A PERIPHERAL CANNABINOID CB$_2$ MECHANISM MODULATES 
THE ACTIVITY OF SPINAL WIDE DYNAMIC RANGE NEURONS 
IN A RAT MODEL OF INFLAMMATION

by
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(Under the Direction of Andrea G. Hohmann)

ABSTRACT

Activation of cannabinoid CB$_2$ receptors attenuates nociception in models of acute and chronic pain, while failing to produce centrally-mediated effects such as hypothermia and catalepsy. The present study was conducted to test the hypothesis that activation of peripheral CB$_2$ receptors suppresses the development of inflammation-evoked excitability of wide dynamic range (WDR) neurons in the spinal dorsal horn. Intraplantar carrageenan increased the responsiveness of WDR neurons to trains of electrical stimuli applied to the cutaneous receptive field of isolated neurons. The CB$_2$ selective cannabinoid agonist AM1241 (330 µg/kg) attenuated the development of carrageenan-evoked neuronal excitability when administered intravenously or locally in the paw. Decreases in WDR neuron responses resulted from a suppression of C-fiber-mediated activity. A$\beta$- and A$\delta$-fiber-mediated responses were not reliably altered during any experiment. Pharmacological specificity was evaluated in studies employing intravenous drug administration. The AM1241-induced suppression of carrageenan-evoked increased neuronal sensitization was blocked by the CB$_2$ antagonist SR144528 but not by the CB$_1$ antagonist SR141716A. Intraplantar administration of AM1241 (33 µg/kg) suppressed the excitability of WDR neurons following administration to the carrageenan-injected paw but was inactive following administration in the contralateral (noninflamed) paw, consistent with a local site of action. Additionally, AM1241 (330 µg/kg, iv and ipl; 33 µg/kg ipl) reduced hindpaw diameter in the carrageenan-injected paw. The AM1241-induced decrease in hindpaw diameter was blocked by the CB$_2$ but not the CB$_1$ antagonist. These data provide evidence that actions at cannabinoid CB$_2$ receptors are sufficient to suppress inflammation-evoked neuronal activity at rostral levels of processing in the spinal dorsal horn. Our findings are consistent with the ability of AM1241 to normalize nociceptive thresholds and produce antinociception in inflammatory pain states.

INDEX WORDS: AM1241, Carrageenan, Cutaneous afferents, Electrophysiology, Hindpaw edema, Pain, Periphery, Windup
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DEDICATION

I would like to dedicate this dissertation to my dearest grandparents, Sam and Norris Nackley. To quote Kahlil Gibran, “It is when you give of yourself that you truly give… These are the believers in life and the bounty of life, and their coffer is never empty.” Unconditionally, you have given to me in so many ways. You have provided me with a lifetime of strength and support, and a love that knows no bounds. I will always hold you close to my heart.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS ................................................................. v</td>
</tr>
<tr>
<td>LIST OF FIGURES ........................................................................ viii</td>
</tr>
</tbody>
</table>

## CHAPTER

1. LITERATURE REVIEW .................................................................. 1
   - History and Functional Significance of the Cannabinoid System ........1
   - Cannabinoid Receptor Subtypes .............................................. 2
   - CB₁ and CB₂ Ligands ................................................................. 3
   - Role of CB₁ in Antinociception and Anti-inflammation ................. 4
   - Role of CB₂ in Antinociception and Anti-inflammation .................. 7
   - Carrageenan Model of Inflammation ......................................... 8
   - Focus of the Present Work ......................................................... 10

2. INTRODUCTION ........................................................................ 11

3. EXPERIMENTAL PROCEDURES .............................................. 16
   - Subjects ....................................................................................... 16
   - Drugs and Chemicals ................................................................. 16
   - Surgical Preparation .................................................................. 16
   - Identification of WDR Neurons and Primary Afferent Inputs .......... 17
   - Electrophysiological Recording and Stimulation ......................... 18
   - Pharmacological Manipulations ................................................. 19
LIST OF FIGURES

Figure 1: Neuronal Activity Evoked in WDR Neurons the Absence of Inflammation..................28
Figure 2: Pre- and Post-injection Levels of Spontaneous Activity ......................................30
Figure 3: Effects of Intravenous AM1241 on Aβ-, Aδ-, C-fiber Activation and Windup in WDR Neurons During the Development of Inflammation.......................................................32
Figure 4: Effects of Intravenous CB1 and CB2 Antagonists on Aβ-, Aδ-, C-fiber Activation and Windup in WDR Neurons During the Development of Inflammation.............34
Figure 5: Effects of Intraplantar AM1241 on Aβ-, Aδ-, C-fiber Activation and Windup in WDR Neurons During the Development of Inflammation.............................................36
Figure 6: Effects of AM1241 on the Development of Inflammation.................................38
CHAPTER 1

LITERATURE REVIEW

*History and Functional Significance of the Cannabinoid System*

Cannabis Sativa, commonly known as marijuana, has been utilized by various cultures for over 4,000 years (Grinspoon & Bakalar, 1993). It has been used recreationally and medicinally in India, China, the Middle East, Southeast Asia, South Africa, South America and North America. Marijuana and synthetic marijuana-like compounds belong to a class of drugs known as cannabinoids. Cannabinoids are known to produce psychoactive effects in humans such as mood alteration, time distortion and short-term memory disruption (Hollister, 1986). In addition to their mind-altering effects, cannabinoids have a wide range of therapeutic uses. They are analgesic, anti-inflammatory, immunosuppressive, anticonvulsive and antiemetic (Porter & Felder, 2001). Furthermore, they relieve intraocular pressure in glaucoma patients and stimulate appetite in chemotherapy patients.

Of the 460 known constituents found in marijuana, $\Delta^9$-THC is the most active and abundant. $\Delta^9$-THC was first isolated by Gaoni and Mechoulam (1964). Subsequent pharmacological studies demonstrated that the effects of $\Delta^9$-THC and other cannabinoids are receptor-mediated (Howlett, 1984; Howlett et al., 1986). Further evidence for a G-protein coupled cannabinoid receptor was provided by Devane’s group (1988) who showed cannabinoids bind to specific receptors in brain tissue with high affinity and stereoselectivity. Mapping of cannabinoid receptors in brain using quantitative autoradiography revealed that these receptors are localized to structures involved in
cognition, movement and sensation (Herkenham et al., 1990; Herkenham, 1991; Tsou et al., 1998). The distribution of cannabinoid receptors in the central nervous system correlates highly with their observed behavioral effects.

Endogenous cannabinoid agonists, such as arachidonylethanolamide (anandamide; Devane et al., 1992) and 2-arachidonoyl glycerol (2-AG; Mechoulam et al., 1995), have been identified. The cloning of the cannabinoid receptor by Matsuda’s group in 1990 facilitated the synthesis of selective antagonists (Rinaldi-Carmona et al., 1994; Rinaldi-Carmona et al., 1998). These ligands provide the pharmacological tools necessary to study the biological roles of the cannabinoid system.

The cannabinoid receptor and its endogenous ligands constitute a physiologically significant system. Cannabinoid receptor function and pharmacology are preserved in various species from the non-mammalian roughskin newt (Soderstrom et al., 2000) to the human (Herkenham, 1991). The high degree of evolutionary conservation apparent in the cannabinoid system denotes the importance of endogenous cannabinoids in the nervous system.

Cannabinoid Receptor Subtypes

Two cannabinoid receptor subtypes have been identified, to date. CB₁ is expressed primarily in the central nervous system (Matsuda et al., 1990; Munro et al., 1993; Zimmer et al., 1999). High densities of CB₁ receptors are located in the basal ganglia, hippocampus and cerebellum (Herkenham et al., 1990) as well as the prefrontal cortex, dorsal spinal cord and amygdala (Mailleux & Vanderhaeghen, 1992). CB₂ is expressed mainly in cells of the immune system (Munro et al., 1993; Lynn & Herkenham, 1994) and is absent in neurons of the central nervous system (Munro et al.,
In immune tissues, levels of CB$_2$ mRNA are 10-100 times greater than that of CB$_1$ mRNA (Galiegue et al., 1995). Both CB$_1$ (Hohmann & Herkenham, 1999a,b; Ahluwalia et al., 2002) and CB$_2$ (Ross et al., 2001; Price et al., 2001; see also Hohmann & Herkenham, 1999a) receptor subtypes have been reported in dorsal root ganglion cells, the source of afferent input to the spinal cord. Activation of CB$_1$ and CB$_2$ modulates intracellular signaling cascades through inhibition of adenyl cyclase via a Gi/o mechanism (Bayewitch et al., 1995). CB$_1$ is negatively coupled to N- and P/Q-type calcium channels (Twitchell et al., 1997) and positively coupled to inwardly rectifying (Mackie et al., 1995) and A-type potassium channels (Deadwyler et al., 1993). Unlike CB$_1$, CB$_2$ is not coupled to either Q-type calcium or inwardly rectifying potassium channels (Felder et al., 1995).

**CB$_1$ and CB$_2$ Ligands**

The role of cannabinoids in pain modulation has been assessed through the development and use of high affinity agonists. The potent cannabinoid agonist WIN55,212-2 shows high affinity for CB$_1$ in rat brain ($K_i = 9.94$ nM) and CB$_2$ in spleen ($K_i = 16.2$ nM; Rinaldi-Carmona et al., 1994). CP55,940 also shows high affinity for both CB$_1$ and CB$_2$ in transfected cell lines ($K_i = 0.6$ nM at both subtypes; Showalter et al., 1996). Anandamide is an endogenous ligand that demonstrates preferential affinity for CB$_1$ in transfected cell lines ($K_i [CB_1 \text{ vs. } CB_2] = 89$ nM vs.371 nM; Showalter et al., 1996). HU-308 is a CB$_2$ selective agonist ($K_i [CB_2 \text{ vs. } CB_1] = 23$ nM vs.10 μM in transfected cells vs. rat brain, respectively; Hanus et al., 1999). AM1241, another CB$_2$ selective agonist, exhibits 340-fold selectivity for CB$_2$ over CB$_1$ ($K_i [CB_2 \text{ vs. } CB_1] = 2$ nM vs. 680 nM in mouse spleen vs. rat brain, respectively).
Pharmacological specificity of cannabinoids has been assessed using competitive antagonists selective for receptor subtype. The selective CB₁ antagonist SR141716A binds to cannabinoid receptors in rat brain with high affinity \( (K_i = 1.98 \text{ nM}) \) and displays minimal affinity for rat spleen or cloned human CB₂ receptors \( (K_i > 1000 \text{ nM}; \text{Rinaldi-Carmona et al., 1994}) \). The selective CB₂ antagonist SR144528 exhibits high affinity for rat spleen and cloned human CB₂ receptors \( (K_i = 0.6 \text{ nM}) \), but does not bind readily to rat brain or cloned human CB₁ receptors \( (K_i = 400 \text{ nM}; \text{Rinaldi-Carmona et al., 1998}) \).

Utilizing selective agonists and selective competitive antagonists with preferential affinity for receptor subtype has allowed us to evaluate the specific roles of CB₁ and CB₂ in modulation of inflammatory nociception.

*Role of CB₁ in Antinociception and Anti-inflammation*

Several lines of evidence implicate a role for CB₁ in the modulation of acute and persistent nociception. Activation of CB₁ at peripheral, spinal and supraspinal sites suppresses nociceptive behavior. Peripheral administration of CB₁ agonists suppresses pain behavior associated with local capsaicin (Ko & Woods, 1999; Li et al., 1999; Johanek et al., 2001) and carrageenan (Richardson et al., 1998; Nackley et al., 2003) application. Intrathecal cannabinoid administration also attenuates formalin-induced pain behavior (Hohmann et al., 1999), mechanical and thermal hyperalgesia (Johanek et al., 2001) and mechanical allodynia (Martin et al., 1999). Additionally, CB₁ agonists increase tail-flick latencies when administered directly to the rostral ventromedial medulla, amygdala, thalamus, superior colliculus or A5 brainstem regioin (Martin et al., 1998, 1999).
The CB₁-mediated suppression of nociceptive behavior is blocked by CB₁ selective antagonists (Richardson et al., 1998; Martin et al., 1999; Nackley et al., 2003). Inhibition of CB₁ by the competitive antagonist SR141716A in the absence of exogenous agonist prolongs and enhances pain responses in naïve (Richardson et al., 1997) and formalin-treated rats (Calignano et al., 1998; Strangman et al., 1998), suggesting cannabinoids act intrinsically to suppress nociception. However, studies using human CB₁-transfected cell lines have shown SR141716A functions as an inverse agonist (Bouaboula et al., 1997; Shire et al., 1999). The possible inverse agonist actions of SR141716A complicate the interpretation that it disrupts tonic cannabinoid activity when administered alone.

The role of CB₁ in the transmission of nociceptive information has been further assessed using electrophysiological and neurochemical studies. Electrophysiological studies demonstrate that cannabinoids suppress noxious stimulus-evoked activity in nociceptive neurons in the spinal dorsal horn in intact (Hohmann et al., 1995, 1998, 1999a; Strangman & Walker, 1999; Drew et al., 2000; Kelly & Chapman, 2001) and inflamed (Drew et al., 2000) rats. The firing of thalamic wide dynamic range (WDR) neurons is also dampened via CB₁ activation (Martin et al., 1996). Additionally, electrical stimulation of the dorsal and lateral periaqueductal gray, a pain modulatory region, produces endogenous anandamide release as well as antinociception (Walker et al., 1999). Results from these studies are consistent with those from behavioral analyses employing similar animal models.

Activation of CB₁ also suppresses nociceptive transmission in neurochemical studies (Tsou et al., 1996; Hohmann et al., 1999; Nackley et al., 2003). Noxious stimuli
evoke the expression of Fos, the protein product of the immediate early gene \( c-fos \) (Hunt et al., 1987), in the superficial and deep nuclei of the lumbar dorsal horn, which are areas involved in the processing of nociceptive information. \( CB_1 \) agonists reduce the number of neurons that express Fos in the presence of inflammation when administered systemically (Tsou et al., 1995), spinally (Hohmann et al., 1999b; Martin et al., 1999) or locally in the paw (Nackley et al., 2003).

Altogether, results from electrophysiological and neurochemical studies suggest that cannabinoids reduce behavioral responses to stimuli by decreasing processing of nociceptive inputs. These data are consistent with neuroanatomical studies which have mapped the distribution of cannabinoid receptors in areas known to control nociceptive tracts, such as the spinal dorsal horn and the periaqueductal gray (Herkenham et al., 1991; Tsou et al., 1998).

In addition to modulating pain, \( CB_1 \) plays a role in the modulation of inflammation. Richardson et al. (1998) showed that local peripheral but not systemic administration of anandamide, a putative endogenous ligand for \( CB_1 \), suppresses edema and inflammation-evoked hyperalgesia in the carrageenan model of inflammation. \( CB_1 \) agonists also act locally to suppress capsaicin-evoked immunoreactive calcitonin gene-related peptide release in rat hind paw skin (Richardson et al., 1998). Moreover, in vitro macrophage spreading and phagocytosis are suppressed by \( CB_1 \) activation in a dose-dependent manner (Lopez-Cepero et al., 1986). Taken together, these data suggest that \( CB_1 \) mechanisms suppress inflammation as well as acute and persistent pain.
Role of CB$_2$ in Antinociception and Anti-inflammation

Recent studies suggest that CB$_2$ is involved in the modulation of cannabinoid antinociception (Hanus et al., 1999; Malan et al., 2001) as well as inflammation (Facci et al., 1995; Mazzari et al., 1996; Farquhar-Smith et al., 2001, 2002). The positioning of CB$_2$ on nonneuronal cells in inflamed tissue may allow CB$_2$ mechanisms to inhibit the release of inflammatory mediators that excite nociceptors (Mazzari et al., 1996).

The notion that CB$_2$ is involved in antinociception stems from initial studies employing the endogenous ligand palmitoylethanolamide (PEA). Exogenous administration of PEA suppresses formalin pain (Calignano et al., 2001) and visceral hyperalgesia (Farquhar-Smith et al., 2001, 2002) through a CB$_2$ mechanism. However, more work is necessary to understand the role of CB$_2$ in the modulation of inflammatory nociception because PEA has little or no affinity for CB$_2$ (Showalter et al., 1996; Lambert et al., 1999). It is plausible that PEA or an active metabolite acts at CB$_2$-like receptors and/or inhibits the inactivation of other endocannabinoids, thus prolonging or enhancing their effects at CB$_2$ (Lambert & DiMarzo, 1999; Petrocellis et al., 2002).

The recent development of selective ligands for CB$_2$ has allowed for evaluation of the role of CB$_2$ in modulating nociception and inflammation. CB$_2$ agonists are antinociceptive in models of acute and tonic pain (Hanus et al., 1999; Malan et al., 2001; Clayton et al., 2002). Hanus et al. (1999) found that systemic administration of the CB$_2$ selective agonist HU-308 suppressed peripheral pain behavior evoked by hindpaw formalin injections. In addition, HU-308 reduced arachidonic acid-induced ear edema, which is a type of inflammation.
Another CB$_2$ selective agonist, AM1241, suppresses thermal nociception in naive rats following systemic and local hindpaw injections (Malan et al., 2001). Unlike mixed CB$_1$/CB$_2$ agonists that act both centrally and peripherally, AM1241 fails to elicit centrally mediated cannabimimetic effects such as hypothermia, catalepsy and hypoactivity.

Recent work in our laboratory has shown that systemic AM1241 dose-dependently suppresses the development of carrageenan-evoked behavioral responses to normally noxious (hyperalgesia) and nonnoxious (allodynia) thermal and mechanical stimulation (Nackley et al., in press). A behaviorally inactive systemic dose of AM1241 suppresses hyperalgesia and allodynia following administration to the carrageenan-injected paw but fails to do so following administration in the contralateral (noninflamed) paw. This observation is consistent with a local site of action. In parallel immunocytochemical studies, AM1241 dose-dependently suppresses spinal Fos-protein expression, a marker of neuronal activity. Pharmacological specificity of AM1241-induced actions was established, as the suppression of pain behavior and Fos-protein expression was blocked by a CB$_2$- but not CB$_1$-selective antagonist. Collectively, these data provide evidence that actions at cannabinoid CB$_2$ receptors are sufficient to increase nociceptive thresholds and suppress inflammation and nociception in models of inflammatory pain.

*Carrageenan Model of Inflammation*

Intraplantar carrageenan has been used to induce inflammation in order to assess the actions of potential analgesic and anti-inflammatory compounds (DiRosa et al., 1971; Kocher et al., 1987). The development of carrageenan-induced hindpaw edema is composed of an early phase beginning immediately and lasting 20-60 min post-injection
and a late phase which is maximal 2-3 hrs post injection (Doherty & Robinson, 1975). The early phase is attributed to the injection trauma, while the late phase corresponds to inflammation. Carrageenan elicits inflammation by prompting neutrophil infiltration (Vinegar et al., 1976) and plasma extravasation of proteins (Labrecque et al., 1984).

Carrageenan-evoked inflammation of peripheral tissues produces sensitization of peripheral and central neurons that results in increased pain sensitivity to noxious stimuli (hyperalgesia) as well as normally innocuous stimuli (alldynia) (Cooper, 1993; Hedo et al., 1998). Peripheral sensitization of Aδ- and C-fiber terminals occurs at the site of inflammation (Reeh, 1994). Central sensitization is apparent at the spinal level and is characterized by a decrease in threshold (Neugebauer & Schaible, 1990; Simone et al., 1991), increase in firing rate (Dougherty et al., 1999) and enlargement of the receptive field (McMahon & Wall, 1984; Ren et al., 1992). Additionally, inflammation causes low threshold Aβ-fibers to undergo a phenotypic change so that they functionally resemble C-fibers: even expressing substance P (Neumann et al., 1996). Sensitization of Aβ-fibers contributes to enhanced spinal neuronal excitability by amplifying normally nonnoxious peripheral tactile stimulation (Nakatsuka et al., 1999; Neumann et al., 1996).

Aδ-, C- and Aβ-fibers provide afferent input to wide dynamic range (WDR) neurons located in the spinal dorsal horn (Light & Perl, 1979; Tsubaki and Yokota, 1983). WDR neurons are prevalent in superficial and deep laminae of the spinal dorsal horn where nociceptive primary afferent fibers terminate. Therefore, sensitization of these afferents can produce subsequent excitability of spinal WDR neurons (Woolf et al., 1994; Hedo et al., 1998; Nakatsuka et al., 1999), which are the cells of interest in the present study.
Focus of the Present Work

Activation of the cannabinoid system significantly diminishes pain. CB₁ agonists attenuate behavioral and physiological responses to painful stimuli (Richardson et al., 1998; Ko and Woods, 1999; Johanek et al., 2001). However, adverse psychoactive effects are associated with CB₁ compounds, thus limiting their use. Although the role of CB₂ is less understood, recent work demonstrates that selective activation of CB₂ suppresses pain behavior in untreated (Malan et al., 2001) and inflamed rats (Clayton et al., 2002; Nackley et al., 2003, in press). Moreover, neuroanatomical studies have confirmed that CB₂ agonists suppress spinal Fos-protein expression, a neurochemical marker of neuronal activity evoked by inflammation (Nackley et al., 2003, in press). In addition, CB₂ selective agonists are believed to be devoid of centrally-mediated effects (Hanus et al., 1999; Malan et al., 2001) and therefore have promising therapeutic potential.

The present work employs electrophysiological methods to directly examine the consequences of local CB₂ activation on central nervous system processing at the level of the lumbar spinal cord under inflammatory conditions. Carrageenan-induced inflammation produces a variety of changes in spinal neuronal excitability such as increased spontaneous activity (neuronal firing occurring in the absence of experimental stimulation) and neuronal sensitization (Chapman & Dickinson, 1997; Hedo et al., 1998; Pertovaara et al., 1998). It was hypothesized that application of AM1241 would decrease the excitability of WDR neurons in the spinal dorsal horn, part of the ascending pain pathway, under inflammatory conditions. Such findings would provide experimental evidence for developing peripherally acting cannabinoids as potential analgesics.
CHAPTER 2

INTRODUCTION

A large body of literature implicates the cannabinoid system in the modulation of pain. Cannabinoids attenuate nociceptive responses in behavioral (Calignano et al., 1998; Martin et al., 1998; Ko & Woods, 1999; Farquhar-Smith & Rice, 2001; Hanus et al., 1999; Malan et al., 2001), neurochemical (Tsou et al., 1998; Hohmann et al., 1999b; Nackley et al., 2003, in press) and electrophysiological (Hohmann et al., 1995, 1998, 1999a; Martin et al., 1996; Drew et al., 2000; Chapman, 2001; Harris et al., 2000) studies employing various animal models. Cannabinoid antinociception is produced at peripheral (Richardson et al., 1998; Malan et al., 2001), spinal (Hohmann et al., 1998; Drew et al., 2000) and supraspinal (Martin et al., 1996, 1999) levels in untreated and inflamed animals. These actions are mediated by both CB$_1$ and CB$_2$ receptor subtypes. CB$_1$ antinociception is well established in models of acute (Martin et al., 1995, 1998; Hohmann et al., 1998, 1999) and chronic pain (Richardson et al., 1998; Ko & Woods, 1999). Recent studies also suggest that CB$_2$ is involved in the modulation of inflammation (Facci et al., 1995; Mazzari et al., 1996; Farquhar-Smith et al., 2001, 2002) as well as antinociception (Hanus et al., 1999; Malan et al., 2001; Nackley et al., 2003, in press).

CB$_1$ is expressed primarily in the central nervous system (Matsuda et al., 1990; Munro et al., 1993; Zimmer et al., 1999). CB$_2$ is expressed mainly in cells of the immune system (Munro et al., 1993; Lynn & Herkenham, 1994) and is absent in neurons of the
central nervous system (Munro et al., 1993; Zimmer et al., 1999; Buckley et al., 2000). In immune tissues, levels of CB$_2$ mRNA are 10-100 times greater than that of CB$_1$ (Galiegue et al., 1995). Ligand binding to Gi/o-coupled CB$_2$ receptors modulates intracellular signaling cascades through adenyl cyclase inhibition (Bayewitch et al., 1995). Unlike CB$_1$, CB$_2$ is not coupled to either Q-type calcium or inwardly rectifying potassium channels (Felder et al., 1995). In vitro and in vivo studies have demonstrated that the proliferation of T cells (Patrini et al., 1997), B cells (Valk et al., 1997) and natural killer cells (Parolaro et al., 1999), which act synergistically to produce a robust immune response, is inhibited by CB$_2$ activation. Furthermore, CB$_2$ agonists suppress the expression of proteins elicited by macrophage immunomodulators (Cabral et al., 1989). Thus, the presence of CB$_2$ on immune cells in inflamed tissue may also allow CB$_2$ mechanisms to prevent the release of inflammatory mediators, such as nerve growth factor, cytokines and ATP, that result in nociceptor sensitization (Mazzari et al., 1996).

The recent development of CB$_2$ selective ligands has provided the means to evaluate the role of CB$_2$ in modulating nociception in the presence and absence of inflammation. CB$_2$ agonists are antinociceptive in models of acute (Malan et al., 2001) and tonic pain (Hanus et al., 1999; Clayton et al., 2002; Nackley et al., in press). Activation of CB$_2$ also reduces arachidonic acid-induced ear edema, which is a type of inflammation (Hanus, 1999). AM1241 is a CB$_2$ selective agonist that exhibits 340-fold selectivity for CB$_2$ over CB$_1$ ($K_{i}$ [CB$_2$ vs. CB$_1$] = 2 nM vs. 680 nM in mouse spleen vs. rat brain, respectively). AM1241 suppresses thermal nociception in naive rats following systemic and local hindpaw injections (Malan et al., 2001). AM1241 fails to elicit centrally mediated cannabimimetic effects such as hypothermia, catalepsy and
hypoactivity (Malan et al., 2001). Thus, CB₂ selective agonists offer considerable therapeutic potential as these agents are devoid of centrally mediated side-effects commonly associated with opiates and CB₁ agonists.

Recent work in our laboratory further demonstrates that selective activation of CB₂ by AM1241 suppresses inflammation-evoked mechanical allodynia and mechanical and thermal hyperalgesia (Nackley et al., in press). In parallel neuroanatomical studies we confirmed that the same treatment suppresses Fos-protein expression, a neurochemical marker of neuronal activity evoked by inflammation, in the spinal dorsal horn. In these studies, immunocytochemical methods were used to map populations of neurons modulated by a CB₂ mechanism. In the present study, electrophysiological methods were used to directly examine the effects of the CB₂ selective agonist AM1241 on pain sensitive neurons in the lumbar dorsal horn under inflammatory conditions.

The presentation of noxious mechanical, thermal and electrical stimuli to the peripheral receptive field stimulates primary afferent fibers, which in turn excite nociceptive specific (NS) and wide dynamic range (WDR) neurons in the spinal dorsal horn. Peripheral nerves mediating pain include thinly myelinated, medium diameter A δ- and unmyelinated, small diameter C-fibers. Aδ-fibers are rapidly conducting (3-30 m/s) afferents associated with first pain (acute, sharp pain with rapid onset), while C-fibers are slowly conducting (0.5-2.0 m/s) afferents associated with second pain (dull, diffuse pain with delayed onset) (Light & Perl, 1979). Repeated activation of C-fibers leads to a phenomena known as windup, in which the number of neuronal responses progressively increases with subsequent stimulation (Mendell, 1966). Windup is generally attributed to C-fiber afterdischarge (resulting 300-800 ms post stimulation)-evoked responses of spinal
dorsal horn neurons and is involved in the maintenance of pain in inflammatory and neuropathic conditions (Dubner, 1991; Gracely et al., 1992). Thickly myelinated, large diameter Aβ-fibers constitute a third class of peripheral nerves that rapidly respond to innocuous stimulation and do not normally contribute to pain (Weidner et al., 1999).

Electrophysiology has been used previously to evaluate cannabinoid modulation of neuronal activity in the spinal dorsal horn (Hohmann et al., 1995, 1998, 1999a; Martin et al., 1996; Strangman & Walker, 1999; Drew et al., 2000; Chapman, 2001; Harris et al., 2001). In untreated animals, cannabinoids suppress the nociceptive responses of primary afferent Aδ- and C-fibers (Strangman & Walker, 1999; Drew et al., 2000; Chapman, 2001; Kelly & Chapman, 2001) and spinal NS and WDR neurons (Hohmann et al., 1995, 1998, 1999a; Strangman & Walker, 1999), thus inhibiting nociceptive transmission to supraspinal sites.

Inflammation of peripheral tissues produces a multitude of neuronal changes that involve both peripheral and central mechanisms (Neumann et al., 1996; Hedo et al., 1998). Peripheral sensitization of Aδ- and C-fiber terminals occurs at the site of inflammation (Reeh, 1994). Central sensitization is apparent at the spinal level and is characterized by a decrease in threshold (Neugebauer & Schaible, 1990; Simone et al., 1991), increase in firing rate (Dougherty et al., 1999; Guilbauld et al., 1986), enlargement of the receptive field (McMahon & Wall, 1984; Ren et al., 1992) and recruitment of low threshold Aβ-fibers (Nakatsuka et al., 1999; Neumann et al., 1996). Previous studies demonstrated that opioids and cannabinoids known to suppress pain behavior, also suppress these inflammation-evoked neuronal changes (Haley et al., 1990; Hylden et al., 1991; Stanfa et al., 1992; Drew et al., 2000; Harris et al., 2000).
The present study was conducted to assess the role of CB$_2$ in modulating the excitability of neurons in the spinal dorsal horn of inflamed rats. It was hypothesized that application of AM1241 would attenuate carrageenan-evoked excitability of WDR spinothalamic tract neuron through a CB$_2$-specific mechanism. Pharmacological specificity was evaluated through the use of competitive antagonists for CB$_1$ and CB$_2$ (SR141716A and SR144528, respectively).
CHAPTER 3

EXPERIMENTAL PROCEDURES

Subjects

Sixty-five adult male Sprague-Dawley rats (245-345g; Harlan, Indianapolis, IN and Charles River Laboratories, Wilmington, MA) were used in these experiments. All procedures were approved by the University of Georgia Animal Care and Use Committee and followed the guidelines for the treatment of animals of the International Association for the Study of Pain (Zimmermann, 1983). All efforts were made to minimize the number of animals and their suffering.

Drugs and Chemicals

Lambda carrageenan was obtained from Sigma Aldrich (St. Louis, MO). AM1241, a potent CB2 selective agonist, was synthesized in the Department of Medicinal Chemistry and Molecular and Cell Biology at The University of Connecticut. SR141716A, a CB1 selective antagonist, and SR144528, a CB2 selective antagonist, were provided by NIDA. Carrageenan (3%) was dissolved in saline and administered in a volume of 100 µl. Drugs were dissolved in dimethylsulfoxide (DMSO) for intraplantar administration (50 µl) and in emulphur, ethanol, saline (1:1:3) for intravenous administration (2 ml/kg body weight).

Surgical Preparation

Rats were anesthetized with urethane (25%, 1.2 g/kg, i.p.). Core body temperature was maintained throughout surgical and experimental procedures using a feedback-
controlled heating pad (Stoelting, Wood Dale, IL). A laminectomy was performed to expose the lumbosacral region of the spinal cord and the dura was retracted. Rats were placed in a stereotaxic frame (Narishige, East Meadow, NY) and the spinal column stabilized with vertebral clamps placed rostral and caudal to the exposed spinal cord. The skin flaps lateral to the cord were pulled taut with surgical staples placed rostral and caudal to the laminectomized region and the spinal cord was covered with a pool of mineral oil. The tail vein was catheterized for intravenous drug administration.

**Identification of Wide Dynamic Range Neurons and Primary Afferent Inputs**

Wide dynamic range (WDR) neurons are located in superficial and deep laminae of the spinal dorsal horn (Mendell, 1966; Tsubaki & Yokota, 1983). WDR neurons were qualitatively identified by responses to mechanical stimulation (brush and pinch) of the receptive field. An innocuous brush stimulus was applied using an artist’s paint brush with a ½ inch long camel-hair tip; the receptive field was gently stroked from heel to toe. Noxious pinch was applied to the receptive field on the plantar surface using teethed forceps at an intensity perceived to be noxious to the rat. After isolating a single WDR neuron and mapping its receptive field on the plantar surface of the hindpaw using a straight point teasing needle, the brush or pinch stimulus was presented for 10 s with a 10 s interstimulus interval. WDR neurons responded with greater frequency to noxious pinch as opposed to non-noxious brush stimulation, demonstrating that they respond differentially to the two types of stimuli (Kenshalo et al., 1979).

Aβ-, Aδ- and C-fiber-mediated responses evoked by transcutaneous electrical stimulation of the peripheral receptive field were characterized using the method of Chapman and Dickenson (1997). Aβ-fiber-mediated responses occurred 0-20 ms post-
stimulation, Aδ-fiber-mediated responses occurred 20-90 ms post-stimulation and C-fiber-mediated responses (early responses) occurred 90-300 ms post-stimulation. The remaining evoked responses occurring 300-800 ms post-stimulation were attributed to C-fiber sensitization (afterdischarge). Spontaneous, or non-elicited, activity was assessed over 100 ms immediately preceding each electrical stimulation within a given train.

*Electrophysiological Recording and Stimulation*

Extracellular recordings from single WDR neurons were obtained using 3 M Ω tungsten microelectrodes (FHC Inc., Brunswick, ME) advanced by a hydraulic microdrive (Narishige, East Meadow, NY). Two stimulating electrodes were placed transcutaneously within the center of the receptive field of isolated WDR neurons. Neuronal activity was evoked by a train of 16 electrical stimuli (2 ms pulses, 2 s apart) applied every 10 min. Stimuli were delivered by a S88 square pulse stimulator (Grass-Telefactor, West Warwick, RI) at approximately three times the C-fiber threshold at 5.0 Hz (Chapman, 2001). Threshold of WDR neurons was identified using the method of limits. Stable baseline responsiveness to electrical stimulation of the receptive field was assessed over a 30 min period prior to drug or vehicle administration. Responsiveness to successive trains of transcutaneous electrical stimulation was subsequently assessed over 90 min following administration of carrageenan as described below. The 90 min time interval was selected based upon the ability of the CB₁/CB₂ agonist WIN55,212-2 to suppress the development of carrageenan-evoked pain behavior and Fos protein expression, a marker of neuronal activity, in the rat spinal dorsal horn through a CB₂ specific mechanism (Nackley et al., 2003). In this work, the WIN55,212-2-induced suppression of pain behavior and Fos protein expression were blocked by the CB₂
antagonist SR144528. These data suggest that the 90 min post-carrageenan time interval is sufficient for observing suppressive effects on inflammatory nociception that results from activation of CB2. Upon conclusion of recording, rats were overdosed by injecting an intravenous bolus of pentobarbital. Recording depth was recorded to the nearest 5 µMs.

**Pharmacological Manipulations**

After stable baseline responsiveness to electrical stimulation was established, drug or vehicle was administered. Drug or vehicle was given either systemically (2 ml/kg i.v.) or locally (50 µl i.pl.) in the plantar surface of the hindpaw just prior to intraplantar administration of carrageenan. Doses of AM1241 were selected based upon the ability of comparable doses of the compound to suppress the development of carrageenan-evoked Fos protein-expression and pain behavior (Nackley et al., in press). Rats received a unilateral intraplantar injection of carrageenan (3%, 100 µl) together with drug or vehicle.

In experiment 1, the effects of systemic administration of AM1241 on the development of inflammation-evoked neuronal excitability were assessed. Separate groups of rats received intravenous injections of AM1241 (330 µg/kg; n = 6), vehicle (n = 6), SR144528 (1 mg/kg; n = 5), SR141716A (1 mg/kg; n = 6), SR144528 (1 mg/kg) together with AM1241 (n = 6) or SR141716A (1 mg/kg) together with AM1241 (n = 6) just prior to intraplantar administration of carrageenan.

In experiment 2, the site of action of AM1241 was examined in inflamed rats. AM1241 (33 or 330 µg/kg n = 6 per group) or vehicle (n = 6) was injected concurrently with carrageenan (3%) in the plantar surface of the hindpaw. In order to control for
systemic leakage of the CB$_2$ agonist, separate groups of rats received AM1241 (33 µg/kg; n = 6) in the contralateral (noninflamed paw). A control group received intraplantar saline together with vehicle (n = 6) to verify that neuronal excitability was induced by carrageenan and not by the injection itself. Paw diameter was measured in duplicate to the nearest mm in experiments 1 and 2 using a caliper square. Hindpaw edema was assessed at the central region of the paw prior to carrageenan administration and at the region of maximal inflammation upon termination of the recording period, approximately 2 hrs subsequent to the induction of inflammation.

Data Analysis

Data were acquired using a CED 1401 interface (Cambridge Electronic Design, Cambridge UK) and a Pentium III computer and Spike-2 software. Spike shapes were templated and monitored throughout the recording interval to confirm that action potentials from the original cell of interest were recorded. Electrophysiological data were analyzed by ANOVA and Fisher’s PLSD post hoc tests. ANOVA was used to assess the statistical significance of experimental differences in the number of spontaneous and stimulation-evoked action potentials attributed to A$\beta$-, A$\delta$- and C-fiber-mediated responses. C-fiber mediated action potentials were assessed in terms of early, afterdischarge and total responses.

Degree of windup was determined using the method of Chapman (2001):

Degree of windup = total number of observed responses in train X - (Number of neuronal responses elicited by stimulation 1 in train X*16)

Data were expressed as percentage of pre-inflammation windup, using the formula:

% Pre-inflammation windup = \frac{\text{Degree of post-inflammation windup in train X}}{\text{Degree of baseline windup responses}} \times 100
Percentage of pre-inflammation windup was calculated for each stimulation train.

Neuronal responsiveness over three successive trains of 16 stimulations (delivered at 10 min intervals) was averaged for data analysis. $P < 0.05$ was considered statistically significant.
CHAPTER 4
RESULTS

General Characteristics of Sampled Neuronal Population

Wide dynamic range neurons were sampled primarily from the superficial dorsal horn in both experiment 1 (Mean ± SEM: 166.53 ± 22.49 μM from the dorsal surface of the spinal cord; Range: 0-500 μM) and experiment 2 (Mean ± SEM: 208.50 ± 36.93 μM from the dorsal surface of the spinal cord; Range: 0-750 μM). All cells responded to brush and pinch and differentiated between stimulus intensity, as they responded with greater frequency to noxious pinch relative to nonnoxious brush in experiment 1 (Mean ± SEM: 108.66 ± 8.29 vs. 391.00 ± 37.30 for brush and pinch-evoked potentials, respectively; \[ F_{1,29} = 61.39, \ P < 0.0002 \]) and experiment 2 (Mean ± SEM: 92.60 ± 8.74 vs. 259.40 ± 29.05 for brush and pinch-evoked potentials, respectively; \[ F_{1,25} = 37.93, \ P < 0.0002 \]). Sampled neurons exhibited windup in response to trains of electrical stimuli applied to the cutaneous receptive field. Current used to activate neurons ranged from 2.5-5.5 mA (Mean ± SEM: 4.52 ± 0.15 mA) in experiment 1 and from 2.0-5.5 mA (Mean ± SEM: 4.55 ± 0.18 mA) in experiment 2 and did not differ between studies.

Rats receiving intraplantar saline together with vehicle failed to show reliably altered Aβ-, Aδ-, C-fiber-mediated neuronal responses or windup (Fig.1). These data suggest that the injection procedure alone failed to produce increases in sensitization of WDR neurons. By contrast, time-dependent changes in Aβ- and early C-fiber-mediated
responses were reliably induced by inflammation in each experiment ($P < 0.03$ for all comparisons).

Prior to inflammation, levels of spontaneous firing were low and did not differ between groups in experiment 1 (Mean ± SEM: 1.08 ± 0.20 action potentials; Fig. 2A,B) or experiment 2 (Mean ± SEM: 12.20 ± 0.37 action potentials; Fig. 2C). Intraplantar carrageenan increased spontaneous firing in experiment 1 ($P < 0.03$ for all comparisons). In experiment 2, spontaneous activity was lower in groups receiving AM1241 (33 or 330 µg/kg ipsi or 33 µg/kg contra) relative to vehicle ($F_{3,20} = 11.46$, $P < 0.0002$). Time-dependent changes were induced by the experimental treatment ($F_{9,60} = 2.63$, $P < 0.03$).

Activation of CB$_2$ by intraplantar administration of AM1241 (33 or 330 µg/kg ipsi) suppressed inflammation-evoked increases in spontaneous activity relative to controls at 70-90 min post-carrageenan ($P < 0.05$ for all comparisons; Fig. 2C). Spontaneous firing in groups treated with AM1241 (33 or 330 µg/kg ipsi or 33 µg/kg contra) was also lower than that observed in groups treated with vehicle from 10-60 min post-carrageenan.

**Experiment 1: CB$_2$ modulation of neuronal excitability in a rat model of inflammation**

Time-dependent changes in early C-fiber-mediated responses were suppressed by AM1241 (330 µg/kg, iv) ($F_{9,60} = 3.51$, $P < 0.004$; Fig. 3A). A trend towards a significant group difference was also observed ($P < 0.07$). At 70-90 min post-carrageenan, AM1241 suppressed early C-fiber-mediated activity relative to vehicle ($P < 0.02$ for all comparisons).

C-fiber-mediated afterdischarge differed between groups receiving AM1241 and control conditions ($F_{3,20} = 5.53$, $P < 0.007$; Fig. 3B). The AM1241-induced suppression of C-fiber-mediated afterdischarge was blocked by SR144528 ($P < 0.02$), but not
SR141716A. The AM1241-induced suppression of C-fiber-mediated afterdischarge became more pronounced as inflammation developed over time ($F_{9,60} = 3.93, P < 0.02$). Preemptive administration of AM1241 attenuated C-fiber-mediated afterdischarge beginning 10 min following the induction of inflammation ($P < 0.02$ for all comparisons). The suppressive effect of AM1241 on C-fiber-mediated afterdischarge was blocked by SR144528 beginning 40 min post-carrageenan ($P < 0.02$ for all comparisons).

Total C-fiber-mediated activity was lower in groups receiving AM1241 relative to vehicle treatment ($F_{3,20} = 3.40, P < 0.04$; Fig. 3C). Intravenous administration of AM1241 suppressed total C-fiber-mediated responses relative to controls at 40-90 min following carrageenan ($F_{9,60} = 4.56, P < 0.002; P < 0.02$ for all comparisons). The CB$_2$ antagonist, SR144528 ($P < 0.02$) blocked the AM1241-induced suppression of total C-fiber-mediated responses, while the CB$_1$ antagonist SR141716A failed to do so.

The percentage of pre-inflammation windup differed between groups receiving AM1241 or vehicle ($F_{3,20} = 4.54, P < 0.02$; Fig. 3D). AM1241 suppressed windup 10-90 min following the induction of inflammation ($F_{9,60} = 3.49; P < 0.02$ for all comparisons).

A$\beta$- and A$\delta$-fiber-mediated responses did not differ reliably between groups before or after carrageenan administration (Fig. 3 E,F). Moreover, no increases in A$\beta$-fiber-mediated activity was observed as a function of inflammation. A trend toward increases in A$\delta$-fiber-mediated activity during the development of inflammation was observed ($P = 0.06$).

Effects of SR144528 or SR141716A administration on A$\beta$-, A$\delta$-, C-fiber-mediated responses and windup did not differ from vehicle treatment (Fig. 4).
Experiment 2: Site of Action of AM1241

Early C-fiber-mediated responses were lower in groups receiving intraplantar administration of AM1241 (33 or 330 µg/kg) to the ipsilateral (ipsi) carrageenan-injected paw relative to control conditions ($F_{3,20} = 10.10$, $P < 0.0004$; Fig. 5A). AM1241 (33 or 330 µg/kg ipsi, ipl) also suppressed early C-fiber-mediated activity relative to controls at 40-90 min following the induction of inflammation ($F_{9,60} = 6.08$, $P < 0.003$; $P < 0.05$ for all comparisons). AM1241 (33 or 330 µg/kg ipsi) reduced early C-fiber-mediated responses relative to administration of AM1241 (33 µg/kg) to the contralateral (contra) noninflamed paw ($P < 0.02$ for all comparisons).

Total C-fiber-mediated activity differed between group receiving AM1241 (33 or 330 µg/kg) in the carrageenan-injected paw and controls ($F_{3,20} = 4.71$, $P < 0.02$; Fig. 5C). AM1241 (33 or 330 µg/kg ipsi, ipl) suppressed total C-fiber-mediated responses at 40-90 min following carrageenan ($F_{9,60} = 5.48$, $P < 0.0004$; $P < 0.05$ for all comparisons).

Time-dependent changes in windup, relative to baseline, were induced by AM1241 during the development of inflammation ($F_{9,60} = 2.36$, $P < 0.04$; Fig. 5D). The percentage of pre-inflammation windup did not differ reliably between groups across the entire observation interval. At 70-90 min post-carrageenan, AM1241 (330 µg/kg ipsi, ipl) suppressed windup relative to controls. AM1241 (33 µg/kg ipsi, ipl) also suppressed windup relative to AM1241 (33 µg/kg contra, ipl) at 70-90 min post-carrageenan ($P < 0.04$ for all comparisons).

The effects of AM1241 were mediated locally in the paw; the attenuation of early and total C-fiber-mediated neuronal responses and windup by local administration of
AM1241 in the ipsilateral (carrageenan-injected) paw was absent when the same dose was applied to the contralateral (noninflamed) paw (Fig. 5).

C-fiber-mediated afterdischarge and Aβ- and Aδ-fiber-mediated responses did not differ reliably between groups before or after carrageenan administration and did not increase as a function of inflammation (Fig. 5 B,E,F).

Peripheral Edema

Prior to administration of carrageenan, paw diameter did not differ between groups (Mean ± SEM: 5.22 ± 0.03 mm and 5.11 ± 0.03 mm in experiments 1 and 2, respectively; Fig. 6). In both studies, intraplantar carrageenan increased hindpaw diameter measured approximately 2 hrs after the induction of inflammation ($P < 0.0002$). Hindpaw diameter was greater in rats receiving carrageenan relative to a control group receiving an equivalent volume of intraplantar saline together with vehicle ($P < 0.0002$ for all comparisons). Hindpaw diameter was lower in groups receiving AM1241 (33 μg/kg ipl or 330 μg/kg iv or ipl) concurrently with carrageenan relative to an equivalent volume of vehicle. Administration of AM1241 (330 μg/kg iv or ipl or 33 μg/kg ipl) reduced hindpaw diameter in the inflamed paw relative to control conditions ($F_{15,87} = 239$, $P < 0.03$ and $F_{9,60} = 32.73$, $P < 0.0001$ in experiments 1 and 2, respectively, $P < 0.03$ for all comparisons). The anti-inflammatory effects of intravenously administered AM1241 were blocked by the CB₂ antagonist SR144528 ($P < 0.02$) but not the CB₁ antagonist SR141716A. No significant differences in paw diameter were observed in the noninflamed contralateral paw before or after carrageenan administration.
Fig. 1. In the absence of inflammation, the activity of WDR neurons remains similar to baseline levels. Rats receiving intraplantar injections of saline together with vehicle failed to exhibit increases in (A) Aβ-, Aδ-, (B) C-fiber-mediated neuronal responses or windup.

Data (Mean ± SEM) represent 3-point blocks. N = 6 rats.
Fig. 1: Neuronal Activity Evoked in WDR Neurons in the Absence of Inflammation
Fig. 2. Prior to inflammation, levels of spontaneous firing did not differ between groups receiving intravenous administration of (A) AM1241 (330 μg/kg), vehicle, AM1241 + SR144528, AM1241 + SR141716A, (B) SR144528 (1 mg/kg) or SR141716A (1 mg/kg). (C) Activation of CB₂ by intraplantar administration of AM1241 suppressed carrageenan-evoked increases in spontaneous activity following administration to the ipsilateral inflamed paw, but not following administration to the contralateral noninflamed paw.

Data (Mean ± SEM) represent the mean of 3 successive stimulation trains delivered at 10 min intervals and are plotted at the midpoint. * P < 0.05 different from vehicle and AM1241 (33 μg/kg contralateral, ipl), **P < 0.01 different from AM1241 (33 μg/kg ipsilateral, contra; 330 μg/kg ipsilateral) by ANOVA and Fisher's PLSD post hoc test. N = 5-6 rats per group.
Fig. 2: Pre- and Post-injection Levels of Spontaneous Activity

A

- Vehicle
- AM1241 (330 µg/kg, iv)
- AM1241 + SR141716A

Mean Number of Neuronal Responses

Time Post-Inflammation (min)

B

- Vehicle
- SR144528 (1 mg/kg, iv)
- SR141716A (1 mg/kg, iv)

Mean Number of Neuronal Responses

Time Post-Inflammation (min)

C

- Vehicle
- AM1241 (330 µg/kg ipsi, ipl)
- AM1241 (33 µg/kg ipsi, ipl)
- AM1241 (33 µg/kg contra, ipl)

Mean Number of Neuronal Responses

Time Post-Inflammation (min)
Fig. 3. Preemptive intravenous administration of the CB$_2$ agonist AM1241 (330 µg/kg) modulates (A) early C-fiber-mediated neuronal excitability, (B) C-fiber-mediated afterdischarge, (C) total C-fiber-mediated neuronal excitability and (D) windup. No reliable differences were observed in inflammation-evoked (E) Aβ- or (F) Aδ-fiber-mediated responses. Data (Mean ± SEM) represent the mean of 3 successive stimulation trains delivered at 10 min intervals and are plotted at the midpoint. **$P < 0.01$, * $P < 0.05$ different from vehicle and AM1241 + SR144528, xxx$P < 0.01$, xxx$P < 0.05$ different from vehicle by ANOVA and Fisher's PLSD post hoc test; $N = 5-6$ per group.
Fig. 3: Effects of Intravenous AM1241 on Aβ-, Aδ-, C-fiber Activation and Windup in WDR Neurons During the Development of Inflammation

A

% of Pre-Inflammation Early C-fiber Activity

Time Post-Inflammation (min)

Vehicle
AM1241 (330 µg/kg, iv)
AM1241 + SR144528
AM1241 + SR141716A

B

% of Pre-Inflammation C-fiber-mediated Afterdischarge

Time Post-Inflammation (min)

C

% of Pre-Inflammation Total C-fiber Activity

Time Post-Inflammation (min)

D

% of Pre-Inflammation Windup

Time Post-Inflammation (min)

E

% of Pre-Inflammation Aβ-fiber Activity

Time Post-Inflammation (min)

F

% of Pre-Inflammation Aδ-fiber Activity

Time Post-Inflammation (min)
Fig. 4. SR144528 (1 mg/kg) and SR141716A (1 mg/kg) did not alter neuronal excitability evoked by (A) early C-fiber-mediated responses, (B) C-fiber-mediated afterdischarge, (C) total C-fiber-mediated responses, (D) windup, (E) Aβ- or (F) Aδ-fiber-mediated responses relative to vehicle in inflamed rats. Data (Mean ± SEM) represent the mean of 3 successive stimulation trains delivered at 10 min intervals and are plotted at the midpoint. N = 5-6 per group.
Fig. 4: Effects of Intravenous CB₁ and CB₂ Antagonists on Aβ-, Aδ-, C-fiber Activation and Windup in WDR Neurons During the Development of Inflammation

A

% of Pre-Inflammation Early C-fiber Activity

% of Pre-Inflammation Total C-fiber Activity

% of Pre-Inflammation Windup

% of Pre-Inflammation Aδ-fiber Activity

% of Pre-Inflammation Aβ-fiber Activity

Time Post-Inflammation (min)
Fig. 5. The effects of AM1241 were mediated locally in the paw. Attenuation of (A) early C-fiber-mediated neuronal responses, (C) total C-fiber-mediated neuronal responses and (D) windup by AM1241 (33 or 330 µg/kg ipsilateral, ipl) was absent when the low (33 µg/kg ipl) dose was applied to the contralateral (noninflamed) paw. Local administration of AM1241 (33 or 330 µg/kg ipsilateral, ipl) did not reliably suppress (B) C-fiber-mediated afterdischarge, (E) Aβ- or (F) Aδ-fiber-mediated responses. Data (Mean ± SEM) represent the mean of 3 successive stimulation trains delivered at 10 min intervals and are plotted at the midpoint. **P < 0.01, *P < 0.05 different from vehicle and AM1241 (33 µg/kg contralateral, ipl), ^P < 0.05 different from AM1241 (33 µg/kg contralateral, ipl) by ANOVA and Fisher’s PLSD post hoc test; N = 6 per group.
Fig. 5: Effects of Intraplantar AM1241 on Aβ-, Aδ-, C-fiber Activation and Windup in WDR Neurons During the Development of Inflammation

A

B

C

D

E

F

% of Pre-Inflammation
Early C-fiber Activity

% of Pre-Inflammation
Total C-fiber Activity

% of Pre-Inflammation
C-fiber-mediated Afterdischarge

% of Pre-Inflammation
Windup

% of Pre-Inflammation
Aβ-fiber Activity

% of Pre-Inflammation
Aδ-fiber Activity

Time Post-Inflammation (min)

Vehicle
AM1241 (330 µg/kg ipsi, ipl)
AM1241 (33 µg/kg ipsi, ipl)
AM1241 (33 µg/kg contra, ipl)

Time Post-Inflammation (min)

Time Post-Inflammation (min)

Time Post-Inflammation (min)

Time Post-Inflammation (min)
Fig. 6. Intraplantar carrageenan increased hindpaw diameter measured 2 hrs after the induction of inflammation. Administration of (A) intravenous AM1241 (330 µg/kg) or (B) intraplantar AM1241 (33 or 330 µg/kg) concurrently with carrageenan reduced inflammation-induced increases in hindpaw diameter. Paw diameter in rats receiving intraplantar saline together with vehicle was similar to pre-carrageenan levels. Data represent paw diameter (Mean ± SEM) to the nearest mm. **$P < 0.01$, * $P < 0.05$ different from control conditions, ##$P < 0.01$ different from all animals receiving carrageenan by ANOVA and Fisher's PLSD post hoc test; $N = 5-6$ per group.
Fig. 6: Effects of AM1241 on the Development of Inflammation

A

B

* * *
CHAPTER 5
DISCUSSION

In the present investigation, transcutaneous electrical stimulation was used to study the effects of the CB$_2$ selective cannabinoid agonist, AM1241, on the responsiveness of primary afferent inputs to WDR neurons recorded in the lumbar spinal cord of rats with carrageenan-inflamed paws. Intraplantar carrageenan increased the responsiveness of neurons to trains of electrical stimuli delivered to their cutaneous receptive fields. Selective activation of CB$_2$ attenuated the development of WDR neuronal excitability in spinal dorsal horn neurons recorded in inflamed rats. The CB$_2$ selective agonist AM1241 suppressed the development of carrageenan-evoked increases in neuronal responsiveness when administered intravenously or locally in the inflamed paw. These data are consistent with earlier work showing that AM1241 suppresses inflammation-evoked Fos protein expression, a marker of neuronal activity, in spinal dorsal horn neurons (Nackley et al., in press) and produces antinociception in acute (Malan et al., 2001) and inflammatory pain states (Nackley et al., in press). In contrast to previous studies demonstrating that the antinociceptive effects of AM1241 are dose-dependent (Malan et al., 2001; Nackley et al., in press), no differences were observed in suppression of carrageenan-evoked neuronal excitability produced by the high (330 $\mu$g/kg, ipl) and low (33 $\mu$g/kg, ipl) dose of AM1241. The low 33 $\mu$g/kg dose was sufficient to produce a maximal inhibition of C-fiber-mediated responses.
Decreases in WDR neuronal excitability resulted from a suppression of C-fiber-mediated activity. Administration of AM1241 attenuated total C-fiber-mediated responses during the development of inflammation; local injections of AM1241 reduced both the early and afterdischarge components while intravenous injections attenuated the early C-fiber-mediated activity. The suppression of C-fiber responses by AM1241 corresponds to unpublished data from our laboratory that show that AM1241 produces anti-nociception in rats receiving intraplantar capsaicin, which is known to activate peripheral C-fiber afferents. By contrast, Aβ- and Aδ-fiber-mediated responses (occurring 0-20 and 20-90 ms post-stimulation, respectively) remained close to baseline levels throughout the recording interval, although a transient difference between AM1241 and controls was observed in Aδ-fiber-mediated activity in experiment 2. These observations are similar to those made by Strangman and Walker (1999) who found that C-, but not Aβ-fiber-mediated responses were modulated by cannabinoids in the carrageenan model of inflammation through a CB₁ mechanism. They additionally found that CB₁ activation modulated carrageenan-evoked Aδ-fiber-mediated responses.

Pharmacological specificity was established in the present work using selective antagonists for cannabinoid CB₁ and CB₂ receptor subtypes. The AM1241-induced suppression of carrageenan-evoked increases in neuronal excitability was blocked by the CB₂ antagonist SR144528 but not by the CB₁ antagonist SR141716A. Thus, CB₁ is unlikely to contribute to the suppressive actions of AM1241 on neuronal responses in the carrageenan model of inflammation.

No antagonist-induced enhancements of carrageenan-evoked increases in neuronal activity were observed. When administered alone, neither SR141716A nor
SR144528 altered carrageenan-evoked excitability of WDR neurons relative to vehicle
treatment. This is consistent with the failure to observe an enhancement of carrageenan-
evoked Fos protein expression in the spinal dorsal horn following intraplantar (Nackley et
al., 2003) or systemic (Nackley et al., in press) administration of SR141716A or
SR144528. These data suggest that a CB$_2$ mechanism does not act tonically to modulate
WDR neuronal activity.

A peripheral cannabinoid mechanism mediated the AM1241-induced suppression
of the development of WDR neuronal excitability in the carrageenan model of
inflammation. Administration of AM1241 directly to the site of inflammation suppressed
the development of C-fiber-mediated responses in spinal WDR neurons, while the same
dose administered to the noninflamed contralateral paw was inactive.

Additionally, activation of peripheral CB$_2$ receptors produced an anti-
inflammatory effect; AM1241 reduced hindpaw diameter in the carrageenan-injected
paw. The AM1241-induced suppression in peripheral edema was blocked by the CB$_2$ but
not the CB$_1$ antagonist. Therefore, it also seems unlikely that CB$_1$ contributes to the anti-
inflammatory actions of AM1241 in the present study.

Spike shapes were templated using Spike-2 software and monitored throughout
the recording interval to confirm that action potentials from the original cells of interest
were recorded. All incoming action potential waveforms were compared to the initial
template for each cell and only recorded in the event of a match. Thus, group differences
cannot be attributed to changes in the population of cells sampled after the induction of
inflammation.

Collectively, these data provide evidence that actions at cannabinoid CB$_2$
receptors are sufficient to suppress inflammation-evoked neuronal excitability at rostral levels of processing in the spinal dorsal horn. Our findings are in agreement with previous work showing that CB$_2$ selective agonists are antinociceptive (Hanus et al., 1999; Clayton et al., 2001; Malan et al., 2001, Nackley et al., in press). The suppression of carrageenan-evoked excitability of WDR neurons in the spinal dorsal horn is likely to reflect the centrifugal consequences of AM1241 actions in the periphery as CB$_2$ is absent in neurons of the central nervous system (Munro et al., 1993; Zimmer et al., 1999; Buckley et al., 2000).

Future studies are required to identify the phenotypes of cells expressing CB$_2$ and the mechanism by which CB$_2$ agonists suppress inflammatory nociception. CB$_2$ agonists may attenuate inflammatory hyperalgesia by inhibiting nerve growth factor (NGF)-induced mast cell degranulation and neutrophil accumulation at the site of inflammation (Rice et al., 2002). Support for this hypothesis is derived from the ability of the endogenous ligand palmitoylethanolamine to attenuate bladder hyperreflexia induced by intra-vesical administration of NGF as well as NGF-induced Fos induction; these effects were blocked by a CB$_2$ antagonist (Farquhar-Smith et al., 2002). However, more work is necessary to understand the role of CB$_2$ in the modulation of inflammatory nociception because palmitoylethanolamide has little or no affinity for CB$_2$ (Showalter et al., 1996; Lambert et al., 1999). It is plausible that palmitoylethanolamide or an active metabolite acts at CB$_2$-like receptors and/or inhibits the inactivation of other endocannabinoids, thus prolonging or enhancing their effects at CB$_2$ (Lambert & DiMarzo, 1999). Nonetheless, if the signal transduction properties of CB$_2$ are consistent with those observed in CB$_2$ transfected cell lines (Felder et al., 1995), it is unclear how activation of CB$_2$ would
suppress neuronal excitability and transmitter release in the absence of coupling to Ca\(^{++}\) or K\(^{+}\) channels.

CB\(_2\) selective agonists offer considerable therapeutic potential as these agents are devoid of centrally mediated side-effects that are commonly associated with CB\(_1\) agonists (Malan et al., 2001). Moreover, such compounds are unlikely to be psychoactive or addictive because CB\(_2\) is not neuronally expressed in the central nervous system (Munro et al., 1993; Zimmer et al., 1999; Buckley et al., 2000). The present work provides evidence that activation of a peripheral cannabinoid CB\(_2\) mechanism is sufficient to suppress the transmission of inflammation-evoked neuronal excitability at the level of the spinal dorsal horn. This suppression was observed in WDR neurons, which are known to modulate nociception and contribute to the ascending pain pathway, the spinothalamic tract. These data collectively suggest that CB\(_2\) agonists may be employed preemptively to attenuate the development of persistent pain in the absence of unwanted central side-effects.
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