CANNABINOID MODULATION OF NOCICEPTION AND NOCICEPTOR ACTIVITY DURING INFLAMMATION

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Dedication

This dissertation is dedicated to my parents, Mario and Loretta Potenzieri, for all their love, support, and encouragement.
Previous studies have demonstrated that peripherally-administered cannabinoids at the site of injury produce antinociception in animal models of acute and persistent pain. Peripheral cannabinoid one (CB₁) receptor-mediated antinociception has been attributed to CB₁ receptors located on nociceptive DRG neurons and their peripheral nerve terminals. Although these studies suggest that activation of peripheral CB₁ receptors located on nociceptive nerve terminals produces antinociception, how cannabinoids modulate nociceptor activity is not known. The overall aim of this thesis was to relate the behavioral antinociceptive effects of locally-administered cannabinoids with changes in the response properties of nociceptors during non-inflamed and inflamed conditions. It was hypothesized that activation of peripheral CB₁ receptors attenuated nociception and nociceptor activity only during inflammation. In behavioral studies, intraplantar administration of complete Freund's adjuvant (CFA), but not saline, produced mechanical allodynia, mechanical hyperalgesia, and heat hyperalgesia. Activation of peripheral CB₁ receptors produced antiallodynia and antihyperalgesia following inflammation, but did not alter nociception during non-inflamed conditions. In electrophysiological studies, only cutaneous nociceptors (Aδ and C) from inflamed skin were sensitized, and not Aβ mechanoreceptors. Local administration of CB₁ receptor agonists attenuated mechanically-evoked responses of Aδ nociceptors from inflamed skin, but did not alter the evoked responses of Aδ nociceptors from non-inflamed skin. The responses of C nociceptors and Aβ mechanoreceptors from either non-inflamed or inflamed skin
were not altered following local administration of cannabinoids. Our results
demonstrated that peripherally-mediated cannabinoid antinociception through
CB₁ receptors is mediated, at least in part, by attenuation of Aδ nociceptor
activity. The results from the present studies suggest that peripherally-acting CB₁
receptor agonists could be administered alone or co-administered with other
analgesic drugs to treat acute and persistent pain in humans and animals.
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Chapter 1

BACKGROUND AND STATEMENT OF PURPOSE
Somatosensation and Sensory Receptors

Somatosensation, sensations produced by stimulation throughout the body, involves activation of distinct sensory receptors originating from the distal processes of sensory ganglion neurons. These terminal endings innervate a vast array of different structures throughout the body including skin, muscle, joints, and visceral structures and are broadly categorized based on the conduction velocity of the nerve fiber from which they originate. Generally, $A_{\alpha}$ fibers have conduction velocities between 120-70 meters/second (m/s), $A_{\beta}$ fibers between 70-25 m/s, $A_{\delta}$ fibers between 25-2.5m/s, and C fibers below 2.5 m/s. The submodalities of somatosensation include proprioception, mechanosensation, thermosensation, and nociception. Each of these submodalities involves specialized sensory receptors responsible for the transduction of specific stimuli.

Proprioception

Proprioception is the sensation of bodily movement and position. Proprioceptors are found inside joint capsules, muscles, and at the junction of tendons and muscle. All proprioceptors have fast conducting, myelinated nerve fibers. Three types of proprioceptors are found inside the joint capsule; Ruffini endings and golgi tendon organs, which are excited by joint flexion and extension (Burgess and Clark 1969), and pacinian corpuscles that respond to vibration (Burgess and Clark 1969). Muscle spindle afferents innervate intrafusal fibers and are excited following muscle stretch (Hunt 1954). Golgi tendon organs are
located at the junction of muscles and are excited during muscle contractions (Hunt 1954; Jansen and Rudjord 1964). Golgi tendon organs and muscle spindle afferents have Aα fibers, while Ruffini endings and Pacinian corpuscles originate from Aβ fibers. Together these proprioceptors provide information regarding the position and movements of the body.

Mechanosensation

Mechanosensation is the sensation of touch produced by innocuous mechanical forces, pressure or stretch. Mechanoreceptors are found throughout the body, localized mostly to cutaneous structures and hollow visceral organs. A majority of mechanoreceptors have myelinated fibers; however, some also arise from unmyleinated fibers. Mechanoreceptors are broadly classified into two categories, slowly adapting (SA) and rapidly adapting (RA), based on their responses to mechanical stimulation (Adrian and Zotterman 1926a, b). Slowly adapting responses to mechanical stimulation include two components, a dynamic and static phase (Adrian and Zotterman 1926a, b). The dynamic phase encodes the velocity of stimulation, while the static phase encodes the duration of stimulation (Adrian and Zotterman 1926a, b). SA mechanoreceptors encode the magnitude and duration of displacement/indentation. Rapidly adapting receptors only exhibit a dynamic phase in response to mechanical stimulation. RA mechanoreceptors encode the frequency of displacement/indentation.
Two SA mechanoreceptors have been identified; Merkel's discs and Ruffini endings. Merkel's discs are excited by indentation, while Ruffini endings respond to stretch of the skin (Munger 1965; Lindblom 1965; Werner and Mountcastle 1965; Iggo and Muir 1969; Chambers et al. 1972). Similarly, two RA mechanoreceptors have been identified; Meissner's and Pacinian corpuscles. Meissner's corpuscles respond to indentation and are important for detection of edges, while Pacinian corpuscles respond optimally to both indentation and vibration (Cauna 1956; Hubbard 1958; Munger et al. 1971; LaMotte and Whitehouse 1986). Merkel discs, Ruffini endings, Meissner's and Pacinian corpuscles have Aβ fibers. Collectivity, SA mechanoreceptors provide sensations of touch-pressure while RA mechanoreceptors give rise to sensations of flutter-vibration (Willis Jr. and Coggeshall 2004).

Other mechanoreceptors have also been identified, but these originate from slowly conducting myelinated fibers and unmyelinated fibers. Hair follicle afferent fibers are excited by mechanical displacement of the hair shaft and typically conduct in the Aδ fiber range (Brown and Iggo 1967). Previous studies have also identified low threshold mechanoreceptors that innervate skin and hollow organs that conduct in the C fiber range (Iggo 1960; Bessou et al. 1971).

**Thermosensation**

Thermosensation is defined as the sensation evoked by non-noxious changes in temperature. Thermoreceptors have either slowly conducting, thinly
myelinated Aδ fibers or unmyelinated C fibers. Thermoreceptors are broadly classified into two types, cold and warm receptors (Hensel et al. 1960; Hensel 1974). Cold receptors typically respond optimally to non-noxious cooling of the skin, while warm receptors are excited by non-noxious warming of the skin (Hensel and Zotterman 1951; Dodt and Zotterman 1952).

Nociception

Nociception is the sensation of pain as well as other events evoked by noxious or potentially damaging stimuli. Nociceptors are classified into a variety of subtypes based on their conduction velocities and the specific modalities (mechanical, heat, cold, or chemical) that excite them. Most nociceptive afferent fibers are thinly myelinated (Aδ) or unmyelinated (C) (Zotterman 1939). Nociceptors are excited by a variety of different stimuli including noxious heat, cold, and chemical mediators. Under normal conditions, nociceptors encode the modality and intensity of noxious stimuli in their discharge rates.

Central Somatosensory Pathways

Following the transduction of peripheral events, primary sensory neurons then relay information to the central nervous system (CNS) for further processing and integration. Typically, somatosensory information from the body ascends in two major pathways, the dorsal column-medial lemniscus (DCML) system and the spinothalamic pathway (Willis Jr. and Coggeshall 2004). Both of these
pathways terminate in different nuclei in the thalamus where thalamic neurons then relay information on to various areas of the cerebral cortex.

Proprioceptive afferent fibers ascend via both the spinomedullothalamic pathway and the DCML system. In the spinomedullothalamic pathway, proprioceptors from the hindlimbs synapse in or around Clarke's column and then ascend and terminate in the ipsilateral nucleus z located in the medulla (Willis Jr. and Coggeshall 2004). From nucleus z, third-order neurons send projections, which decussate via the medial lemniscus, to the contralateral thalamus (Willis Jr. and Coggeshall 2004). Some forelimb proprioceptive afferents also synapse in the spinal cord while others project directly to the cuneate nucleus via the dorsal columns.

Mechanosensory afferent fibers enter the spinal cord and ascend via the DCML pathway. The terminals of these afferents ascend in the dorsal funiculus or dorsal columns and terminate in the dorsal column nuclei in the medulla. Afferents from the hindlimbs and the lower parts of the body terminate in gracile nucleus, while afferents from the upper parts of the body and forelimbs terminate in the cuneate nucleus (Willis Jr. and Coggeshall 2004). The secondary neurons in the cuneate and gracile nuclei project to the contralateral thalamus via the medial lemniscus.

Visceral afferent fibers originating from the vagus nerve project to the nucleus of the solitary tract which sends projections to thalamic structures sometimes with intermediate connections to the thalamus via the parabrachial
nucleus (Willis Jr. and Coggeshall 2004). Visceral afferents originating from dorsal root ganglion neurons ascend in both the DCML pathway and the spinal thalamic tract.

Nociceptive and thermoreceptive information is primarily conveyed to the CNS via the spinothalamic pathway. Nociceptors and thermoreceptors typically terminate in the dorsal horn of the spinal cord. Following their termination, secondary nociceptive spinal cord neurons decussate and ascend to the contralateral thalamus via the spinal thalamic tract located in the ventral lateral funiculus of the spinal cord (Willis Jr. and Coggeshall 2004).

**Neural Mechanisms of Nociception**

*Acute Pain and Hyperalgesia*

Pain is a complex perception that is influenced by a variety of physical and psychological factors. Generally, pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP 2008). Typically, pain is felt following the application of a noxious stimulus and subsequent activation of nociceptors. Nociceptive pain is defined as pain resulting from noxious or potentially damaging stimuli. However, pain can also occur without any detectable activation of nociceptors or identifiable pathology. To account for this complex situation, the use of the term pain in this text will refer to nociceptive pain.
Types of Pain

The sensation of pain has been divided into first pain (sharp, pricking) and second pain (dull, burning) relative to the onset of a noxious stimulus (Lewis 1942). The nerve fibers responsible for first and second pains were determined by the use of cuff-block to transiently disrupt blood supply to the nerve and suppress A fiber conduction (Sinclair and Hinshaw 1950; Mackenzie et al. 1975; Burke et al. 1975; Price et al. 1977). First pain, or the pain felt immediately after an experience with a noxious stimulus, is characterized by a sharp, stabbing quality and is due to activation of Aδ nociceptors. Second pain or the pain felt following first pain is characterized by a dull, burning quality and is due to activity of C nociceptors. These earlier observations were later confirmed by stimulating cutaneous nerve fibers during human microneurography experiments (Konietzny et al. 1981; Ochoa and Torebjörk 1983; Schmelz et al. 1997).

Nociceptor Subtypes

Aδ nociceptors are broadly classified based on their responsiveness to mechanical and thermal stimuli. Mechanically sensitive Aδ nociceptors (AM) are further grouped if they are heat responsive (AMH), cold responsive (AMC) or heat and cold responsive (AMHC). AMH nociceptors are further classified into type I and II based on their heat response thresholds. Type I Aδ nociceptors typically have high heat response threshold (>53°C), while type II Aδ nociceptors have lower heat response thresholds (Treede et al. 1995). Further studies have
also classified another subclass of Aδ nociceptors that are mechanically insensitive (AMIA) and may be excited by thermal and chemical stimuli under normal conditions (Treede et al. 1998).

C nociceptors are also broadly classified based on their responsiveness to mechanical and thermal stimuli. Mechanically sensitive C nociceptors (CM) are further divided if they are heat responsive (CMH), cold responsive (CMC), or heat and cold responsive (CMHC). Mechanically insensitive C nociceptors (C-MIA) are further divided if they are heat responsive (CH) or heat insensitive (CMiHi). Similar to Aδ nociceptors, many C nociceptors are also excited by algesic chemical stimuli.

Neural Mechanisms of Hyperalgesia

Pain serves a vital protective function by preventing bodily injury; however, persistent pain can lead to suffering and a reduction in the quality of life. Under certain conditions, there can be enhanced pain states that include hyperalgesia and allodynia. Hyperalgesia was classically defined as a decrease in pain threshold and/or increased sensitivity to painful stimuli (Hardy et al. 1952). Currently, hyperalgesia is defined as "an increased response to a stimulus which is normally painful" (IASP 2008). Allodynia is currently used to describe a decrease in pain threshold or "pain due to a stimulus which does not normally provoke pain" (IASP 2008). The terms hyperalgesia and allodynia represent a
continuum of enhanced nociception that often occurs following tissue injury and inflammation.

Following tissue injury or damage, nociceptors can become sensitized. Nociceptor sensitization is physiological correlate of hyperalgesia and characterized by a decrease in response threshold, increased responses to suprathreshold stimuli, and ongoing activity (Bessou and Perl 1969; Fitzgerald and Lynn 1977; Campbell et al. 1979). Sensitization of both Aδ and C nociceptors has been demonstrated following exposure to a variety of chemical mediators and experimental injuries. For example, following an experimental burn-injury to non-glabrous skin, cutaneous C nociceptors develop sensitization. Specifically, CMH nociceptors from both humans and non-human primates exhibited decreased heat response thresholds, enhanced suprathreshold responses to heat, and ongoing activity following a mild burn injury (LaMotte et al. 1982, 1983, 1984; Torebjörk et al. 1984). The sensitization of CMH nociceptors from humans and non-human primates correlated with psychophysical measures of hyperalgesia to heat in humans following the same mild burn injury. Some AMH nociceptors developed mild sensitization to heat stimuli, but their enhanced responses did not correlate with psychophysical measures of heat hyperalgesia. However, AMH nociceptors from glabrous skin developed sensitization following a mild burn injury that correlated with psychophysical measures of hyperalgesia to heat (Campbell and Meyer 1981; Campbell and Meyer 1983). Interesting, CMH nociceptors from glabrous skin
developed fatigue to heat stimuli following the mild burn injury. Overall these studies indicate that following injury, nociceptor sensitization is a neurophysiological correlate of hyperalgesia.

Nociceptor sensitization also correlates with behavioral hyperalgesia in experimental pain models in laboratory animals. For example, cutaneous nociceptors are sensitized following inflammation produced by complete Freund's adjuvant (Andrew and Greenspan 1999; Djouhri and Lawson 1999; Du et al. 2003, 2006; Wenk et al. 2006; Xiao and Bennett 2007), carrageenan (Kocher et al. 1987; Kirchhoff et al. 1990; Koltzenburg et al. 1999), formalin (Puig and Sokrin 1996), and UV-irradiation (Rukwied et al. 2008). Following nerve injury produced by chemotherapeutic agents (Xiao and Bennett 2008), STZ-induced diabetes (Khan et al. 2002; Suzuki et al. 2002; Chen and Levine 2003), or direct trauma (Wu et al. 2002; Shim et al. 2005; Djouhri et al. 2006) nociceptors also exhibit sensitization. Sensitization of nociceptors also correlates with behavioral hyperalgesia following an incision on the hindpaw (Hämäläinen et al. 2002; Pogatzki et al. 2002).

Nociceptor sensitization is likely to due to a complex milieu of neuronal and non-neuronal events. Following injury, non-neuronal cells respond by releasing a variety of inflammatory mediators that can act directly on nociceptors to produce sensitization. Some of these mediators include prostaglandins (Martin et al. 1987; Schaible and Schmidt 1988; Cohen and Perl 1990; Chen et al. 1999), cytokines (Martin and Murphy 1995; Sorkin et al. 1997; Junger and Sorkin 2000;
Obreja et al. 2002; Yu et al. 2007), bradykinin (Beck and Handwerker 1974; Liang et al. 2001), endothelins (Gokin et al. 2001), ATP (Cook and McCleskey 2002), and serotonin (Zeitz et al. 2002). Nociceptors also sensitize following exposure to acidic conditions (Steen et al. 1992, 1993; Kress et al. 1997; Jiang et al. 2006).

The neural mechanisms underlying sensitization also involve changes in nociceptor excitability through modulation of ion channel activity. Tetrodotoxin-resistant (TTX-R) voltage-gated sodium channels are predominately expressed by nociceptive DRG neurons and previous studies have demonstrated that following inflammation, TTX-R voltage-gated sodium channel expression is increased (Coggeshall et al. 2004) with enhanced activation following exposure to inflammatory mediators (Gold et al. 1996; Zhou et al. 2002). Previous studies have also demonstrated that calcium channel activity is increased following inflammation and after exposure to inflammatory mediators (Lu and Gold 2008; Czeschik et al. 2008).

The transduction of thermal and algesic chemical stimuli occur, in part, through the activation of transient receptor potential (TRP) channels located on nociceptors. The transient receptor potential vanilloid type one (TRPV1) receptor is activated by algesic chemicals such as capsaicin (Caterina et al. 1997) and resiniferatoxin (Szallasi et al. 1999) as well as and noxious heat (Caterina et al. 1997). The TRPV2 receptor is activated by noxious heat >52°C (Caterina et al. 1999). Previous studies have also shown that vanilloid receptor expression and function are increased following inflammation. Transient receptor potential vanilloid type one (TRPV1) and two (TRV2) receptors expression is increased following inflammation and hyperalgesia (Amaya et al. 2003, 2004, 2006; Ji et al. 2002; Shimosato et al. 2005; Breese et al. 2005).

Sensitization of nociceptors is not the only physiological change that contributes to hyperalgesia. Nociceptive spinal cord neurons can also become sensitized, a process referred to as central sensitization (Wolf 1983; Dubner and Ruda 1992). Like nociceptors, nociceptive spinal cord neurons also develop enhanced responses to suprathreshold stimuli, decreased thresholds for activation, increased ongoing activity, and sometimes enlarged receptive field areas following injury and inflammation (Menétrey and Besson 1982; Cook et al. 1987; Hylden et al. 1989; Simone et al. 1991; Wolf and Thompson 1991).

Although much information regarding the neural mechanisms underlying nociception has been obtained, therapeutic advances for treating chronic pain have been limited. Opiates and non-steroid anti-inflammatory drugs (NSAIDS)
remain the primary analgesics administered; however, it is estimated that between 40-50% of patients do not receive adequate analgesia while under clinical care (Glajchen 2001). This deficiency in pharmacological treatments for chronic pain has driven the search for novel analgesic agents. One novel class of compounds that has recently been explored for analgesic properties are cannabinoids. The term cannabinoid is derived from the scientific name for the plant *Cannabis sativa* and is used to describe compounds that exhibit bioactivity similar to those isolated from the plant.

**Historical Use of Cannabis**

Archaeological evidence dating back over 5000 years ago demonstrates that *Cannabis sativa* (cannabis) was cultivated for a variety of purposes. Evidence suggests that the ancient Chinese were the first to cultivate cannabis where oil from its seeds was used in cooking and its fiber was used to make paper and rope (Booth 2003). Writings from the earliest known pharmacopoeia, from China, indicate that cannabis was also used medicinally (Touw 1981). Cannabis was used to treat a variety of ailments including gastrointestinal disorders, rheumatism, headaches, and parasites. Cannabis was also used medicinally in ancient India and Egypt for its antibiotic, analgesic, anti-inflammatory, and hypnotic effects (Russo 2007). For the treatment of burns, bruising, and inflammatory lesions, cannabis preparations were often applied topically to the affected site to alleviate pain and inflammation (Zuardi 2006).
Cannabis was also used for religious purposes in ancient civilizations. Assyrians, Indians, Scythians, and Tibetans used cannabis during religious ceremonies for its mood-altering, psychoactive effects (Touw 1981). During these ceremonies cannabis was often ingested orally or burned to inhale the smoke to procure its psychoactive effects. The effects of cannabis during these ceremonies included seeing visions, communicating with spirits, and leaving one's body (Zuardi 2006).

As ancient peoples migrated and traded, the cultivation of cannabis eventually spread throughout Asia, the Middle East, and Africa where it continued to be used for its fiber. Cannabis cultivation spread into Europe around the 5th century C.E. and eventually into North and South America by the mid 15th century C.E. (Booth 2003). Accounts of its medicinal and intoxicant usage remained as cannabis spread throughout the world.

**Cannabinoids and Cannabinoid Receptors**

Despite its continued use for thousands of years, the main psychoactive constituent of cannabis was not discovered until (-)-\(\Delta^9\)-tetrahydrocannabinol (\(\Delta^9\)-THC) was isolated in the mid 1960's (Mechoulam and Gaoni 1965; Gaoni and Mechoulam 1971). Over 60 plant-derived cannabinoids (phytocannabinoids) have been isolated, prominent among these are (-)-\(\Delta^8\)-tetrahydrocannabinol (\(\Delta^8\)-THC), cannabidiol, (-)-\(\Delta^9\)-tetrahydrocannabivarin, and cannabigerol (Turner et al. 1980). Of these phytocannabinoids, (-)-\(\Delta^9\)-tetrahydrocannabivarin and (-)-\(\Delta^8\)-
tetrohydrocannabinol have shown psychoactivity or cannabimimetic properties (Turner et al. 1980; Pertwee 2008). Following the derivation of phytocannabinoids, synthetic analogs of these naturally occurring compounds were developed that exhibited cannabimimetic properties. The synthetic cannabinoid receptor agonists HU-210 and CP55940 were developed as analogs of THC (Howlett et al. 2002, 2004).

The mechanism through which cannabinoids produce their biological effects was not determined until the first cannabinoid receptor was isolated and cloned. This cannabinoid receptor, named cannabinoid one (CB₁) receptor, is a G-protein coupled receptor that is mainly localized to neurons (Matsuda et al. 1990). Typically, CB₁ receptors associate with G_{i/o} proteins and activation of these receptors decreases cAMP production by inhibiting adenylyl cyclase (Matsuda et al. 1990). Activation of CB₁ receptors has been shown to modulate ion channel activity. Through decreases in cAMP concentrations, CB₁ receptors activate A-type potassium channel current which results from decreased phosphorylation of potassium channels by protein kinase A (Childers and Deadwyler 1996). CB₁ receptors also activate G-protein coupled inwardly rectifying potassium (GIRK) channels via G_{βγ} subunits (Mackie et al. 1995; McAllister et al. 1999). Further studies have also demonstrated that activation of CB₁ receptors inhibits L-(Gebremedhin et al. 1999), N- (Mackie and Hille 1992; Caulfield and Brown 1992; Pan et al. 1996; Khasabova et al. 2004), P- (Twitchell...
et al. 1997; Fisyunov et al. 2006), and Q-type (Twitchell et al. 1997) calcium channels.

A later study characterized a second cannabinoid receptor, named cannabinoid two (CB₂) receptor, which has 48% homology with the CB₁ receptor (Munro et al. 1993). CB₂ receptors are mainly localized to leukocytes; however, CB₂ receptors have been localized to small subsets of neurons (Van Sickle et al. 2005; Onaivi et al. 2006). CB₂ receptors also associate with G_i/o G-proteins and activation of CB₂ receptors produces decreases in cAMP concentrations (Slipetz et al. 1995). Activation of CB₂ receptors also modulates cytokine secretion to produce a shift from cell-mediated (Th1) to humoral (Th2) immunity (Klein et al. 2003). Activation of CB₂ receptors has also been shown to reduce proliferation of T- and B-cells to mitogens through activation of apoptotic pathways (Lombard et al. 2007). Monocyte chemotaxis has also been shown to be suppressed following CB₂ receptor activation through PI3K/Akt and ERK1/2 signaling (Montecucco et al. 2008).

Recently a third cannabinoid receptor was discovered, named GPR55, which has 13.5% and 14.4% homology to CB₁ and CB₂ receptors, respectively (Ryberg et al. 2007). GPR55 receptors are localized to adrenal glands, gastrointestinal tract, and the central nervous system. Unlike CB₁ and CB₂ receptors, GPR55 receptors associate with G₁₃, G₁₂, and G₉ G-proteins (Ryberg et al. 2007; Lauckner et al. 2008). Activation of GRP55 receptors increases intracellular calcium concentrations through release of internal stores and inhibits
M-type potassium current (Lauckner et al. 2008). Other G-proteins such as RhoA, cdc42, and rac1 are also activated by GRP55 receptors (Ryberg et al. 2007).

Following the isolation and cloning of cannabinoid receptors, endogenous cannabinoid receptor ligands or endocannabinoids, were discovered. The first and most thoroughly studied endocannabinoid, arachidonylethanolamide (anandamide), is synthesized on-demand from membrane phospholipids. So far all other endocannabinoids isolated are also polyunsaturated fatty acids and include: 2-arachidonoyl glycerol (2-AG) (Mechoulam et al. 1995; Sugiura et al. 1995), 2-arachidonoylglyceryl ether (noladin ether) (Hanus et al. 2001), O-arachidoyl-ethanolamine (virdodhamine) (Porter et al. 2002), and N-arachidonoyl-ethanolamine (NADA) (Bisogno et al. 2000; Huang et al. 2002).

**Behavioral Pharmacology of Cannabinoids**

*Human Studies*

The oft cited effect of consuming cannabis is the subjective "high" experienced by the user. This "high" is generally reported as feelings of euphoria or well-being, followed by a state of sedation (Abood and Martin 1992). These subjective effects are thought to be primarily due to $\Delta^9$-THC, which produces similar effects when administrated alone to human participants (Abood and Martin 1992). Excessive consumption of cannabis has been reported to produce paranoia, hallucinations, and depersonalization (Abood and Martin 1992).
Consumption of cannabis also produces mild cognitive and psychomotor impairment in humans with the severity of impairment dependent on the $\Delta^9$-THC content. Studies have demonstrated that cannabis can impair learning, attention, reaction time, and psychomotor function (Abel 1971; Abood and Martin 1992). Reports of these cognitive and psychomotor deficits are not consistent across all studies, likely due to differences between participants, $\Delta^9$-THC content, and route of administration. Participants' level of experience as moderate or heavy cannabis-users likely also influences performance on cognitive and psychomotor tasks as does the amount of $\Delta^9$-THC administered (Ramaekers et al. 2006).

**Animal Studies**

Administration of cannabinoids to laboratory animals generally produces central nervous system depression and a series of behavioral cannabimimetic effects. Typically most behavioral assays have employed the use of canines, non-human primates, and rodents. Administration of cannabinoids to canines produces static ataxia, hyperreflexia, tucked-tail, and decreases in prancing and spontaneous activity (Razdan 1986). Non-human primates given cannabinoids display a reduction of aggressiveness and motor activity and develop stupor and ataxia (Razdan 1986). In rodents these cannabimimetic effects include antinociception, catalepsy, hypothermia, and decreased spontaneous locomotor activity (Martin 1985). Previous studies have also documented that cannabinoids impair learning and memory in laboratory animals (Carlini et al. 1970; Ferraro
CNS expression of Cannabinoid Receptors

Autoradiographic, in situ hybridization, and immunohistochemical studies have demonstrated that CB₁ receptors are broadly expressed throughout the central nervous system. In these studies, CB₁ receptors are most densely expressed in the cerebral cortex, hippocampal formation, striatum, and cerebellum (Herkenham et al. 1990, 1991; Mailleux and Vanderhaeghen 1992; Matsuda et al. 1993; Tsou et al. 1998; Egertová and Elphick 2000). The localization of CB₁ receptors in the central nervous system likely reflects the behavioral cannabimimetic effects observed following administration of cannabinoids (Howlett et al. 2002, 2004). In addition to this broad localization, further studies revealed that these receptors are associated with a low density of GTP-ases compared to opioid receptors (3 versus 20) (Sim et al. 1996; Breivogel et al. 1997; Gifford et al. 1999).

Analgesic Properties of Cannabinoids

Human Studies

Archaeological evidence indicates that cannabis was used for its analgesic properties. Despite these historical claims, few controlled trials have been conducted. The lack of controlled studies on cannabis is due to its legal
prohibition in most countries including the United States. Smoked cannabis has been shown to produce analgesia in patients suffering from neuropathic pain (Abrams et al. 2007; Wilsey et al. 2008), while cannabis-extracts were found to produce analgesia for treating post-operative pain (Holdcroft et al. 2006) and neuropathic pain (Rog et al. 2005). Despite these few studies, a survey of patients with chronic non-cancer pain patients in Canada who used marijuana to treat their pain indicated that 47% of patients reported it very effective at relieving their symptoms (Ware et al. 2003).

Other studies have examined the effects of synthetic cannabinoids on pain in humans and have produced mixed results. The analgesic efficacy of \( \Delta^9 \)-THC was similar to codeine in patients with chronic cancer pain (Noyes Jr. et al. 1975a,b). CT-3 (Karst et al. 2003) and ajulemic acid (Salim et al. 2005), both analogs of \( \Delta^9 \)-THC, have been shown to have analgesic efficacy in patients with chronic neuropathic pain. Sativex, a mixture of \( \Delta^9 \)-THC and cannabidiol, was also found to reduce neuropathic pain in humans (Nurmikko et al. 2007). However, previous studies found that synthetic analogs of \( \Delta^9 \)-THC did not produce analgesia for treating post-operative pain (Jain et al. 1981; Buggy et al. 2003; Beaulieu 2006). Synthetic cannabinoids have also been shown to act synergistically when co-administered with opioids in humans (Roberts et al. 2006; Narang et al. 2008).

The testing of cannabinoids on experimental pain models in humans has also been limited. Smoked cannabis or synthetic cannabinoid receptor agonist
HU210 attenuated the hyperalgesia produced by capsaicin (Rukwied et al. 2003; Wallace et al. 2007). However, oral administration of Δ⁹-THC failed to alter hyperalgesia produced by UV irradiation or capsaicin (Kraft et al. 2008).

Animal Studies

Systemic administration of cannabinoids to lab animals also produces analgesia. Previous studies have demonstrated that administration of Δ⁹-THC produces antinociception in rodents (Buxbaum 1972; Sofia et al. 1975; Wilson and May 1975; Moss and Johnson 1980; Varvel et al. 2005), dogs (Kaymakçalan et al. 1974) and non-human primates (Vivian et al. 1998). Systemically administered synthetic cannabinoid receptor agonists have also been shown to attenuate hyperalgesia produced by neuropathic injury (Herzberg et al. 1997; Fox et al. 2001; Bridges et al. 2001; Costa et al. 2004; Doğrul et al. 2004), formalin (Jaggar et al. 1998), capsaicin (Li et al. 1999), carrageenan (Conti et al. 2002; Elmes et al. 2005), complete Freund's adjuvant (Dyson et al. 2005), and cancer (Kehl et al. 2003; Hamamoto et al. 2007).

Peripheral Antihyperalgesic Effects of Cannabinoids

Behavioral Studies

The analgesic actions produced by systemically administered cannabinoids are likely due to activation of cannabinoid receptors throughout the CNS. Since systemically administered cannabinoids alter motor function, the analgesic effects could potentially result from motor impairment. This is unlikely,
since previous studies have demonstrated that systemically administered cannabinoid attenuate the responses of nociceptive spinal cord neurons and thalamic neurons (Martin et al. 1996; Hohmann et al. 1999). To avoid these centrally-mediated effects, further studies have employed site-directed administration of cannabinoids to the periphery.

Previous studies have demonstrated that locally-administered cannabinoids produce analgesia in animal models of acute and persistent pain through activation of peripheral CB₁ receptors. Local administration of cannabinoids produced antihyperalgesia induced by capsaicin (Ko and Woods 1999; Johanek et al. 2001), formalin (Calignano et al. 1998; Guindon et al. 2006), carrageenan (Richardson et al. 1998; Gutierrez et al. 2007), complete Freund's adjuvant (Amaya et al. 2006; Potenzieri et al. 2008b), cancer (Guerrero et al. 2008; Khasabova et al. 2008; Potenzieri et al. 2008a) and nerve injury (Fox et al. 2001; Ulugol et al. 2004; Guindon and Beaulieu 2006; Agarwal et al. 2007).

Further studies have also demonstrated that locally-administered cannabinoids produce antinociception through activation of CB₂ receptors. Activation of peripheral CB₂ receptors results in antinociception to noxious heat (Malan Jr. et al. 2001; Ibrahim et al. 2005; Ibrahim et al. 2006). Local administration of CB₂ receptor agonists attenuated hyperalgesia produced by carrageenan (Nackley et al. 2003; Quartilho et al. 2003; Elmes et al. 2005; Gutierrez et al. 2007), capsaicin (Hohmann et al. 2004), and nerve pain (Ibrahim et al. 2003). The exact mechanism responsible CB₂ receptor-mediated...
antinociception is unclear, but likely involve both neuronal and non-neuronal components.

Peripheral Neural Mechanisms of Cannabinoid Antihyperalgesia

The peripherally-mediated antihyperalgesia produced by locally-administered cannabinoid is likely mediated, in part, by activation of CB₁ receptors located on primary afferent nerve terminals. Previous studies have demonstrated that DRG neurons express CB₁ receptors (Hohmann and Herkenham 1999; Ahluwalia et al. 2000; Bridges et al. 2003). These studies indicate that CB₁ receptors are localized mainly to large diameter, DRG neurons the give rise to myelinated nerve fibers (presumably both nociceptive and non-nociceptive). Activation of CB₁ receptors on DRG neurons decreased high-voltage activated calcium currents (Ross et al 2001; Khasabova et al. 2002, 2004) and reduced capsaicin-evoked calcium transients (Millns et al. 2001; Sagar et al. 2005). Targeted deletion of CB₁ receptors from Nav1.8 expressing nociceptive sensory neurons produced a loss peripherally-mediated cannabinoid analgesia, indicating that nociceptors are affected by cannabinoids (Agarwal et al. 2007). Although numerous studies indicate that activation of peripheral CB₁ receptors mediates the antihyperalgesia produced by locally-administered cannabinoids, the extent to which cannabinoids alter responses of nociceptors to decrease hyperalgesia is not currently known.
Statement of Purpose

Numerous studies have demonstrated that activation of peripheral cannabinoid receptors produced antinociception in a variety of animal models of acute and persistent pain. Further in vitro studies have demonstrated that cannabinoids decrease the responses of nociceptive neurons. Taken together, these studies suggest that cannabinoids act on nociceptors to produce antinociception. The specific mechanisms of how cannabinoids modulate nociceptor activity and the types of nociceptors sensitive to cannabinoids, are not known. Using correlative behavioral and in vivo teased-fiber electrophysiological methods, the overall aim of this thesis is to determine the peripheral neural mechanisms by which cannabinoids attenuate nociception.

Although previous studies have demonstrated that locally-administered cannabinoids produce antinociception, very few studies have examined the effects of cannabinoids during naive, control conditions. To account for this state-dependent effect, the first specific aim was formulated:

**To determine if peripheral CB₁ receptor activation decreases mechanical and thermal sensitivity during non-inflamed and inflamed conditions.**

To assess the ability of locally-administered cannabinoids to alter mechanical and thermal sensitivity, a model of inflammatory pain was employed. Complete Freund’s adjuvant (CFA) was administered into the hindpaw to produce inflammation, allodynia, and hyperalgesia. The non-inflamed condition was induced by an intraplantar injection of an equal volume of saline. Paw
withdrawal thresholds and frequencies evoked by von Frey monofilaments applied to the plantar surface of the hindpaw were determined to assay mechanical sensitivity. Latencies to paw withdrawal from radiant heat were determined to assay thermal sensitivity. The effect of the cannabinoid receptor agonists arachidonyl-2’-chloroethylamide (ACEA) and (R)-(+) methanandamide (methAEA) on mechanical and thermal sensitivity was determined before and at several time-points following injection into the affected hindpaw. It is hypothesized that neither ACEA nor methAEA will have an effect on mechanical and thermal sensitivity during control, non-inflamed conditions, but will attenuate nociception during CFA-evoked inflammation via CB$_1$ receptors.

Despite previous studies demonstrating a reduction in the excitability of nociceptive DRG neurons following CB$_1$ receptor activation, how cannabinoids modulate the response properties of nociceptors is not known. Thus, the second specific aim was formulated:

To determine if peripheral CB$_1$ receptor activation decreases evoked responses of cutaneous nociceptors during non-inflamed inflamed conditions.

Cutaneous A$\delta$ and C nociceptors were isolated from the tibial nerve following intraplantar injection of saline or CFA into the hindpaw, in the same manner as in behavioral studies. To assess the ability of locally-administered cannabinoids to alter the response properties of nociceptors, responses to controlled mechanical and heat stimuli were determined before and at several
time-points after injection of ACEA, methAEA into the receptive field (RF) located on the plantar surface of the hindpaw. It is hypothesized that neither ACEA nor methAEA will alter evoked responses of Aδ and C nociceptors in non-inflamed conditions. However, it is hypothesized that ACEA and methAEA will decrease evoked responses of both Aδ and C nociceptors from inflamed skin via CB₁ receptors.

Previous studies have demonstrated that CB₁ receptors are mainly expressed on large diameter DRG neurons that give rise to myelinated nerve fibers (Bridges et al. 2003). Since these expression studies cannot specifically determine if these large diameter DRG neurons are nociceptive or non-nociceptive, it is possible that Aβ mechanoreceptors are also express CB₁ receptors. Thus, the third specific aim was formulated:

**To determine if peripheral CB₁ receptor activation decreases evoked responses of cutaneous mechanoreceptors during non-inflamed and inflamed conditions.**

Cutaneous Aβ mechanoreceptors were isolated from the tibial nerve following intraplantar injection of saline or CFA into the hindpaw, in the same manner as in behavioral studies. To assess the ability of locally-administered cannabinoids to alter the response properties of mechanoreceptors, responses to controlled mechanical stimuli were determined before and at several time-points after injection of ACEA into the receptive field (RF) located on the plantar surface
of the hindpaw. It is hypothesized that ACEA will not alter evoked responses of 
Aβ mechanoreceptors from either non-inflamed or inflamed skin.

Although prior studies employed the cannabinoid receptor agonists ACEA and methAEA, preliminary studies tested the ability of the endogenous cannabinoid receptor agonist, arachidonylethanolamide (AEA) to attenuate inflammatory hyperalgesia. Unlike ACEA and methAEA, AEA did not attenuate inflammatory hyperalgesia. AEA has also been characterized as an endogenous vanilloid receptor agonist, and to further characterize its effects, a fourth specific aim was formulated:

**To determine if peripheral administration of anandamide excites cutaneous nociceptors and produces nocifensive behaviors through activation of transient receptor potential vanilloid type one (TRPV1) receptors.**

AEA was injected into the RFs of cutaneous Aδ and C nociceptors isolated from the tibial nerve to determine if excitation occurred. The presence of nocifensive behavior was assessed following intraplantar injection of AEA. TRPV1 receptor antagonists were pre-administered prior to anandamide to determine receptor selectivity. It is hypothesized that AEA will excite cutaneous nociceptors and produce nocifensive behavior through activation TRPV1 receptors.
Chapter 2

CANNABINOID MODULATION OF NOCICEPTION DURING NON-INFLAMED AND INFLAMED CONDITIONS
Introduction

Systemic administration of cannabinoids produces antinociception and attenuates hyperalgesia and allodynia in animal models of acute and chronic pain (for reviews see Walker et al. 1999; Hohmann 2002; Walker and Huang, 2002; Mbvundula et al. 2004). The efficacy of systemically delivered cannabinoids is limited by adverse cannabimimetic effects (catalepsy, hypolocomotion, and disruptions in memory) which are mediated through CB1 receptor activation in the central nervous system (for review see Iversen 2003). One way to avoid these centrally-mediated cannabimimetic effects, while still providing analgesia, is through the selective targeting of peripheral CB1 receptors expressed on nociceptive primary afferent dorsal root ganglion (DRG) neurons (Hohmann and Hekenham, 1999; Ahluwalia et al. 2000) and cutaneous nerve terminals (Ständer et al. 2005; Amaya et al. 2006; Agarwal et al. 2007).

Peripheral administration of cannabinoids attenuated hyperalgesia in animal models of inflammatory pain (Richardson et al. 1998; Amaya et al. 2006; Gutierrez et al. 2007), neuropathic pain (Fox et al. 2001; Guidon and Beaulieu 2006), heat injury (Johanek and Simone 2004), and capsaicin-evoked hyperalgesia (Johanek at al. 2001) through activation of CB1 receptors. Although previous studies have demonstrated that locally-administered cannabinoids produce antinociception, few studies have examined the effects of cannabinoids during naive, control states. To account for this state-dependent effect, the overall aim of the present study was to determine if peripheral CB1 receptor
activation decreases mechanical and thermal sensitivity during non-inflamed and inflamed conditions. A report of this study has been published and is included in this thesis with permission from the American Physiological Society (Potenzieri et al. 2008b).
Methods

Subjects

Adult, male, Sprague–Dawley rats weighing 280–350 g were used in this study. Animals were obtained from Harlan (Indianapolis, IN), housed on a 12-hour light/dark schedule, and allowed *ad libitum* access to food and water. Experiments were performed during the light cycle. All procedures were approved by the Animal Care Committee at the University of Minnesota, and experiments were conducted according to the guidelines established by the International Association for the Study of Pain.

Induction of inflammation

Rats were anesthetized with a mixture of isoflurane gas (Phoenix Pharmaceuticals, St. Joseph, MO) in air (2% for induction and maintenance) and received a single intraplantar injection of complete Freund’s adjuvant (CFA) (Sigma Chemical, St. Louis, MO) or sterile isotonic saline as a control (Baxter, Deerfield, IL). CFA (1 mg/mL) and saline were given in a volume of 50 µl using a 28-guage needle. Behavioral experiments were performed 24 hours after injection of CFA or saline.

Drug preparation and administration

The cannabinoid receptor agonists were N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA) and (R)-N-(2-Hydroxy-1-
methylethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (methAEA). The CB\textsubscript{1} receptor antagonist used was N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) which exhibits \( \approx 300 \)-fold selectivity over CB\textsubscript{2} receptors (Gatley et al. 1996). The CB\textsubscript{2} receptor antagonist was 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl(4-methoxyphenyl)methanone (AM630) which exhibits \( \approx 160 \)-fold selectivity over CB\textsubscript{1} receptors (Pertwee et al. 1995; Hosohata et al. 1997). All drugs were obtained from Tocris Bioscience (Ellisville, MO). ACEA was supplied pre-dissolved in ethanol (5 mg/mL). MethAEA was supplied pre-dissolved in a water soluble emulsion (Tocrisolve\textsuperscript{TM}) (5 mg/mL). AM251 was dissolved in anhydrous ethanol (25 \( \mu \)g/\( \mu \)l). AM630 was dissolved in a vehicle containing 5% Tween80 and 5% DMSO in isotonic saline (20 \( \mu \)g/\( \mu \)l). All drugs were diluted to their final concentration in sterile isotonic saline and administered via subcutaneous intraplantar injection in a volume of 50 \( \mu \)l.

**Paw withdrawal thresholds**

Paw withdrawal thresholds to mechanical stimuli were used as a measure of mechanical allodynia. Withdrawal thresholds were determined using a series of eight calibrated Semmes-Weinstein von Frey monofilaments of logarithmic incremental stiffness (0.40-15 g) (Stoelting, Wood Dale, IL). Animals were placed on an elevated plastic mesh platform under individual plastic cages and allowed to acclimate to the testing environment for 30 minutes prior to testing. The
monofilaments were applied to the mid-plantar surface of the hindpaw for 1-2 s with an interstimulus interval of 5-6 s. The paw withdrawal threshold (g) was calculated according to the methods described by Chaplan et al. (1994). Baseline measures were determined for each animal for three consecutive days prior to injection of saline or CFA. Mechanical allodynia was defined as a decrease in paw withdrawal threshold.

**Paw withdrawal frequencies**

The frequency of paw withdrawal evoked by a standard mechanical stimulus was used to measure mechanical hyperalgesia. The frequency of paw withdrawal evoked by a Semmes-Weinstein von Frey monofilament (Stoelting, Wood Dale, IL) with a weight of 26 g was determined. Animals were placed on an elevated plastic mesh platform under individual plastic cages and allowed to acclimate to the testing environment for 30 minutes prior to testing. The filament was applied to the mid-plantar surface of the hindpaw ten times for 1-2 s each with an interstimulus interval of 5-6 s and the paw withdrawal frequency (%) was determined. Baseline measures were determined for each animal for three consecutive days prior to injection of saline or CFA. Mechanical hyperalgesia was defined as an increase in the paw withdrawal frequency.

**Paw withdrawal latencies**
The latency of paw withdrawal (s) from radiant heat stimulation was used to measure heat hyperalgesia. Withdrawal responses to radiant heat stimuli were determined using a custom built device that uses an encased 50W bulb to deliver a radiant heat source similar to methods described by Hargreaves et al. (1988). Rats were placed under a clear plastic cage on a 3-mm thick glass plate that was elevated to allow maneuvering of the radiant heat source beneath it. Rats were acclimated to the testing environment for at least 15 min prior to stimulation. The heat source was positioned such that the focused beam of radiant heat (8 mm diameter) was applied to the mid-plantar surface. Withdrawal latencies to the nearest 0.1 s were measured automatically by use of a photocell that terminated each trial and stopped the timer upon withdrawal of the hindpaw. A 19 s cutoff was imposed to prevent tissue damage. Four stimuli were applied to each hindpaw, alternating between paws, with an interstimulus interval of at least 60 s. Withdrawal latency for each paw was defined as the average of the last three trials. Withdrawal latencies were obtained daily for each hindpaw during a training period of 3 consecutive days. Hyperalgesia to heat was defined as a decrease in paw withdrawal latency.

Experimental design

Paw withdrawal thresholds and latencies were determined concurrently, while paw withdrawal frequencies were determined in separate groups of animals. Following three consecutive days of baseline testing, animals received
an intraplantar injection of either CFA or saline into the left hindpaw. Twenty-four hours following injection, animals were randomly divided into groups of eight to ten rats each. Paw withdrawal thresholds, frequencies, and latencies were determined for both hindpaws before and 30, 60, and 120 minutes after cannabinoid or vehicle administration into the left hindpaw. In separate groups of rats, ACEA or methAEA was injected into the contralateral hindpaw and paw withdrawal thresholds, frequencies, and latencies were determined in the inflamed (ipsilateral) hindpaw. The doses of either ACEA or methAEA given were 0.1, 1, or 10 µg. AM251 or AM630 were each given in doses of 30 µg and were co-injected with either ACEA (10 µg) or methAEA (10 µg). Each animal was used in only one experiment and the experimenter was blinded to the identity of the drug administered.

Data analysis

To determine the effect of cannabinoids or their vehicles on withdrawal thresholds, frequencies, and latencies to heat compared to baseline measures, comparisons were made using one-way repeated-measures ANOVA followed by paired t-tests with the Bonferroni correction for multiple comparisons. A one-way ANOVA followed by unpaired t-tests with the Bonferroni correction for multiple comparisons was used to determine the effect of cannabinoids or their vehicles on withdrawal thresholds, frequencies, and latencies between groups. Within group comparisons of the main effect of CFA and saline injection on withdrawal
thresholds, frequencies, and latencies were made using paired t-tests. For all statistical analyses, a probability value <0.05 was considered significant. All data are presented as mean (±S.E.M).
Results

Mechanical allodynia, mechanical hyperalgesia, and heat hyperalgesia following inflammation

Twenty-four hours after intraplantar injection of CFA, rats exhibited mechanical allodynia, mechanical hyperalgesia, and heat hyperalgesia in the injected hindpaw. Paw withdrawal thresholds decreased from 13.6±0.2 g to 5.0±0.2 g (n=140, p<0.001), paw withdrawal frequencies increased from 26.9±0.7% to 94.8±0.6% (n=188, p<0.0001), and paw withdrawal latencies decreased from 11.3±0.1 s to 6.0±0.1 s (n=140, p<0.0001). Withdrawal thresholds (n=37), withdrawal frequencies (n=32), and withdrawal latencies (n=32) did not change 24 hours after intraplantar injection of saline (from 13.1±0.3 g to 12.9±0.4 g, 24.6±1.7% to 25.7±1.3%, and 10.9±0.2 s to 10.4±0.3 s, respectively). No changes in withdrawal thresholds, frequencies, or latencies were observed in the contralateral hindpaw in either CFA- or saline-treated rats.

Effects of ACEA and methAEA on mechanical allodynia

Intraplantar injection of ACEA or methAEA, but not vehicle, attenuated mechanical allodynia produced by CFA. Increases in withdrawal threshold occurred following the 1 and 10 µg doses of both cannabinoids (Figure 1-2). The antiallodynic effects of both cannabinoids peaked 30 minutes after administration and withdrawal thresholds returned to baseline values by 60 minutes after administration (Figure 1-2). Mean paw withdrawal thresholds 30 minutes after
administration of 1 and 10 µg of ACEA were 9.2±1.6 g and 14.3±0.7 g, respectively (p<0.05). Mean paw withdrawal thresholds 30 minutes after administration of 1 and 10 µg of methAEA were 9.6±1.8 g and 13.8±1.1 g, respectively (p<0.05).

To determine if the antiallodynia produced by ACEA and methAEA was mediated by peripheral cannabinoid receptors, rather than through a systemic mechanism, ACEA (10 µg) or methAEA (10 µg) was injected into the contralateral hindpaw and paw withdrawal thresholds were determined in the inflamed (ipsilateral) hindpaw. Withdrawal thresholds 30, 60, and 120 minutes after administration of ACEA into the contralateral paw (4.1±0.9 g, 3.5±0.3 g, 3.7±0.4 g, respectively) were not different from baseline withdrawal thresholds (5.0±0.5 g). Similarly, withdrawal thresholds 30, 60, 120 minutes after administration of methAEA into the contralateral paw (3.1±0.4 g, 3.1±0.8 g, 3.8±0.5 g, respectively) also did not differ from baseline withdrawal thresholds (4.4±0.6 g). These results indicate that the antiallodynia following administration of ACEA and methAEA was mediated by peripheral cannabinoid receptors.

ACEA and methAEA were co-administered with either the CB₁ receptor antagonist, AM251, or the CB₂ receptor antagonist, AM630, to determine which cannabinoid receptor subtype mediated the antiallodynic effects produced by either cannabinoid. Co-administration of either ACEA (10 µg) or methAEA (10 µg) with AM251 (30 µg), but not with AM630 (30 µg), blocked the increase in withdrawal thresholds produced by ACEA and methAEA (Figure 1-2). These
results suggest that the antiallodynia following administration of ACEA and methAEA are mediated by peripheral CB₁ receptors. Administration of AM251 or AM630 alone, or their vehicles, did not alter withdrawal thresholds (data not shown).

Effects of ACEA and methAEA on mechanical hyperalgesia

Intraplantar injection of ACEA or methAEA, but not vehicle, dose-dependently attenuated mechanical hyperalgesia. Decreases in withdrawal frequencies occurred following administration of the 1 or 10 µg doses of each cannabinoid (Figure 3-4). The antihyperalgesic effects of both cannabinoids peaked 30 minutes after administration and paw withdrawal frequencies returned to baseline values by 60 minutes after administration (Figure 3-4). Mean paw withdrawal frequencies 30 minutes after administration of 1 or 10µg of ACEA were 66.3±2.6 % and 38.8±5.6 %, respectively. Mean paw withdrawal thresholds 30 minutes after administration of 1 or 10µg of methAEA were 70.0±2.5 % and 44.0±6.1 %, respectively.

Similar to the results for mechanical allodynia, intraplantar injection of either ACEA (10 µg) or methAEA (10 µg) into the contralateral hindpaw did not alter paw withdrawal frequencies in the inflamed (ipsilateral) hindpaw. Withdrawal frequencies 30, 60, and 120 minutes after administration of ACEA into the contralateral paw (96.0±2.0 %, 92.0±3.7 %, 98.0±2.0 %, respectively) were not different from baseline withdrawal thresholds (96.0±2.4 %). Similarly, withdrawal
frequencies 30, 60, and 120 minutes after administration of methAEA into the contralateral paw (95.0±1.6 %, 96.6±1.6 %, 95.0±1.6 %, respectively) were not different from baseline withdrawal thresholds (94.0±2.2 %). Again, these results indicate that the antihyperalgesic effects of locally-administered ACEA and methAEA are mediated by peripheral cannabinoid receptors.

The decrease in withdrawal frequencies produced by either ACEA (10 µg) or methAEA (10µg) was blocked by co-administration of the CB₁ receptor antagonist AM251 (30 µg), but not by CB₂ receptor antagonist AM630 (30 µg) (Figure 3-4). These results suggest that the antihyperalgesia produced by ACEA and methAEA are mediated by peripheral CB₁ receptors. Administration of AM251 or AM630 alone, or their vehicles, did not alter paw withdrawal frequencies (data not shown).

Effects of ACEA and methAEA on heat hyperalgesia

Similar to results obtained for mechanical hyperalgesia, intraplantar administration of ACEA or methAEA also attenuated heat hyperalgesia. Decreases in withdrawal latencies occurred following administration of the 10 µg dose of either drug (Figure 5-6). The antihyperalgesic effects of both cannabinoids peaked 30 minutes after administration and paw withdrawal latencies returned to baseline values by 60 minutes after administration (Figure 5-6). The mean withdrawal latencies 30 minutes after administration of 10 µg of ACEA and methAEA were 10.0±0.86 s and 9.1±0.6 s, respectively.
Administration either ACEA (10 µg) or methAEA (10 µg) into the contralateral hindpaw did not alter paw withdrawal latencies in the inflamed (ipsilateral) hindpaw. Withdrawal latencies 30, 60, and 120 minutes after administration of ACEA into the contralateral paw (6.0±0.3 s, 5.6±0.3 s, 5.8±0.3 s, respectively) were not different from baseline withdrawal thresholds (6.7±0.3 s). Similarly, withdrawal frequencies 30, 60, and 120 minutes after administration of methAEA into the contralateral paw (5.2±0.2 s, 5.8±0.3 s, 5.7±0.3 s, respectively) were not different from baseline withdrawal thresholds (5.5±0.3 s). Again, these results indicate that the antihyperalgesic effects of locally-administered ACEA and methAEA into the inflamed hindpaw are mediated by peripheral cannabinoid receptors.

The decrease in withdrawal latencies produced by either ACEA (10 µg) or methAEA (10 µg) was blocked by the CB₁ receptor antagonist AM251 (30 µg), but not by CB₂ receptor antagonist AM630 (30 µg) (Figure 5-6). These results suggest that the antihyperalgesia produced by ACEA and methAEA is mediated by peripheral CB₁ receptors. Administration of AM251 or AM630 alone, or their vehicles, did not alter paw withdrawal latencies (data not shown).

Effects of ACEA and methAEA in control, non-inflamed rats

Twenty-four hours after intraplantar injection of saline, rats received an intraplantar injection of ACEA (10 µg), methAEA (10 µg), or vehicle. In contrast to the antinociceptive effects observed when given into the inflamed hindpaw,
ACEA and methAEA, as well as their vehicles, produced a small decrease in paw withdrawal thresholds and a trend for an increase paw withdrawal frequencies (Figure 7-8). The decrease in withdrawal thresholds produced by ACEA and methAEA did not differ from their vehicles at any time point tested. Withdrawal latencies also decreased following administration of ACEA, but did not differ from its vehicle at any time point test (Figure 9). Intraplantar administration of methAEA did not alter withdrawal latencies, but withdrawal latencies decreased 60 and 120 minutes after administration of its vehicle (Figure 9). These results suggest that peripheral administration of ACEA or methAEA did not produce antinociception to mechanical and heat stimuli during control, non-inflamed conditions.
Discussion

In the present study, local administration of the cannabinoid receptor agonists, methAEA or ACEA, into the inflamed hindpaw attenuated mechanical allodynia, mechanical hyperalgesia, and heat hyperalgesia. In contrast, administration of ACEA or methAEA into the contralateral hindpaw did not alter allodynia and hyperalgesia in the ipsilateral inflamed hindpaw. The antiallodynic and antihyperalgesic effects of ACEA and methAEA were blocked by co-administration with the CB₁ receptor antagonist AM251, strongly suggesting that activation of peripheral CB₁ receptors underlies these effects. Administration of neither methAEA nor ACEA produced antinociception to mechanical or heat stimuli in control, non-inflamed rats. Together, these results suggest that local injection of ACEA and methAEA attenuated inflammatory allodynia and hyperalgesia through activation peripheral CB₁.

Attenuation of allodynia and hyperalgesia by cannabinoids

The results of our behavioral studies agree with prior studies demonstrating antiallodynia/antihyperalgesia following local administration of cannabinoids into inflamed tissue occurs through activation of peripheral CB₁ receptors (Richardson et al. 1998; Amaya et al. 2006; Gutierrez et al. 2007). Local administration of cannabinoids have also been shown to attenuate hyperalgesia produced by nerve injury (Fox et al. 2001; Guindon and Beaulieu 2006), cutaneous heat injury (Johanek and Simone 2004), and cancer (Guerrero
et al. 2008; Potenzieri et al. 2008a) through activation of peripheral CB₁ receptors.

Previous studies have demonstrated that ACEA (Hillard et al. 1999; Meybohm et al. 2008) and methAEA (Abadji et al. 1994) given systemically produced typical cannabinemic effects such as hypothermia, hypolocomotion, catalepsy, and antinociception through activation of CB₁ receptors in the central nervous system. The doses required to produce these cannabinemic effects were 3 to 10 fold greater than the highest doses of ACEA and methAEA used in the present study (Abadji et al. 1994; Hillard et al. 1999; Meybohm et al. 2008). No antiallodynia or antihyperalgesia occurred when the doses of cannabinoids used in the present study were injected into the paw contralateral to the inflamed paw, demonstrating that the effects of cannabinoids occurred through peripheral, and not central, mechanisms. The antiallodynia and antihyperalgesia produced by ACEA and methAEA were blocked by co-administration with the CB₁ receptor antagonist AM251, but not with the CB₂ receptor antagonist AM630. The contribution of CB₁ receptors to the antiallodynia/antihyperalgesia produced by ACEA and methAEA is consistent with their higher affinity for CB₁ over CB₂ receptors, 1400- and 40-fold, respectively (Abadji et al. 1994; Hillard et al. 1999).

We found that intraplantar administration of ACEA and methAEA attenuated withdrawal responses to mechanical and heat stimuli only during inflammation. Similarly, the ability of ACEA to attenuate behavioral responses to heat was greater during inflammation (Amaya et al. 2006). The enhancement of
ACEA’s antinociceptive effects was related to increased CB₁ receptor labeling in both nociceptive DRG neurons and their peripheral nerve terminals (Amaya et al. 2006). Similarly, up-regulation of CB₁ receptors also occurred in DRG neurons two weeks following spinal nerve ligation (SNL) (Mitrirattanakul et al. 2006) and was related to the enhanced antinociception produced by locally-administered cannabinoids in this model of neuropathic pain (Fox et al. 2001). These studies suggest that peripherally-mediated antinociception produced by locally-administered cannabinoids may result from increased CB₁ receptor expression in nociceptive DRG neurons; however, the specific nociceptor subtypes involved have not been determined. It is possible that acute changes also exist to regulate CB₁ receptor activity, since locally-administered cannabinoids also produce peripherally-mediated antinociception in animal models of acute pain such as intraplantar injection of capsaicin (Johanek et al. 2001), cutaneous heat injury (Johanek and Simone 2004), and intraplantar injection of formalin (Calignano et al. 1998; Guindon et al. 2006) through activation of peripheral CB₁ receptors.

We found that administration of CB₁ receptor antagonist AM251 or CB₂ receptor antagonist AM630 alone did not alter mechanical allodynia or hyperalgesia following inflammation. These results suggest that changes in endocannabinoid tone do not occur or are not functionally observable following inflammation. A similar observation was noted by Gutierrez et al. (2007) using different CB₁ and CB₂ receptors antagonist (SR141716A and SR144528, respectively).
Summary

In conclusion, allodynia and hyperalgesia produced by inflammation was attenuated by local administration of ACEA or methAEA into the inflamed hindpaw. The antiallodynia and antihyperalgesia produced by ACEA and methAEA was mediated by CB₁ receptors and was not due to systemic effects. Administration of ACEA and methAEA did not produce antinociception to mechanical and heat stimuli under non-inflamed conditions. Based on results from the present study, peripherally-acting cannabinoid receptor agonists may be useful to treat inflammatory pain and hyperalgesia.
Figure 1

A

![Bar graph showing PW T (grams) vs. ACEA (µg) concentrations.](image)

- BL
- CFA
- Veh
- 0.1
- 1
- 10

B

![Line graph showing PW T (grams) over time.](image)

- BL
- CFA
- 30
- 60
- 120

Lines:
- ACEA
- AM251 + ACEA
- AM630 + ACEA

**Annotations:**
- *: Significant difference
- #: Significant difference
**Figure 1.** Attenuation of mechanical allodynia by ACEA through activation of peripheral CB₁ receptors. Peripheral administration of ACEA (A) dose-dependently increased paw withdrawal thresholds at doses of 1 and 10 µg (values shown indicate 30 minutes after administration). Co-administration with the CB₁ receptor antagonist AM251 (30 µg), but not the CB₂ receptor antagonist AM630 (30 µg), blocked the antiallodynic effects of 10 µg ACEA. BL: mean baseline paw withdrawal thresholds 24 hours prior to injection of CFA. CFA: mean paw withdrawal thresholds 24 hours after intraplantar injection of CFA, and also serves as the pre-drug baseline. PWT: paw withdrawal threshold. Time: time after intraplantar injection of drugs. * indicates a significant difference from vehicle (p<0.05). # indicates a significant difference from 1 µg of ACEA (p<0.05). ‡ indicates significant difference from 10 µg of ACEA (p<0.05). n= 8-10 animals per dose.
Figure 2

A

[Bar chart showing PWT (grams) vs. methAEA (µg) for different conditions: BL, CFA, Veh, 0.1, 1, 10.]

B

[Line graph showing PWT (grams) over time (minutes) for different treatments: methAEA, AM251 + methAEA, AM630 + methAEA.]
Figure 2. Attenuation of mechanical allodynia by methAEA through activation of peripheral CB₁ receptors. Peripheral administration of methAEA (A) dose-dependently increased paw withdrawal thresholds at doses of 1 and 10 µg (values shown indicate 30 minutes after administration). Co-administration with the CB₁ receptor antagonist AM251 (30 µg), but not the CB₂ receptor antagonist AM630 (30 µg), blocked the antiallodynic effects of 10 µg methAEA (B). BL: mean baseline paw withdrawal thresholds 24 hours prior to injection of CFA. CFA: mean paw withdrawal thresholds 24 hours after intraplantar injection of CFA, and also serves as the pre-drug baseline. PWT: paw withdrawal threshold. Time: time after intraplantar injection of drugs. * indicates a significant difference from vehicle (p<0.05). # indicates a significant difference from 1 µg of methAEA (p<0.05). ‡ indicates significant difference from 10 µg of methAEA (p<0.05). n= 8-10 animals per dose.
Figure 3

A

B

P W F (%)

ACEA (µg)

BL  CFA  Veh  0.1  1  10

0  20  40  60  80  100

P W F (%)

BL  CFA  30  60  120

0  20  40  60  80

ACEA  AM251 + ACEA  AM630 + ACEA

Time (minutes)

‡

*  #
**Figure 3.** Attenuation of mechanical hyperalgesia by ACEA through activation of peripheral CB₁ receptors. Peripheral administration of ACEA (A) dose-dependently decreased paw withdrawal frequencies at doses of 1 and 10µg (values shown indicate 30 minutes after administration). Co-administration with the CB₁ receptor antagonist AM251 (30 µg), but not the CB₂ receptor antagonist AM630 (30 µg), blocked the antihyperalgesic effects of 10 µg ACEA (B). BL: mean baseline paw withdrawal frequency 24 hours prior to injection of CFA. CFA: mean paw withdrawal frequency 24 hours after intraplantar injection of CFA, and also serves as the pre-drug baseline. PWF: paw withdrawal frequency. Time: time after intraplantar injection of drugs. * indicates a significant difference from vehicle (p<0.05). # indicates a significant difference from 1 µg of ACEA (p<0.05). ‡ indicates significant difference from 10 µg ACEA (p<0.05). n= 8-10 animals per dose.
Figure 4

A

B

methAEA (µg)

methAEA

AM251 + methAEA

AM630 + methAEA
Figure 4. Attenuation of mechanical hyperalgesia by methAEA through activation of peripheral $\text{CB}_1$ receptors. Peripheral administration of methAEA (A) dose-dependently decreased paw withdrawal frequencies at doses of 1 and 10$\mu$g (values shown indicate 30 minutes after administration). Co-administration with the $\text{CB}_1$ receptor antagonist AM251 (30 $\mu$g), but not the $\text{CB}_2$ receptor antagonist AM630 (30 $\mu$g), blocked the antihyperalgesic effects of 10 $\mu$g methAEA (B). BL: mean baseline paw withdrawal frequency 24 hours prior to injection of CFA. CFA: mean paw withdrawal frequency 24 hours after intraplantar injection of CFA, and also serves as the pre-drug baseline. PWF: paw withdrawal frequency. Time: time after intraplantar injection of drugs. * indicates a significant difference from vehicle ($p<0.05$). # indicates a significant difference from 1 $\mu$g of methAEA ($p<0.05$). ‡ indicates significant difference from 10 $\mu$g methAEA ($p<0.05$). n= 8-10 animals per dose.
**Figure 5.** Attenuation of heat hyperalgesia by ACEA through activation of peripheral CB₁ receptors. Peripheral administration of ACEA (A) increased paw withdrawal latencies at a dose of 10 µg (values shown indicate 30 minutes after administration). Co-administration with the CB₁ receptor antagonist AM251 (30 µg), but not the CB₂ receptor antagonist AM630 (30 µg), blocked the antihyperalgesic effects of 10 µg ACEA (B). BL: mean baseline paw withdrawal latencies 24 hours prior to injection of CFA. CFA: mean paw withdrawal latencies 24 hours after intraplantar injection of CFA, and also serves as the pre-drug baseline. PWL: paw withdrawal latency. Time: time after intraplantar injection of drugs. * indicates a significant difference from vehicle (p<0.05). ‡ indicates significant difference from 10 µg of ACEA (p<0.05). n= 8-10 animals per dose.
Figure 6

A

B

methAEA (µg)

Time (minutes)

P WL (s)

methAEA

AM251 + methAEA

AM630 + methAEA

Time (minutes)
Figure 6. Attenuation of heat hyperalgesia by methAEA through activation of peripheral CB₁ receptors. Peripheral administration of methAEA (A) increased paw withdrawal latencies at a dose of 10 µg (values shown indicate 30 minutes after administration). Co-administration with the CB₁ receptor antagonist AM251 (30 µg), but not the CB₂ receptor antagonist AM630 (30 µg), blocked the antihyperalgesic effects of 10 µg methAEA (B). BL: mean baseline paw withdrawal latencies 24 hours prior to injection of CFA. CFA: mean paw withdrawal latencies 24 hours after intraplantar injection of CFA, and also serves as the pre-drug baseline. PWL: paw withdrawal latency. Time: time after intraplantar injection of drugs. * indicates a significant difference from vehicle (p<0.05). ‡ indicates significant difference from 10 µg of ACEA (p<0.05). n= 8-10 animals per dose.
Figure 7. Effects of ACEA and methAEA on paw withdrawal thresholds during non-inflamed conditions. Effects of intraplantar injection of ACEA (10 µg) or vehicle on mean paw withdrawal thresholds (A) 24 hours after intraplantar injection of saline. Effects of intraplantar injection of methAEA (10 µg) or vehicle (B) on mean paw withdrawal thresholds 24 hours after intraplantar injection of saline. BL: mean baseline paw withdrawal threshold and withdrawal frequency 24 hours before intraplantar injection of saline. SAL: mean paw withdrawal threshold and mean withdrawal frequency 24 hours after intraplantar injection of saline, and also serves as the pre-drug baseline. * indicates a significant difference from SAL (p<0.05). n=8-10 animals per dose.
Figure 8

A

![Graph A](image)

B

![Graph B](image)
Figure 8. Effects of ACEA and methAEA on paw withdrawal frequencies during non-inflamed conditions. Effects of intraplantar injection of ACEA (10 µg) or vehicle on mean paw withdrawal frequencies (A) 24 hours after intraplantar injection of saline. Effects of intraplantar injection of methAEA (10 µg) or vehicle (B) on mean paw withdrawal frequencies 24 hours after intraplantar injection of saline. BL: mean baseline paw withdrawal frequency 24 hours before intraplantar injection of saline. SAL: mean paw withdrawal frequency 24 hours after intraplantar injection of saline, and also serves as the pre-drug baseline. * indicates a significant difference from SAL (p<0.05). n=8-10 animals per dose.
Figure 9

A

![Graph A](image)

B

![Graph B](image)

Time (minutes)

PWL (s)

10µg ACEA

10µg methAEA

vehicle

vehicle

* * *
Figure 9. Effects of ACEA and methAEA on paw withdrawal latencies during non-inflamed conditions. Effects of intraplantar injection of ACEA (10 µg) or vehicle on mean paw withdrawal latencies (A) 24 hours after intraplantar injection of saline. Effects of intraplantar injection of methAEA (10 µg) or vehicle (B) on mean paw withdrawal latencies 24 hours after intraplantar injection of saline. BL: mean baseline paw withdrawal latency 24 hours before intraplantar injection of saline. SAL: mean paw withdrawal latency 24 hours after intraplantar injection of saline, and also serves as the pre-drug baseline. * indicates a significant difference from SAL (p<0.05). n=8-10 animals per dose.
Chapter 3

CANNABINOID MODULATION OF CUTANEOUS NOCICEPTORS DURING NON-INFLAMED AND INFLAMED CONDITIONS
Introduction

Several studies have demonstrated that locally-administered cannabinoids produce antinociception in animal models of both acute and persistent pain through peripheral mechanisms (for reviews see Walker et al. 1999; Hohmann 2002; Walker and Huang 2002; Mbvundula et al. 2004). Two main receptors for cannabinoids have been isolated, cannabinoid 1 (CB₁) and cannabinoid 2 (CB₂), and both are G-protein coupled receptors (Matsuda et al. 1990; Munro et al. 1993) which have been localized to various tissues. CB₁ receptors are most commonly expressed on neurons, and their activation can decrease neuronal excitability by decreasing calcium channel conductance and increasing potassium channel conductance (for review see Howlett et al. 2004). CB₂ receptors are predominately expressed on leukocytes, and their activation can produce a variety of different immunological effects (for review see Klein et al. 2003; Massi et al. 2006).

Locally-administered cannabinoids produce antinociception through activation of peripheral CB₁ and CB₂ receptors. CB₁ receptor-mediated antinociception has been attributed to activation of CB₁ receptors expressed by nociceptive dorsal root ganglion neurons (Hohmann and Herkenham 1999; Ahluwalia et al. 2000) and their peripheral nerve terminals (Ständer et al. 2005; Amaya et al. 2006). Conditional knockdown of CB₁ receptors in Nav1.8-expressing nociceptive sensory neurons prevented cannabinoids applied to the periphery from producing antinociception in models of neuropathic and
inflammatory pain (Agarwal et al. 2007). Consistent with these observations, activation of CB₁ receptors decreased high-voltage activated calcium currents (Ross et al. 2001; Khasabova et al. 2002, 2004) and reduced capsaicin-evoked calcium transients (Millns et al. 2001; Sagar et al. 2005) in nociceptive dorsal root ganglion neurons in vitro. A recent study also showed that mechanically-evoked responses of primary afferent fibers were decreased by CB₁ receptor activation in vivo; however, the types of afferent fibers affected were not known (Kelly and Donaldson 2008). The precise mechanisms underlying CB₂ receptor-mediated antinociception remain unclear, but likely involve both indirect and direct actions on neuronal tissue (for review see Guidon and Hohmann 2008).

Although behavioral studies have indicated that activation of peripheral CB₁ produces antinociception, it is currently unknown if cannabinoids alter the response properties of nociceptors and which subtypes of functionally-identified nociceptors are cannabinoid-sensitive. In the present study, electrophysiological studies were conducted to determine the effects of cannabinoids on nociceptor activity. In our behavioral studies, we showed that intraplantar administration of the cannabinoid receptor agonists arachidonyl-2'-chloroethylamide (ACEA) and (R)-(−)-methanandamide (methAEA) attenuated inflammatory mechanical allodynia, mechanical hyperalgesia, and heat hyperalgesia through activation of peripheral CB₁ receptors. In these correlative studies, we investigated the effects of intraplantar administration of ACEA and methAEA on evoked responses of cutaneous nociceptors from inflamed and control, non-inflamed
skin. A report of this study has been published and is included in this thesis with permission from the American Physiological Society (Potenzieri et al. 2008b).
Methods

Subjects

Adult, male, Sprague–Dawley rats weighing 280–350 g were used. Animals were obtained from Harlan (Indianapolis, IN), housed on a 12-hour light/dark schedule, and allowed ad libitum access to food and water. Experiments were performed during the light cycle. All procedures were approved by the Animal Care Committee at the University of Minnesota, and experiments were conducted according to the guidelines established by the International Association for the Study of Pain.

Induction of inflammation

Rats were anesthetized with a mixture of isoflurane gas in air (2% for induction and maintenance) (Phoenix Pharmaceuticals, St. Joseph, MO) and received a single intraplantar injection of complete Freund’s adjuvant (CFA) (Sigma Chemical, St. Louis, MO) or sterile isotonic saline as a control (Baxter, Deerfield, IL). CFA (1 mg/mL) and saline were given in a volume of 50 µl using a 28-guage needle. Behavioral experiments were performed 24 hours after injection of CFA or saline.

Drug preparation and administration

The cannabinoid receptor agonists used were N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA) and (R)-N-(2-Hydroxy-1-
methylethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (methAEA). All drugs were obtained from Tocris Bioscience (Ellisville, MO). ACEA was supplied pre-dissolved in ethanol (5 mg/mL). MethAEA was supplied pre-dissolved in a water soluble emulsion (Tocrisolve™) (5 mg/mL). The CB₁ receptor antagonist used was N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) which exhibits ≈ 300-fold selectivity over CB₂ receptors (Gatley et al. 1996). AM251 was dissolved in anhydrous ethanol (25 µg/µl). All drugs were diluted to their final concentration in sterile isotonic saline and administered via subcutaneous intraplantar injection in a volume of 20 µl.

Electrophysiological studies

Surgical preparation. Rats were initially anesthetized by intramuscular injection of ketamine (100 mg/kg) and xylazine (45 mg/kg). The trachea was cannulated and a catheter was placed in the external jugular vein to provide supplemental anesthesia with sodium pentobarbital (10 mg/kg/h). Core body temperature was maintained at 37°C using a feedback-controlled heating pad (Harvard Apparatus, Holliston, MA).

Electrophysiological recording. Recordings were made from cutaneous afferent fibers of the left tibial nerve using a teased-fiber approach. The tibial nerve was dissected from the surrounding tissue and the overlaying skin was sewn to a metal ring to form a pool that was filled with warm mineral oil. The tibial
nerve was placed onto a mirror platform for fine dissection with sharpened Dumont # 5 forceps (Fine Science Tools, Foster City, CA). Teased fibers were placed onto a tungsten wire electrode and action potentials were recorded extracellularly. Action potentials were amplified, audio monitored, displayed on an oscilloscope, and stored on a PC computer for data analysis. Only fibers with clearly discriminated single unitary action potentials (units) were studied. Responses of individual units were analyzed off-line using a customized data analysis program (LabVIEW, version 5.1; National Instruments, Austin, TX).

*Identification of units.* Afferent units were found by mechanically stimulating the plantar surface of the hindpaw with the experimenter's finger or by stimulation with calibrated von Frey monofilaments. Once a single unit was identified, its mechanical receptive field (RF) was marked on the skin using a felt-tipped pen.

*Conduction velocity.* The conduction velocity of each unit was determined by electrically stimulating the skin outside the unit’s RF with pin electrodes to electrically activate the unit (200 µs pulse width at 0.5 Hz). The unit was stimulated 1.5X its electrical threshold and the conduction latency was measured from the time of the electrical stimulus artifact to the evoked unitary action potential. Conduction distance was determined by measuring the distance from
the unit's RF to the recording electrode. Conduction velocity (m/s) was calculated by dividing conduction distance by conduction latency.

**Functional classification of nociceptors.** Units were classified functionally according to their responses evoked by mechanical and heat stimuli applied to the RF. Mechanical stimuli units included light brushing with the tip of a cotton swab, mildly pinching with a pair of forceps, and application of von Frey filaments. Mechanical response thresholds were determined using a series of calibrated von Frey monofilaments and defined as the weight (g) required to evoke at least one impulse when applied to the RF for 1 s. Heat stimuli were delivered using a feedback-controlled Peltier device (Yale Electronics, New Haven, CT) with a contact area of 1 cm². A unit was considered heat-responsive if it responded with at least one impulse to a stimulus temperature of 51°C for 5 s. Units were classified as nociceptors if they exhibited a slowly adapting response to noxious pinch but not to light touch (Leem et al. 1993). Units were further classified as Aδ nociceptors if they had a conduction velocity between 2.5 - 25.0 m/s and as C nociceptors if the conduction velocity < 2.5 m/s.

**Responses evoked by mechanical stimuli.** Once a nociceptor was characterized, baseline responses evoked by a 26 g von Frey monofilament were determined. This was the same filament used to determine mechanical hyperalgesia in our behavioral studies. The monofilament was secured in a
manipulator and lowered onto the mechanical RF for 5 s. The monofilament was applied twice to the same location with an interstimulus interval of 180 s. The number of evoked impulses and the discharge rate (from the first to the last evoked impulse) were averaged over the two trials. For nociceptors that exhibited ongoing activity, the number of impulses that occurred 5 s prior to the stimulus was subtracted from the number of impulses evoked during stimulation. To assess the variability between stimulus trials, the number of impulses elicited during the second stimulus trial was expressed as a percent of the number of impulses elicited during the first stimulus trial (Wenk et al. 2006).

Responses evoked by heat stimuli. Heat stimuli were applied to the RF of isolated nociceptors using a feedback-controlled Peltier device. Heat stimuli consisted of a ramp from 32°C to 49°C and then immediately back down to 32°C at a constant rate of 1°C (total stimulus time of 34 s). This heat ramp was applied twice with an interstimulus interval of 600 s. The number of evoked impulses and the discharge rate (from the first to the last evoked impulse) were averaged over the two trials. Heat response thresholds were defined as the temperature at which the first impulse was evoked. For nociceptors that exhibited ongoing activity, the number of impulses that occurred 34 s prior to the stimulus was subtracted from the number of impulses evoked during heat stimulation. To assess the variability between stimulus trials, the number of impulses elicited
during the second stimulus trial was expressed as a percent of the number of impulses elicited during the first stimulus trial.

**Experimental design**

After baseline responses to mechanical and heat stimuli were determined, cannabinoid or vehicle was injected into the RF. The needle was inserted outside the RF and the injectate was observed as a bleb of fluid centered in the unit's RF. Ongoing activity was recorded before, during, and for 300 s after injection of drug or vehicle. Responses evoked by injection were separated into injection responses, the response during injection, and post-injection responses, the response after injection of drug and withdrawal of the needle from the skin. Injection and post-injection response magnitudes are indicated as both the number of impulses elicited and discharge rate (Hz).

The effects of cannabinoids on responses to mechanical and heat stimuli were studied in separate groups of nociceptors. Mechanical response thresholds, heat response thresholds, mechanically-evoked responses, heat-evoked responses, and the variability in responses between stimulus trials were determined at 30, 60, 90, and 120 minutes after injection of drug or vehicle as described above. The dose of either ACEA or methAEA given was 10 µg. In some experiments, the CB1 receptor antagonist, AM251 (30 µg), was injected 5 minutes prior to injection of ACEA (10 µg) or methAEA (10 µg). Only one nociceptor was studied per animal.
**Data analysis**

To determine the effect of cannabinoids or their vehicles on the number of evoked impulses (heat and mechanical), heat response thresholds, and the variability between stimulus trials compared to baseline measures, comparisons were made using a one-way ANOVA with repeated-measures followed by paired t-tests with the Bonferroni correction for multiple comparisons. Two-way ANOVAs followed by unpaired t-tests with the Bonferroni correction for multiple comparisons was used to determine the effect of cannabinoids or their vehicles on evoked responses between groups. Between group comparisons of conduction velocity and evoked responses (# of impulses) of nociceptors isolated from CFA- and saline-injected hindpaws were made using unpaired t-tests. To determine the effect of cannabinoids or their vehicles on mechanical response thresholds compared to baseline measures, comparisons were made using the Kruskal-Wallis ANOVA followed by Mann-Whitney rank sum tests. The effects of drug or vehicle on mechanical response thresholds between groups were compared using Mann-Whitney rank sum tests. Injection and post-injection response magnitudes (# of impulses and average discharge rate) were compared between groups using one-way ANOVA followed by un-paired t-tests with the Bonferroni correction for multiple comparisons. The proportions of units exhibiting injection and post-injection responses between groups were made using Chi-square test followed by pair-wise comparisons using the Fisher Exact
test. For all statistical analyses, a probability value <0.05 was considered significant. All data are presented as mean (±S.E.M). All statistical analysis was performed using Sigma Stat software (Systat Software, San Jose, CA).
Results

Responses of Aδ nociceptors to mechanical stimuli

A total of 145 Aδ nociceptors were studied for their responses to mechanical stimuli: 40 from control, non-inflamed (saline-injected) skin and 105 from inflamed (CFA-injected) skin. The mean conduction velocity of Aδ nociceptors isolated from non-inflamed skin was 15.7±0.6 m/s with a range of 4.2-20.8 m/s and was similar to the mean conduction velocity of Aδ nociceptors in inflamed skin (15.1±0.4 m/s with a range of 3.1-21.8 m/s). Examples of conduction latency traces are displayed in Figure 1. The median mechanical response threshold of Aδ nociceptors from inflamed skin was 2.5 g (interquartile range =3.4 g), which was lower than the median mechanical response threshold of Aδ nociceptors from non-inflamed skin (4.7 g; interquartile range =4.3 g) (p<0.001). None of the Aδ nociceptors from control, non-inflamed skin exhibited ongoing activity, whereas 25% (26/105) of Aδ nociceptors from inflamed skin exhibited ongoing activity with an average discharge rate of 0.16±0.03 Hz (range = 0.02 to 0.61 Hz). None of the Aδ nociceptors from non-inflamed skin were excited by noxious heat; however, 4% (4/105) of Aδ nociceptors from inflamed skin were excited by heat. Examples of responses to heat for a single Aδ nociceptor from inflamed skin are shown in Figure 1.

We determined the effects of cannabinoids or vehicle on responses evoked by the 26 g von Frey filament, the same stimulus used in behavioral studies to characterize mechanical hyperalgesia (see Chapter 2). A concern was
the potential variability of responses to repeated application of the stimulus, since responses at each time point were averaged over two stimulus trials (see methods). We determined the variability of responses by expressing the number of impulses evoked during the second stimulus trial as a percent of the number of impulses evoked during the first stimulus trial. It was found that the variability between stimulus trials for baseline responses of Aδ nociceptors isolated from non-inflamed and inflamed hindpaws did not differ, and were 110.8±4.9% (n=40) and 105.6±3.0% (n=105), respectively. Units were classified as cannabinoid-sensitive if the evoked responses after cannabinoid administration were two standard deviations below the baseline response (Wenk et al. 2006). Thus, units that had a decrease in response of ≤ 44% after cannabinoid administration were considered cannabinoid-sensitive.

The mean number of impulses evoked by the 26 g von Frey filament was 33.4±1.9 impulses (6.6±0.3 Hz) for all Aδ nociceptors from non-inflamed skin. For Aδ nociceptors from inflamed skin (n=105), the mean number of impulses was 78.4±3.9 impulses (16.0±0.8 Hz), and was greater than the responses of nociceptors from non-inflamed skin (p<0.001). Aδ nociceptors from inflamed skin also exhibited enhanced responses to stimulation with 10 and 60 g von Frey monofilaments compared to Aδ nociceptors from non-inflamed skin (n=6 per group; p<0.001; Figure 2).

*Effects of methAEA on Aδ nociceptors from inflamed skin*
Administration of the cannabinoid receptor agonist methAEA (10 µg) into the RF decreased mechanically-evoked responses. The number of impulses evoked by the 26 g von Frey monofilament decreased from 81.1±10.1 impulses to 42.3±6.6 impulses (a decrease of ≈ 48%) at 30 minutes after administration and returned to baseline values by 60 minutes. (Figure 3). Unlike the decreases in mechanically-evoked responses observed after injection of methAEA (10 µg), injection of vehicle produced a small increase in mechanically-evoked responses at all time points tested (p<0.01) (Figure 3). Seven of the 20 (35%) Aδ nociceptors treated with methAEA were cannabinoid-sensitive. Neither vehicle nor methAEA altered mechanical response thresholds or the variability in responses (# of evoked impulses) between stimulus trials at any time point tested compared to baseline measures (data not shown).

To determine if the attenuation of mechanically-evoked responses by methAEA was mediated by CB₁ receptors, the CB₁ receptor antagonist AM251 (30 µg) was administered into the RF five minutes prior to injection of methAEA (10 µg). Pretreatment with AM251 blocked the attenuation of mechanically-evoked responses produced by methAEA (Figure 3). No changes in mechanical response thresholds or the variability in responses between stimulus trials occurred at any time point tested after administration of AM251 followed by methAEA compared to baseline measures (data not shown).

Effects of ACEA on Aδ nociceptors from inflamed skin
Administration of the cannabinoid receptor agonist ACEA (10 µg) also attenuated mechanically-evoked responses of Aδ nociceptors. Before administration, the 26 g von Frey filament evoked 80.0±9.1 impulses and this decreased to 51.3±6.1 impulses (a decrease of ≈36%) 30 minutes after administration and returned to baseline values 60 minutes after administration (Figure 4). Six of the 17 (35%) Aδ nociceptors treated with ACEA were cannabinoid-sensitive. Neither vehicle nor ACEA altered mechanical response thresholds or the variability in responses between stimulus trials at any time point tested compared to baseline measures (data not shown). The injection of vehicle did not alter mechanically-evoked responses.

As with methAEA, AM251 (30 µg) also blocked the attenuation of mechanically-evoked responses produced by ACEA (Figure 4). No changes in mechanical response thresholds or the variability in responses between stimulus trials occurred at any time point tested after administration of AM251 followed by ACEA compared to baseline measures (data not shown).

Effects of ACEA and methAEA on Aδ nociceptors from non-inflamed skin

In contrast to the decrease in responses of Aδ nociceptors from inflamed skin, responses of Aδ nociceptors from non-inflamed skin evoked by the 26 g monofilament increased after methAEA (10 µg) or its vehicle (Figure 5). The small increase in mechanically-evoked responses produced by methAEA and vehicle did not differ from each other at any time point tested. Neither methAEA
nor its vehicle altered mechanical response thresholds or variability in responses between stimulus trials at any time point tested. Similarly, administration of ACEA (10 µg), but not vehicle, increased mechanically-evoked responses compared to baseline measures (Figure 6). As with methAEA, the changes in mechanically-evoked responses produced by ACEA and vehicle did not differ at any time point tested. Neither ACEA nor vehicle altered mechanical response thresholds or the variability in responses between stimulus trials at any time point tested compared to baseline measures (data not shown).

Responses of Aδ nociceptors evoked by methAEA, ACEA, and their vehicles

Previous studies have demonstrated that both ACEA (Price et al. 2004) and methAEA (Ralevic et al. 2001; Roberts et al. 2002) can activate TRPV1 receptors. To determine if Aδ nociceptors were excited by ACEA or methEA, we recorded nociceptor activity during injection of drug (injection response) and for a 5 minute period after injection (post-injection response). Injection and post-injection response magnitudes are indicated as both the number of impulses evoked and discharge rate (Hz). Injection responses of Aδ nociceptors from non-inflamed skin did not differ in either proportion or magnitude regardless of the cannabinoid or vehicle injected. Similarly, injection responses of Aδ nociceptors from inflamed skin did not differ in either proportion or magnitude regardless of the cannabinoid or vehicle injected. The magnitude of injection responses of Aδ nociceptors from inflamed skin, but not the proportion excited, were greater than
those of A\(\delta\) nociceptors from non-inflamed skin (p<0.01). Since only a small proportion of A\(\delta\) nociceptors from non-inflamed skin exhibited post-injection responses (5/40), differences between cannabinoids and vehicle could not be ascertained. Post-injection responses of A\(\delta\) nociceptors (53/105) from inflamed skin did not differ in either proportion or magnitude regardless of cannabinoid or vehicle injected. Overall, the magnitude (p<0.01) and proportion (p<0.0001) of post-injection responses of A\(\delta\) nociceptors from inflamed skin were greater than those of A\(\delta\) nociceptors from non-inflamed skin. These data show that injection of cannabinoids or their vehicles into the plantar surface of the hindpaw evokes non-specific excitation of A\(\delta\) nociceptors, and inflammation increases the magnitude of this response.

Responses of C nociceptors to mechanical stimuli

A total of 56 C nociceptors were studied for their responses to mechanical stimuli: 20 from control, non-inflamed (saline-injected) skin and 36 from inflamed (CFA-injected) skin. The mean conduction velocity of C nociceptors from non-inflamed skin was 0.75±0.03 m/s with a range of 0.53-1.1 m/s and was similar to the mean conduction velocity of C nociceptors from inflamed skin (0.93±0.07 m/s with a range of 0.43-2.1 m/s). The median mechanical response threshold of C nociceptors from inflamed skin was 6.7 g (interquartile range =3.1 g), which was lower than the median mechanical response threshold of C nociceptors from non-inflamed skin (10.5 g; interquartile range =6.87 g) (p<0.001). None of the C
nociceptors from non-inflamed skin exhibited ongoing activity, while 22% (8/36) of C nociceptors from inflamed skin exhibited ongoing activity with an average discharge rate of 0.26±0.06 Hz (range = 0.04 to 0.50 Hz). A similar proportion of C nociceptors from non-inflamed and inflamed skin were excited by heat (45% or 9/20 and 42% or 15/36, respectively.

As described previously for Aδ nociceptors, the effects of cannabinoids or vehicle on mechanically-evoked responses of C nociceptors were studied using a 26 g von Frey filament. The variability between stimulus trials for baseline responses of C nociceptors isolated from non-inflamed and inflamed hindpaws did not differ, and were 119.6±16.0% (n=20) and 103.7±6.1% (n=36), respectively. As for Aδ nociceptors, C nociceptors were classified as cannabinoid-sensitive if the evoked responses after cannabinoid administration were two standard deviations below the baseline response (Wenk et al. 2006). Thus, units that had a decrease in response of ≤ 97% after cannabinoid administration were considered cannabinoid-sensitive.

Across all C nociceptors from non-inflamed skin, the mean number of impulses evoked by the 26 g von Frey filament was 16.9±1.3 impulses (3.4±0.2 Hz). For C nociceptors from inflamed skin (n=36), the mean number of impulses was 37.8±5.3 impulses (7.4±0.9 Hz), which was greater than the responses of nociceptors from non-inflamed skin (p<0.001).

Effects of ACEA and methAEA on C nociceptors from inflamed skin
Administration of either ACEA (10 µg) or its vehicle into the RF did not alter mechanically-evoked responses of C nociceptors at any time point tested (Figure 7A). Similarly, administration of methAEA (10 µg) or its vehicle did not alter mechanically-evoked responses of C nociceptors (Figure 7B). Neither ACEA, methAEA, nor their vehicles altered mechanical response thresholds or the variability between stimulus trials at any time compared to baseline measures.

Effects of ACEA and methAEA on C nociceptors from non-inflamed skin

Administration of ACEA (10 µg) did not alter mechanically-evoked responses, whereas injection of vehicle increased the number of mechanically-evoked impulses at 30 and 60 minutes after administration (p<0.05; Figure 8A). Administration of methAEA (10 µg) or its vehicle did not alter mechanically-evoked responses at any time point tested (Figure 8B). Neither ACEA, methAEA, nor their vehicles altered mechanical response thresholds or the variability between stimulus trials at any time point tested compared to baseline measures.

Responses of C nociceptors to heat stimuli

A total of 20 additional C nociceptors were studied for their responses to heat stimuli: 10 from non-inflamed skin and 10 from inflamed skin. Only nociceptors that discharged ≥ 1 impulse to a stimulus of 48°C for 3 s were studied. The mean conduction velocity of C nociceptors isolated from non-
inflamed skin was 0.62±0.03 m/s with a range of 0.49-0.77 m/s and was similar to the mean conduction velocity of C nociceptors from inflamed skin (0.93±0.15 m/s with a range of 0.51-1.7 m/s). Examples of conduction latency traces are displayed in Figure 9A. Responses to heat were determined using a heat ramp of 32°C to 49°C and then immediately back down to 32°C at a constant rate of 1°C. Examples of a C nociceptor's response to the heat ramp stimulus are displayed in Figure 9B. Heat response thresholds were defined as the temperature at which the first impulse was evoked. The mean heat response threshold of C nociceptors from inflamed skin was 39.5±1.2°C, which did not differ from the heat response threshold of C nociceptors from non-inflamed skin (41.7±1.2°C). A total of 20% (2/10) of C nociceptors from inflamed skin were unresponsive to mechanical stimuli, while none of C nociceptors from non-inflamed were unresponsive to mechanical stimuli. None of the C nociceptors from non-inflamed skin exhibited ongoing activity, while 30% (3/10) of C nociceptors from inflamed skin exhibited ongoing activity.

To determine the effect of ACEA or vehicle on response to heat stimuli, the heat ramp was applied twice with an interstimulus interval of 600 s. To assess the variability between stimulus trials, the number of impulses elicited during the second stimulus trial was expressed as a percent of the number of impulses elicited during the first stimulus trial. Responses to heat for each nociceptor were defined as the number of evoked impulses and the discharge rate (from the first to the last evoked impulse) averaged over the two trials.
The variability between stimulus trials for baseline responses of C nociceptors isolated from non-inflamed and inflamed hindpaws did not differ and was 116.4±82.0%. Units were classified as cannabinoid-sensitive if the evoked responses after cannabinoid administration were two standard deviations below the baseline response (Wenk et al. 2006). Thus, units that had a decrease in response of ≤ 98% after cannabinoid administration were considered cannabinoid-sensitive.

Prior to injection, the response to the heat ramp was a mean of 8.3±1.6 impulses (0.24±0.04 Hz). This did not differ from the response of C nociceptors from non-inflamed skin (8.3±2.4 impulses or 0.29±0.12 Hz).

**Effect of ACEA and its vehicle on heat-evoked responses of C nociceptors**

Administration of either ACEA (10 µg), or its vehicle, did not alter heat-evoked responses of C nociceptors from non-inflamed or inflamed skin at any time point tested (Figure 10A). Neither ACEA nor its vehicle altered heat response thresholds or the variability between stimulus trials at any time point tested compared to baseline measures (Figure 10B).

**Responses evoked by methAEA, ACEA, and their vehicles**

To determine if methAEA, ACEA, or their vehicles excited C nociceptors, activity was recorded during injection (injection response) and for 300 s after injection (post-injection response). Injection and post-injection response
magnitudes are indicated as both the number of impulses elicited and discharge rate (Hz). Injection responses of C nociceptors from non-inflamed skin did not differ in either proportion or magnitude regardless of the cannabinoid or vehicle injected. Similarly, injection responses of C nociceptors from inflamed skin did not differ in either proportion or magnitude regardless of the cannabinoid or vehicle injected. The magnitude of injection responses of C nociceptors from inflamed skin, but not proportions, were greater than injection responses of C nociceptors from non-inflamed skin (p<0.05). Since only a small proportion of C nociceptors from non-inflamed skin exhibited post-injection responses (2/20), differences between cannabinoids and vehicle could not be ascertained. Post-injection responses of C nociceptors (23/36) from inflamed skin did not differ in either proportion or magnitude regardless of cannabinoid or vehicle injected. The proportion (p<0.0001), but not magnitude, of post-injection responses of C nociceptors from inflamed skin were greater than those of C nociceptors from non-inflamed skin. These data show that injection of cannabinoids or their vehicles into the plantar surface of the hindpaw evokes non-specific excitation of C nociceptors, and inflammation increases the likelihood of this response.
Discussion

In the present study, we found that 24 hours after intraplantar injection of CFA, a proportion of cutaneous A\(\delta\) and C nociceptors from inflamed skin exhibited enhanced responses to mechanical stimuli. Local administration of the cannabinoid receptor agonists, methAEA or ACEA decreased mechanically-evoked responses of A\(\delta\), but not C, nociceptors from inflamed skin. The decrease in mechanically-evoked responses produced by methAEA and ACEA were blocked by the CB\(_1\) receptor antagonist AM251, suggesting that activation of peripheral CB\(_1\) receptors underlies this effect. Administration of either methAEA or ACEA did not decrease mechanically-evoked responses of A\(\delta\) nociceptors from non-inflamed skin. Evoked responses (heat and mechanical) of C nociceptors from inflamed or non-inflamed skin were not altered following administration of ACEA and methAEA. These data suggest that antinociception produced by activation of peripheral CB\(_1\) receptors during inflammation is due in part to the attenuation of evoked-responses of A\(\delta\) nociceptors.

Sensitization of nociceptors during inflammation

Under pathological conditions nociceptors can become sensitized which is characterized by a decrease in response threshold, increased responses to suprathreshold stimuli, and ongoing activity (Bessou and Perl 1969; for review see Raja et al. 1988; Treede et al. 1992). Nociceptor sensitization has been shown to correlate with psychophysical measures of primary hyperalgesia in
humans (Meyer and Campbell 1981; LaMotte et al. 1982; LaMotte et al. 1983; Torebjörk et al. 1984). Previous studies in vivo (Andrew and Greenspan 1999; Djouhri et al. 2006) and in vitro (Du et al. 2003; Du et al. 2006; Wenk et al. 2006) have demonstrated that cutaneous Aδ and C nociceptors innervating glabrous skin can become sensitized following intraplantar injection of CFA. Other studies of inflammatory pain using carrageenan have also demonstrated that Aδ and C nociceptors innervating non-glabrous (hairy) skin also exhibit enhanced responses to natural stimuli (Kocher et al. 1987; Kirchhoff et al. 1990; Koltzenburg et al. 1999).

We found that Aδ nociceptors innervating inflamed skin exhibited ongoing activity, a decrease in mechanical response thresholds, and enhanced responses to suprathreshold mechanical stimuli. Similar findings were reported by Andrew and Greenspan (1999), with the exception that they did not observe decreases in mechanical response thresholds, perhaps due to differences in sample size (40 versus 145 in the present study). Similar to that study, we found no heat-responsive Aδ nociceptors from non-inflamed skin, whereas a small proportion of Aδ nociceptors from inflamed skin were sensitive to heat, as also found by Wenk et al. (2006). However, a higher proportion of heat-responsive Aδ nociceptors were reported innervating the plantar surface of the hindpaw of naive rats as compared to our sample (Leem et al. 1993). The low proportion of heat-responsive Aδ nociceptors in our study was likely due to heat stimuli used (51°C
for 5 s versus 52°C for 20 s) which would have excluded Aδ nociceptors with higher heat response thresholds (Leem et al. 1993).

Similar to Aδ nociceptors, C nociceptors from inflamed skin exhibited ongoing activity, a decrease in mechanical response thresholds, and enhanced responses to suprathreshold mechanical stimuli. As with Aδ nociceptors, our findings agree with those reported by Andrew and Greenspan (1999), with the exception that they did not observe decreases in mechanical response thresholds, perhaps also due to differences in sample size (10 versus 36 in the present study). We also found that heat-evoked responses and heat response thresholds of C nociceptors from inflamed skin were not different from C nociceptors from non-inflamed skin. This observation is similar to those reported by Andrew and Greenspan (1999).

Contributions of nociceptor sensitization to allodynia and hyperalgesia after CFA

Consistent with mechanical hyperalgesia observed 24 hours after intraplantar injection of CFA, responses of Aδ nociceptors from inflamed skin evoked by the 26 g von Frey filament, the same filament used to characterize hyperalgesia in our behavioral studies, were enhanced as compared to responses of Aδ nociceptors from non-inflamed skin. This enhanced responsiveness of Aδ nociceptors during inflammation suggests a contribution of Aδ nociceptors to the mechanical hyperalgesia. Mechanical response thresholds of Aδ nociceptors from inflamed skin were lower than thresholds of Aδ
nociceptors from non-inflamed skin; however, their respective thresholds were still below paw withdrawal thresholds in behavioral studies of non-inflamed and inflamed rats. The contribution of A\(\delta\) nociceptors to mechanical allodynia following intraplantar injection of CFA likely resides in their enhanced evoked responses, rather than changes in thresholds. In a similar study following an incision-injury to the plantar surface of the rat hindpaw, a decrease in mechanical response thresholds and increased responses to suprathreshold stimuli also correlated with decreases in paw withdrawal thresholds and increases in paw withdrawal frequencies (Hämäläinen et al. 2002; Pogatzki et al. 2002).

Also consistent with mechanical hyperalgesia observed 24 hours after intraplantar injection of CFA, responses of C nociceptors from inflamed skin evoked by the 26 g von Frey filament were also enhanced compared to responses of C nociceptors from non-inflamed skin. This suggests a contribution of C nociceptors to mechanical hyperalgesia produced by CFA. Mechanical response thresholds of C nociceptors from inflamed skin were lower than thresholds of C nociceptors from non-inflamed skin; however, their respective thresholds were slightly higher than paw withdrawal thresholds in behavioral studies of inflamed rats. Therefore it is unlikely that C nociceptors contribute to the mechanical allodynia following intraplantar injection of CFA.

Although rats developed hyperalgesia to heat following intraplantar injection of CFA, the contribution of cutaneous nociceptors to heat hyperalgesia was not readily apparent. None of the A\(\delta\) nociceptors from non-inflamed skin
were heat-responsive, while only small proportions (4%) of Aδ nociceptors from inflamed skin were sensitive to heat. It is likely that these heat-responsive Aδ nociceptors contribute to the heat hyperalgesia during inflamed conditions.

We also found that the heat-evoked responses of C nociceptors from non-inflamed and inflamed skin were not different. A similar observation was also reported by Andrew and Greenspan (1999) using multiple heat stimuli to characterize heat-evoked responses. It is possible that C nociceptors contribute to heat hyperalgesia following inflammation, but this likely occurs through modulation of their central input to nociceptive spinal cord neurons (Hylden et al. 1989; Ren et al. 1992).

Responses of nociceptors to intraplantar injection

Injection of cannabinoids or their vehicles into the mechanical RF of Aδ nociceptors from both non-inflamed and inflamed skin produced excitation during the injection, termed injection responses. There were no within-group differences of injection responses exhibited by Aδ nociceptors regardless of the cannabinoid or vehicle injected. These results suggest that injection responses are a non-specific effect, possibly due to mechanical distention within RFs (Hilliges et al. 2002). Responses during injections were greater in magnitude in Aδ nociceptors from inflamed skin than those of Aδ nociceptors from non-inflamed skin. This increase in the response during the injection is likely related to the enhanced sensitivity to mechanical stimulation during inflammation.
Injection of cannabinoids or vehicle into mechanical RFs of both Aδ nociceptors from non-inflamed and inflamed skin produced excitation that persisted after injection, termed post-injection responses. A greater proportion Aδ nociceptors from inflamed skin exhibited post-injection impulses compared to Aδ nociceptors from non-inflamed skin. Post-injection responses of Aδ nociceptors from inflamed skin did not differ in proportion and magnitude regardless of the cannabinoid or vehicle injected, also suggesting a non-specific effect, and probably also reflects enhanced sensitivity to mechanical stimulation.

Similar to results for Aδ nociceptors, there were no within-group differences of injection responses exhibited by C nociceptors regardless of the cannabinoid or vehicle injected. These results further support the idea that injection responses are a non-specific effect. Responses during injections were greater in magnitude in C nociceptors from inflamed skin than those of C nociceptors from non-inflamed skin.

Injection of cannabinoid or vehicle into mechanical RFs of both C nociceptors from non-inflamed and inflamed skin also produced post-injection responses. A greater proportion of C nociceptors from inflamed skin exhibited post-injection impulses compared to C nociceptors from non-inflamed skin. However, unlike Aδ nociceptors, post-injection response magnitudes of C nociceptors from inflamed and non-inflamed skin did not differ.

Cannabinoid modulation of nociceptors
Following injection of either ACEA or methAEA into the RFs of A\(\delta\) nociceptors from inflamed skin, mechanically-evoked responses were attenuated and returned to baseline levels by 60 minutes after injection. This is similar to the time-course of antiallodynia/antihyperalgesia following injection of ACEA or methAEA in our behavioral studies. Additionally, administration of the CB\(_1\) receptor antagonist, AM251, attenuated both the decrease in mechanically-evoked responses and antiallodynia/antihyperalgesia produced by ACEA and methAEA, suggesting that activation of peripheral CB\(_1\) receptors underlies these effects. We did not administer the CB\(_2\) receptor antagonist AM630 in the electrophysiological studies, since AM630 did not alter the antihyperalgesic/antiallodynic effects of ACEA and methAEA in behavioral studies. Surprisingly, injection of cannabinoids or vehicles did not alter mechanical response thresholds of A\(\delta\) nociceptors from inflamed skin at any time point tested. This lack of change in mechanical response thresholds likely reflects the greater contributions of the magnitude of evoked responses in mediating changes in mechanical sensitivity.

The decrease in mechanically-evoked responses following administration of ACEA and methAEA was likely due in part to direct activation of CB\(_1\) receptors located on A\(\delta\) nociceptors. Previous studies using immunohistochemical methods have localized CB\(_1\) receptors to DRG neurons with nociceptive phenotypes that have either myelinated or unmyelinated fibers indicating that both A\(\delta\) and C nociceptors express CB\(_1\) receptors (Khasabova et al. 2002;
Bridges et al. 2003; Amaya et al. 2006; Agarwal et al. 2007). Despite these expression studies, we found that only the evoked responses of Aδ nociceptors were attenuated by cannabinoids. It is not possible to completely rule out the actions of ACEA and methAEA on other cell types in the cutaneous environment, which could potentially affect nociceptive sensitivity and nociceptor activity. Fibroblasts (Ständer et al. 2005), endothelial cells (Liu et al. 2000), lymphocytes (Parolaro 1999), mast cells (Samson et al. 2003), keratinocytes (Maccarrone et al. 2003), T-cells (Maccarrone et al. 2001), and dendritic cells (Matias et al. 2002) all express CB₁ receptors.

In contrast to the effects on Aδ nociceptors from inflamed skin, administration of methAEA or ACEA transiently increased mechanically-evoked responses of non-inflamed Aδ nociceptors. This small increase in evoked responses was related to decreased paw withdrawal thresholds and a trend for an increase in paw withdrawal frequencies in behavioral studies following intraplantar injection of either cannabinoid into non-inflamed hindpaws. This disparity between decreased paw withdrawal thresholds and a trend for an increase in paw withdrawal frequencies suggests that paw withdrawal threshold may be a more sensitive measure than withdrawal frequency testing using the 26 g von Frey monofilament. The enhanced responses of Aδ nociceptors and increased mechanical sensitivity in behavioral studies likely resulted from irritation produced by the injection.
We did not find any evidence that CB₂ receptors mediated the antinociceptive effects of cannabinoids used in our study, since the CB₂ receptor antagonist, AM630, did not block the antinociceptive effects produced by ACEA or methAEA. This was likely due to the higher selectivity of ACEA and methAEA for CB₁ receptors over CB₂ receptors (1400- and 40-fold, respectively). However, previous studies using selective CB₂ receptor agonists have shown that these drugs produce antinociception to noxious heat (Malan Jr. et al. 2001; Ibrahim et al. 2005; Ibrahim et al. 2006) and in a variety of pain models including hyperalgesia produced by carrageenan (Nackley et al. 2003; Quartilho et al. 2003; Elmes et al. 2005; Gutierrez et al. 2007), capsaicin (Hohmann et al. 2004), and neuropathic pain (Ibrahim et al. 2003). Locally-administered CB₂ receptor agonists have also been shown to decrease evoked responses of nociceptive spinal cord neurons through activation of peripheral CB₂ receptors (Sokal et al. 2003; Elmes et al. 2004; Nackley et al. 2004). Although CB₂ receptors are mainly expressed on leukocytes, studies have demonstrated that nociceptive DRG neurons express functional CB₂ receptors (Sagar et al. 2005; Anand et al. 2008). Further studies are needed to determine how selective activation of peripheral CB₂ receptors affects the excitability and response properties of nociceptors.

Summary

Local administration of cannabinoids, ACEA or methAEA, into inflamed hindpaws attenuated mechanically-evoked responses of cutaneous Aδ
nociceptors from inflamed skin through activation of CB₁ receptors. This effect was state-dependent, since ACEA and methAEA did not attenuate evoked responses of Aδ nociceptors from non-inflamed skin. Administration of ACEA or methAEA did not affect evoked responses (heat and mechanical) of cutaneous C nociceptors from inflamed and non-inflamed skin. Together, these results suggest cannabinoids differentially affect Aδ nociceptors’ responses which contribute to the antinociception produced by activation of peripheral CB₁ receptors following local administration of ACEA and methAEA during inflammation. Our data suggest that peripherally-acting cannabinoids could be a potential therapeutic treatment for chronic inflammatory pain.
Figure 1

A

BRUSH PINCH

1 ms

B

BRUSH

PINCH

C

45°C 47°C 49°C 51°C

5 s
**Figure 1.** Examples of Aδ nociceptor activity. (A) Three overlaying conduction latency traces of a single Aδ nociceptor from non-inflamed skin. Arrowhead indicates electrical stimulus artifact. (B) The response of this nociceptor to noxious pinch but not brushing in its RF. The line above each trace in (B) represents 2 s. (C) Responses of a single Aδ nociceptor from inflamed skin to increasing heat stimuli applied to the unit's RF.
Figure 2

A

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<td>10 grams</td>
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<td>26 grams</td>
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B

# of imp/stim

von Frey Filament (grams)
Figure 2. Responses of Aδ nociceptors to mechanical stimulation. (A) Examples of responses of Aδ nociceptors evoked by stimulation with 10, 26, and 60 g von Frey monofilaments from non-inflamed (left) and inflamed (right) skin (line above each traces represent stimulation for 5 s). (B) The mean number impulses evoked by stimulation with 10, 26 and 60 g von Frey filaments of Aδ nociceptors are shown. # of imp/stim: number of impulses elicited by stimulation with a von Frey monofilament for 5 seconds. Groups that do not share letters are significantly different (p<0.05). n=6 units per group.
Figure 3

A

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<td>![60 min Chart]</td>
<td>![60 min Chart]</td>
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</table>

B

![Graph showing the number of imp/stim over time for different conditions]
**Figure 3.** Local administration of methAE A decreases mechanically-evoked responses of Aδ nociceptors from inflamed skin by activation of CB₁ receptors. (A) Examples of mechanically-evoked responses via stimulation with a 26 g von Frey filament before and after administration of methAE A (10 µg), vehicle, or AM251 (30 µg) followed by methAE A (10 µg) are shown. Each column represents responses of different Aδ nociceptors and each row represents a different time point indicated on the left. The line above each trace represents the time of stimulation (5 s). (B) The mean number impulses evoked by stimulation with a 26 g von Frey filament before and after administration of methAE A (10 µg), vehicle, or AM251 (30 µg) followed by methAE A (10 µg) are shown. BL: mean baseline pre-drug number of impulses. Time: time after administration of drug. # of imp/stim: number of impulses elicited by stimulation with a von Frey monofilament for 5 seconds.* indicates a significant difference from vehicle (p<0.05). n=15-20 units per group.
Figure 4. Local administration of ACEA decreases mechanically-evoked responses of Aδ nociceptors from inflamed skin by activation of CB₁ receptors. (A) Examples of mechanically-evoked responses via stimulation with a 26 g von Frey filament before and after administration of ACEA (10 µg), vehicle, or AM251 (30 µg) followed by ACEA (10 µg) are shown. Each column represents responses of different Aδ nociceptors and each row represents a different time point indicated on the left. The line above each trace represents the time of stimulation (5 s). (B) The mean number impulses evoked by stimulation with a 26 g von Frey filament before and after administration of ACEA (10 µg), vehicle, or AM251 (30 µg) followed by ACEA (10 µg) are shown. BL: mean baseline pre-drug number of impulses. Time: time after administration of drug. # of imp/stim: number of impulses elicited by stimulation with a von Frey monofilament for 5 seconds. * indicates a significant difference from vehicle (p<0.05). n=15-20 units per group.
Figure 5

A

<table>
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<tr>
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<tr>
<td>60 min</td>
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<td><img src="image6" alt="Graph" /></td>
</tr>
</tbody>
</table>

B

![Graph](image7)

- **# of imp/stim**
- **Time (minutes)**
- **10µg methAEA**
- **vehicle**
Figure 5. Effect of methAEA on mechanically-evoked responses of Aδ nociceptors from non-inflamed skin. (A) Examples of responses evoked by stimulation with the 26 g monofilament before and after administration of methAEA (10 µg) or vehicle. Each column represents responses of different Aδ nociceptors and each row represents a different time point indicated on the left. The line above each trace represents the time of stimulation (5 s). (B) The mean number impulses evoked by the monofilament before and after administration of methAEA (10 µg) or vehicle are shown. BL: baseline pre-drug number of impulses. Time: time after administration of drug. # of imp/stim: number of impulses elicited by stimulation with a von Frey monofilament for 5 seconds * indicates a significant difference from BL (p<0.05). n=10 units per group.
Figure 6

A

Vehicle

ACEA

BL
30 min
60 min

B

# of imp/stim

10µg ACEA
vehicle

Time (minutes)
**Figure 6.** Effect of ACEA on mechanically-evoked responses of Aδ nociceptors from non-inflamed skin. (A) Examples of responses evoked by stimulation with the 26 g monofilament before and after administration of ACEA (10 µg) or vehicle. Each column represents responses of different Aδ nociceptors and each row represents a different time point indicated on the left. The line above each trace represents the time of stimulation (5 s). (B) The mean number impulses evoked by the monofilament before and after administration of ACEA (10 µg) or vehicle are shown. BL: pre-drug number of impulses. Time: time after administration of drug. # of imp/stim: number of impulses elicited by stimulation with a von Frey monofilament for 5 seconds. * indicates a significant difference from BL (p<0.05). n=10 units per group.
Figure 7

A

- 10µg ACEA
- Vehicle

Time (minutes)

Number of imp/stim

B

- 10µg methAEA
- Vehicle

Number of imp/stim

Time (minutes)
Figure 7. Effect of ACEA and methAEA on mechanically-evoked responses of C nociceptors from inflamed skin. (A) The mean number impulses evoked by the monofilament before and after administration of ACEA (10 µg) or vehicle are shown. (B) The mean number impulses evoked by the monofilament before and after administration of methAEA (10 µg) or vehicle are shown. BL: pre-drug number of impulses. Time: time after administration of drug. # of imp/stim: number of impulses elicited by stimulation with a von Frey monofilament for 5 seconds. n=8-10 units per group.
Figure 8

A

- 10µg ACEA
- vehicle

B

- 10µg methAEA
- vehicle

Time (minutes)

BL 30 60 90 120

# of imp/stim
Figure 8. Effect of ACEA and methAEA on mechanically-evoked responses of C nociceptors from non-inflamed skin. (A) The mean number impulses evoked by the monofilament before and after administration of ACEA (10 µg) or vehicle are shown. (B) The mean number impulses evoked by the monofilament before and after administration of methAEA (10 µg) or vehicle are shown. BL: pre-drug number of impulses. Time: time after administration of drug. # of imp/stim: number of impulses elicited by stimulation with a von Frey monofilament for 5 seconds. * indicates a significant difference from BL (p<0.05). n=5 units per group.
Figure 9. Examples of C nociceptor activity. (A) Three overlaying conduction latency traces of a single nociceptor from inflamed skin. Arrowhead indicates electrical stimulus artifact. (B) The response of the same nociceptor to application of 2 consecutive heat ramp stimuli applied to its RF.
Figure 10

A

$\# \text{ of imp/stim}$

- $10\mu g$ ACEA
- Vehicle

B

$\# \text{ of imp/stim}$

- $10\mu g$ ACEA
- Vehicle
**Figure 10.** Effect of ACEA on heat-evoked responses of C nociceptors from non-inflamed and inflamed skin. (A) The mean number impulses evoked by heat ramp stimuli before and after administration of ACEA (10 µg) or vehicle are shown for C nociceptors from inflamed skin. (B) The mean number impulses evoked by heat ramp stimuli before and after administration of ACEA (10 µg) or vehicle are shown for C nociceptors from non-inflamed skin. BL: pre-drug number of impulses. Time: time after administration of drug. # of imp/stim: number of impulses elicited by stimulation with a heat ramp stimulus (32°C→49°C at a rate of 1°C) for 34 seconds. n=5 units per group.
Chapter 4

CANNABINOID MODULATION OF CUTANEOUS MECHANORECEPTORS
DURING NON-INFLAMED AND INFLAMED CONDITIONS
**Introduction**

Previous studies have demonstrated that locally-administered cannabinoids attenuate mechanical hyperalgesia and mechanical allodynia in a variety of animal models of acute and persistent pain (Hohmann 2002). The attenuation of hyperalgesia and allodynia has been attributed to activation of CB₁ receptors located on primary afferent nerve terminals (for review see Kress and Kuner 2009).

CB₁ receptors are located on primary afferent dorsal root ganglion (DRG) neurons. Expression studies have localized CB₁ receptors to medium and large diameter DRG neurons (Hohmann and Herkenham 1999; Ahluwalia et al. 2000; Bridges et al. 2003), indicating that DRG neurons that give rise to myelinated nerve fibers express CB₁ receptors. Other studies showed that CB₁ receptors are also expressed on small diameter neurons (Ahluwalia et al. 2000; Amaya et al. 2006; Agarwal et al. 2007), indicating that DRG neurons that give rise to unmyelinated nerve fibers also express CB₁ receptors. Taken together, these studies suggest that nociceptors (both Aδ and C) express CB₁ receptors. However, it is probable that DRG neurons that give rise to non-nociceptive Aβ mechanoreceptors also express CB₁ receptors, since expression studies cannot exclusively determine non-nociceptive phenotypes in large diameter DRG neurons.

Prior behavioral studies demonstrated that local administration of ACEA attenuated inflammatory mechanical allodynia and mechanical hyperalgesia (see
Moreover, subsequent electrophysiological experiments showed that locally-administered ACEA attenuated mechanically-evoked responses of Aδ nociceptors from inflamed skin, but not from control, non-inflamed skin (see Chapter 2). Since CB₁ receptors are located on large diameter DRG neurons, we sought to determine if peripherally-administered cannabinoid receptor agonist ACEA could attenuate mechanically-evoked responses of Aβ mechanoreceptors from non-inflamed and inflamed skin.
Methods

Subjects

Adult, male, Sprague–Dawley rats weighing 280–350 g were used. Animals were obtained from Harlan (Indianapolis, IN), housed on a 12-hour light/dark schedule, and allowed *ad libitum* access to food and water. Experiments were performed during the light cycle. All procedures were approved by the Animal Care Committee at the University of Minnesota, and experiments were conducted according to the guidelines established by the International Association for the Study of Pain.

Induction of inflammation

Rats were anesthetized with a mixture of isoflurane gas in air (2% for induction and maintenance) (Phoenix Pharmaceuticals, St. Joseph, MO) and received a single intraplantar injection of complete Freund’s adjuvant (CFA) (Sigma Chemical, St. Louis, MO) or sterile isotonic saline as a control (Baxter, Deerfield, IL). CFA (1 mg/mL) and saline were given in a volume of 50 µl using a 28-guage needle. Behavioral experiments were performed 24 hours after injection of CFA or saline to document the development of mechanical hyperalgesia and mechanical allodynia.

Drug preparation and administration
The cannabinoid receptor agonist used was N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA) which was obtained from Tocris Bioscience (Ellisville, MO). ACEA was supplied pre-dissolved in ethanol (5 mg/mL), diluted to its final concentration in sterile isotonic saline, and administered via subcutaneous intraplantar injection in a volume of 20 µL.

**Electrophysiological studies**

**Surgical preparation.** Rats were initially anesthetized by intramuscular injection of ketamine (100 mg/kg) and xylazine (45 mg/kg). The trachea was cannulated to maintain an unobstructed airway and a catheter was placed in the external jugular vein to provide supplemental anesthesia with sodium pentobarbital (10 mg/kg/h). Core body temperature was maintained at 37°C using a feedback-controlled heating pad (Harvard Apparatus, Holliston, MA).

**Electrophysiological recording.** Recordings were made from cutaneous afferent fibers of the left tibial nerve using a teased-fiber approach. The tibial nerve was dissected from the surrounding tissue and the overlaying skin was sewn to a metal ring to form a pool that was filled with warm mineral oil. The tibial nerve was placed onto a mirror platform for fine dissection with sharpened Dumont # 5 forceps (Fine Science Tools, Foster City, CA). Teased fibers were placed onto a tungsten wire recording electrode and action potentials were recorded extracellularly. Action potentials were amplified, audio monitored,
displayed on an oscilloscope, and stored on a PC computer for data analysis. Only fibers with clearly discriminated single unitary action potentials (units) were studied. Responses of individual units were analyzed off-line using a customized data analysis program (LabVIEW, version 5.1; National Instruments, Austin, TX).

Identification of units. Afferent units were found by mechanically stimulating the plantar surface of the hindpaw with the experimenter's finger or with calibrated von Frey monofilaments. Once a single unit was identified, its mechanical receptive field (RF) was marked on the skin using a felt-tipped pen.

Conduction velocity. The conduction velocity was determined by stimulating the skin electrically with pin electrodes placed outside the unit’s RF to electrically activate the unit (200 µs pulse width at 0.5 Hz). The unit was stimulated 1.5X its electrical threshold and the conduction latency was measured from the time of the electrical stimulus artifact to the evoked unitary action potential. Conduction distance was determined by measuring the distance from the unit’s RF to the recording electrode. Conduction velocity (m/s) was calculated by dividing conduction distance by conduction latency. Units were classified as Aβ mechanoreceptors if they had a conduction velocity > 25.0 m/s.
Functional classification of mechanoreceptors. Units were classified functionally according to their responsiveness to mechanical stimulation. Mechanical stimuli used to classify units included light brushing with the tip of a cotton swab and application of von Frey filaments. Mechanical response thresholds were determined using a series of calibrated von Frey monofilaments and defined as the weight (g) required to evoke at least one impulse when applied to a unit's RF for 1 s. Units were classified mechanoreceptors if they responded to light touch (Leem et al. 1993). Mechanoreceptors were further classified as slowly adapting (SA) and rapidly adapting (RA) based on sustained mechanical stimulation with a von Frey filament (4 g) applied for 5 s. Units were classified as RA if the exhibited dynamic responses to application and removal of the stimulus only and as SA if they exhibited a dynamic and static response.

Mechanically-evoked responses. Once a mechanoreceptor was characterized, baseline responses evoked by 4 and 26 g von Frey monofilaments were determined. The monofilament was secured in a manipulator and lowered onto the mechanical RF for 5 s. The monofilament was applied twice to the same location with an interstimulus interval of 120 s. The number of evoked impulses and the discharge rate (from the first to the last evoked impulse) were averaged over the two trials. For mechanoreceptors that exhibited ongoing activity, the number of impulses that occurred 5 s prior to the stimulus was subtracted from the number of impulses evoked during stimulation. To assess the variability between stimulus trials for each von Frey filament, the number of impulses...
elicited during the second stimulus trial was expressed as a percent of the number of impulses elicited during the first stimulus trial (Wenk et al. 2006).

*Experimental design for electrophysiological studies*

After baseline responses were determined, ACEA (10 µg) or vehicle was injected into the unit's RF. The needle was inserted outside the RF and the injectate was observed as a bleb of fluid centered in the unit's RF. Ongoing activity was recorded before, during, and for 300 s after injection. Responses were separated into injection responses, the response during injection, and post-injection responses, defined as the response after injection of drug and withdrawal of the needle from the skin. Injection and post-injection response magnitudes are indicated as both the number of impulses elicited and discharge rate (Hz).

Mechanical response thresholds, mechanically-evoked responses, and the variability between stimulus trials were determined 30, 60, 90, and 120 minutes after injection of ACEA or vehicle as described above. Only one mechanoreceptor was studied per animal.

*Data analysis*

To determine the effect of ACEA or vehicle on mechanically-evoked responses and the variability between stimulus trials compared to baseline measures, comparisons were made using a one-way repeated-measures
ANOVA followed by paired t-tests with the Bonferroni correction for multiple comparisons. A two-way ANOVA followed by unpaired t-tests with the Bonferroni correction for multiple comparisons was used to determine the effect of ACEA or vehicle on evoked responses between groups. Between group comparisons of conduction velocity and evoked responses of mechanoreceptors isolated from CFA- and saline-injected hindpaws were made using unpaired t-tests. To determine the effect of ACEA or vehicle on mechanical response thresholds compared to baseline measures, comparisons were made using the Kruskal-Wallis ANOVA followed by Mann-Whitney rank sum tests. To compare the effect of ACEA or vehicle on mechanical response thresholds between groups, Mann-Whitney rank sum tests were used. Injection and post-injection response magnitudes were compared between groups using one-way ANOVA followed by un-paired t-tests with the Bonferroni correction for multiple comparisons. The proportions of units exhibiting injection and post-injection responses between groups were made using Chi-square test followed by pair-wise comparisons using the Fisher Exact test. For all statistical analyses, a probability value <0.05 was considered significant. All data are presented as mean (±S.E.M). All statistical analysis was performed using Sigma Stat software (Systat Software, San Jose, CA).
Results

General properties of Aβ mechanoreceptors

A total of 71 Aβ mechanoreceptors were studied: 34 from control, non-inflamed (saline-injected) skin and 37 from inflamed (CFA-injected) skin. The mean conduction velocity of Aβ mechanoreceptors isolated from non-inflamed skin was 39.4±0.8 m/s (range of 29.0-47.5 m/s) which did not differ from the mean conduction velocity of Aβ mechanoreceptors from inflamed skin (37.1±0.8 m/s with a range of 29.1-46.6 m/s). Examples of conduction latencies from SA and RA mechanoreceptors are displayed in Figure 1A and 2A, respectively. Of the 34 mechanoreceptors from non-inflamed skin, 53% (18/34) were SA and 47% (16/34) were RA. Of the 37 mechanoreceptors from inflamed skin, 54% (20/34) were SA and 46% (17/37) were RA. 27% (5/18) of SA mechanoreceptors from non-inflamed skin exhibited ongoing activity with a mean discharge rate of 0.13±0.03 Hz (range 0.08-0.24 Hz). Similar to mechanoreceptors from non-inflamed skin, 20% (4/20) SA mechanoreceptors from inflamed skin also exhibited ongoing with a mean discharge rate of 0.25±0.08 Hz (range 0.07-0.46 Hz). None of the RA mechanoreceptors from inflamed skin exhibited ongoing activity, while 1/16 (6%) of RA mechanoreceptors from non-inflamed skin exhibited ongoing activity with a discharge rate of 0.1 Hz. The median mechanical response threshold of SA mechanoreceptors from inflamed skin was 1.2 g (interquartile range =0.6 g), which was similar to the median mechanical response threshold of SA mechanoreceptors from non-inflamed skin (1.2 g;
The median mechanical response threshold of RA mechanoreceptors from non-inflamed and inflamed skin did not differ and were 0.6 g (interquartile range =0.4 g) and 0.6 g (interquartile range =0.8 g), respectively.

**Mechanically-evoked responses of Aβ nociceptors**

We determined the effects of ACEA or vehicle on responses evoked by 4 and 26 g von Frey filaments, the same filaments used in behavioral studies to characterize allodynia and mechanical hyperalgesia, respectively (see Chapter 2). A concern was the potential variability of responses to repeated application of stimuli, since responses at each time point were averaged over two stimulus trials (see methods). We determined the variability of responses by expressing the number of impulses evoked during the second stimulus trial as a percent of the number of impulses evoked during the first stimulus trial. Overall, the variability between stimulus trials for baseline responses of SA mechanoreceptors isolated from non-inflamed and inflamed skin did not differ, and were 99.9±4.4% (n=40) and 100.2±6.0% (n=105), respectively. Similarly, variability between stimulus trials for baseline responses of RA mechanoreceptors isolated from non-inflamed and inflamed hindpaws also did not differ, and were 116.3±8.5% (n=16) and 111.0±8.2% (n=17), respectively. Units were classified as cannabinoid-sensitive if the evoked responses after cannabinoid administration were two standard deviations below the baseline.
response (Wenk et al. 2006). Thus, SA mechanoreceptors that had a decrease in response of \( \leq 64\% \) and RA mechanoreceptors that had a decrease in response \( \leq 96\% \) after cannabinoid administration were considered cannabinoid-sensitive.

Across all 18 SA mechanoreceptors from non-inflamed skin, the mean number of impulses evoked by the 4 g filament prior to injection was 50.7±9.4 impulses (10.1±1.8 Hz). The mean baseline number of impulses evoked by the 26 g filament was 141.8±10.6 impulses (28.3±2.1 Hz) which was greater than responses to the 4 g von Frey monofilament (p<0.01). For SA mechanoreceptors from inflamed skin (n=20), the mean number of impulses evoked by the 4 g filament was 37.5±7.2 impulses (7.5±1.5 Hz). The mean number of impulses evoked by the 26 g filament was 137.5±17.5 impulses (27.5±3.5 Hz) which was also greater than responses to the 4 g monofilament (p<0.01). Examples of a SA mechanoreceptor’s responses evoked by 4 and 26 g filaments are displayed in Figure 1 B and C, respectively.

Across all 16 RA mechanoreceptors from non-inflamed skin, the mean number of impulses evoked by the 4 g von Frey filament was 5.7±0.8 impulses (1.1±0.1 Hz) prior to injection. The mean baseline number of impulses evoked by the 26 g filament were 13.7±1.7 impulses (2.7±0.3 Hz) which was greater than responses to the 4 g von Frey filament (p<0.01). For RA mechanoreceptors from inflamed skin (n=17), the mean number of impulses evoked by the 4 g filament was 6.1±0.7 impulses (1.2±0.1 Hz). The mean number of impulses evoked by the 26 g filament was of 15.2±1.4 impulses (3.0±0.2 Hz) which was also greater than
responses to the 4 g monofilament. Examples of a RA mechanoreceptor’s responses evoked by 4 and 26 g filaments are displayed in Figure 2 B and C, respectively.

*Effects of ACEA on evoked responses of Aβ mechanoreceptors*

Administration of ACEA (10 µg) or its vehicle did not alter mechanically-evoked responses of SA mechanoreceptors from inflamed (Figure 3) or non-inflamed skin (Figure 4). Likewise, neither ACEA nor vehicle altered mechanically-evoked responses of RA mechanoreceptors from inflamed (Figure 5) and non-inflamed skin (Figure 6), except for a slight increase in mechanically evoked responses of RA mechanoreceptors from inflamed skin (Figure 5). Neither vehicle nor ACEA altered mechanical response thresholds or the variability in responses (# of evoked impulses) between stimulus trials at any time point tested (data not shown).

*Responses evoked by ACEA and its vehicle*

To determine any potential excitatory effect of ACEA or vehicle on mechanoreceptors, we recorded action potential activity during injection of drug (injection response) and for a 5 minute period after injection (post-injection response). Overall, injection and post-injection responses of SA and RA mechanoreceptors from non-inflamed and inflamed skin did not differ in either proportion or magnitude. However, RA mechanoreceptors from inflamed skin
exhibited a greater proportion of post-injection responses (10/17) compared to mechanoreceptors from non-inflamed skin (2/16). Injection and post-injection responses of SA mechanoreceptors did not differ in proportion or magnitude regardless of injection (ACEA or vehicle). Injection responses of RA mechanoreceptors did not differ in either proportion or magnitude regardless of injection (ACEA or vehicle). Post-injection responses evoked by ACEA or vehicle from RA mechanoreceptors from inflamed skin did not differ. Post-injection responses of RA mechanoreceptors from non-inflamed skin could not be compared since too few exhibited post-injection impulses (one per group). These data demonstrate that injection of ACEA or its vehicle into the plantar surface of the hindpaw evokes a non-specific excitation of Aβ mechanoreceptors that does not change during inflammation.
Discussion

In the present study, we found response properties of cutaneous Aβ mechanoreceptors 24 hours after intraplantar injection of CFA did not differ from mechanoreceptors from control, non-inflamed skin. Local administration of the CB₁ receptor agonist ACEA or its vehicle did not alter mechanically-evoked responses of mechanoreceptors from either non-inflamed or inflamed skin. These results suggest that antihyperalgesia and antiallodynia produced by activation of peripheral CB₁ receptors during inflammation is not likely mediated by changes in evoked responses of Aβ mechanoreceptors.

Effects of Cannabinoids on Aβ mechanoreceptors

Previous studies have shown that both large (Hohmann and Herkenham 1999; Ahluwalia et al. 2000; Bridges et al. 2003) and small diameter (Ahluwalia et al. 2000; Amaya et al. 2006; Agarwal et al. 2007) DRG neurons express CB₁ receptors. Since DRG neuron cell body diameter is related to conduction velocity (Harper and Lawson 1985), DRG neurons that give rise to both myelinated (large diameter) and unmyelinated (small diameter) nerve fibers likely express CB₁ receptors. In the present study, we found that administration of a CB₁ receptor agonist (ACEA) into the RF did not alter response properties of Aβ mechanoreceptors. Overall, 25% of all DRG neurons express CB₁ receptors, and 69-82% of CB₁ receptor-expressing DRG neurons give rise to myelinated nerve fibers (Bridges et al. 2003). Although we were unable to determine if the Aβ
mechanoreceptors we studied expressed CB$_1$ receptors, our results suggest they do not express functional CB$_1$ receptors. Moreover, it is not possible to rule out that CB$_1$ receptors found on the terminal endings of A$\beta$ mechanoreceptors couple to other cellular processes besides ion channel excitability. Prior studies have demonstrated that local administration of CB$_1$ receptor agonists attenuate evoked responses of A$\delta$ nociceptors, but not C nociceptors (see Chapter 3). Taken together, these results agree with an earlier study that demonstrated conditional knockdown of CB$_1$ receptors from Nav1.8-expressing nociceptive sensory neurons produced a loss of peripherally-mediated cannabinoid analgesia suggesting a differential effect of cannabinoids on nociceptors (Agarwal et al. 2007).

**Peripheral contributions of A$\beta$ mechanoreceptors to allodynia and hyperalgesia**

Typically, A$\beta$ mechanoreceptors encode low-threshold mechanical forces applied to bodily structures. Following damage or injury, nociceptors become sensitized, characterized as decreased threshold for activation, development of ongoing activity, and enhanced suprathreshold responses (Bessou and Perl 1969; Fitzgerald and Lynn 1977; Campbell et al. 1979). Few previous studies have determined if A$\beta$ mechanoreceptors sensitize following injury or inflammation. No differences in the response properties of A$\beta$ mechanoreceptors occurred following an incision-injury, (Hämäläinen et al. 2002), ischemic nerve injury (Bulka et al. 2002; Bulka and Wiesenfeld-Hallin 2003), or CFA-evoked
inflammation (Djouhri et al. 2006; Wenk et al. 2006). A greater proportion of modified rapidly-adapting receptors were found following spinal-nerve ligation; however, the response properties of other cutaneous mechanoreceptors subtypes were not altered (Na et al. 1993). Results from the present study agree with these prior studies, since the response properties of mechanoreceptors from non-inflamed and inflamed skin did not differ in our study. Taken together, these studies suggest that Aβ mechanoreceptor do not sensitize following injury or damage.

Previous studies described nociceptors that have conduction velocities in the Aβ range, the so-called "Aβ nociceptors" (for review see Djouhri and Lawson 2004). Although it has been suggested that Aβ nociceptors represent a majority of nociceptors found in somatic DRG, few studies have systematically examined their response properties during pathological states. Although we did not exclusively search for such nociceptors in the present study, we did not find any nociceptors that had conduction velocities in the Aβ range in previous study characterizing nociceptive afferents (Chapter 3).

Central contributions of Aβ mechanoreceptors to allodynia and hyperalgesia

Following damage or injury, nociceptive spinal cord neurons become sensitized, termed central sensitization (Wolf 1983; Dubner and Ruda 1992). Although previous studies do not indicate that Aβ mechanoreceptors become
sensitized during pathological states, further studies have shown that Aβ mechanoreceptors contribute to central sensitization.

The exact mechanism though which Aβ mechanoreceptors contribute to central sensitization is not known. Aβ mechanoreceptor input is thought to become facilitated and coupled to nociceptive spinal cord neurons during central sensitization. Previous studies have demonstrated that Aβ mechanoreceptor input to nociceptive spinal cord neurons is facilitated during inflammation (Baba et al. 1999), nerve injury (Palecek et al. 1992), and after intradermal injection of capsaicin (Simone et al. 1991). Human studies have shown that A-fiber conduction block attenuated capsaicin-evoked secondary hyperalgesia (Wasner et al. 1999; Ziegler et al. 1999) and mechanical allodynia in humans with neuropathic injuries (Campbell et al. 1988). Collectively, these studies suggest the Aβ mechanoreceptors contribute to hyperalgesia and allodynia through modulation of their central synaptic input in the spinal cord.

**Summary**

Local administration of cannabinoid receptor agonist ACEA into either inflamed or non-inflamed hindpaws did not alter mechanically-evoked responses of cutaneous Aβ mechanoreceptors. Also, the response properties of Aβ mechanoreceptors from non-inflamed and inflamed skin are not different. These results suggest that local administration of cannabinoids at the site of injury do not affect the response properties of Aβ mechanoreceptors. Previous studies
have demonstrated that local administration of cannabinoids attenuate hyperalgesia and Aδ nociceptor activity. Taken together, it is unlikely that Aβ mechanoreceptors contribute to the peripherally-mediated antinociceptive effects of cannabinoids.
**Figure 1.** Examples of SA mechanoreceptor activity. (A) Three overlaying conduction latency traces of a single SA mechanoreceptor from non-inflamed skin. Arrowhead indicates electrical stimulus artifact. Examples of responses of the same mechanoreceptor to stimulation with a 4 g (B) and 26 g (C) von Frey monofilament (line above each traces represent stimulation for 5 s).
Figure 2

A

B

C

1 ms
**Figure 2.** Examples of RA mechanoreceptor activity. (A) Three overlaying conduction latency traces of a single RA mechanoreceptor from non-inflamed skin. Arrowhead indicates electrical stimulus artifact. Examples of responses of the same mechanoreceptor to stimulation with a 4 g (B) and 26 g (C) von Frey monofilament (line above each traces represent stimulation for 5 s).
Figure 3

A

![Graph A](image)

- **10µg ACEA**
- **Vehicle**

B

![Graph B](image)

- **10µg ACEA**
- **Vehicle**

# of imp/stim

Time (minutes)
Figure 3. Effect of ACEA on mechanically-evoked responses of SA mechanoreceptors from inflamed skin. The mean number impulses evoked by stimulation with a 4 g (A) and 26 g (B) von Frey filament before and after administration of ACEA or its vehicle. BL: mean baseline pre-drug number of impulses. Time: time after administration of drug. # of imp/stim: number of impulses elicited by stimulation with a von Frey monofilament for 5 seconds. n=10 units per group.
Figure 4

A

B

BL 30 60 90 120
0 20 40 60 80
# of imp/stim

- 10µg ACEA
- vehicle

Time (minutes)

# of imp/stim

- 10µg ACEA
- vehicle

Time (minutes)

144
Figure 4. Effect of ACEA on mechanically-evoked responses of SA mechanoreceptors from non-inflamed skin. The mean number impulses evoked by stimulation with a 4 g (A) and 26 g (B) von Frey filament before and after administration of ACEA or its vehicle. BL: mean baseline pre-drug number of impulses. Time: time after administration of drug. # of imp/stim: number of impulses elicited by stimulation with a von Frey monofilament for 5 seconds. n=10 units per group. # indicates a significant difference from vehicle (p<0.05). ## indicates a significant difference from vehicle (p<0.01). n=9 per group.
Figure 5

**Panel A**

- # of imp/stim vs. Time (minutes)
- 10µg ACEA
- Vehicle
- BL 30 60 90 120
- 0 5 10 15
- * * *

**Panel B**

- # of imp/stim vs. Time (minutes)
- 10µg ACEA
- Vehicle
- BL 30 60 90 120
- 0 10 20 30 40
**Figure 5.** Effect of ACEA on mechanically-evoked responses of RA mechanoreceptors from inflamed skin. The mean number impulses evoked by stimulation with a 4 g (A) and 26 g (B) von Frey filament before and after administration of ACEA or its vehicle. BL: mean baseline pre-drug number of impulses. Time: time after administration of drug. # of imp/stim: number of impulses elicited by stimulation with a von Frey monofilament for 5 seconds. n=10 units per group. * indicates a significant difference from BL (p<0.05). n=8-9 per group.
Figure 6

A

\[ \text{# of imp/stim} \]

\[ \begin{align*}
10 & \mu g \text{ ACEA} \\
\text{vehicle} \\
\end{align*} \]

B

\[ \text{# of imp/stim} \]

\[ \begin{align*}
10 & \mu g \text{ ACEA} \\
\text{vehicle} \\
\end{align*} \]
Figure 6. Effect of ACEA on mechanically-evoked responses of RA mechanoreceptors from non-inflamed skin. The mean number impulses evoked by stimulation with a 4 g (A) and 26 g (B) von Frey filament before and after administration of ACEA or its vehicle. BL: mean baseline pre-drug number of impulses. Time: time after administration of drug. # of imp/stim: number of impulses elicited by stimulation with a von Frey monofilament for 5 seconds. n=10 units per group. n=8 per group.
Chapter 5

EXICITATION OF CUTANEOUS C NOCICEPTORS BY INTRAPLANTAR ADMINISTRATION OF ANANDAMIDE
Introduction

Anandamide (AEA) is a membrane-derived fatty acid amide and was the first identified endogenous cannabinoid receptor agonist, or endocannabinoid (Devane et al. 1992). Currently, two receptors for cannabinoids have been isolated and cloned, cannabinoid one (CB₁) and cannabinoid two (CB₂) receptors (Matsuda et al. 1990; Munro et al. 1993), both being G-protein coupled receptors localized to various neuronal and non-neuronal tissues. CB₁ receptors are most commonly expressed on neurons, and activation of these receptors has been shown to be inhibitory by decreasing calcium channel conductance and increasing potassium channel conductance (for review see Howlett et al. 2004; Demuth and Molleman 2006). AEA has affinity for both CB₁ (Devane et al. 1992) and CB₂ (Felder et al. 1996; Slipetz et al. 1995) receptors, with slightly higher affinity for CB₁ receptors. Previous studies in laboratory animals have demonstrated that systemic administration of anandamide produces typical cannabimimetic effects such as hypothermia, hypolocomotion, catalepsy, and antinociception (Fride and Mechoulam 1993; Smith et al. 1994) primarily through activation of CB₁ receptors (Wise et al. 2007). Additionally, peripheral administration of anandamide attenuates formalin-evoked nociception (Calignano et al. 1998; Guindon et al. 2006) and hyperalgesia following inflammation (Richardson et al. 1998) and nerve injury (Guindon and Beaulieu 2006) through activation of peripheral CB₁ receptors.
In contrast to these inhibitory actions through CB₁ receptors, AEA has also been identified as an endogenous ligand for the transient receptor potential vanilloid type one (TRPV1) receptor, and is part of a growing class of endovanilloids (Melck et al. 1999; Zygmunt et al. 1999; Smart et al. 2000). The TRPV1 receptor is a non-selective cationic channel that is activated by capsaicin (Caterina et al. 1997), resiniferatoxin (Szallasi et al. 1999), protons (Caterina et al. 1997; Tominaga et al. 1998), and noxious heat (Caterina et al. 1997). Unlike its inhibitory actions via cannabinoid receptors, high concentrations of AEA excite isolated nociceptive dorsal root ganglion neurons through activation of TRPV1 receptors resulting in depolarizing inward current, increased intracellular calcium, and release of calcitonin-gene related peptide (CGRP) (Tognetto et al. 2001; Olah et al. 2001; Jerman et al. 2002; Ahluwalia et al. 2003; Fischbach et al. 2007). Similar excitatory effects were observed for isolated nociceptive trigeminal ganglion neurons (Roberts et al. 2002; Price et al. 2004). Additional studies demonstrated that AEA excited bronchopulmonary (Lin and Lee, 2002; Kollarik and Undem, 2004; Lee et al. 2005), mesenteric (Zygmunt et al. 1999), and articular (Gauldie et al. 2001) C fibers through interactions with TRPV1 receptors.

Although in vitro studies have demonstrated that AEA can excite dorsal root ganglion neurons and visceral C fibers, it is not known whether AEA excites cutaneous nociceptors in vivo. Therefore, the aim of the present study was to determine if local injections of anandamide into the hindpaw excited cutaneous C nociceptors in vivo, and if so, whether activation of C nociceptors by AEA
produced nocifensive behaviors. A preliminary report of this study has been previously published and is included in this thesis with permission from the Elsevier Limited (Potenzieri et al. 2009).
Methods

Subjects

A total of 130 adult, male, Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 280-350 g were used in this study. Animals were housed on a 12-hour light/dark schedule and allowed ad libitum access to food and water. All animal procedures were approved by the Animal Care Committee at the University of Minnesota, and experiments were conducted according to the guidelines established by the International Association for the Study of Pain.

Drug preparations and administration

Anandamide (N-(2-Hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide), the TRPV1 receptor antagonists capsazepine (N-[2-(4-Chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihy droxy-2H-2-benzazepine-2-carbothioamide) and SB 366791 (4'-Chloro-3-methoxycinnamani lide), and Tocrisolve™100 were acquired from Tocris Bioscience (Ellisville, MO). AEA was supplied pre-dissolved in Tocrisolve™100 (5 µg/µl). Capsazepine and SB 366791 were both prepared in a stock solution of 100% ethanol (20 µg/µl). Capsazepine is a vanilloid receptor antagonist with an IC₅₀ ≈ 230 - 600 nM (Dickenson and Dray, 1991; Seabrook et al. 2002). SB 366791 is a selective TRPV1 receptor antagonist with an IC₅₀ ≈ 6 nM (Gunthorpe et al. 2004). The doses of capsazepine (26.5 nmol) and SB 366791 (10.4 nmol) used here were similar, but slightly higher than doses (1 nmol each) used to attenuate capsaicin-evoked nocifensive behaviors in a
previous study (Andrade et al. 2008). All drugs were diluted to their final concentrations in sterile isotonic saline and were administered by subcutaneous injection into the plantar surface of the hindpaw in a total volume of 20 µl using a 0.3 ml insulin syringe. The vehicle used corresponded to the highest dose of AEA (100 µg).

Electrophysiological recording

All animals were initially anesthetized by intramuscular injection of ketamine (100 mg/kg) and xylazine (45 mg/kg). A catheter was placed into the external jugular vein to provide supplemental anesthesia with sodium pentobarbital (10 mg/kg per hour) to maintain areflexia. Core body temperature was maintained at 37°C using a feedback-controlled heating pad (Harvard Apparatus, Holliston, MA). Animals were euthanized at the end of each experiment with an overdose of sodium pentobarbital.

Electrophysiological recordings were made from identified cutaneous afferent fibers from the left tibial nerve using a teased-fiber approach. The tibial nerve was dissected from the surrounding tissue and the overlaying skin was sewn to a metal ring to form a pool that was filled with warm mineral oil. The tibial nerve was placed onto a mirror platform for fine dissection with sharpened Dumont # 5 forceps (Fine Science Tools, Foster City, CA). Teased fibers were placed onto a tungsten electrode and action potentials were recorded extracellularly. Action potentials were amplified, audio monitored, displayed on
an oscilloscope, and stored on a PC computer for data analysis. Only fibers with clearly discriminated single unitary action potentials (units) were studied. Responses of individual units were analyzed off-line using a customized data analysis program (LabVIEW, version 5.1; National Instruments, Austin, TX).

*Identification of Afferent Units*

Afferent units were found by mechanically stimulating the plantar surface of the hindpaw with the experimenter's finger or by stimulation with calibrated von Frey monofilaments. Once a single unit was identified, the location of its mechanical receptive field (RF) was identified with von Frey filaments and marked on the skin using a felt-tipped pen.

*Conduction Velocity*

The conduction velocity was determined by electrically stimulating the skin with pin electrodes inserted just outside the RF. Electrical stimuli consisted of 200-µs pulses delivered at a rate of 0.5 Hz. Units were excited at 1.5X their electrical threshold and the conduction latency was measured from the time of the electrical stimulus artifact to the evoked action potential. Conduction distance was determined by measuring the distance from the RF to the recording electrode. Conduction velocity (m/s) was calculated by dividing conduction distance by conduction latency.
Functional Classification of Nociceptors

Units were classified functionally according to their responses to mechanical, heat, and cold stimuli applied to the RF. Mechanical stimuli used to classify units included light brushing with the tip of a cotton swab, mildly pinching with a pair of forceps, and application of von Frey filaments. Mechanical response thresholds were determined using a series of calibrated von Frey monofilaments and defined as the smallest force (mN) that reliably evoked at least one impulse. Heat stimuli were delivered using a feedback-controlled Peltier device (Yale Electronics, New Haven, CT) with a contact area of 1 cm². Heat response thresholds were determined by stimulating the RF for 5 s using an ascending series of heat stimuli from 35 - 51°C with 2°C intervals and an interstimulus interval of 120 s. Heat response thresholds were defined as the temperature (°C) to evoke at least one impulse. A unit was considered heat-responsive if it responded with at least one impulse to a stimulus temperature of 51°C or less. A unit was classified as cold responsive if it discharged at least one impulse in response to placement of a small piece of ice on its receptive field for 20 s. Units were classified as nociceptors if they exhibited a slowly adapting response to noxious pinch but not to light touch (Leem et al. 1993). Units were further classified as Aδ nociceptors if they had a conduction velocity between 2.5 - 25.0 m/s and as C nociceptors if they has a conduction velocity < 2.5 m/s.
Nociceptor responses to intraplantar injection

Following the classification of a unit as a nociceptor, activity over 300 s in the absence of stimulation was recorded to determine if the unit exhibited ongoing activity. Drug was administered by inserting the needle outside the unit's RF and the injectate was observed as a bleb of fluid centered within the RF. Ongoing activity was also recorded during and for 300 s after injection. Responses were separated into the response during injection (injection responses), and responses that occurred after injection of drug and withdrawal of the needle from the skin (post-injection responses). Injection and post-injection responses are indicated as both the evoked number of impulses and mean discharge rate (Hz). A unit was considered responsive to drug if it discharged ≥ 2 post-injection impulses. Only one nociceptor was studied per animal.

Nocifensive behaviors

After three consecutive days of acclimation to the testing environment, animals were briefly restrained in a cloth towel and received an intraplantar injection of AEA or vehicle into one hindpaw. Animals were then placed under a clear plastic cage (23x3x13 cm³) on an elevated wire mesh platform for observation of nocifensive behaviors. Nocifensive behaviors consisted of spontaneous lifting, flinching, and licking of the hindpaw. Immediately following injection, a timer was started and the latency to onset (s) and the duration (s) of
nocifensive behaviors was recorded over a 5 minute period. The experimenter was blinded to the identity of drug given.

Withdrawal responses to radiant heat

Paw withdrawal latency (s) was determined according to a method similar to that described by Hargreaves et al. (1988). Withdrawal responses to radiant heat stimuli were determined using a custom built device that uses an encased 50W bulb to deliver a radiant heat source. Rats were placed under a clear plastic cage on a 3-mm thick glass plate that was elevated to allow maneuvering of the radiant heat source beneath it. Rats were acclimated to the testing environment for at least 15 min prior to stimulation. The heat source was positioned such that the focused beam of radiant heat (8 mm diameter) was applied to the mid-plantar surface. Withdrawal latencies to the nearest 0.1 s were measured automatically by use of a photocell that terminated each trial and stopped the timer upon withdrawal of the hindpaw. A 19 s cutoff was imposed to prevent tissue damage. Four stimuli were applied to each hindpaw, alternating between paws, with an interstimulus interval of at least 60 s. Withdrawal latency for each paw was defined as the average of the last three trials. Withdrawal latencies were obtained daily for each hindpaw during a training period of 3 consecutive days. Hyperalgesia and analgesia to heat were defined as a decrease or an increase in paw withdrawal latency, respectively. The experimenter was blinded to the identity of drug given.
Data analyses

All data are presented as mean±S.E.M. Injection responses produced by vehicles, AEA, and antagonists; dose-response relationships of anandamide on the number of post-injection impulses and mean discharge rates; and between group comparisons on the duration of nocifensive behaviors and paw withdrawal latencies to radiant heat were all made using one-way ANOVA followed by un-paired t-tests with the Bonferroni correction for multiple comparisons. Analysis of the proportions of nociceptors exhibiting injection and post-injection impulses following vehicles, AEA, and antagonists were compared using the Chi-square test followed by pair-wise comparisons using the Fisher Exact test. The effect of antagonists on the number of post-injection impulses and mean discharge rate and were made using un-paired t-tests. The effect of antagonist pre-treatment on the proportion of C nociceptors exhibiting post-injection impulses was determined using the Fisher exact test. All statistical analyses were performed using Sigma Stat software (Systat Software, San Jose, CA). A probability value <0.05 was considered significant.
Results

General properties of C nociceptors

A total of 91 cutaneous C nociceptors with mechanical RFs located on the plantar surface of the hindpaw were studied. Examples of conduction latency and responses to noxious pinch and heat are shown for a single nociceptor in Figure 1. The mean conduction velocity of all C nociceptors was 0.66±0.01 m/s (range of 0.43-1.5 m/s), the mean mechanical threshold was 104.3±9.0 mN (range of 24.1-674.2 mN), and the mean heat response threshold was 47.1±0.5°C (range of 41-51°C). Of the 91 C nociceptors studied, 40 (44%) responded to heat and mechanical stimuli (CMH nociceptors), 37 (41%) were excited by only mechanical stimuli (CM nociceptors), 8 (9%) responded to mechanical and cold stimuli (CMC nociceptors) and 6 (6%) exhibited responses to heat, mechanical, and cold stimuli (CMHC nociceptors). None of the C nociceptors had ongoing activity prior to any drug injection.

Responses of C nociceptors to injection of vehicle

Injection of vehicle (Tocrisolve™:saline) into the RFs excited 4 of 10 C nociceptors, probably due to mechanical distention of the RF (Hilliges et al. 2002). Overall, injection of vehicle evoked 13.5±2.5 impulses with a mean discharge rate of 2.9±0.8 Hz. Importantly, responses only occurred during injection and no post-injection impulses were evoked once the needle was
removed from the skin. An example of a typical response to vehicle for a single C nociceptor is displayed in Figure 2a.

*Dose-dependent excitation of C nociceptors by anandamide*

Injection of AEA at doses from 0.001 to 100 µg into the RF excited 24 out of 52 C nociceptors (n=10-11 per dose). Injection responses did not vary as a function of AEA dose and the combined injection response produced by all doses of AEA was 10.3±1.8 impulses with a mean discharge rate of 3.0±0.1 Hz. This was similar to the proportion and magnitude of injection responses produced by vehicle. However, unlike vehicle, injection of AEA at doses greater than 0.001 µg evoked responses after the needle was withdrawn from the skin (post-injection impulses). Examples of post-injection impulses evoked by injection of AEA at doses of 0.10 µg and 100 µg are shown for individual C nociceptors in Figure 2b and 2c, respectively. The proportion of nociceptors that exhibited post-injection impulses, as well as the number of impulses and discharge rate, were greater after AEA doses of 10 and 100 µg as compared to vehicle (p<0.05; Figure 3). Post-injection discharge rates produced by 100 µg AEA were greater than those produced by 10 µg AEA (p<0.01; Figure 3). The latency to onset and the duration of post-injection impulse firing did not differ between the doses. The mean latency to onset of post-injection impulses was 60.3±2.8 s and the mean duration of response was 133.6±4.1 s across all doses. The proportion of C nociceptors excited by AEA was not related to functional subtype of C nociceptor. Overall,
these data demonstrate that peripheral administration of AEA excited cutaneous C nociceptors in a dose-dependent manner.

Excitation of cutaneous nociceptors by AEA appears to be restricted primarily to C nociceptors since in a small sample of mechanosensitive Aδ nociceptors studied, none were excited by 100 µg AEA (n=5) or vehicle (n=5) (data not shown).

Attenuation of AEA-evoked responses by TRPV1 receptor antagonists

To determine whether excitation of nociceptors by AEA occurred through a TRPV1-dependent mechanism, either capsazepine or SB 366791, both competitive TRPV1 receptor antagonists, was injected into the RF 5 minutes prior to 100 µg AEA. Injection of capsazepine (10 µg), SB 366791 (3 µg), or vehicle (ethanol:saline) produced injection responses that did not differ in either proportion or magnitude from each other or AEA and its vehicle (data not shown). Capsazepine, SB 366791, or vehicle did not produce any post-injection impulses. Pre-treatment with either capsazepine (10 µg) or SB 366791 (3 µg) attenuated the excitation produced by 100 µg AEA. Pre-treatment with either capsazepine or SB 366791 attenuated the number of post-injection impulses (Figure 4b) and the mean discharge rates (Figure 4c) evoked by AEA. These data suggest that excitation of C nociceptors by AEA occurs, at least in part, via TRPV1 receptors.
**Nocifensive behaviors produced by AEA**

Since AEA excited cutaneous nociceptors, we determined if intraplantar injection of AEA also evoked nocifensive behaviors. Rats received a single intraplantar injection of 10 or 100 µg AEA, or vehicle, and the onset and duration of nocifensive behaviors were recorded. AEA, but not vehicle, produced nocifensive behaviors. Doses of 10 and 100 µg produced nocifensive behaviors that lasted for a duration of 15.0±7.5 s and 65.3±7.0 s, respectively (p<0.001; Figure 5). To determine if the nocifensive behaviors produced by AEA were mediated by TRPV1 receptors, rats received an intraplantar injection of capsazepine (10 µg), SB 366791 (3 µg), or vehicle prior to injection of 100 µg AEA. Capsazepine, SB 366791, or vehicle did not produce any nocifensive behaviors (data not shown). However, nocifensive behaviors produced by AEA were attenuated following pre-treatment with capsazepine or SB 366791, but not vehicle (Figure 5). The onset of nocifensive behavior produced by AEA did not differ between the groups, and the mean onset of nocifensive behavior across all groups was 63.5±5.9 s. These data are consistent with our electrophysiological studies and suggest that nocifensive behaviors produced by AEA occur, at least in part, through activation of TRPV1 receptors.

**Withdrawal responses to radiant heat following AEA**

Since other TRPV1 receptor agonists produce nocifensive behaviors and hyperalgesia to heat, we determined whether AEA also produced heat
hyperlgesia. Paw withdrawal latencies from radiant heat were determined before and after intraplantar injection of vehicle or 100 µg AEA (n=6-7 per group). Vehicle produced a small decrease in the paw withdrawal latencies from 10.7±0.6 s to a peak decrease of 8.3±0.5 s at 30 minutes after injection (p<0.05). AEA produced a similar decrease in paw withdrawal latency. Mean withdrawal latencies decreased from 10.9±0.8 s before injection to a peak decrease of 8.4±0.8 s at 30 minutes after injection (p<0.05), which did not differ at any time point from the vehicle treated group (data not shown). Withdrawal latencies returned to baseline values by 60 minutes after injection of AEA or vehicle. These data suggest that intraplantar injection of AEA does not produce either hyperalgesia or antinociception to radiant heat.
Discussion

The present study demonstrated that intraplantar injection of AEA produced dose-dependent excitation of cutaneous C nociceptors which was attenuated by pre-treatment with TRPV1 receptor antagonists capsazepine and SB 366791. Similarly, intraplantar injection of AEA produced nocifensive behaviors that were also attenuated by either capsazepine or SB 366791. Unlike other TRPV1 receptor agonists, intraplantar injection of AEA alone did not alter withdrawal responses to radiant heat. Together, these results demonstrate that AEA excites cutaneous C nociceptors and produces nocifensive behaviors, in part, through activation of TRPV1 receptors.

Activation of TRPV1 receptors by AEA

In previous studies conducted in vitro, AEA excited isolated DRG neurons (Tognetto et al. 2001; Olah et al. 2001; Jerman et al. 2002; Ahluwalia et al. 2003; Fischbach et al. 2007). The present study extends these observations and shows that cutaneous C nociceptors in vivo are also excited by AEA. The doses of AEA used here to excite cutaneous nociceptors were similar to those used to excite bronchopulmonary (Lin and Lee, 2002; Kollarik and Undem, 2004; Lee et al. 2005), mesenteric (Zygmunt et al. 1999), and articular (Gauldie et al. 2001) C fibers.

Excitation of C nociceptors by AEA was attenuated by pre-treatment with the TRPV1 receptor antagonists capsazepine and SB 366791, consistent with
earlier studies in vitro (Tognetto et al. 2001; Olah et al. 2001; Jerman et al. 2002; Ahluwalia et al. 2003; Fischbach et al. 2007). Despite the higher affinity and selectivity of SB 366791 for TRPV1 receptors compared to capsazepine, both attenuated the excitation produced by AEA to a similar degree. The results of the present study are in agreement with and extend earlier studies demonstrating that AEA can excite nociceptive dorsal root ganglion neurons through TRPV1 receptors. Interestingly, synthetic cannabinoid receptor agonists, such as arachidonyl-2-chloroethylamide (ACEA), WIN 55,212-2, and AM1241 can also activate TRP channels (Price et al. 2004; Jeske et al. 2006; Akopian et al. 2008).

Although our results demonstrate that excitation of cutaneous C nociceptors by AEA is at least partly mediated by activation of TRPV1 receptors, some nociceptors were still excited by AEA after pre-treatment with capsazepine or SB 366791. This suggests that other mechanisms in addition to TRPV1 receptors may be involved in the excitation of nociceptors by AEA. In a previous study it was found that AEA activated TRPV4 receptors following metabolism by cytochrome P450 epoxygenase (Watanabe et al. 2003). Additional studies have shown that other enzymes can oxidize AEA, with unknown actions on nociceptors (for review see Burstein et al. 2000; Woodward et al. 2008).

Although responses of C nociceptors to capsaicin were not determined in the present study, prior studies have clearly documented the excitatory effects of capsaicin on cutaneous C nociceptors in rats (Ren et al. 2005), non-human primates (Baumann et al. 1991) and humans (LaMotte et al. 1992). Intradermal
injection of capsaicin produces excitation of C nociceptors immediately following injection that lasts for several minutes (Baumann et al. 1991; LaMotte et al. 1992; Ren et al. 2005). The excitation of C nociceptors following intradermal injection of capsaicin is consistent with the sensation of burning pain in humans (Simone et al. 1989; LaMotte et al. 1992) and nocifensive behaviors exhibited by rats (Gilchrist et al. 1996). In contrast to capsaicin, AEA did not produce hyperalgesia to radiant heat and had a longer onset (≈ 60 s) to excitation of C nociceptors. The decrease in paw withdrawal latencies likely resulted from irritation produced by injection, and we have also observed increased mechanical sensitivity following intraplantar injections (Potenzieri et al. 2008). Although administration of AEA into the RFs of C nociceptors evoked responses during injection, this likely represents a non-specific effect since the magnitude of these responses did not differ between doses of AEA, antagonists, or vehicles.

Nocifensive behaviors produced by intraplantar injection of AEA

The excitation of cutaneous C nociceptors by AEA likely underlies the nocifensive behaviors evoked by AEA. A strong correlation was found between the onset of nociceptor excitation and the onset of nocifensive behavior after administration of 100 µg AEA. The mean onset of nocifensive behaviors after injection was 64.6±9.4 s, which was similar to the onset of C nociceptor excitation (60.3±2.8 s). Although the duration of excitation of cutaneous C nociceptors by AEA did not differ between the doses used, the magnitude of
excitation was greater at higher doses. This increased magnitude of excitation likely contributes to the nocifensive behaviors produced by the higher doses of AEA. In a similar study, nocifensive behaviors following intraplantar injection of ATP in rats also correlated with the magnitude of C nociceptor excitation by ATP (Hamilton et al. 2001). The decrease in both nociceptor excitation and the attenuation of nocifensive behaviors by pre-treatment with capsazepine or SB 366791 also demonstrates the importance of C nociceptors to the nocifensive behaviors following AEA. Prior studies have demonstrated that both capsazepine and SB 366791 can also attenuate nocifensive behaviors evoked by intraplantar injection of capsaicin (Andrade et al. 2008).

Although intraplantar injection of 100 µg AEA produced nocifensive behaviors, it did not alter withdrawal latencies to radiant heat. A similar result was recently reported in which intrathecal administration of 100 µg AEA produced a temporary pro-nociceptive effect described as "vocalization and excitation" without subsequent hyperalgesia to heat (Horvath et al. 2008). This lack of hyperalgesia to heat following AEA differs from that of other endogenous TRPV1 agonists, such as N-arachidonoyldopamine (NADA) (Huang et al. 2002) and N-oleoyldopamine (OLDA) (Chu et al. 2003). The lack of hyperalgesia to heat after AEA may be related to its lower efficacy or its partial agonist activity at rat TRPV1 receptors (Zygmunt et al. 1999; Sprague et al. 2001; Smart et al. 2001; Jerman et al. 2002).
Peripheral administration of AEA does not always produce nocifensive behaviors. For example, application of NADA, but not AEA at doses of 61 to 608 µg, to the cornea of rats elicited nocifensive behavior (Price et al. 2004). This observation is somewhat surprising given the dose-dependent activation of trigeminal ganglion neurons by AEA (Price et al. 2004). In humans, local application of 30 mM AEA into the skin of the forearm produced vasodilatation without sensations of pain (Movahed et al. 2005). The absence of pain sensation following injection of AEA could be related to the route of administration, since AEA was applied to the superficial skin using a lancet (Movahed et al. 2005), whereas in the present study AEA was administered via subcutaneous injection.

Peripheral injection of AEA has traditionally been shown to produce analgesia in rodent pain models (Richardson et al. 1998; Guindon and Beaulieu, 2006; Guindon et al. 2006). This is a dose-dependent effect since the analgesic doses of AEA used in those studies were all in nanogram range and much lower than those needed to excite nociceptors in the present study. These results are consistent with a concentration-dependent, biphasic model for AEA, where at low concentrations AEA acts as an agonist at CB1 receptors to inhibit nociceptive transmission, whereas higher concentrations produce excitation of nociceptors via activation of TRPV1 receptors (Tognetto et al. 2001; Ahluwalia et al. 2003). Thus, it is possible that AEA can produce peripherally-mediated analgesia at low doses without activating C nociceptors, but produce nocifensive behaviors at higher doses.
Physiological implications for excitation of C nociceptors by AEA

Previous studies have demonstrated that AEA is found in the skin of rodents at low levels (Felder et al. 1996; Calignano et al. 1998; Beaulieu et al. 2000). Therefore, it is unlikely that the concentrations of AEA which excite cutaneous C nociceptors in this study exist under basal conditions. However, under certain conditions the effect of AEA on TRPV1 receptors may be potentiated. For example, the affinity of AEA for TRPV1 receptors increased following activation of PKA (de Petrocellis et al. 2001) and PKC (Vellani et al. 2001), during acidification (pH ≤ 6), and after exposure to inflammatory mediators (Singh Tahim et al. 2005). Thus, under pathological conditions, endogenous AEA may activate TRPV1 receptors. In addition, concentrations of AEA have been shown to increase during cystitis and contractions of the urinary bladder by AEA via TRPV1 receptors were greatly enhanced during cystitis, suggesting that AEA contributes to this pathological state (Harrison et al. 2003; Dinis et al. 2004; Saitoh et al. 2007). During inflammatory conditions, it is possible that elevated concentrations of AEA could serve to promote nociceptive signaling by activating nociceptors via TRPV1 receptors. Further studies are needed to determine the contribution of endogenous AEA to the excitation and sensitization of nociceptors following injury and inflammation.
Summary

The present study demonstrated that AEA excited cutaneous C nociceptors dose-dependently and produced correlative nocifensive behaviors. Excitation of C nociceptors and nocifensive behaviors produced by AEA were attenuated by pre-treatment with TRPV1 receptor antagonists capsazepine or SB 366791, suggesting that excitation by AEA occurs, at least in part, through activation of TRPV1 receptors. Additional studies are needed to determine the functional roles of AEA in modulating nociceptor activity under normal and pathological conditions.
Figure 1

A

BRUSH PINCH

B

BRUSH

PINCHE

C

45°C 47°C 49°C 51°C

5 s
**Figure 1.** Functional classification of cutaneous C nociceptors. (A) An example of a conduction latency trace for a single C nociceptor illustrating the latency of action potential discharge following electrical stimulation at the RF (arrow head). (B) An example of a response of a C nociceptor evoked by application of a cotton swab brushed across the RF followed by lifting the skin and pinching the RF with a pair of forceps. (C) Responses of a C nociceptor to increasing intensities of heat stimuli applied to the RF.
Figure 2

A

Vehicle (Tocrisolve™: saline)

B

AEA 0.10 µg

C

AEA 100 µg

50 s
Figure 2. Responses of cutaneous C nociceptors to vehicle or AEA. (A) An example of a response of a single cutaneous C nociceptor to injection of vehicle (Tocrisolve™:saline). (B) An example of excitation produced by injection of 100 ng AEA. (C) An example of the response evoked by injection of 100 µg AEA. Downward arrow designates the time of injection.
Figure 3

A

% Responsive

Post-Injection Impulses (mean discharge rate: Hz)

B

Post-Injection Impulses (number of impulses)

C

Post-Injection Impulses (mean discharge rate: Hz)

AE log(g)
Figure 3. Dose-dependent excitation of cutaneous C nociceptors by AEA. (A) The percent of C nociceptors responsive to each dose of AEA. (B) The mean number of post-injection impulses evoked by each dose of AEA. (C) The mean discharge rate of post-injection impulses evoked by AEA. * indicates a significant difference from vehicle (p<0.05). ** indicates a significant difference from vehicle (p<0.01). ## indicates a significant difference from 10 µg AEA (p<0.01). n=10-11 per group.
Figure 4

A

B

C

% Responsive

Post-Injection Impulses

(mean discharge rate: Hz)

0
10
20
30
40
50
60
70
80
90
100

Post-Injection Impulses

(number of Impulses)

0
10
20
30
40

AEA 100µg
CAPZ 10µg+
AEA 100µg

SB 3µg+
AEA 100µg

* *
****
Figure 4. Capsazepine and SB 366791 attenuate excitation of C nociceptors evoked by AEA. (A) The change in the percent of C nociceptors responsive to 100 µg AEA following pre-treatment with either capsazepine (10 µg) or SB 366791 (3µg). Capsazepine and SB 366791 produced a significant decrease in both the number of post-injection impulses (B) and the mean discharge rate of post-injection impulses (C) evoked by injection of 100 µg AEA. * indicates a significant difference from 100 µg AEA (p<0.05). CAPZ: capsazepine. SB: SB 366791 ** indicates a significant difference from 100 µg AEA (p<0.01). n=11–12 per group.
Figure 5

![Bar chart showing nocifensive behavior (s) with different treatments.]

- AEA 10 µg
- AEA 100 µg
- CAPZ 10 µg + AEA 100 µg
- SB 3 µg + AEA 100 µg
- Vehicle + AEA 100 µg

Statistical significance marked with asterisks: ***, indicating p < 0.001.
Figure 5. Nocifensive behavior following intraplantar injection of AEA is attenuated by capsazepine and SB 366791. The duration of nocifensive behavior (s) was quantified following injection of AEA at doses of 10 or 100 µg alone or 100 µg AEA following pre-treatment with capsazepine (10 µg), SB 366791 (3 µg), or vehicle. CAPZ: capsazepine. SB: SB 366791. Vehicle: vehicle for capsazepine and SB 366791. Note: neither anandamide's vehicle (Tocrisolve:saline), CAPZ/SB's vehicle (ethanol:saline), CAPZ alone, or SB alone evoked nocifensive behaviors. *** indicates a significant difference from 100 µg AEA (p<0.001). n=6–10 per group.
Chapter 6

SUMMARY OF RESULTS AND DISCUSSION
Summary of Results

The main objective of these studies was to determine how activation of peripheral CB₁ receptors affects both nociception and nociceptor activity during non-inflamed and inflamed conditions. Activation of peripheral CB₁ receptors attenuated allodynia and hyperalgesia following inflammation, but had no effect on nociception during non-inflamed conditions. Activation of peripheral CB₁ receptors also attenuated the responses of Aδ nociceptors only during inflammation. The responses of C nociceptors and Aβ mechanoreceptors were not altered following administration of CB₁ receptor agonists during either non-inflamed or inflamed conditions. The dual TRPV1 and cannabinoid receptor agonist, AEA, excited C nociceptors and produced transient nocifensive behaviors through TRPV1 receptors, but did not alter sensitivity to radiant heat. Our results demonstrated that peripherally-mediated cannabinoid antinociception through CB₁ receptors is mediated, at least in part, by attenuation of Aδ nociceptor activity. Below is a detailed account of the major findings of these studies.

Behavioral studies: modulation of nociception by cannabinoids

1. Intraplantar administration of CFA (inflamed condition), but not saline (non-inflamed condition) produced mechanical hyperalgesia, mechanical allodynia, and heat hyperalgesia.
2. Local administration of either ACEA or methAEA attenuated hyperalgesia and allodynia through peripheral CB$_1$ receptors.

3. Local administration of either ACEA or methAEA did not alter nociception (mechanical or heat) during non-inflamed conditions.

*Electrophysiological studies: modulation of cutaneous nociceptors and mechanoreceptors by cannabinoids*

1. Cutaneous A$\delta$ and C nociceptors were sensitized only after intraplantar administration of CFA, but not saline.

2. Local administration of either ACEA or methAEA attenuated mechanically-evoked responses of cutaneous A$\delta$ nociceptors from inflamed skin, but not from non-inflamed skin, through activation of CB$_1$ receptors.

3. Neither ACEA nor methAEA attenuated evoked responses of C nociceptors from non-inflamed and inflamed skin.

4. The response properties of A$\beta$ mechanoreceptors from non-inflamed and inflamed skin did not differ.

5. Local administration of ACEA did not alter evoked responses of A$\beta$ mechanoreceptors from either non-inflamed or inflamed skin.

*Behavioral and electrophysiological studies: effects of AEA on nociception and nociceptor activity*
1. Local administration of AEA dose-dependently excited C nociceptors, but not Aδ nociceptors, through TRPV1 receptors.

2. Intraplantar administration of AEA produced nocifensive behaviors through activation of TRPV1 receptors, but not either analgesia or hyperalgesia to heat.

Discussion

Archaeological evidence dating back over 5000 years ago indicates that cannabis was used medicinally for its analgesic properties (Touw 1981; Zuardi 2006; Russo 2007). The mechanism through which cannabis and other cannabinoids produce their biological effects was not determined until the first cannabinoid receptor was isolated and cloned in 1990 (Matsuda et al. 1990). Since then, a total three cannabinoid receptors have been isolated: CB₁ (Matsuda et al. 1990), CB₂ (Munro et al. 1993), and GPR55 receptors (Ryberg et al. 2007). Numerous studies have demonstrated that systemic administration of cannabinoids produce antinociception in laboratory animals through activation of CB₁ receptors located in the central nervous system.

Local administration of cannabinoids at the site of injury also produces antinociception; however, the underlying mechanisms mediating these effects have not been determined. Although these effects have been attributed to both CB₁ and CB₂ receptors, the primary aim of this thesis was to determine the mechanism underlying peripheral CB₁ receptor-mediated antinociception. Targeted deletion of CB₁ receptors from Nav1.8 expressing DRG neurons
prevented locally-administered cannabinoids from producing analgesia, but did not block systemically-administered cannabinoids from producing analgesia (Agarwal et al. 2007). Peripheral CB₁ receptor-mediated antinociception has been attributed to CB₁ receptors located on Nav1.8 expressing DRG neurons, which are primarily found on nociceptive neurons or nociceptors (Djouhri et al. 2003). Although deletion of CB₁ receptors from nociceptors prevented peripheral cannabinoid antinociception, how cannabinoids modulate nociceptors is not known. The overall aim of this thesis was to relate the behavioral antinociceptive effects of locally-administered cannabinoids with changes in the response properties of cutaneous nociceptors.

**Peripheral CB₁ Receptor-Mediated Analgesia**

Administration of either ACEA or methAEA dose-dependently attenuated mechanical hyperalgesia and mechanical allodynia through activation of peripheral CB₁ receptors. The attenuation of heat hyperalgesia by ACEA/methAEA was not dose-dependent and of lower magnitude compared to the antinociception to mechanical stimuli. One possible reason for this difference is through the actions of cannabinoids on different subtypes of nociceptors. We found that ACEA or methAEA only attenuated the responses of Aδ nociceptors, but not C nociceptors. A greater proportion of C nociceptors were heat-responsive compared to Aδ nociceptors (42% versus 4%, respectively) during inflammation. Since the responses of C nociceptors to heat were not attenuated,
it is likely the overall magnitude of noxious heat input to nociceptive spinal cord neurons was not reduced enough to completely attenuate hyperalgesia to heat after administration of ACEA/methAEA. Although we did not determine if $A\delta$ nociceptor responses to heat were affected by cannabinoids, the partial attenuation of heat hyperalgesia following administration of ACEA/methAEA was likely to due attenuation of heat-responsive $A\delta$ nociceptors.

Although previous studies have attributed the antinociception of peripherally-administered cannabinoids to direct actions on nociceptors, our studies could not exclude the involvement of other cell-types in the skin. CB$_1$ receptors are expressed on numerous cell-types within the cutaneous environment, including: fibroblasts (Ständer et al. 2005), endothelial cells (Liu et al. 2000), lymphocytes (Parolaro 1999), mast cells (Samson et al. 2003), keratinocytes (Maccarrone et al. 2003), T-cells (Maccarrone et al. 2001), and dendritic cells (Matias et al. 2002). Following inflammation, these cells could contribute to the changes in nociception and nociceptor activity by cannabinoids. To date, no studies have addressed whether activation of CB$_1$ receptors found on these cells influence the excitability of nociceptors. The site of action mediating the attenuation of nociception and nociceptor activity was likely due, in part, to activation of CB$_1$ receptors expressed on the terminal endings of nociceptors.

Local administration of either ACEA or methAEA decreased the number of evoked impulses of $A\delta$ nociceptors during application of von Frey monofilaments.
by activation of CB₁ receptors. Although our studies did not determine the mechanism mediating the decrease in evoked impulses, it is likely that this effect was due to modulation of current through potassium ion channels. Activation of CB₁ receptors on hippocampal neurons activates A-type potassium channel current through inhibition of adenylyl cyclase and subsequent decreases in cAMP concentrations (Childers and Deadwyler 1996). The activation of A-type potassium channel current decreased the firing frequency of these neurons through a lengthening of the interspike interval (Childers and Deadwyler 1996). Other potassium channels might also contribute to the decreased firing of nociceptors, since CB₁ receptors also couple to G-protein-coupled inwardly rectifying potassium (GIRK) channels via Gβγ subunits (Mackie et al. 1995; McAllister et al. 1999). Although we did not determine if modulation of potassium channel current mediated the decrease in evoked impulses following administration of CB₁ receptor agonists, it is likely that modulation of potassium current through CB₁ receptors mediated the attenuation of nociceptor firing.

Activation of peripheral CB₁ receptors attenuated nociception and nociceptor responses only following inflammation, and not under normal conditions. The mechanism responsible for this differential effect is not known. One possible mechanism mediating this result is increased CB₁ receptor expression on peripheral nociceptor nerve terminals following inflammation. A previous study demonstrated that both CB₁ receptor mRNA and protein expression increased 48 hours following inflammation in nociceptive DRG
neurons (Amaya et al. 2006). Increased expression of CB₁ receptors was correlated to enhanced antihyperalgesic effects of peripherally administered ACEA (Amaya et al. 2006). Results from this study suggest that the antihyperalgesic effects of ACEA during inflammation are due to increased anterograde transport of CB₁ receptors from the DRG neuron cell bodies to their peripheral terminals (Amaya et al. 2006).

It is unlikely that increased anterograde transport of newly synthesized CB₁ receptors from DRG neuron cell bodies underlie the antinociceptive effects of cannabinoids seen in our studies. We found that peripherally administered cannabinoids produced antihyperalgesic and antiallodynic effects 24 hours following inflammation. Assuming newly synthesized CB₁ receptors are transported via fast axonal transport at rate of 400 mm/day (Vallee and Bloom 1991; Shah and Cleveland 2002), and the distance from the L4/L5 DRG neurons to the plantar surface of the hindpaw is at least 10 cm; these receptors would reach their terminals in glabrous skin of the hindpaw in approximately 2.5 days. Given the time it would take for these newly synthesized receptors to reach their targets, it is therefore unlikely that newly synthesized CB₁ receptors contribute to peripherally-mediated antinociceptive effects of ACEA/methAEA seen in our studies.

The differential antinociceptive effect of cannabinoids during inflammation could also result from short-term changes in the properties of CB₁ receptors located on nociceptor terminals. Previous studies have demonstrated activation
of peripheral CB₁ receptors in animal models of acute pain such as capsaicin (Johanek et al. 2001), cutaneous heat injury (Johanek and Simone 2004), and formalin (Calignano et al. 1998; Guindon et al. 2006) produce antinociception. Potential mechanisms mediating short-term changes in the properties of CB₁ receptors include insertion of vesicular stores of CB₁ receptors or through increased G-protein coupling to CB₁ receptors at nerve terminals.

The antihyperalgesic and antiallodynic effects of peripherally-administered cannabinoids could also result from activation of "ionotropic cannabinoid receptors" (Akopian et al. 2009). Ionotropic cannabinoid receptors are TRP channels and their classification as such reflect the properties of some cannabinoid receptor ligands that have affinity for both TRP channels and cannabinoid receptors (Akopian et al. 2009). Activation of ionotropic cannabinoid receptors desensitized the responses of sensory ganglion neurons to noxious chemical stimuli in vitro (Akopian et al. 2009). ACEA excited DRG neurons through TRPV1 receptors (Price et al. 2004) and attenuated capsaicin-evoked responses in vitro (Akopian et al. 2008). The CB₁/2 receptor agonist WIN 55,212-2 and CB₂ receptor agonist AM1241 excited DRG neurons through TRPA1 receptors and attenuated responses of capsaicin and mustard oil, respectively (Akopian et al. 2008). Peripheral administration of WIN 55,212-2 attenuated capsaicin-evoked nocifensive behaviors, but not in TRP1A knock-out mice (Akopian et al. 2008). The mechanism underlying the antinociceptive effects of WIN 55,212-2 through TRPV1 receptors occur, in part, through activation of
calcineurin leading to dephosphorylation of TRPV1 receptors (Patwardhan et al. 2006), which is dependent on TRPA1 co-expression (Jeske et al. 2006).

Although these studies suggest that certain cannabinoids excite sensory neurons through TRPA1 and TRPV1 receptors in vitro, cannabinoids overwhelmingly produce antinociception following local administration in vivo. The lack of nociception following local administration of cannabinoids, despite excitation of sensory neurons in vitro, has been suggested to result from the excitation being below the threshold to evoke nociception (Akopian et al. 2009). CB₁ receptors are predominately expressed on large diameter sensory neurons, while small diameter sensory neurons express a low level of CB₁ receptors, but high levels of TRPV1 and TRPA1 (Kobayashi et al. 2005; Patwardhan et al. 2006). Overall, these studies suggest the inhibitory actions of cannabinoids on small diameter DRG neurons are likely mediated through ionotropic cannabinoid receptors leading to desensitization of TRP channels (Patwardhan et al. 2006; Akopian et al. 2009).

Ionotropic cannabinoid receptors do not likely mediate the antinociception observed in our studies. Although studies in vitro demonstrated excitation of nociceptive sensory neurons by cannabinoids, the cannabinoids we used (ACEA and methAEA) produced non-specific excitation of Aδ and C nociceptors that did not differ from their vehicles. Ionotropic cannabinoid receptors desensitized responses of TRP receptors on small diameter DRG neurons; however, the evoked responses of C nociceptors (heat and mechanical) from either non-
inflamed or inflamed skin were not altered by cannabinoids. Moreover, the attenuation of nociceptor responses, allodynia, and hyperalgesia by ACEA and methAEA were blocked by CB₁ receptor antagonist AM251 strongly suggesting that these effects were mediated by CB₁ receptors and not ionotropic cannabinoid receptors.

Unlike ACEA and methAEA, we found that the dual CB and TRPV1 receptor agonist, AEA, excited C nociceptors and evoked nocifensive behaviors through TRPV1 receptors in vivo. The doses of AEA that produced nociceptor excitation and nocifensive behaviors were higher than those previously used to produce analgesia through cannabinoid receptors, suggesting that only high doses of AEA activate TRPV1 receptors. No changes in heat sensitivity were observed following administration of AEA compared to vehicle indicating desensitization of heat-sensitive TRP receptors was unlikely. Further studies are needed to determine the functional significance of ionotropic cannabinoid receptors in modulating the response properties of nociceptors in vivo.

Another potential mechanism mediating the antinociception following peripheral administration of ACEA and methAEA is through activation of GPR55 receptors. Recent studies have demonstrated that GPR55 receptors are expressed on large diameter DRG neurons and activation of these receptors increased intracellular calcium concentrations and inhibited M-types potassium current (Ryberg et al. 2007). M-type potassium current, which is involved in maintaining resting membrane potential, has been implicated in nociception.
Inhibition of M-type potassium current excited DRG neurons and intraplantar administration of XE991 (M-channel blocker) produced hyperalgesia (Linley et al. 2008). Pharmacological characterization of GPR55 receptors remains incomplete; however, both methAEA and the CB1 receptor antagonist AM251 are GPR55 receptor agonists (Ryberg et al. 2007; Lauckner et al. 2008). Administration of AM251 alone did not alter nociception following intraplantar injection CFA. Since commercially available GPR55 receptor antagonists are currently unavailable, we were unable to determine if this receptor contributes to the antinociceptive effects of ACEA and methAEA. Further studies are needed to determine the functions of GPR55 receptors in modulating nociceptor excitability.

**CB2 Receptor-Mediated Analgesia**

Peripheral administration of either ACEA or methAEA attenuated nociception only through CB1 receptors. Although we did not find any CB2 receptor-mediated effects in our work, previous studies using selective CB2 receptor agonists have demonstrated that these drugs produce peripherally-mediated antinociception (for review see Guindon and Hohmann 2008). CB2 receptors are mainly expressed on leukocytes; however, some brain stem neurons (Van Sickle et al. 2005; Onaivi et al. 2006) and dorsal root ganglion neurons express CB2 receptors (Sagar et al. 2005; Anand et al. 2008).

The exact mechanism underlying CB2 receptor-mediated antinociception is unclear, but likely involves both neuronal and non-neuronal components.
Activation of CB$_2$ receptors on keratinocytes resulted in release of β-endorphin which mediated the antinociception produced by CB$_2$ receptor agonist AM1241 (Ibrahim et al. 2005). Another CB$_2$ receptor agonist, GW405833 (L768242), produced peripherally-mediated antihyperalgesia that was not dependent on release of endogenous opioids (Whiteside et al. 2005). Direct activation of CB$_2$ receptors on DRG neurons by both JW-133 (Sagar et al. 2005) and GW405833 (Anand et al. 2008) attenuated capsaicin-evoked excitation of DRG neurons. Locally-administered CB$_2$ receptor agonists also decreased evoked responses of nociceptive spinal cord neurons through activation of peripheral CB$_2$ receptors (Sokal et al. 2003; Elmes et al. 2004; Nackley et al. 2004). Overall, these studies suggest that activation of peripheral CB$_2$ receptors produces antinociception through attenuation of nociceptor responses.

_Peripheral Opioid and Cannabinoid Synergy_

Peripheral administration of opiates also produces antinociception following inflammation through opioid receptors (Stein et al. 1989; Joris et al. 1990; Nozaki-Taguchi et al. 1999; Nandi et al. 2004; Obara et al. 2007; Obara et al. 2009; Hernández et al. 2009). The peripheral antinociceptive effects of opiates have been attributed to activation of μ-, δ-, κ-opioid receptors located on nociceptive DRG neurons and their peripheral nerve terminals (Ji et al. 1995; Coggeshall et al. 1997; Wenk and Honda 1999; Beland et al. 2001; Ständler et al. 2002; Nandi et al. 2004; Endres-Becker et al. 2007). Exposure of peripheral
nerve terminals to cytokines and nerve growth factor following inflammation results in an increase in opioid receptor synthesis and trafficking from DRG neurons to their peripheral nerve terminals (Rittner and Stein 2005; Stein et al. 2003; Vetter et al. 2006).

Previous studies have demonstrated that activation of opioid receptors decreased the excitability of nociceptive DRG neurons in vitro (Khasabova et al. 2004; Nandi et al. 2004; Endres-Becker et al. 2007). Opioid receptor agonist morphine attenuated the evoked responses of cutaneous nociceptors (heat and mechanical) from inflamed skin, but not nociceptors from non-inflamed skin ex vivo. Collectively, these studies suggest that activation of peripheral opioid receptors on nociceptors produces antinociception.

Systemic co-administration of opiates and cannabinoids produced synergistic antinociception in lab animals (Cichewicz 2004; Cichewicz et al. 2005; Smith et al. 2007; Cox et al. 2007) and humans (Roberts et al. 2006). Since cannabinoid and opioid receptors are expressed on nociceptors, it is possible that peripheral co-administration of cannabinoids and opiates could also produce synergistic antinociception. This idea is supported by the finding that topical administration of opiates and cannabinoids produced synergistic antinociception to heat in rodents (Yesilyurt et al. 2003). To date, no studies have demonstrated synergistic antinociception in animal models of acute and persistent pain following co-administration of cannabinoids and opiates. Results from this study and others demonstrated that cannabinoids and opiates individually produce
peripherally-mediated antinociception and attenuation of nociceptor responses during inflammation. Thus, it is possible that co-administration of opiates and cannabinoids will produce synergistic antinociception and attenuation of nociceptor activity during inflammation.

**Future Directions**

*Tolerance to peripheral cannabinoid antinociception*

Although our studies demonstrated that peripheral administration of either ACEA or methAEA attenuated nociception and nociceptor activity through peripheral CB1 receptors, we only determined these effects following a single administration. Future studies should determine how chronic peripheral administration of cannabinoids affects both nociception and nociceptor activity. If tolerance does develop, this could significantly impact the clinical applicability of these studies. The cannabinoids used here were derived from the molecular structure of the putative endocannabinoid AEA. Further studies should determine how other classes of cannabinoid receptor agonists affect nociception and nociceptor activity.

*Cannabinoid modulation of nociceptors in other pain models*

Previous studies have demonstrated that cannabinoids produce antinociception in animal models of acute and persistent pain. Both the type and
duration of injury likely affects the response properties of nociceptors and their
sensitivity to cannabinoids. Newly synthesized CB₁ and CB₂ receptors could
mediate peripheral cannabinoid analgesia in animal models of persistent pain.
Further studies should determine how cannabinoids modulate nociceptors in
other models of acute and persistent pain.

Conclusion

The results from the present studies suggest that peripherally-acting CB₁
receptor agonists produce antinociception during inflammation. Collectively,
these studies suggest peripherally-acting cannabinoids could be employed to
attenuate chronic pain. To date, no studies have reported the development of
peripherally-acting CB₁ receptor agonists in treating pain in humans. Ideally,
peripherally-acting CB₁ receptor agonists that do not cross the blood-brain barrier
could be developed to reduce possible systemic cannabimimetic effects.
Peripherally-acting CB₁ receptor agonists could be administered alone or co-
administered with other analgesic drugs to treat acute and persistent pain in
humans and animals.
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