink et al., 2012). The analgesic effects of CBR agonists in these clinical pathologies may also be related to their anti-inflammatory properties.

There were two main goals in this study. First, we sought to evaluate the effects of chronic and acute inflammatory challenges (i.e. repeated or acute IP LPS, respectively) on pain-related stimulation of mechanical allodynia and pain-related

depression of behavior. Administration of LPS or direct administration of pro-inflammatory cytokines has previously been shown to produce mechanical allodynia in a Von Frey paw-withdrawal procedure and hyperalgesia in a warm water tail-flick procedure (Watkins et al., 1994; Cahill et al., 1998). Furthermore, other studies have shown that LPS or pro-inflammatory cytokine administration produces inflammation-related decreases in behavior such as ICSS (Anisman et al., 1996; Anisman et al., 1998; Borowski et al., 1998; Barr et al., 2003; van Heesch et al., 2013), feeding (Kubera et al., 2013), and social interaction (Konsman et al., 2008). The second goal of this study was to assess the effects of the prototype cannabinoid agonist, THC, on LPS-stimulated mechanical allodynia (assay of pain-stimulated behavior) and LPS-induced depression of ICSS (assay of pain-depressed behavior), as cannabinoid agonists have been shown to produce robust anti-inflammatory effects in many preclinical studies (Croxford and Yamamura, 2005; Valenzano et al., 2005; Whiteside et al., 2005; Burstein and Zurier, 2009) and may also be effective in several clinical chronic inflammatory pain-related disorders (i.e. rheumatoid arthritis and multiple sclerosis) (Blake et al., 2006; Leussink et al., 2012). We have previously used assays of pain-stimulated and pain-depressed behavior to assess the effects of opioids, nonsteroidal anti-inflammatory drugs, and other drug classes (Pereira Do Carmo et al., 2009; Negus et al., 2010b; Kwilasz and Negus, 2012; Negus et al., 2012; Rosenberg et al., 2013). Specifically, we have shown that the mixed CB1R/CB2R agonists THC and CP55940 failed to produce antinociception on acute pain-induced depression of behavior (Kwilasz and Negus, 2012) elicited by IP lactic acid administration, a finding concordant with the poor clinical efficacy of CBR agonists as analgesics against acute pain in humans (Raft et al., 1977;

Rice, 2006; Karst et al., 2010; Kraft, 2012). Chapters three and four of this dissertation also show that the FAAH inhibitor URB597 and the CB2R agonist GW405833 produce non-CBR-mediated antinociception in the assay of pain-depressed behavior. Taken together, these data suggest that assays of pain-depressed behavior may be useful for the assessment of candidate cannabinoid analgesics, and furthermore may provide insight into new mechanisms of cannabinoid-mediated antinociception. Insofar as CBR agonists produce anti-inflammatory effects and may be effective to treat several clinical inflammatory pain-related disorders (Blake et al., 2006; Leussink et al., 2012), we predicted that a CBR agonist such as THC might be more effective against an inflammatory noxious stimulus such as IP LPS versus a chemically-induced noxious stimulus such as IP acid. Body temperature was also assessed as a physiological indicator of LPS exposure.

5.2. Methods

Subjects

Twenty-eight male Sprague-Dawley rats (Harlan, Frederick, MD, USA) weighing approximately 300-320 g (age 10-11 weeks) were used for these studies. All housing, maintenance, and research conditions in this chapter are identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Assay of LPS-induced changes in body temperature

Behavioral procedure. Eighteen rats were used for studies of LPS-induced changes in body temperature. During test sessions, rats were wrapped in a 2 ft. x 3 ft. length of surgical drape and held firmly horizontally against the chest of the experimenter. A 2 mm diameter wire probe attached to a Thermometer (VWR International; Radnor, PA) was inserted into the subject's rectum to a marked line that indicated approximately 3.5 cm from the tip of the probe and was held in place for 6 seconds or until a stable temperature was obtained. Rats were tested for body temperatures immediately after determining mechanical thresholds in the assay of LPS-stimulated mechanical allodynia as described below.

To assess the effect of THC on acute LPS-induced changes in body temperature, THC (0.32-1 mg/kg or vehicle) was administered 90 min after LPS or saline. Body temperatures were determined immediately prior to THC administration (LPS/saline baseline), and then 30 and 100 min thereafter. Tests were conducted weekly, and animals received LPS and saline once every two weeks, alternating LPS and saline treatments each week for a total of 6 weeks. THC was delivered in Latin-Square dose order and doses were separated by one week.

Data Analysis. Drug effects on LPS-induced changes in body temperature were evaluated by repeated measures two-way analysis of variance (ANOVA). A significant ANOVA was followed by Holm-Sidak post hoc test, and the criterion for significance was set at $p < 0.05$.

Assay of LPS-stimulated mechanical allodynia

  Behavioral procedure. Twenty-three rats were used for studies of LPS-stimulated mechanical allodynia. During test sessions, rats were placed in an elevated, custom-built chamber composed of acrylic walls and a wire mesh floor (15 cm high x 41 cm deep x 91 cm across) to permit access to the plantar surfaced of the right rear paw from below. The up-and-down method was used to determine mechanical thresholds (Chaplan et al., 1994). Rats were habituated in the chamber for at least 15 min prior to testing.

  Studies with LPS were conducted in two phases. First, the effect of chronic LPS administration on mechanical thresholds was determined by administering LPS (0.1 mg/kg) 30 min and 100 min before mechanical threshold determination for 7 consecutive days. Saline was also administered for three days prior to LPS and data from these days were averaged to determine a saline baseline. Rats in the first phase had intracranial ICSS implants and were also subjects in ICSS studies that were conducted immediately after the mechanical threshold determination. Second, in a separate group of rats without intracranial ICSS implants, to assess the effect of THC on acute LPS-stimulated mechanical allodynia, THC (0.32-1 mg/kg or vehicle) was administered 90 min after LPS or saline. Mechanical thresholds were determined immediately prior to THC administration (baseline), and then 30 and 100 min thereafter. Tests were conducted weekly, and animals received LPS and saline once every two weeks, alternating LPS and saline treatments each week for a total of 6 weeks. THC was delivered in Latin-Square dose order and doses were separated by one week. Animals in phase two tested in the mechanical allodynia procedure were also tested for

LPS-induced changes in body temperature as described above immediately following determination of mechanical thresholds.

Data Analysis. Drug effects on LPS-stimulated mechanical were evaluated by repeated measures one-way or two-way analysis of variance (ANOVA). A significant ANOVA was followed by Dunnett's (one-way) or Holm-Sidak (two-way) post hoc test, and the criterion for significance was set at $p < 0.05$. In phase one, the saline baseline (average of 3 days prior to LPS injection) served as the baseline for the rest of the study. In phase two, baselines were determined before LPS/saline injection on each test day.

Assay of intracranial self-stimulation (ICSS)

Surgery. All surgical procedures in this chapter are identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Apparatus. All apparatus and materials used in this chapter are identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Behavioral procedure. After initial shaping of lever press responding, rats were trained under a continuous reinforcement schedule of brain stimulation using procedures identical to those described previously (Kwilasz and Negus, 2012; Rosenberg et al., 2013) and in Chapter Two of this dissertation.

Studies with LPS were conducted in two phases. In phase one, the effects of chronic LPS on ICSS were studied. In this phase, LPS (0.1 mg/kg) was administered

consecutively for seven days 30 min and 100 min before the ICSS session. Subjects were placed in the chamber to determine mechanical thresholds after administration of LPS (0.1 mg/kg) and then transferred back and forth to the operant chambers at the designated times (30 and 100 min) for three consecutive "test" components, totaling 30 min at each time point. Saline was also administered for three days prior to LPS and data from these days were averaged to determine a saline baseline. In the second phase, the effects of THC on acute LPS-induced depression of ICSS were studied. In this phase, LPS (0.1 mg/kg or saline) was administered immediately after removing the subjects from the operant chamber after the third baseline component. Subjects were placed in their home cages after administration of LPS and then transferred back to the operant chambers after 90 min for two consecutive "test" components, for a total of 20 min. This initial test was used to determine the baseline effect of LPS. Immediately after determination of this baseline, THC (0.32-1 mg/kg or vehicle) was administered and subjects were placed in their home cages and transferred back to the operant chambers at the designated times (30 and 100 min) for two consecutive "test" components, totaling 20 min at each time point. Tests were conducted weekly, and animals received LPS and saline once every two weeks, alternating LPS and saline treatments each week for a total of 6 weeks. THC was delivered in Latin-Square dose order and doses were separated by one week.

   Data Analysis. The primary dependent variable in this ICSS procedure was the total number of stimulations per component, which was calculated as the sum of stimulations delivered across all 10 frequency-trials of each component. Test data were then normalized to individual baseline data using the equation Percent Baseline Total

Stimulations per Component = (Mean Total Stimulations per Test Component ÷ Mean Total Stimulations per Baseline Component) x 100. Data were then averaged across rats in each experimental condition and compared by repeated measures one-way ANOVA or two-way ANOVA where appropriate. A significant one-way ANOVA was followed by Dunnett's post hoc test, a significant two-way ANOVA was followed by Holm-Sidak post hoc test, and the criterion for significance was set at $p < 0.05$. In phase one, the saline baseline (average of 3 days prior to LPS injection) served as the baseline for the rest of the study, and subsequent data collected with LPS were normalized to this baseline. In phase two, baselines were determined before LPS/saline injection on each test day, and data for each test day were normalized to its respective baseline.

Drugs

LPS was purchased from Sigma Chemical Co. (St. Louis, MO). THC was provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). LPS was prepared in sterile saline. THC was prepared in a vehicle consisting of ethanol, cremophor (Sigma), and sterile saline in a ratio of 1:1:18, respectively. All solutions were injected intraperitoneally in a volume of 1 ml/kg.

5.3. Results

Effects LPS and THC on body temperature. Figure 5.2a shows that acute IP administration of LPS (0.1 mg/kg) induced treatment-dependent hypothermia 90 min after administration. This effect was treatment dependent, as subsequent treatments

with LPS failed to induce hypothermia (Figure 5.2a). Figure 5.3 (panels a and d) show that administration of THC (0.32-1 mg/kg or vehicle) did not significantly alter body temperature alone or in combination with LPS at either 30 min (Fig. 5.3a) or 100 min (Fig. 5.3d) post-administration, and both Figs. 5.3a and 5.3d further illustrate the variable and weak effects of LPS on body temperature.

Effects of THC on LPS-stimulated mechanical allodynia. Figure 5.1a shows that chronic administration of LPS (0.1 mg/kg) produced a significant decrease in mechanical thresholds for the first two consecutive days at 30 min post-administration. This effect was no longer seen on days 3-7 of LPS treatment, however. Moreover, this same regimen of LPS failed to significantly decrease mechanical thresholds on any day at 100 min post-administration (Fig 5.1c). Figure 5.2b shows that acute IP administration of LPS (0.1 mg/kg) did not produce a significant effect on mechanical thresholds 90 min after administration on any of the three times it was tested. Figure 5.3 shows that administration of THC (0.32-1 mg/kg or vehicle) did not significantly alter mechanical thresholds alone or in combination with LPS at either 30 min (Fig. 5.3b) or 100 min (Fig. 5.3e) post-administration.

Effects of THC on LPS-induced depression of ICSS. Figure 5.1 (panels b and d) show that chronic administration of LPS (0.1 mg/kg) produced a time-dependent decrease in total stimulations of ICSS delivered across all frequencies. LPS significantly decreased ICSS at 30 min post-administration on the second day of chronic treatment (Fig. 5.1b), whereas at 100 min post-administration, ICSS was significantly decreased on days 1, 2, and 4 of chronic LPS treatment (Fig. 5.1d). Figure 5.2c shows that acute IP administration of LPS (0.1 mg/kg) produced variable and weak decreases in total

stimulations of ICSS delivered across all frequencies compared to administration of IP lactic acid used in previous chapters. Specifically, acute LPS treatment significantly decreased total stimulations of ICSS after the first and third administration, but not after the second administration (2-week washout between LPS treatments) (Fig. 5.2c). Figure 5.3 shows that administration of THC (0.32-1 mg/kg or vehicle) did not significantly alter LPS-induced depression of ICSS at either 30 min (Fig. 5.3c) or 100 min (Fig. 5.3f) post-administration.

Figure 5.1
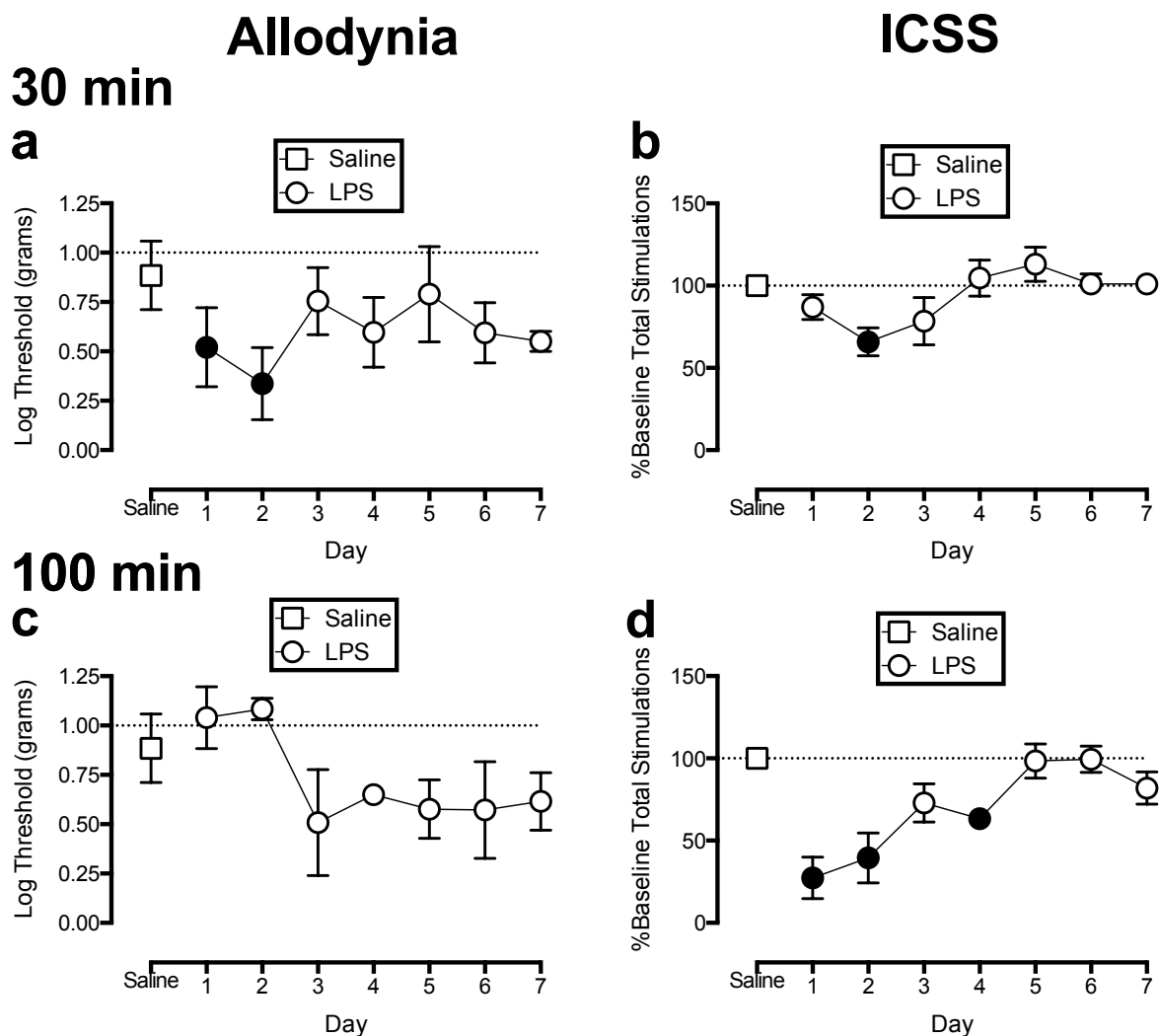
Figure 5.1. Chronic LPS produces a treatment- and time-dependent decrease in mechanical thresholds and ICSS. Figure 5.1a shows the effect of LPS (0.1 mg/kg) or saline administered 30 min before mechanical threshold determination. Abscissa (all panels): day of LPS treatment. Ordinate (panels a and c): Log mechanical threshold in grams. One-way ANOVA indicated a significant main effect of LPS treatment [F(7,21)=3.92; *p*=0.007]. Figure 5.1c shows the effect of LPS (0.1 mg/kg) or saline administered 100 min before mechanical threshold determination. One-way ANOVA indicated a significant main effect of LPS treatment [F(7,21)=2.69; *p*=0.038]. Figure 5.1b shows the effect of LPS (0.1 mg/kg) or saline administered 30 min before ICSS.

Ordinate (panels b and d): percent baseline total number of stimulations per component. One-way ANOVA indicated a significant main effect of LPS treatment [$F_{(7,28)}=3.73$; *p*=0.005]. Figure 5.1d shows the effect of LPS (0.1 mg/kg) or saline administered 100 min before ICSS. One-way ANOVA indicated a significant main effect of LPS treatment [$F_{(7,28)}=10.22$; *p*<0.001]. *Filled symbols* indicate significantly different from saline treatment as determined by Dunnett's post hoc test, *p* < 0.05. All *bars* show mean ± SEM in five rats.

Figure 5.2
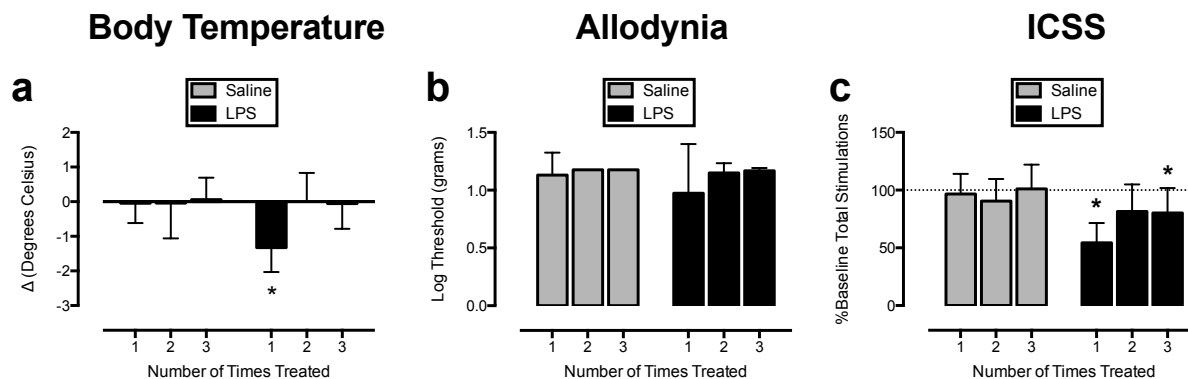
Figure 5.2. Acute LPS administration produces unreliable and treatment-dependent effects on body temperature, mechanical thresholds, and ICSS. Figure 5.2a shows the effect of LPS (0.1 mg/kg) or saline administered 90 min before determination of body temperatures. Abscissa (all panels): Number of times treated with LPS or saline. Ordinate: Change in body temperature from pre-LPS/saline baseline in degrees Celsius. Two-way ANOVA indicated a significant main effect of LPS treatment [$F_{(1,17)}=7.91$; $p=0.012$], a significant main effect of times treated [$F_{(2,34)}=16.14$; $p<0.001$], and a significant treatment x times treated interaction [$F_{(2,34)}=9.13$; $p<0.001$]. All *bars* show mean ± SEM in 18 rats. 5.2b shows the effect of LPS (0.1 mg/kg) or saline administered 90 min before determination of mechanical thresholds. Ordinate: Log mechanical threshold in grams. Two-way ANOVA indicated a significant main effect of times treated [$F_{(2,34)}=4.48$; $p=0.019$]. All *bars* show mean ± SEM in 18 rats. Figure 5.2c shows the effect of LPS (0.1 mg/kg) or saline administered 90 min before determination of ICSS. Ordinate: percent baseline total number of stimulations per component. Two-way ANOVA indicated a significant main effect of LPS treatment [$F_{(1,4)}=12,16$; $p=0.025$] and a significant treatment x times treated interaction [$F_{(2,8)}=5.27$; $p<0.035$]. All *bars* show mean ± SEM in six rats. *Asterisks* (*) in all panels indicate significantly different than respective saline control bar as determined by Holm-Sidak post hoc test, $p < 0.05$.

Figure 5.3
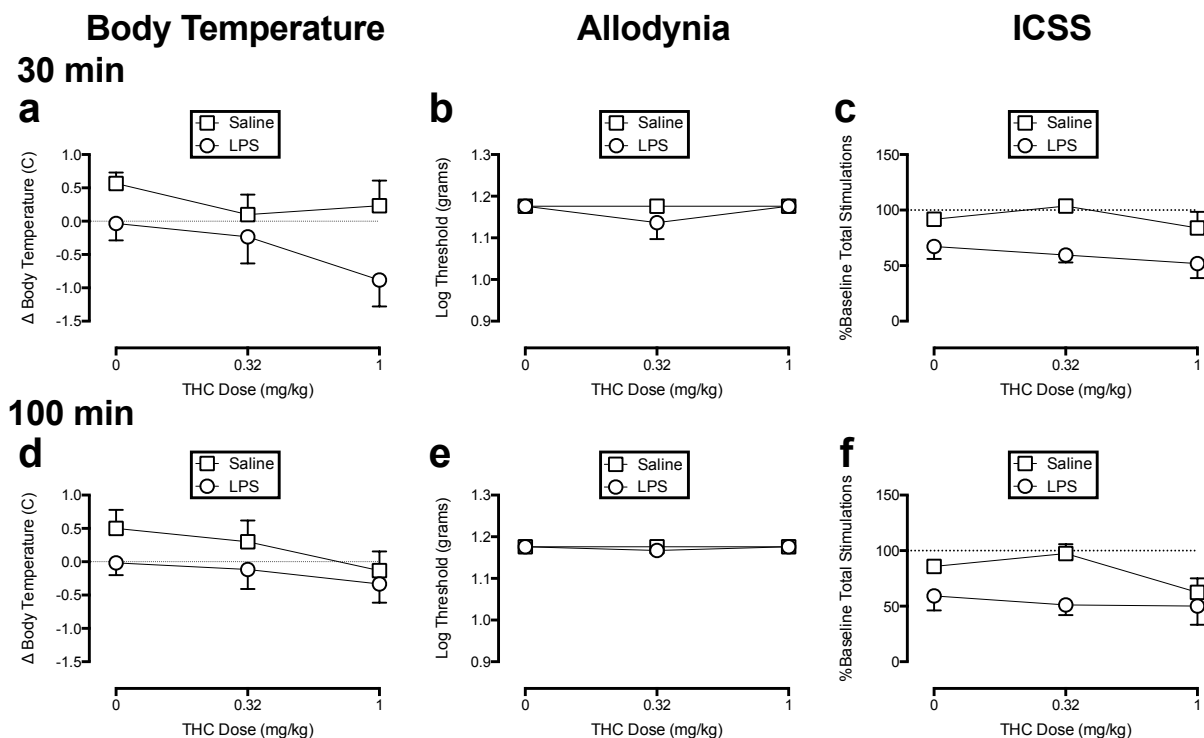
Figure 5.3. THC administration does not alter acute LPS-induced effects on body temperature, mechanical thresholds, and ICSS. 5.3a shows the effect of THC (0.32-1 mg/kg or vehicle) administered 30 min after LPS or saline on body temperature. Abscissa (all panels): Dose THC in milligrams per kilogram. Ordinate (panels a and d): Change in body temperature from pre-LPS/saline baseline in degrees Celsius. Two-way ANOVA indicated a significant main effect of LPS treatment [F(1,5)=13.00; *p*=0.015]. Figure 5.3d shows the effect of THC (0.32-1 mg/kg or vehicle) administered 100 min after LPS or saline on body temperature. There was no effect of LPS or THC on body temperature at this time point as indicated by two-way ANOVA. Figure 5.3 shows the effect of THC (0.32-1 mg/kg or vehicle) administered 30 min (Fig. 5.3b) and 100 min (Fig. 5.3e) after LPS or saline on mechanical thresholds. Ordinate (panels b and e): Log mechanical threshold in grams. There was no effect of LPS or THC on mechanical thresholds at either time point. All *bars* in panels a-d show mean ± SEM in 18 rats. Figure 5.3c shows the effect of THC (0.32-1 mg/kg or vehicle) administered 30 min after LPS or saline on LPS-depressed ICSS. Ordinate (panels c and f): percent baseline total

number of stimulations per component. Two-way ANOVA indicated a significant main effect of LPS treatment [$F(1,4)=25.01$; $p=0.008$]. Figure 5.3f shows the effect of THC (0.32-1 mg/kg or vehicle) administered 100 min after LPS or saline on LPS-depressed ICSS. Two-way ANOVA indicated a significant main effect of LPS treatment [$F(1,4)=17.54$; $p=0.014$].

5.4. Discussion

The goal of these studies was to assess the effects of THC in assays of LPS-stimulated and LPS-depressed behavior. Body temperature was also included as a physiological marker of LPS effects. There were four main findings. First, chronic LPS administration produced treatment- and time-dependent stimulation of mechanical allodynia as well as treatment- and time-dependent decreases in ICSS. In general, however, these effects of LPS were transient and unreliable. Second, acute LPS administration produced no effect on mechanical thresholds, and as with chronic administration, also produced transient and unreliable decreases in ICSS. The discrepancy between acute and chronic LPS administration in the assay of mechanical allodynia may be due to a number of dose, time, or subject-related variables. There is no literature, however, that supports this profile of effects of LPS on mechanical thresholds. Furthermore, some literature suggests an initial inflammatory insult may be necessary to produce (or "prime") LPS-stimulated mechanical allodynia (Cahill et al., 1998; Hains et al., 2010). In this regard, animals studied with THC in the assays of mechanical allodynia and body temperature did not have intracranial implants, which also may have influenced behavioral responses to LPS administration, as the intracranial surgery could have served at least as a "priming" inflammatory insult. Third, acute LPS produced a reliable hypothermic effect after the first administration, but this effect was absent after the second and third LPS administrations. LPS has previously been shown to produce both hyperthermic effects in rats (Klir et al., 1993, unpublished observations); however, it has only been shown to produce hypothermic effects at higher doses in rats (Steiner et al., 2011; Al-Saffar et al., 2013). The dose chosen for

LPS was 0.1 mg/kg, which is moderately high compared to other studies. Thus although these data were not predicted, they also were not completely unexpected. Lastly, THC administration did not alter the rather weak effects of LPS on body temperature, mechanical thresholds, and ICSS. Taken together, these results suggest that LPS was a poor noxious stimulus in this set of experiments compared to IP lactic acid used in previous chapters. Future studies would benefit from a more extensive manipulation of dose-, time-, and subject-related variables in regard to LPS administration, and/or assessment of other inflammatory-related noxious stimuli.

CHAPTER SIX

Discussion

6.1. Summary

The experiments described in this dissertation evaluated the antinociceptive effects of cannabinoid receptor agonists and of FAAH inhibitors in preclinical assays of pain-stimulated and pain-depressed behaviors in rats. In chapters 2-4, an IP injection of dilute lactic acid served as the noxious stimulus to model acute pain. All cannabinoids tested produced CB1R-mediated antinociception in the assay of acid-stimulated stretching with the exception of PF3845, which for unknown reasons produced pronociception. In contrast, cannabinoids produced distinct effects in assays of acid-depressed behavior. The mixed-action CB1R/CB2R agonists THC and CP55940 failed to produce antinociception in assays of pain-depressed behavior, whereas the FAAH inhibitor URB597 and the CB2R agonist GW405833 both produced submaximal though statistically significant antinociception. Intriguingly, the effects of both of URB597 and GW405833 were not mediated by CB1Rs or CB2Rs. Moreover the other FAAH inhibitor, PF3845, failed to produce antinociception in the assay of acid-depressed ICSS; a finding concordant with its lack of effect in the assay of pain-stimulated behavior, but discrepant with the antinociceptive effects of the other FAAH inhibitor URB597 in the same assays. Taken together, the results with URB597 and GW40583 suggest alternative non-cannabinoid mechanisms may mediate the antinociceptive effects of these two compounds in assays of pain-depressed behavior. In chapter 5, we also

attempted to model more chronic inflammatory-related pain through acute/repeated IP injection of LPS and subsequent assessment of mechanical allodynia and depression of ICSS. LPS produced weak and unreliable pronociceptive effects in assays of both LPS-stimulated mechanical allodynia or LPS-depressed ICSS, and these weak effects of LPS made it difficult to assess the effects of cannabinoids on LPS-induced pronociceptive behavior. THC was nevertheless tested in these assays but did not show any indication that it altered these weak effects of LPS. These results provide weak evidence that THC may be ineffective against LPS-stimulated mechanical allodynia and LPS-depressed ICSS; however, further studies are needed with a more reliable chronic inflammatory-related noxious stimulus to fully determine the effects of cannabinoids in assays of chronic inflammatory pain-depressed behavior.

6.2. Effects of the mixed CB1R/CB2R agonists THC and CP55940 on acute pain-stimulated and pain-depressed behavior.

Cannabinoid agonist effects on pain-stimulated behavior. In assays of pain-stimulated behavior, delivery of a noxious stimulus increases the rate or intensity of the target behavior, and drug-induced antinociception is inferred from drug-induced decreases in the target behavior (Negus et al., 2010a). In the present study, acid stimulated a stretching response in rats, and THC and CP55940 produced antinociception insofar as they decreased acid-stimulated stretching. The antinociceptive effects of THC and CP55940 in the present study agree with a large literature showing that cannabinoid agonists produce antinociception in nearly all assays of pain-stimulated behavior (for recent reviews, see Rice, 2006; Karst et al.,

2010). For example, previous studies in rodents have shown that cannabinoid agonists decreased stretching elicited by IP acid administration (Sofia et al., 1975; Anikwue et al., 2002; Booker et al., 2009), first and second phases of nociceptive behavior elicited by intraplantar formalin injection (Finn et al., 2004; Khodayar et al., 2006), tail-flick/paw-withdrawal responses elicited by noxious heat (Lichtman and Martin, 1991; De Vry et al., 2004; Wiley et al., 2007), and hypersensitive withdrawal responses elicited by thermal/mechanical stimuli in inflammatory or neuropathic pain models (Cheng and Hitchcock, 2007; Elikkottil et al., 2009; Sain et al., 2009). As in the present study, cannabinoid antinociception is often shown to be dose and/or time dependent, and sensitivity to rimonabant antagonism or genetic knockout of cannabinoid 1 receptors has been interpreted as evidence of cannabinoid 1 receptor mediation (Monory et al., 2007; Booker et al., 2009). It is generally appreciated that nonselective behavioral depression may confound measures of cannabinoid antinociception in assays of pain-stimulated behavior (De Vry et al., 2004; Finn et al., 2004), and in the present study, THC and CP55940 produced evidence of nonselective behavioral depression insofar as they decreased ICSS in the absence of pain. However, THC-induced depression of acid-stimulated stretching was longer lasting than THC-induced depression of control ICSS. Furthermore, chronic THC administration produced complete tolerance to THC-induced depression of control ICSS, while only partial tolerance developed to THC-induced depression of acid-stimulated stretching. Lastly, CP55940 produced antinociception in the assay of acid-stimulated stretching at doses that produced no effect on control ICSS. These findings provide evidence for a selective antinociceptive effect of cannabinoids in assays of pain-stimulated behavior. Similarly, other studies of

pain-stimulated behavior on THC and CP55940 have found comparable dose selectivity for antinociception versus nonselective behavioral depression (Fox et al., 2001; Booker et al., 2009). Overall, the robust and reliable antinociceptive effects of cannabinoid agonists in preclinical assays of pain-stimulated behavior have encouraged development of cannabinoids as candidate analgesics.

Cannabinoid agonist effects on pain-depressed behavior. In assays of pain-depressed behavior, delivery of a noxious stimulus decreases the rate or intensity of the target behavior, and drug-induced antinociception is inferred from drug-induced increases in the target behavior (Negus et al., 2010a). In the present study, acid-induced depression of ICSS and of feeding served as assays of pain-depressed behavior, and in these assays, THC and CP55940 failed to produce antinociception. Rather, THC and CP55940 only exacerbated acid-induced depression of ICSS and were ineffective in the assay of acid-depressed-feeding. This lack of cannabinoid antinociception cannot be attributed to a lack of assay sensitivity. In the present study and in a previous study (Negus et al., 2011), the NSAID ketoprofen blocked acid-stimulated stretching and acid-induced depression of ICSS and feeding, and these data agree with the clinical efficacy of ketoprofen for treatment of acute pain in animals (Flecknell, 2009) and humans (Sarzi-Puttini et al., 2010). Similarly, the mu opioid receptor agonist and clinically effective analgesic morphine also blocked acid-stimulated stretching and acid-induced depression of ICSS in rats (Pereira Do Carmo et al., 2009; Negus et al., 2010b) and acid-induced depression of feeding in mice (Stevenson et al., 2006). Both NSAID and mu-opioid analgesics have also been shown to block other examples of pain-depressed behavior including acid-induced depression of locomotion

and wheel running in mice (Stevenson et al., 2009; Miller et al., 2011), laparotomy-induced depression of locomotion and food-maintained operant responding in rats (Martin et al., 2007), and depression of locomotion and wheel running induced by bilateral inflammation of the knee joints by complete Freund's adjuvant in rats (Matson et al., 2007; Cobos et al., 2012). Taken together, these results suggest that cannabinoid agonist effects on pain-depressed behavior are opposite to those produced by clinically effective NSAID and opioid analgesics.

Determinants of the poor efficacy of cannabinoids in assays of pain-depressed behavior remain to be understood. However, three points warrant mention. First, cannabinoids can produce general behavioral depressant effects manifested in this study as decreases in control ICSS, and such general behavioral depressant effects could obscure expression of antinociception in assays of pain-depressed behavior (Negus et al., 2010a). However, several findings argue against a major influence of this factor. For example, THC/CP55940 failed to block acid-induced depression of ICSS even at times/doses that did not decrease control ICSS but did block acid-stimulated stretching. Moreover, in contrast to previous results with a delta opioid receptor agonist (Negus et al., 2012), chronic administration of THC did not unmask antinociceptive effects of THC in the assay of acid-depressed ICSS, despite producing complete tolerance to THC-induced rate-decreasing effects on control ICSS and only partial tolerance to THC-induced antinociception in the assay of acid-stimulated stretching. Lastly, THC failed to block pain-related depression of feeding at a dose that did attenuate satiation-related depression of feeding in this study and that stimulated feeding by rats in other studies (Williams et al., 1998; Jarbe and DiPatrizio, 2005).

Consequently, just as nonselective behavioral depression could not account entirely for the apparent *presence* of cannabinoid antinociception in the assay of pain-stimulated behavior, it also cannot account entirely for the *absence* of cannabinoid antinociception in the assays of pain-depressed behavior.

Second, the neural circuits that mediate acid-induced stimulation of stretching and depression of ICSS are incompletely mapped but may be dissociable (Willis, 2009), and the present results provide support for this contention. For example, noxious stimuli activate both serial and parallel spinal and supraspinal pathways, and different neural circuits have been associated with different components of pain (e.g. sensory vs. affective components of pain) (Price, 2002; Borsook and Becerra, 2011). Results of the present study suggest that THC and other cannabinoids may be more effective in modulating neural circuits that mediate acid-induced stimulation of stretching than those mediating acid-induced depression of ICSS.

Lastly, the present study evaluated effects of systemic cannabinoid administration on pain-related behaviors produced by an acute chemical noxious stimulus delivered to the abdominal cavity, and poor cannabinoid antinociception may be related to these or other experimental variables. To expand the scope of these data, we assessed the efficacy of other cannabinoid drugs (i.e. inhibitors of endocannabinoid hydrolysis and agonists selective for cannabinoid 2 receptors) to produce antinociception in assays acute acid-stimulated stretching and acid-depressed ICSS in *Chapters Three* and *Four* of this dissertation. We furthermore examined the effects of THC on LPS-stimulated mechanical allodynia and LPS-depressed ICSS in *Chapter Five* of this dissertation, to assess the efficacy of a cannabinoid receptor agonist against a

more inflammatory-related pain state. Other studies would also benefit from assessing other modalities of noxious stimuli, or by noxious stimulation of other parts of the body, as well as by administration of cannabinoids via other routes of administration (e.g. intraplantar, intravenous, inhalation, etc.). However, the present results from this chapter demonstrate the potential for diametrically opposite effects of cannabinoid receptor agonists on pain-stimulated and pain-depressed behaviors elicited by the same noxious stimulus. Moreover, these results distinguish these cannabinoids from clinically effective analgesics and suggest that cannabinoid antinociception in assays of acute pain-stimulated behavior cannot be attributed to a simple and selective blockade of sensitivity to noxious stimuli.

Abuse-related effects of mixed CB1/CB2R cannabinoid agonists. In control experiments for this study, cannabinoid effects on ICSS were evaluated in the absence of the acid noxious stimulus. Drug-induced facilitation of ICSS under these conditions is often interpreted as an abuse-related effect (Carlezon and Chartoff, 2007), but THC and CP55940 produced only decreases in ICSS. These results are consistent with previous studies showing only rate-decreasing effects of cannabinoid agonists on ICSS (Vlachou et al., 2005; Vlachou et al., 2007), although other studies have found weak facilitation of ICSS by THC under certain conditions (Gardner et al., 1988; Lepore et al., 1996). THC and other cannabinoid agonists also often fail to produce place conditioning as well as reinforcing effects in assays of cannabinoid drug self-administration (see Panagis et al., 2008 for review). Taken together, these findings suggest that THC and related cannabinoids often fail to produce abuse-related facilitation of ICSS under conditions that are sensitive to facilitation by other classes of abused drugs.

6.3. Effects of the FAAH inhibitors URB597 and PF3845 on acute pain-stimulated and pain-depressed behavior.

FAAH inhibitor effects on pain-stimulated behavior. Chapter 3 described effects of the FAAH inhibitors URB597 and PF3845 in the same assays of acid-stimulated stretching and acid-depressed ICSS that were used in testing with THC and CP55940. URB597 produced dose-dependent and CB1R-mediated antinociception in the assay of acid-stimulated stretching insofar as it decreased this stretching behavior at 1 h and 4 h after administration. The antinociceptive effects of URB597 are in agreement with previous studies showing that URB597 produces dose-dependent and CB1R-mediated antinociception in other preclinical assays of pain-stimulated behavior, such as stretching elicited by intraperitoneal acetic acid administration (Naidu et al., 2009; Clapper et al., 2010; Miller et al., 2012), first and second phases of nociceptive behavior elicited by intraplantar formalin injection (Hasanein et al., 2009), tail-flick responses elicited by noxious heat (Hasanein et al., 2009), and hypersensitive withdrawal responses elicited by thermal/mechanical stimuli in inflammatory or neuropathic pain models (Jayamanne et al., 2006; Jhaveri et al., 2006; Guindon et al., 2013). These results thus add to a growing body of literature that supports the antinociceptive effects of URB597 in various preclinical models of pain-stimulated behavior.

In contrast to the results with URB597, PF3845 decreased the mean number of acid-stimulated stretches when tested 1 h after PF3845 administration, but this effect did not achieve statistical significance. Moreover, PF3845 produced a pronociceptive increase in acid-stimulated stretching 4 h post-administration. These effects of PF3845 contrast not only with the present results with URB597, but also with previous studies

reporting antinociceptive effects of PF3845 in other preclinical assays of pain-stimulated behavior where PF3845 was tested anywhere from 2-8 h post-administration (Ahn et al., 2009; Long et al., 2009; Kinsey et al., 2010; Booker et al., 2012; Ghosh et al., 2012). There are at least two possible reasons for this discrepancy. First, expression of PF3845 antinociception may be influenced by experimental variables such as the type of noxious stimulus, the time tested after administration, and species of subject. This is the first study to examine effects of PF3845 on acid-stimulated stretching in rats, and previous studies have been conducted primarily in mice using chronic inflammatory or neuropathic pain models (Sagar et al., 2010a; Sagar et al., 2010b; Guindon et al., 2013). Second, drugs that produce nonselective behavioral depression can produce false-positive antinociception in assays of pain-stimulated behavior by impairing the subject's ability to perform in the assay (Negus et al., 2010a). For example, in this study, both URB597 and PF3845 significantly depressed control ICSS at doses and early treatment times similar to those at which they produced their greatest reductions in acid-stimulated stretching. Moreover, URB597 produced greater decreases than PF3845 both in control ICSS and in acid-stimulated stretching. Lastly, the present data with URB597 are consistent with previous studies showing that URB597 at high doses can decrease locomotor activity (Lee et al., 2006) and ICSS (Vlachou et al., 2006). Taken together, these results suggest that, in assays of pain-stimulated behavior, any apparent dissociation in antinociceptive effects of URB597 and PF3845 at short post-treatment times could be mediated in part by a dissociation in their efficacies to produce nonselective behavioral depression. However, this interpretation does not account for

the antinociceptive effect of URB597 and pronociceptive effect of PF3845 4 h post-administration, because neither URB597 nor PF3845 altered control ICSS at this time.

FAAH inhibitor effects on pain-depressed behavior. URB597 partially attenuated acid-induced depression of ICSS in the present study, and these data agree with a previous study that reported partial efficacy of URB597 in assays of acid-depressed wheel-running and acid-depressed feeding in mice (Miller et al., 2012). Moreover, the present finding that URB597 produced antinociception in assays of both acid-stimulated stretching and acid-depressed ICSS without altering control ICSS at the 4 h test time further suggests that URB597 effects included analgesic attenuation of sensitivity to the noxious stimulus rather than (or in addition to) non-selective motor effects. This profile supports further consideration of URB597 as a candidate analgesic. Intriguingly, though, URB597 antinociception in the assay of acid-depressed ICSS was not blocked by the CB1R antagonist rimonabant, suggesting that URB597 effects in this assay were not mediated by CB1 receptors. This contrasts with findings in the assay of acid-stimulated stretching (present study) and with rimonabant antagonism of URB597 antinociception in assays of acid-depressed behavior in mice (Miller et al., 2012). Taken together, these findings suggest a potential for URB597 to produce antinociception via both CB1R-mediated and non-CB1R-mediated mechanisms. The present study evaluated a potential role of CB2Rs and PPAR-α as two possible non-CB1R mechanisms, but results did not support the role of either CB2Rs or PPAR-α in mediating URB597-induced antinociception in the assay of acid-depressed ICSS. Other possible non-CB1R mechanisms might include effects mediated by TRPV1 ion channels (Maione et al., 2006), abnormal-cannabidiol-sensitive receptors (Bosier et al., 2012), or altered

arachidonate metabolism to reduce synthesis of cyclooxygenase-mediated products (Fowler, 2007).

In contrast to URB597, PF3845 produced no effect on acid-induced depression of ICSS. Although these results contrast with previous reports of PF3845 antinociception as discussed above (Ahn et al., 2009; Long et al., 2009; Kinsey et al., 2010; Booker et al., 2012; Ghosh et al., 2012), they agree with the lack of PF3845 antinociception in the present assay of acid-stimulated stretching. The lack of effect with PF3845 in the assay of pain-depressed behavior cannot be attributed to a lack of assay sensitivity, because URB597 produced antinociception in the present study, and both mu opioid agonists and nonsteroidal anti-inflammatory drugs have produced antinociception in previous studies (Pereira Do Carmo et al., 2009; Kwilasz and Negus, 2012). Furthermore, as with URB597, PF3845 was tested at a time when initial behavioral depressant effects on control ICSS had dissipated and could not confound assessment of antinociception. The failure of PF3845 to produce antinociception in assays of either pain-stimulated or pain-depressed behavior is also consistent with the recent clinical failure of the structurally-related FAAH inhibitor PF7845 for the treatment of osteoarthritis-related pain (Huggins et al., 2012).

Relationship of FAAH-inhibitor effects to fatty acid ethanolamine levels. There was no clear association between drug effects on behavior and fatty acid ethanolamine levels. Both URB597 and PF3845 produced qualitatively similar increases in AEA, OEA, and PEA, consistent with their classification as FAAH inhibitors; however, these two drugs produced different and sometimes diametrically opposite effects on behavior. For example, both URB597 and PF3845 produced similar increases in brain levels of the

endocannabinoid AEA after 240 min, but URB597 produced antinociception at this time in both behavioral assays, whereas PF3845 produced pronociception (assay of acid-stimulated stretching) or no effect (assay of acid-depressed ICSS). The only plausible biochemical correlate of the different behavioral effects of URB597 and PF3845 was the weaker effects of URB597 in increasing PEA brain levels. In support of this proposition, PEA has been shown in some studies to increase the ability of AEA to activate TRPV1 ion channels (De Petrocellis et al., 2001; Ho et al., 2008; Garcia Mdel et al., 2009), and this increased activity at TRPV1 might potentiate the effects of acid that have also been shown to be mediated at least in part through TRPV1 (Tang et al., 2007). A more parsimonious conclusion might be that antinociceptive effects of URB597 are mediated at least in part by mechanisms other than FAAH inhibition and associated increased levels of AEA, PEA, and/or OEA. This conclusion is also consistent with the finding that antinociceptive effects of URB597 in the assay of acid-depressed ICSS were not blocked by CB1R, CB2R, or PPAR-α antagonists, and that other effects of URB597 may also be independent of CBR/PPAR-α activation by endocannabinoids/fatty acid ethanolamines (Maione et al., 2006; Fowler, 2007; Bosier et al., 2012). For example, URB597 been shown to decrease tyrosine hydroxylase in mouse brain through FAAH-independent mechanisms (Bosier et al., 2012) and also may alter arachidonate metabolism to reduce synthesis of cyclooxygenase-mediated products (Fowler, 2007). Another less parsimonious explanation is that multiple receptors may contribute to the antinociceptive effects of URB597 in a synergistic fashion and that one or more of these receptors is not activated to the same level by PF3845. Overall, the present results suggest that URB597 is superior to PF3845 as a candidate analgesic for the treatment

of acute pain, and that URB597 antinociception may be mediated in part by mechanisms independent of FAAH inhibition, increased AEA levels, and CB1R activation.

Abuse-related effects of FAAH inhibitors. As in the previous chapter with mixed CB1R/CB2R agonists, FAAH inhibitors were also evaluated on ICSS performance in the absence of noxious stimulation. Similar to the effects of the mixed CB1/CB2R agonists, FAAH inhibitors did not produce facilitation of ICSS commonly interpreted as an abuse-related effect (Carlezon and Chartoff, 2007; Bauer et al., 2013). Rather, FAAH inhibitors only produced rate-decreasing effects on control ICSS at higher doses and early pre-treatment times. These results are consistent with a previous study, which also found that URB597 and several other FAAH inhibitors only depressed ICSS in rats at similar doses and pre-treatment times (Vlachou et al., 2006). Overall, these results are consistent with the notion that FAAH inhibitors do not produce abuse-related effects (Schlosburg et al., 2009; Alvarez-Jaimes and Palmer, 2011); however, these data should be interpreted cautiously, as mixed CB1R/CB2R agonists that are abused by humans also commonly do not produce abuse-related effects in this assay (Vlachou et al., 2007; Kwilasz and Negus, 2012).

6.4. Effects of the CB2R agonist GW405833 on acute pain-stimulated and pain-depressed behavior.

CB2R agonist effects on pain-stimulated behavior. The CB2R agonist GW405833 was tested in the same assays of acid-stimulated stretching and acid-depressed ICSS that were used for studies with the other cannabinoids described

above. In the assay of acid-stimulated stretching, a high dose of the CB2R agonist GW405833 produced antinociception (i.e. 32 mg/kg), whereas lower doses (i.e. 3.2-10 mg/kg) did not. CB2R agonists including GW405833 have been shown to produce antinociception in many preclinical assays of pain-stimulated behavior in rodents, including mechanical allodynia following paw incision (LaBuda et al., 2005; Valenzano et al., 2005), intraplantar injection of CFA (Valenzano et al., 2005; Whiteside et al., 2005), or partial sciatic nerve ligation (Whiteside et al., 2005), as well as in assays of IP p-phenylquinone (PPQ)-stimulated stretching (Anikwue et al., 2002), intraplantar formalin-induced nociceptive behavior (Jafari et al., 2007), and intraplantar carrageenan-induced changes in rear hind-paw weight bearing (Clayton et al., 2002; Elmes et al., 2005). In addition to their antinociceptive effects, CB2R agonists have also been shown to reduce paw edema after intraplantar injection of carrageenan (Clayton et al., 2002; Elmes et al., 2005) and are thought to exert at least some of their anti-inflammatory effects through inhibition of inflammatory cells such as microglia and macrophages (Cabral et al., 2008; Wilkerson and Milligan, 2011). Indeed, studies have shown that higher doses of CB2R agonists are commonly required to produce antinociception in assays using acute noxious stimulation such as PPQ-induced stretching or paw incision-induced mechanical allodynia versus more inflammatory-related stimuli such as CFA-induced mechanical allodynia (Anikwue et al., 2002; Valenzano et al., 2005). These studies thus further support the notion that CB2R agonists can produce antinociception through anti-inflammatory-related mechanisms, and add to a growing body of literature that supports the antinociceptive properties of CB2R agonists in nearly all assays of pain-stimulated behavior.

CB2R agonist effects on pain-depressed behavior. As in the assay of acid-stimulated stretching, the CB2R agonist GW405833 produced antinociception in the assay of acid-depressed ICSS at a high dose of 32 mg/kg, whereas lower doses were ineffective. No dose of GW405833 significantly altered responding for control ICSS in the absence of the noxious stimulus, including the dose that produced antinociception; thus, GW405833-induced antinociception in either assay cannot be explained by nonspecific rate-altering effects of GW405833. The present study is the first to report on effects of a CB2R agonist in an assay of pain-depressed behavior. In general, this study supports results from assays of pain-stimulated behavior; however, further studies will need to be conducted with different CB2R agonists and different noxious stimuli to determine the specific circumstances under which CB2R agonists produce antinociception in assays of pain-depressed behavior. The present results support the proposition that CB2R agonists may be effective analgesics for the treatment of mild acute pain in clinical settings.

Mechanisms of CB2R agonist-induced antinociception. Despite its primary mechanism of action as a CB2R agonist, GW405833 did not produce CB2R-mediated antinociception in either assay of acid-stimulated stretching or acid-depressed ICSS. Rather, in the assay of acid-stimulated stretching, GW405833-induced antinociception was blocked by the CB1R antagonist, rimonabant, but not by the CB2R antagonist, SR144528. These results indicated GW405833-induced antinociception in the assay of acid-stimulated stretching was mediated by CB1Rs but not CB2Rs. Indeed, studies have shown that CB2R agonists administered at high doses produce CB1R-mediated antinociception and/or cannabimimetic side-effects associated with activation of the

CB1R (Anikwue et al., 2002; Valenzano et al., 2005; Whiteside et al., 2005). Furthermore, drug development efforts at Merck found that two structurally different classes of CB2R agonists (i.e. imidazopyridines and decahydroquinolines) were ineffective in the preclinical assay of intraplantar CFA-induced mechanical allodynia in rats, unless some functional activity at the CB1R was also present (Manley et al., 2011; Trotter et al., 2011). The present data and existing literature thus fully support the notion that CB2R agonists can produce CB1R-mediated antinociception in assays of pain-stimulated behavior, especially when administered at high doses, and some studies even suggest CB1R activation is essential for CB2R agonist-induced antinociception.

In contrast to the assay of acid-stimulated stretching, in the assay of acid-depressed ICSS, GW405833 produced non-CB1R/CB2R-mediated antinociception. These results suggest that GW405833-induced antinociception in the assay of acid-depressed ICSS is mediated by either a) unknown cannabinoid targets and/or b) known or unknown non-cannabinoid-targets. For example, GW405833 has been shown to interact with a known putative cannabinoid receptor target, G-protein coupled receptor 55 (GPR55) (Anavi-Goffer et al., 2012). CB2R agonists have also been shown to produce off-target effects mediated by mu-opioid receptors (Ibrahim et al., 2005; Whiteside et al., 2005) and TRPV1 (Schuelert et al., 2010). Further studies will need to be conducted to determine mechanisms of GW405833-induced antinociception in the assay of acid-depressed ICSS, as well as CB2R agonist effects on other pain-depressed behaviors in general. Nonetheless, these results demonstrate that a CB2R agonist can function through two different non-CB2R-mediated mechanisms to produce different antinociceptive effects. Moreover, these results demonstrate that neural

pathways mediating acid-stimulated stretching and acid-depressed ICSS can be dissociated, as we have also demonstrated in a previous study (Kwilasz and Negus, 2012), and could suggest that they involve different components of pain-processing. Although neural pathways are incompletely mapped (Willis, 2009), noxious stimulation by an acute IP injection of lactic acid likely involves spinal and supraspinal pathways of pain-processing (Price, 2002; Borsook and Becerra, 2011; Jarcho et al., 2012). GW405833 thus appears to alter the components of pain-processing that mediate both acid-stimulated stretching and acid-depressed ICSS. Taken together, these results support complementing assays of pain-depressed behavior with assays of pain-stimulated behavior in preclinical analgesic drug development and suggest that CB2R agonists may be useful for the treatment of acute pain. Moreover, these results suggest that the therapeutic effects of CB2R agonists in acute pain settings may not be solely related to their activity at CB2Rs.

Abuse-related effects of CB2R-agonists. To assess abuse-liability as in previous chapters, effects of GW405833 on control ICSS responding were assessed in the absence of noxious stimulation. Similar to results with mixed CB1R/CB2R agonists and FAAH inhibitors, GW405833 did not produce increased responding for control ICSS, which is commonly interpreted as abuse-related effect (Carlezon and Chartoff, 2007; Bauer et al., 2013). This is the first study to report on the effects of a CB2R agonist in an assay of ICSS. These results support results from other studies that have demonstrated CB2R agonists lack abuse-liability and the side effect profile commonly associated with activation of the CB1R (Anikwue et al., 2002; Valenzano et al., 2005; Whiteside et al., 2005; Karst and Wippermann, 2009). Furthermore, a recent study has

demonstrated that CB2R agonists may be an effective treatment for cocaine abuse, demonstrating that CB2R agonists can block cocaine-induced self-administration and associated increases in dopamine in the nucleus accumbens (Xi et al., 2011), which are also commonly interpreted as abuse-related effects (Carlezon and Chartoff, 2007; Blum et al., 2011; Bauer et al., 2013). In summary, CB2R agonists do not produce abuse-related effects in preclinical studies, and some studies suggest they may useful to block abuse-related effects of other drugs.

6.5. Effects of THC on acute and repeated LPS-induced stimulation of mechanical allodynia and depression of ICSS.

LPS effects on pain-stimulated behavior. In these studies, LPS, a constituent of bacterial cell walls, was administered IP as a pro-inflammatory noxious stimulus to stimulate mechanical allodynia. Daily LPS administration produced mechanical allodynia at 30 min but not at 100 min. However, this effect was variable between subjects and only persisted for two of the seven days of LPS administration. Interestingly, a trend for LPS to produce mechanical allodynia at the later time point of 100 min was also observed, and this effect began on the third day of LPS treatment and persisted for the remaining three days of the experiment. This effect coincided with a dramatic tolerance to the "sickness-like" signs produced by LPS on the first two days of administration (observed by the experimenter at the later 100 min time point, although not quantified). These signs included unkempt fur with mild porphyrin staining, hunched posture, weak muscle tone, sedation, and ptosis, which appeared to interfere with behavioral responses to mechanical stimulation of the hind paw. Most animals in this

group did not respond to any Von Frey filament even up to those that lifted their paw due to the force applied by the filament alone while showing these signs. This could suggest that animals may have displayed mechanical allodynia at this later time point on the first two days of LPS administration if these sickness signs had not interfered, although this is a purely speculative suggestion. These results are in agreement with previous studies, which have shown that IP administration of LPS produces mechanical allodynia in a Von Frey paw-withdrawal procedure (Cahill et al., 1998) and hyperalgesia in a warm-water tail-flick procedure in rats (Watkins et al., 1994).

In contrast to the group that received chronic LPS treatment, acute administration of LPS failed to produce mechanical allodynia in a separate group of rats. In this group, LPS was administered acutely a total of three times with a two-week interval between doses. One possibility for this discrepancy may be related to differences in prior exposure to inflammatory-related noxious stimuli between the two experimental groups. For example, previous studies have shown that mechanical allodynia following IP LPS is dependent upon a prior immunological insult, such as an earlier injection of LPS (Cahill et al., 1998), or surgical procedure, such as laparotomy (Hains et al., 2010). Due to different methodologies between studies, the first group that received chronic LPS treatment had surgically-implanted intracranial electrodes for ICSS procedures, which have been shown to produce associated neuroinflammation (Hirshler et al., 2010). Furthermore, these rats also participated in daily ICSS sessions, which also may have influenced ongoing neuroinflammation. In contrast, rats that received acute LPS treatment did not have intracranial implants, and two-week intervals between LPS injections may have been too long to sustain an immunological "priming" effect that has

been reported to exacerbate responses to LPS in other studies. Collectively, these studies suggest that subject- and/or other experimental-related variables considerably influenced the results. Moreover, LPS was a rather weak and unreliable noxious stimulus, although a more rigorous manipulation of subject-, time-, and dose-related variables that determine LPS-induced mechanical allodynia could reveal more useful results.

LPS effects on pain-depressed behavior. In these studies, IP LPS served as the noxious stimulus to decrease ICSS. The group of animals that received daily LPS injections displayed significantly decreased responding for ICSS at the later 100 min time point on days one, two, and four of the seven-day chronic LPS regimen; however, this effect was rather unreliable across all animals and other time points. For example, ICSS was also significantly decreased at the early 30 min time point on day two. The animals also displayed apparent "sickness-like" behaviors on the first two days of LPS treatment at the later 100 min time point (described in the previous section). This "sickness-like" behavior seemed to correlate with the most profound decreases in ICSS behavior. In a separate group of rats, acute LPS administration also depressed ICSS behavior 90 min post-LPS administration, albeit this effect was also weak relative to the effects of IP acid and was not reliable between subjects or when administered multiple times at a 2-week dosing interval. LPS has been shown in previous studies to stimulate pro-inflammatory cytokine production, and previous studies have shown that LPS and cytokine administration can depress a wide variety of different behaviors including ICSS (Anisman et al., 1996; Anisman et al., 1998; Borowski et al., 1998; Barr et al., 2003; van Heesch et al., 2013), feeding (Kubera et al., 2013), and social interaction (Konsman et

al., 2008). The results of the present study are thus concordant with previous studies and suggest that LPS can be used as a pro-inflammatory noxious stimulus to decrease behavior. However, the present results also suggest that LPS effects are rather weak compared to an IP acid noxious stimulus and unreliable across subjects and repeated treatments.

THC effects on LPS-induced nociceptive behaviors. It was impossible to determine whether THC was able to produce antinociception in the assay of LPS-stimulated mechanical allodynia, because LPS did not produce mechanical allodynia in the group of animals tested with THC. THC also did not produce evidence of antinociception in the assay of LPS-induced depression of ICSS; however, LPS effects were unreliable in that they did not depress behavior in all animals and were not stable when administered a second and third time at two-week dosing intervals. These properties of LPS effects on ICSS behavior made assessment of THC effects difficult. For example, LPS only significantly decreased ICSS on the first and third administrations, but not on the second administration. Previous studies have shown that THC and other cannabinoids produce robust anti-inflammatory effects and block LPS-induced stimulation of pro-inflammatory cytokines (Puffenbarger et al., 2000; Roche et al., 2006), and these effects are thought to be related to their ability to produce antinociception and anti-inflammatory effects in preclinical assays of inflammatory-related pain (Croxford and Yamamura, 2005; Burstein and Zurier, 2009; Stein and Machelska, 2011). The present results cannot accurately conclude whether THC effects in the assay of LPS-depressed ICSS agree with previous studies; however, they do suggest a possible disagreement, showing THC may be ineffective in the assay of LPS-

depressed ICSS. These results should be reproduced under more stable experimental conditions to accurately discern whether THC produces antinociception in the assay of LPS-depressed ICSS.

6.6. General Discussion and Summary

In chapters 2-4, an IP injection of lactic acid served as an acute noxious stimulus to stimulate stretching or depress ICSS. Cannabinoids produced antinociception in all assays of acid-stimulated stretching, with the exception of the FAAH inhibitor PF3845 for unknown reasons. In contrast, cannabinoids displayed no efficacy or submaximal efficacy to produce antinociception in the assay of acid-depressed ICSS, and even drugs that produced weak antinociception (i.e. FAAH inhibitor URB597 and CB2R agonist GW405833) did not produce their effects through CBRs. In chapter 5, chronic/acute IP injections of LPS served as chronic/acute inflammatory-related noxious stimuli to stimulate mechanical allodynia or depress ICSS. However, the effects of LPS on both endpoints were weak compared to an IP acid injection and unreliable across subjects and repeated treatments, which made prediction of THC effects on LPS-induced nociceptive behaviors difficult. In general, these data are concordant with the clinical data on cannabinoid effects on acute pain (Rice, 2006; Karst et al., 2010; Kraft, 2012), but data from the LPS studies are not adequate to predict cannabinoid effects in an assay of inflammatory-related pain-depressed behavior.

Implications for preclinical strategies of drug development. Preclinical assays of pain and analgesia play a critical role in analgesic drug development, but there is a growing appreciation that drug effects in conventional preclinical assays of pain-

stimulated behavior are often poor predictors of clinical analgesic efficacy in humans (Blackburn-Munro, 2004; Negus et al., 2006; Whiteside et al., 2008; Mogil, 2009). Results with THC and CP55940 have illustrated this discordance most clearly insofar as mixed cannabinoid agonists produce robust and reliable antinociception in most assays of acute pain-stimulated behavior but little or no analgesia against acute pain in humans (Rice, 2006; Karst et al., 2010; Kraft, 2012). For example, oral delivery of THC or other cannabinoids lacked analgesic efficacy or exacerbated pain in most well-controlled clinical studies of postoperative or acute experimental pain [(Raft et al., 1977; Buggy et al., 2003; Naef et al., 2003; Beaulieu, 2006; Kraft et al., 2008; Klooker et al., 2011); for the lone exception, see (Campbell et al., 2001)]. Similarly, smoked marijuana at doses up to those producing untoward motor/cognitive/subjective effects produced little or no change in sensitivity to acute thermal, mechanical, or chemical noxious stimuli in clinical laboratory studies, and as with oral cannabinoids, pain ratings were sometimes worsened by smoked cannabis (Greenwald and Stitzer, 2000; Wallace et al., 2007). Moreover, the failure of PF3845 to produce antinociception in assays of either pain-stimulated or pain-depressed behavior is also consistent with the recent clinical failure of the structurally-related FAAH inhibitor PF7845 for the treatment of osteoarthritis-related pain (Huggins et al., 2012). The present studies suggest that preclinical assays of pain-depressed behavior may yield results that complement results from more conventional assays of pain-stimulated behavior and improve predictions of clinical cannabinoid effects. As such, these studies support the utility of assays of pain-depressed behavior in development of cannabinoid analgesics.

Particularly interesting are the effects of the FAAH inhibitor URB597 and the CB2R agonist GW405833 in the assay of pain-depressed behavior, which produced antinociceptive effects mediated by different mechanisms than in the assay of pain-stimulated behavior. Both URB597 and GW405833 produced CB1R-mediated antinociception in the assay of pain-stimulated behavior but produced non-CBR-mediated antinociception in the assay of acid-depressed ICSS. URB597-induced antinociception was also not mediated by PPAR-α, another related target of FAAH. These results demonstrate the potential for assays of pain-depressed behavior to reveal potential new mechanisms of antinociception/analgesia produced by putative cannabinoid drugs.

Summary, conclusions, and future directions. The goal of this study was to characterize the efficacy of cannabinoid drugs on pain-stimulated versus pain-depressed behavior. Although some of the drugs tested produced CB1R-mediated antinociception in the assay of pain-stimulated behavior (i.e. THC, CP55940, URB597 and GW4085833), these drugs either failed to produce antinociception in the assay of pain-depressed behavior (i.e. THC and CP5594), or they produced non-CB1/2R-mediated antinociception in the assay of pain-depressed behavior (i.e. URB597 and GW405833). These results challenge prevailing notions about the efficacy and mechanisms of cannabinoids to produce analgesia. Consequently, one conclusion from these studies is that assays of pain-depressed behavior provide a useful complement to assays of pain-stimulated behavior, as they are able to measure behaviors that have different underlying substrates than those underlying assays of pain-stimulated behavior. The substrates of pain-depressed behavior are not fully understood, but are

thought to involve traditional pain pathway interaction with reward-related brain areas, with the ultimate result of noxious stimulation decreasing mesolimbic DA. This suggests that the inclusion of assays of pain-depressed behavior in preclinical analgesic drug development might provide the ability to discover drugs that are effective against substrates underlying pain-depressed behavior. Moreover, these studies may ultimately lead to a more thorough characterization of the substrates of pain, which may encompass other facets of pain besides sensory components (e.g. affective and/or cognitive) that are not measured in traditional assays of pain-stimulated behavior. This will lead to development of preclinical assays of pain that are more predictive of clinical outcome, and will ultimately lead to more efficient analgesic drug development.

The research in this dissertation has identified several cannabinoid drugs that block pain-induced depression of behavior; however, the mechanisms underlying the effects of these drugs have not yet been determined. These studies investigated the traditional receptor mechanisms by which these drugs are thought to act but were unsuccessful in determining a mechansim. The model in Figure 1.4 will thus require revision to include non-CBR-mediated mechanisms of antinociception that are mediated by URB597 and GW405833. A common receptor target of these two compounds is TRPV1 (Maione et al., 2006; Schuelert et al., 2010), which may be included in the model in Figure 1.4. TRPV1 is present on primary afferent nociceptors and may be desensitized by activation by cannabinoids, which could result in an overall decrease in sensory transmission (Ralevic and Kendall, 2009). Some future studies that could be done would be to attempt to block the effects of URB597 and GW405833 with a TRPV1 antagonist, such as capsazepine, and to try to recapitulate the effects these drugs with

a TRPV1 agonist, such as capsaicin. Capsaicin has already been shown to desensitize primary afferent nociceptors following activation of TRPV1 (Brederson et al., 2013), providing a proof of concept for this mechanism. Moving forward, TRPV1 will be an important mechanism to test, however, future studies may also be necessary to determine other mechanisms underlying the antinociceptive effects of these drugs. Until future studies can more extensively characterize the effects of other cannabinoid-related drugs on acid-induced pain-related behavior as well as behavior stimulated and depressed by other noxious stimuli, the results of these studies should also be qualified by their experimental parameters.

One important future study that should be done is to test other CB2R agonists besides GW405833, as well as other FAAH inhibitors besides URB597 and PF3845, in the assays of acid-stimulated stretching and acid-depressed ICSS. These studies will help to determine if the antinociceptive effects of these drugs are common between CB2R agonists/FAAH inhibitors or only specific to GW405833 and URB597. From here, it would be useful to determine commonalities or differences between the effects of drugs that produce antinociception in assays of pain-depressed behavior, and then individually probe each one of these mechanisms as specifically as possible to determine if they can alter the effects of these drugs. Ultimately, once the mechanisms underlying the antinociceptive properties of these drugs are relatively understood, drugs could be developed if not already available to test if these mechanisms are sufficient to produce antinociception in both assays of pain-stimulated and pain-depressed behavior. If promising, this could lead to clinical trials and effective new analgesics with reduced

side effects, improved efficacy, and/or that treat refractory pain conditions. These new analgesics may or may not have cannabinoid-related mechanisms.

Another direction to take this line of research would be to assess other noxious stimuli. We made an attempt to study another noxious stimulus in this dissertation (i.e. IP LPS); however under the experimental parameters that we studied, IP LPS was a rather poor noxious stimulus compared to IP lactic acid. We had hypothesized that cannabinoid drugs may be more effective to produce antinociception against a highly inflammatory-related noxious stimulus such as IP LPS, versus IP lactic acid. The results of our studies were inconclusive; however future studies could examine different doses of LPS as well as different subject-related variables, such as whether "priming" the animals with a noxious stimulus is important in producing future pain-related behaviors. Other noxious stimuli besides LPS and lactic acid must also be studied, and to date other studies have also shown that complete Freund's adjuvant produces pain-related depression of locomotion, rearing, and wheel-running behavior (Matson et al., 2007; Cobos et al., 2012). Future studies examining different noxious stimuli should first examine whether other acute, inflammatory, neuropathic, and cancer-related pain manipulations produce depression of behavior and other signs of pain-stimulated behavior alone. Next, studies should determine whether cannabinoid-related drugs can either block/reverse the effects of the noxious stimulus on some behavior. If a noxious stimulus does not produce depression of behavior alone, cannabinoid-related drugs could still be tested in combination with this noxious stimulus to determine if the combination alters the effects of cannabinoids on various cannabinoid-mediated behaviors. Other studies could determine whether cannabinoids were effective by other

routes of administration, including the inhaled route, which might prove to be more similar to consumption of smoked marijuana. Lastly, other behaviors not currently considered pain-related behaviors should be tested for their sensitivity to noxious stimuli and analgesic drug treatment, as they may also prove to be useful new assays of pain-related behavior. These new assays may encompass other facets of pain not measured by assays of pain-stimulated or pain-depressed behavior, further expanding our ability to predict the clinical efficacy of candidate analgesics.

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♦ *Pharmacology Ph.D. Graduate Student,* Virginia Commonwealth University, Richmond, VA
  o August 2009-present
  o Mentor: Dr. Steve Negus, Ph.D.
  o Funded by NIDA R01 NS070715, PI: Steve Negus and NIDA F31 DA032267, PI: A. J. Kwilasz
♦ *Laboratory Specialist,* Virginia Commonwealth University, Richmond, VA
  o July 2007-August 2009
  o Mentor: Dr. Louis Harris, Ph.D.
  o Funded by NIDA contract DA 7-8859, PI: Dr. Louis S. Harris

♦ *Neurobiology Research Apprentice,* University of Wisconsin Eau Claire, Eau Claire, WI
  o June 2006-May 2007
  o Mentor: Dr. Daniel Janik, Ph.D.
♦ *Behavioral Pharmacology Research Apprentice,* University of Wisconsin Eau Claire, Eau Claire, WI
  o January 2004-September 2006
  o Mentor: Dr. David Jewett, Ph.D., Professor

RESEARCH SKILLS:
♦ Stereotaxic surgery (rodent)
♦ Intravenous jugular vein catheterization (rat)
♦ Osmotic minipump implantation (rodent)
♦ Animal care (rodent and nonhuman primate)
♦ Formulating drug solutions in complex vehicles
♦ Drug delivery by various routes of administration (rodent: SC, IP, IM, IV, PO, intraplantar, inhalation, intracerebral microinjection)
♦ Installation/programming/operation/maintenance of computer-operated hardware for behavioral testing
♦ Behavioral and physiological testing procedures (rodent)
    *Operant behavior (food-reinforced operant responding, drug self-administration, drug-discrimination, intracranial self-stimulation)
    *Unconditioned behavior (nociceptive behaviors, locomotor activity, wheel running, feeding)
    *Body temperature
♦ Euthanasia (CO_2 exposure, rapid live decapitation)
♦ Brain and plasma collection
♦ Basic experience with histological brain sectioning and cell staining
♦ Basic experience with polymerase chain reaction

OTHER SKILLS:
♦ Excellent written and oral communication skills
♦ Excellent computer skills (Apple OS X, Microsoft Windows, Linux)
♦ Extensive experience with research design and data analysis

RESEARCH SUPPORT:
Ongoing Research Support:
NIH/NIDA F31 DA032267; 4/25/12-4/25/14
Effects of cannabinoids on pain-stimulated and pain-depressed behavior in rats
Goals: Determine efficacy of various cannabinoid and cannabinoid-related compounds to produce antinociception in assays of acute and chronic pain-stimulated and pain-depressed behavior in rats.
Role: PI

PUBLICATIONS:

Kwilasz, A.J., & Negus, S.S. (2012). Dissociable effects of the cannabinoid receptor agonists delta 9-tetrahydrocannabinol and CP55940 on pain-stimulated versus pain-depressed behaviors in rats. *Journal of Pharmacology and Experimental Therapeutics.* 343, 389-400.

Vann, R.E., Tobey, K.M., Lobe, S.L., Kipps, B., **Kwilasz, A.J.**, Aceto, M.D., & Harris, L.S. (2011) Varenicline does not alter brain stimulation reward thresholds and reverses nicotine-facilitated thresholds in rats. *Drug Development Research.* 72, 310-314.

Kwilasz, A.J., Harris, L.S., & Vann, R.E. (2009) Removal of continuous nicotine infusion produces somatic but not behavioral signs of withdrawal in mice. *Pharmacology, Biochemistry, and Behavior*. 94, 114-118.

Manuscripts In Preparation:

Kwilasz, A.J., Lichtman, A.H., & Negus, S.S. (2012). The FAAH inhibitors PF3845 and URB597 produce distinct effects on acute pain-stimulated and pain-depressed behavior in rats. *Submitted to the Journal of Pain*.

Kwilasz, A.J. & Negus, S.S. (2013). Effects of the cannabinoid-2 receptor agonist GW405833 on acute pain-stimulated and pain-depressed behavior in rats. *In preparation*.

INVITED PRESENTATIONS:

2012-The FAAH inhibitors PF3845 and URB597 produce distinct effects on acute pain-stimulated and pain-depressed behavior in rats. 22nd Annual Symposium of the International Cannabinoid Research Society

2012-Dissociable effects of the cannabinoid receptor agonists delta 9-tetrahydrocannabinol and CP55940 on pain-stimulated vs. pain-depressed behavior in rats. National Institute of Health Pain Consortium 7th Annual Symposium on Advances in Pain Research

ACADEMIC LECTURES:

2013-Cannabinoids and their effects on intracranial self-stimulation.
Course: Intracranial Self-Stimulation as an Experimental Tool in Behavioral Pharmacology
Instructor: Dr. Steve Negus

2012-Effects of cannabinoids on pain.
Course: Neuropharmacology of Pain and Analgesia
Instructor: Dr. Steve Negus

PUBLISHED ABSTRACTS:

Kwilasz, A. J., & Negus, S.S. The cannabinoid 2 receptor agonist GW405833 produces antinociception in assays of acute pain-stimulated and pain-depressed behavior in rats. *American Society of Pharmacology and Experimental Therapeutics No 4145*.

Kwilasz, A. J., & Negus, S.S. Dissociable effects of the FAAH inhibitors PF3845 and URB597 on acute pain-stimulated and pain-depressed behavior in rats. *Society for Neuroscience Abstract Program No. 376.22, 2012.*

Kwilasz, A. J., Lichtman, A.H., & Negus, S.S. The FAAH inhibitors PF3845 and URB597 produce distinct effects on acute pain-stimulated and pain-depressed behavior in rats. *International Cannabinoid Research Society, 2012.*

Kwilasz, A. J, & Negus, S.S. Dissociable effects of the cannabinoid receptor agonists delta 9-tetrahydrocannabinol and CP55940 on pain-stimulated vs. pain-depressed behavior in rats. *National Institute of Health Pain Consortium 7th Annual Symposium on Advances in Pain Research. 2012.*

Kwilasz, A. J., & Negus, S.S. Dissociable effects of the cannabinoid receptor agonists delta 9-tetrahydrocannabinol and CP55940 on pain-stimulated vs. pain-depressed behavior in rats. *Carolina Cannabinoid Collaborative. No. P1-3, 2011.*

Kwilasz, A. J., & Negus, S.S. Effects of delta 9-tetrahydrocannabinol on pain-stimulated and pain-depressed behavior in rats. *International Cannabinoid Research Society No. P1-13, 2011.*

Kwilasz, A. J., & Negus, S.S. Effects of delta 9-tetrahydrocannabinol in assays of pain-stimulated and pain-depressed behavior in rats. *American Society of Pharmacology and Experimental Therapeutics No. 617.7, 2011.*

Vann, R.E., **Kwilasz, A.J.**, & Walentiny, D.M. The role of delta 9-tetrahydrocannabinol in inhaled marijuana's rewarding and discriminative stimulus effects in mice. *Society for Neuroscience Abstracts Program No. 884.19, 2009.*

Rolfes, M.A., **Kwilasz, A.J.**, Harris, L.S., & Vann, R.E. The effects of delta 9-tetrahydrocannibinol, the major psychoactive component of marijuana, on food and brain reward. *Virginia Academy of Science Abstracts, 2009.*

Kwilasz, A.J., Vann, R.E., Kipps, B., Lobe, S.L., Aceto, M.D., & Harris, L.S. Evaluation of nicotine dependence in mice in an operant model of spontaneous and precipitated withdrawal. *Society for Neuroscience Abstracts Program No. 61.7, 2008.*

Vann, R.E., **Kwilasz, A.J.**, Kipps, B., Lobe, S.L., Aceto, M.D., & Harris, L.S. Evaluating operant contingencies of brain reward self stimulation in mice. *Society for Neuroscience Abstracts Program No. 767.19, 2008.*

Leung, A.L. & **Kwilasz, A.J.** Methamphetamine-induced nonphotic-like circadian clock resetting. *Midbrains Undergraduate Neuroscience Abstracts, 2007.*

Wiebelhaus, J.M., **Kwilasz, A.J.**, Tham, R.L, Grace, M. K., Levine, A.S., & Jewett, D.C. Effects of opioids in subjects trained to discriminate between 2 hours and 22 hours food deprivation. *Society for Stimulus Properties of Drugs, 2006.*

Tham, R.L., **Kwilasz, A.J.**, Wiebelhaus, J.M., Hahn, T.W., Smith, T.R., Grace, M.K., Levine, A.S., & Jewett, D.C. Effects of a high-fat, high carbohydrate diet on the discriminative stimulus effects produced by 22 hour food deprivation. *Society for Neuroscience Abstracts Program No. 360.2, 2006.*

Jewett, D.C., Carrol, R.A., Hahn, T.W., Mack-Olsen, E.J., Schweiner, T.A., **Kwilasz, A.J.**, Dunn, A.T., Wiebelhaus, J.M., Grace, M.K., & Levine, A.S. Effects of food, sucrose, and saccharin on the discriminative stimulus effects produced by 22 hours food deprivation. *Society for Neuroscience Abstracts Program No. 529.14, 2005.*

AWARDS:

◆ Finalist for Mitchell Max Best Poster Award at National Institute of Health Pain Consortium 7th Annual Symposium on Advances in Pain Research (2012)

◆ Travel grant to present at the International Cannabinoid Research Society annual meeting. National Institute on Drug Abuse 2R13DA016280 (2012)

◆ Travel grant to present at the Carolina Cannabinoid Collaborative annual meeting. Virginia Commonwealth University Graduate Student Organization (2011)

◆ Travel grant to present at the International Cannabinoid Research Society annual meeting. National Institute on Drug Abuse 2R13DA016280 (2011)

◆ Travel grant to present at the Society for Neuroscience annual meeting. Virginia Commonwealth University Department of Pharmacology (2008)

◆ Best undergraduate poster award at Society for Stimulus Properties of Drugs Annual Meeting (2006)

◆ Travel grant to present at the Society for Neuroscience annual meeting. UW-Eau Claire Office of Research and Sponsored Programs (2006)

◆ Travel grant to present at the Society for Neuroscience annual meeting. UW-Eau Claire Office of Research and Sponsored Programs (2005)

PROFESSIONAL ASSOCIATIONS:

◆ Society for Neuroscience (2006-present)

◆ Society for Stimulus Properties of Drugs (2007-present)

◆ The American Society for Pharmacology and Experimental Therapeutics (2010-present)

◆ International Cannabinoid Research Society (2011-present)

◆ International Association for the Study of Pain (2013-present)