ACTIVATION OF CANNABINOID CB₂ RECEPTORS SUPPRESSES CHEMOTHERAPY-INDUCED NEUROPATHY IN RATS: LACK OF CNS SIDE EFFECTS

by

ELIZABETH JOCELYN RAHN

(Under the Direction of Andrea G. Hohmann)

ABSTRACT

The cannabinoid system is a recently discovered neurotransmitter system that shares the same pharmacological target as the primary active constituent in cannabis, Δ⁹-tetrahydrocannabinol. The cannabinoid system consists of two primary endogenous neurotransmitters (anandamide and 2-arachidonoylglycerol) as well as at least two metabotropic receptors (CB₁ and CB₂). Activation of the CB₁ receptor is associated with psychotropic and motor effects, whereas activation of the CB₂ receptor lacks these central nervous system side effects. The cannabinoid receptors have disparate distributions in the body, which may account for their differential side effect profiles. The CB₁ receptor is localized primarily within the central nervous system (CNS). The CB₂ receptor was originally believed to be restricted to immune cells in the periphery; however recent suggests indicates the presence of this receptor at low levels within the CNS. The present studies focus on the cannabinoid CB₂ receptor as a potential target for novel analgesics. Antinociceptive properties of the purported cannabinoid CB₂ receptor agonists, (R,S)-AM1241, (R)-AM1241, (S)-AM1241, AM1714, and AM1710 were assessed in response
to both mechanical and thermal stimulation to better characterize these drugs. All four
drugs produced robust antinociception in response to thermal, but not mechanical
stimulation. The antinociceptive profile of AM1714 and at high doses, AM1710,
involved activation of the cannabinoid CB$_1$ receptor, whereas effects of ($R,S$)-AM1241
and its enantiomers were mediated exclusively by the cannabinoid CB$_2$ receptor. ($R,S$)-
AM1241, AM1714, and AM1710 failed to show CNS side effects at doses that produced
robust antinociception to thermal stimulation. The final series of experiments assessed the
efficacy of cannabinoid CB$_2$-preferring agonists in a model of chemotherapy-induced
neuropathy. ($R,S$)-AM1241, ($R$)-AM1241, and AM1714 attenuated established
mechanical allodynia observed in animals treated with the chemotherapeutic agent
paclitaxel. Prophylactic administration of the CB$_2$-preferring agonist, AM1710, the mixed
cannabinoid CB$_1$/CB$_2$ agonist, WIN55,212-2, and the neuropathic pain medication,
gabapentin, suppressed the development of paclitaxel-induced neuropathic nociception
with equal efficacy. All three drugs produced a protective effect against the development
of neuropathy that was present up to two weeks following removal of the drug.
Collectively, the data presented herein suggests that the cannabinoid CB$_2$ receptor is a
viable pharmacological target with immense potential for analgesic development with
limited adverse side effects.

INDEX WORDS: Antinociception, cannabinoid, cancer, CB$_1$, CB$_2$, chemotherapy, cold
allodynia, mechanical allodynia, naloxone, neuropathic pain, opioid, tetrad
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DEDICATION

I would like to dedicate this dissertation to four individuals who each touched my life in their own unique way and who I lost during my time in graduate school. This dissertation is dedicated in loving memory to Thomas Stephen Canerday, Ollie Mae Newnam Crawford, Annie Ruth McElroy England, and Brian Christopher Tackett.
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CHAPTER 1

GENERAL INTRODUCTION

Cannabinoids are drugs that share the same molecular target as Δ⁹-
tetrahydrocannabinol, the active ingredient in cannabis. Although the medicinal
properties of the cannabis plant have been common knowledge for thousands of years it
was not until the discovery of receptors (CB₁ and CB₂) and endogenous ligands
(anandamide and 2-arachidonoylglycerol) that the scientific birth of the cannabinoid
system took place.

Cannabinoids, although a relatively young member of the pharmacology field,
have amassed a rich literature and scientific enclave. Researchers have reported
numerous physiological effects associated with activation of the cannabinoid system,
including analgesia. Cannabinoid receptors are pre-synaptic receptors that can modulate
neurotransmission of both inhibitory and excitatory mediators. Receptors are localized
densely in areas of the brain and spinal cord associated with pain modulation (e.g.
periaqueductal gray, rostral ventral medulla, dorsal root ganglia). Both endogenous and
exogenous cannabinoid mediators exhibit analgesic properties in a variety of acute and
chronic pain models.

The purpose of the first series of studies (Chapters 3 and 4) was to further
characterize the antinociceptive properties of cannabinoids from two distinct chemical
classes. The cannabinoid CB₂ agonists from the aminoalkylindole class of cannabinoids,
(R,S)-AM1241, (R)-AM1241, and (S)-AM1241, were tested for antinociceptive
properties in naive rats following both thermal and mechanical stimulation (Chapter 3). Further tests were performed to assess potential interactions with the endogenous opioid system that have been previously implicated as the underlying cause of antinociceptive properties reported for this drug. Antinociceptive properties of two cannabinoid CB₂-prefering agonists from the novel cannabilactones class, AM1714 and AM1710, were assessed in naive rats in response to thermal and mechanical stimulation in a later study (Chapter 4). Central nervous system side effects (e.g. hypothermia, tail-flick antinociception, motor ataxia, and hypoactivity) for both cannabilactones, as well as the aminoalkylindole, (R,S)-AM1241, were also evaluated.

The first two studies provide evidence that cannabinoid CB₂ agonists from two distinct classes are effective analgesics that lack unwanted central nervous system side effects. The final two studies assessed the analgesic properties of these compounds in a model of neuropathic pain. The cannabinoid CB₂ agonists, (R,S)-AM1241, (R)-AM1241, (S)-AM1241 and AM1714, were administered acutely following induction of chemotherapy-induced neuropathy in the first study (Chapter 5). The effects of these drugs were compared to a commonly administered analgesic (morphine). In a follow-up study cannabinoid agonists with distinct mechanisms of action were administered prophylactically to assess the preventive efficacy of these drugs in a model of chemotherapy-induced neuropathy (Chapter 6). In this final study, the anti-allodynic efficacy of the CB₂-prefering agonist, AM1710 and the mixed CB₁/CB₂ cannabinoid agonist, WIN55,212-2, were compared with the commonly administered neuropathic pain medication, gabapentin (Neurontin®), when drugs were administered prior to and throughout, and following chemotherapeutic treatment.
CHAPTER 2

LITERATURE REVIEW:

CANNABINOID PHARMACOTHERAPIES FOR NEUROPATHIC PAIN:
FROM THE BENCH TO THE BEDSIDE

Abstract

Reprinted from Neurotherapeutics, 6(4), Rahn, E. J. & Hohmann, A. G. Cannabinoid pharmacotherapies for neuropathic pain: From the bench to the bedside, 713-737., Copyright (2009), with permission from Elsevier.
Neuropathic pain is a debilitating form of chronic pain resulting from nerve injury, disease states, or toxic insults. Neuropathic pain is often refractory to conventional pharmacotherapies, necessitating validation of novel analgesics. Cannabinoids, drugs that share the same target as Δ⁹-tetrahydrocannabinol (Δ⁹-THC), the psychoactive ingredient in cannabis, have the potential to address this unmet need. Here, we review studies evaluating cannabinoids for neuropathic pain management in the clinical and preclinical literature. Neuropathic pain associated with nerve injury, diabetes, chemotherapeutic treatment, human immunodeficiency virus (HIV), multiple sclerosis (MS), and herpes zoster infection is considered. In animals, cannabinoids attenuate neuropathic nociception produced by traumatic nerve injury, disease, and toxic insults. Effects of mixed cannabinoid CB₁/CB₂ agonists, CB₂-selective agonists, and modulators of the endocannabinoid system (i.e. inhibitors of transport or degradation) are compared. Effects of genetic disruption of cannabinoid receptors or enzymes controlling endocannabinoid degradation on neuropathic nociception are described. Specific forms of allodynia and hyperalgesia modulated by cannabinoids are also considered. In humans, effects of smoked marijuana, synthetic Δ⁹-THC analogs (e.g. Marinol®, Cesamet®) and medicinal cannabis preparations containing both Δ⁹-THC and cannabidiol (e.g. Sativex®, Cannador®) in neuropathic pain states are reviewed. Clinical studies largely affirm that neuropathic pain patients derive benefits from cannabinoid treatment. Subjective (i.e. rating scales) and objective (i.e. stimulus-evoked) measures of pain and quality of life are considered. Finally, limitations of cannabinoid pharmacotherapies are discussed together with directions for future research.
Key words: Endocannabinoid, marijuana, neuropathy, multiple sclerosis, chemotherapy, diabetes
Neuropathic Pain

Neuropathic pain is a debilitating form of treatment-resistant chronic pain caused by damage to the nervous system. Neuropathic pain may result from peripheral nerve injury, toxic insults, and disease states. Neuropathic pain remains a significant clinical problem because it responds poorly to available therapies. Moreover, adverse side-effect profiles may limit therapeutic dosing and contribute to inadequate pain relief. Drug discovery efforts have consequently been directed towards identifying novel analgesic targets for drug development. This review will evaluate the efficacy of cannabinoids as analgesics for the treatment of neuropathic pain from the bench to the bedside.

Cannabinoid Receptor Pharmacology

Evidence for the use of Cannabis sativa as a treatment for pain can be traced back to the beginnings of recorded history. The discovery by Gaoni and Mechoulam\(^1\) of \(\Delta^9\)-tetrahydrocannabinol (\(\Delta^9\)-THC), the primary psychoactive ingredient in cannabis, set the stage for the identification of an endogenous cannabinoid (endocannabinoid) transmitter system in the brain. The endocannabinoid signaling system includes cannabinoid receptors (e.g. CB\(_1\) and CB\(_2\)), their endogenous ligands (e.g. anandamide and 2-arachidonoylglycerol) and the synthetic and hydrolytic enzymes which control the bioavailability of the endocannabinoids. Both CB\(_1\)\(^2\) and CB\(_2\)\(^3\) receptors are G-coupled protein receptors that are negatively coupled to adenylate cyclase. Activation of CB\(_1\) receptors suppresses calcium conductance and inhibits inward rectifying potassium conductance, thereby suppressing neuronal excitability and transmitter release. CB\(_2\) receptor activation stimulates MAPK activity but does not modulate calcium or potassium conductances.\(^4\) The development of CB\(_1\)\(^5\) and CB\(_2\)\(^6\) receptor knockout mice
has helped elucidate the physiological roles of cannabinoid receptors in the nervous system. Generation of CB₁⁻/⁻ mice that lack CB₁ receptors in nociceptive neurons in the peripheral nervous system while retaining CNS expression (SNS-CB₁⁻) has also documented a role for these receptors in controlling nociception.⁷

CB₁ and CB₂ receptors exhibit disparate anatomical distributions.³ CB₁ receptors are localized to the central nervous system (CNS) and the periphery. CB₁ receptors are found in sites associated with pain processing, including the periaqueductal gray (PAG),⁸ rostral ventromedial medulla (RVM),⁸ thalamus,⁹ dorsal root ganglia (DRG),¹⁰ amygdala,⁸ and cortex⁸. Densities of CB₁ receptors are low in brainstem sites critical for controlling heart rate and respiration. This distribution explains the low toxicity and absence of lethality following marijuana intoxication. Activation of the CB₁ receptor also results in hypothermia, sedation, catalepsy, and altered mental status.¹¹ Thus, it is critical for any cannabinoid-based pharmacotherapy targeting CB₁ receptors to balance clinically-relevant therapeutic effects with unwanted side-effects. The CB₂ receptor was originally believed to be restricted to the periphery, primarily to immune cells (e.g. mast cells),¹² although they may be present neuronally in some species. CB₂ receptor protein has been reported in the DRG,¹³ brainstem,¹⁴ thalamus,¹⁵ PAG,¹⁵ and cerebellum¹⁵, ¹⁶ of naive rats. CB₂ receptor levels in most CNS sites are present at only low levels under basal conditions (or are below the threshold for detection). However, an upregulation of CB₂ receptor immunoreactivity or mRNA is observed in sites implicated in nociceptive processing under conditions of induced neuropathy.¹⁷, ¹⁸ CB₂ receptors are localized to microglia, a resident population of macrophages within the CNS that are functionally and anatomically similar to mast cells. Microglia secrete pro-inflammatory factors and induce
the release of several mediators (e.g. nitric oxide (NO), neurotrophins, free radicals) that are associated with synaptogenesis and plasticity, leading to changes in neuronal excitability.

**Endocannabinoids**

The first endogenous ligand for cannabinoid receptors\(^\text{19}\) was named anandamide (AEA) after the Sanskrit word for bliss. Several other endocannabinoids including 2-arachydonoylglycerol (2-AG),\(^\text{20,21}\) noladin ether,\(^\text{22}\) virodhamine,\(^\text{23}\) and N-arachidonolys-dopamine (NADA)\(^\text{24}\) have been described. Fatty-acid amide hydrolase (FAAH) is the principle catabolic enzyme for fatty-acid amides including AEA and \(N\)-palmitoylethanolamine (PEA).\(^\text{25}\) PEA does not bind cannabinoid receptors and has recently been described as an endogenous ligand for peroxisome proliferator receptor-\(\alpha\) (PPAR-\(\alpha\)).\(^\text{26}\) PEA may indirectly alter levels of endocannabinoids by competing with anandamide and other fatty-acid amides for degradation by FAAH or by suppressing FAAH expression at the transcriptional level.\(^\text{27,28}\) FAAH\(^/-\) mice are hypoalgesic in models of acute and inflammatory pain; these effects are blocked by a CB\(_1\) antagonist.\(^\text{29,30}\) This basal hypoalgesia is absent in FAAH\(^/-\) mice subjected to nerve injury, where genotype differences in evoked neuropathic pain behaviors are not apparent.\(^\text{30}\)

Anandamide also acts as an endovanalloid at the transient receptor potential cation channel (TRPV1) receptor.\(^\text{31}\) AEA shows affinity for TRPV1 that is 5-20 fold lower than its affinity for CB\(_1\). TRPV1 is not activated by classical, nonclassical, or aminoalkylindole cannabinoid agonists. AEA can also activate the peroxisome proliferator receptor-\(\gamma\) (PPAR\(\gamma\)) receptor.\(^\text{32}\) Thus, not all effects of AEA are mediated by cannabinoid receptors.
The metabolic pathways responsible for endocannabinoid degradation are well-characterized. Several FAAH inhibitors (e.g. OL135, URB597) have been developed and used to investigate physiological effects of increasing accumulation of AEA and other fatty-acid amides. Monoacylglycerol lipase (MGL) is a key enzyme implicated in the hydrolysis of 2-AG.\textsuperscript{33, 34} MGL inhibitors (e.g. URB602, JZL184) have been developed and can be employed to selectively increase accumulation of this endocannabinoid. The endocannabinoid system has complex relationships with other metabolic pathways. Both AEA and 2-AG can be metabolized by cyclooxygenase-2 (COX-2), a phenomenon that may contribute to the antinociceptive properties of non-steroidal anti-inflammatory drugs (NSAIDS) that act through inhibition of COX-2.\textsuperscript{4} Table 2.1 provides a summary of cannabinoids and related compounds that have been evaluated for efficacy in preclinical and clinical studies of neuropathic pain.

**Cannabinoid Modulation of Neuropathic Nociception in Animal Models**

W. E. Dixon was the first scientist to systematically study the antinociceptive properties of *Cannabis sativa*. Dixon reported that cannabis smoke delivered to dogs attenuated their responsiveness to pin-pricks.\textsuperscript{35} He observed that normally “evil-tempered and savage” dogs became “docile and affectionate” following exposure to cannabis – reflecting the psychotropic and mood-altering effects of cannabinoids. Motor effects observed following high doses of cannabinoids included drowsiness, awkward gate, and ataxia. Work by Walker’s group subsequently demonstrated that cannabinoids suppress nociceptive transmission (for review see\textsuperscript{36}). Early observations of the antinociceptive properties of cannabinoids laid a foundation for future research examining the impact of cannabinoids and modulation of the endocannabinoid system on neuropathic pain.
Models of Surgically-induced Traumatic Nerve Injury. Cannabinoids suppress neuropathic nociception in at least nine different animal models of surgically-induced traumatic nerve or nervous system injury. Here, we review the literature with a focus on uncovering effects of different classes of cannabinoids on both neuropathic nociception and central sensitization in each model. We also consider the impact of nerve injury on the endocannabinoid signaling system. Where applicable, we review effects of neuropathic injury on levels of endocannabinoids and related lipid mediators and describe regulatory changes in CB₁ and CB₂ receptors induced by nerve injury. Finally, we will consider implications of the preclinical findings for cannabinoid-based pharmacotherapies for neuropathic pain in humans.

Chronic Constriction Injury (CCI)³⁷. CCI produces mechanical allodynia as well as thermal alldynia and hyperalgesia in the ipsilateral paw as early as two days post-surgery.³⁷ Initial reports failed to find mechanical hyperalgesia, although several of the reviewed papers report its presence following surgery. Very few studies have investigated the presence of cold alldynia following this nerve injury; however those that have evaluated its presence uniformly demonstrate efficacy of cannabinoids in suppressing cold alldynia. CB₁ receptors are upregulated in the spinal cord following CCI; these effects are believed to be modulated by tyrosine kinase³⁸ and glucocorticoid³⁹ receptors. Not surprisingly, several classes of cannabinoids have been shown to suppress CCI-induced neuropathic nociception in rodents and include mixed cannabinoid agonists which target both CB₁ and CB₂ receptors, CB₂-selective agonists and modulators of the endocannabinoid system that inhibit FAAH or MGL (Tables 2.2 and 2.3).
Chronic administration of synthetic analogues of natural cannabinoid ligands containing cannabidiol attenuate or reverse established thermal and mechanical hyperalgesia in the CCI model. However, anti-hyperalgesic effects observed with these compounds are likely to be independent of cannabinoid receptors, and may be mediated through TRPV1. Those studies investigating pharmacological specificity have demonstrated blockade with the TRPV1 antagonist capsazepine, but not a cannabinoid CB1 or CB2 antagonist.40, 41 The CB1-specific antagonist SR141716 has been tested in this model with disparate results. SR141716, administered acutely, is pro-hyperalgesic and pro-allodynic in this model. 42 However, SR141716 (p.o.), administered chronically, suppresses thermal and mechanical hyperalgesia in both rats and CB1+/− mice, while failing to produce an effect in CB1−/− mice. 43 These reports are interspersed with a host of papers that indicate no antinociceptive or pronociceptive effects of either CB1 or CB2 antagonists, administered alone. Thus, it is important to emphasize that the behavioral phenotype induced by antagonist treatment may depend upon level of endocannabinoid tone present in the system, the injection paradigm (chronic vs. acute), and presence of regulatory changes in cannabinoid receptors or endocannabinoids.

Several mixed cannabinoid CB1/CB2 agonists have been shown to suppress all forms of neuropathic nociception observed in the CCI model, primarily through CB1-mediated mechanisms. Several studies, including the original study by Herzberg and colleagues42 were conducted before the development of a CB2 antagonist and recognition that CB2 receptor mechanisms modulate neuropathic pain.44 Mixed CB1/CB2 agonists, such as CP55,940 or WIN55,212-2, typically act as CB1-selective agonists following systemic administration,45 although CB2-mediated effects may be unmasked following
administration of CB2-selective agents or following local administration of the same compounds. A neurophysiological basis for these findings is derived from the observation that WIN55,212-2 (i.v.) dose-dependently inhibits windup as well as CCI-induced increases in spontaneous firing of spinal wide dynamic range (WDR) neurons through a CB1-dependent mechanism. Spontaneous firing of WDR neurons is believed to contribute to behavioral hypersensitivity and neuronal sensitization in neuropathic pain states. WIN55,212-2 also normalizes prostaglandin E2 (PGE2) levels and nitric oxide (NO) activity, two mediators of neuropathic pain that are increased following CCI.

Multiple CB2-selective agonists have been demonstrated to suppress CCI-induced mechanical allodynia, although pharmacological specificity has not been consistently assessed (Table 2.2). Thus, it is noteworthy that CB2 receptor mRNA is upregulated in the lumbar spinal cord following CCI. This upregulation is restricted to non-neuronal cells (e.g. glia). Interestingly, GW405833, a CB2-specific agonist, also reduces depression-like behavior associated with this mononeuropathy in the forced swim test. Tolerance, a feature which may contribute to loss of analgesic efficacy of currently available analgesics, failed to develop following repeated administration the CB2-specific agonist of A-836339. Thus, CB2 agonists may show therapeutic potential for suppressing neuropathic pain without producing tolerance when administered either alone or as adjuncts to existing treatments.

Endocannabinoid modulators suppress neuropathic pain symptoms associated with CCI (Tables 2.2 and 2.3). AM404, an endocannabinoid transport inhibitor, increases accumulation and, hence, bioavailability, of anandamide (and potentially other endocannabinoids) through a mechanism that remains incompletely understood. AM404
also normalizes CCI-induced changes in NO activity,\textsuperscript{52, 53} COX-2\textsuperscript{53} activity, cytokine levels (e.g. TNF-\(\alpha\) and IL10),\textsuperscript{52} and NF-\(\kappa\)B\textsuperscript{52} levels. In CCI rats, chronic administration of either AM404 or URB597 suppresses plasma extravasation, a condition associated with neuropeptide release at peripheral levels.\textsuperscript{54, 55} AM404, administered chronically or acutely, does not affect locomotor behavior, indicating a low propensity of this agent to produce unwanted motor side-effects associated with direct activation of CB\(_1\) receptors.\textsuperscript{52, 53}

CCI produces regulatory changes in endocannabinoid levels. CCI increases AEA and 2-AG levels in the PAG and RVM, sites implicated in the descending modulation of pain.\textsuperscript{56} CCI also increases levels of endogenous AEA, but not 2-AG, in the dorsal raphe – an observation which may help explain the anti-hyperalgesic efficacy of an anandamide transport inhibitor in this model.\textsuperscript{57} CCI increases serotonin (5-HT) levels in the dorsal raphe and this effect was suppressed by both WIN55,212-2 and AM404 in a CB\(_1\)-dependent manner.\textsuperscript{57} CCI-induced Fos expression was observed in response to non-noxious mechanical stimulation in spinal cord laminae I and II, the site of termination of A\(\delta\) and C fibers, which carry nociceptive sensory information from the periphery to the CNS. Lower levels of evoked Fos expression were observed in laminae III and IV of CCI rats. Chronic administration of AM404 significantly decreased CCI-induced Fos expression in the lumbar spinal cord through CB\(_1/\)CB\(_2\) and TRPV1-mediated mechanisms.\textsuperscript{58} Antinociceptive effects of FAAH inhibitors (OL135 and URB597) have also been reported in mice following CCI. OL135 and URB597 attenuate cold and mechanical allodynia in a manner that is dependent upon activation of both CB\(_1\) and CB\(_2\) receptors.\textsuperscript{59} Additionally both OL135 and URB597 are antinociceptive in FAAH\(^{+/+}\) mice,
but fail to produce an effect in FAAH-/- mice.59 The novel MGL inhibitor, JZL184, attenuates CCI-induced mechanical and cold allodynia through indirect activation of the CB1 receptor; JZL184 was efficacious in attenuating neuropathic nociception in both FAAH+/+ and FAAH-/- mice.59 The fatty acid PEA, administered chronically, attenuated the development of thermal hyperalgesia and mechanical allodynia in the CCI model through CB1, PPARγ and TRPV1-mediated mechanisms.60 Chronic administration of PEA also normalized levels of three neutrophic factors (NGF, GDNF, and NT-3) that were increased by CCI.60 Thus, activation of CB1 and CB2 receptors as well as pharmacological manipulation of endocannabinoid accumulation or breakdown suppresses neuropathic nociception in rodents.

Partial Sciatic Nerve Ligation (Seltzer Model)61. Mechanical hyperalgesia and allodynia are observed following partial ligation of the sciatic nerve. Thermal hyperalgesia was present in all studies reviewed here that evaluated this measure with one exception.62 Only two studies we reviewed examined the presence of cold allodynia following partial sciatic nerve ligation; the first study found that both CB2+/+ and CB2-/- mice showed evidence of cold alldynia following surgery.63 Cold allodynia has also been reported in rats following partial sciatic nerve ligation.64 All classes of cannabinoids evaluated produced anti-allodynic and anti-hyperalgesic effects in the Seltzer model (Table 2.4).

Pro-hyperalgesic effects of SR141716 and SR144528 have been reported in the Seltzer model,65 indicating a potential alteration in endocannabinoid tone following nerve injury. No other papers we reviewed reported similar effects of cannabinoid antagonists administered alone in this model. Exogenously applied endocannabinoids, AEA and 2-
AG, suppress changes in neuropathic nociception induced by partial sciatic nerve ligation. Interestingly, anandamide produced anti-hyperalgesic and anti-allodynic effects through a CB$_1$ mechanism, whereas 2-AG produced anti-hyperalgesic and anti-allodynic effects through activation of both peripheral CB$_1$ and CB$_2$ receptors. Anandamide and PEA exerts effects, at least in part, through a peripheral mechanism; both fatty-acid amides suppressed release of calcitonin gene-related peptide and somatostatin evoked by the irritant resiniferotoxin without altering peptide release under basal conditions. Anti-hyperalgesic effects of AEA and PEA were blocked by a CB$_1$ and CB$_2$ antagonist, respectively. One limitation with studies employing exogenous administration of endocannabinoids is that they do not imply that endocannabinoids are released under physiological conditions to produce these effects. Several studies report efficacy of mixed cannabinoid CB$_1$/CB$_2$ agonists in this model, although CNS side-effects were nonetheless observed in the same dose range that resulted in full reversal of neuropathic nociception. Ajulemic acid (CT-3), which was developed as a peripherally restricted cannabinoid analogue, also produced activity in the tetrad but anti-hyperalgesic effects occurred at doses lower than those producing side-effects.

Structurally distinct CB$_2$-specific agonists are efficacious in suppressing neuropathic nociception in this model. Moreover, CB$_2$ receptors in the spinal cord contribute to CB$_2$-mediated suppression of mechanical allodynia. CB$_2$ -/- mice reportedly develop thermal hyperalgesia and mechanical allodynia in the contralateral paw following surgery, whereas CB$_2$ +/+ do not. Microglia and astrocyte expression in the spinal dorsal horn is observed in both CB$_2$ -/- and CB$_2$ +/+ ipsilateral to nerve injury. However, CB$_2$ -/- mice notably exhibit increased microglial and astrocyte expression in the
contralateral spinal dorsal horn – a mechanism which may help to explain differences in neuropathic nociception between wild-types and knockouts. Further support for this hypothesis is derived from the observation that overexpression of the CB2 receptor attenuated enhanced expression of microglia. These results suggest that genetic disruption of the CB2 receptor has a disinhibitory effect on the responses of glial cells following partial sciatic nerve ligation. The cytokine, interferon-gamma (IFN-γ), is produced by astrocytes and neurons ipsilateral to injury in both CB2\(^{+/+}\) and CB2\(^{-/-}\) mice. However, CB2\(^{-/-}\) mice exposed to partial sciatic nerve ligation exhibit IFN-γ immunoreactivity in the spinal dorsal horn contralateral to injury. IFN-γ\(^{-/-}\)/CB2\(^{-/-}\) mice showed no evidence of neuropathic nociception when the contralateral paw was stimulated following surgery, suggesting that immune responses underlie neuropathic pain responses observable in the contralateral paw of CB2\(^{-/-}\) mice. Deletion of a putative novel cannabinoid receptor, GPR55, is also associated with the failure to develop mechanical hyperalgesia following partial sciatic nerve ligation.

Compounds targeting three distinct mechanisms for modulating endocannabinoid levels also suppress neuropathic nociception following partial sciatic nerve ligation. The transport inhibitor AM404, administered systemically, suppressed mechanical allodynia in a CB\(_1\)-dependent manner, without producing motor effects. The FAAH inhibitor URB597, administered locally in the paw, but not systemically suppressed both thermal hyperalgesia and mechanical allodynia through a CB\(_1\) mechanism. The MGL inhibitor URB602 (which cannot be used systemically as a selective MGL inhibitor), administered locally in the paw, also suppressed neuropathic nociception in this model through activation of both CB\(_1\) and CB\(_2\) receptors. The fatty-acid analogue of PEA, L-
29, also suppressed thermal hyperalgesia and mechanical allodynia in the Seltzer model. The L29-induced suppression of thermal hyperalgesia was mediated by both the CB₁ receptor and PPAR-α, whereas suppression of mechanical allodynia was mediated by CB₁/CB₂ and PPAR-α receptors. PEA abolished mechanical hyperalgesia following partial sciatic nerve ligation through a mechanism that was blocked by a CB₂ antagonist. When considering the effects of PEA it is important to emphasize that PEA does not bind directly to CB₂ receptors; therefore, blockade by a CB₂-specific antagonist could indicate indirect modulation of receptor activity (e.g. via activation of PPAR-α or entourage effects) or blockade of an uncharacterized cannabinoid receptor that binds the CB₂ antagonist SR144528. Intrathecal N-arachidonoyl glycine (NaGly), the arachidonic acid conjugate, also attenuated mechanical allodynia in this model, however, the anti-hyperalgesic actions of this compound are independent of spinal cannabinoid receptors. Locally injected (i.paw) paracetamol suppressed mechanical alldynia and thermal hyperalgesia present following partial sciatic nerve ligation and these effects are blocked by local administration of either a CB₁ or a CB₂ antagonist. Paracetamol may undergo local metabolic transformation into AM404, resulting in increased levels of endocannabinoids.

**Spinal Nerve Ligation (SNL)**. All studies reviewed here documented the presence of mechanical allodynia following SNL. All studies with the exception of one indicated the presence of thermal hyperalgesia when animals were tested. One study evaluated the presence of cold alldynia and confirmed that animals with this injury display hypersensitivity to non-noxious levels of cold stimulation. Gabapentin successfully attenuated mechanical allodynia in this model, however, several other commonly
prescribed neuropathic pain medications including amitriptyline, fluoxetine and indomethacin failed to show similar effects.\textsuperscript{80} Thus, it is noteworthy that mixed cannabinoid agonists, cannabinoid CB\textsubscript{2}-selective agonists and FAAH inhibitors all attenuated neuropathic nociception induced by SNL (Table 2.5).

As with other nerve injury models, several mixed cannabinoid CB\textsubscript{1}/CB\textsubscript{2} agonists suppress hyperalgesia and allodynia produced by SNL. Acute WIN55,212-2 suppresses all forms of neuropathic nociception tested in this model. Chronic administration of WIN55,212-2 also attenuates the development of mechanical allodynia and suppresses glial activation in the spinal cord following SNL with no overt motor side-effects.\textsuperscript{81} Chronic administration of WIN55,212-2 produced anti-allodynic effects up to six days following the final injection. A reappearance of glial activation was also associated with return of neuropathic nociception in this study.\textsuperscript{81} CP55,940 produces antinociception in CB\textsubscript{1}\textsuperscript{+/+}, CB\textsubscript{2}\textsuperscript{+/+}, CB\textsubscript{2}\textsuperscript{-/-}, but not CB\textsubscript{1}\textsuperscript{-/-} mice subjected to SNL, suggesting that activity at CB\textsubscript{1} dominates the antinociceptive profile of mixed CB\textsubscript{1}/CB\textsubscript{2} agonists following systemic administration.\textsuperscript{45} Spinal, but not systemic, administration of HU-210 has been reported to reduce A\textdelta fiber-evoked responses on spinal WDR neurons in both shams and SNL rats, whereas HU-210 showed no effect on C-fiber responses of SNL rats.\textsuperscript{82}

SNL produces regulatory changes in CB\textsubscript{1} mRNA and endocannabinoid levels. Increases in CB\textsubscript{1} mRNA are observed in the uninjured (but abnormal) L4 DRG ipsilateral to injury.\textsuperscript{83} Increases in both AEA and 2-AG have also been reported in the ipsilateral injured L5, but not the uninjured L4 DRG.\textsuperscript{83} These findings collectively document the presence of regulatory changes in endocannabinoid levels associated with SNL, a finding
which may contribute to the efficacy of peripherally administered cannabinoid agonists that activate CB₁ receptors in this model.

Noxious stimulation (e.g. C-fiber mediated activity) induces phosphorylation of extracellular signal-regulated protein kinase (ERK) in dorsal horn neurons. The CB₁-specific agonist ACEA inhibits pERK expression induced by *in vitro* application of capsaicin to the spinal cords of SNL rats. This observation contrasts with effects of opioids (i.e. morphine and DAMGO) which lose the ability to inhibit C-fiber induced ERK activation in the L5 spinal cord following SNL.⁸⁴

Multiple CB₂-specific agonists suppress neuropathic nociception induced by SNL. The CB₂ agonist AM1241 suppresses both thermal hyperalgesia and mechanical allodynia following SNL in both rats⁷³,⁷⁴,⁸⁵ and mice⁴⁴. CB₁⁻/⁻ mice receiving AM1241 showed enhanced anti-hyperalgesia.⁴⁴ An emerging body of literature now suggests that antinociceptive effects of CB₂ agonists may be mediated by suppression of microglial activation.⁴

Evidence for upregulation of CB₂ following SNL has been reported by several groups. CB₂ mRNA was upregulated in the lumbar spinal cord following SNL,⁴⁹ coincident with the expression of activated microglia. Colocalization studies, however, were not performed. Upregulation of CB₂ receptor immunoreactivity on sensory afferent terminals in the spinal cord has also been reported following SNL.¹⁸ This group failed to find co-localization of CB₂ with markers for glial cells in SNL rats, and concluded that CB₂ receptors were upregulated on sensory neurons following spinal nerve ligation.¹⁸ CB₂ mRNA was also shown to be upregulated in the ipsilateral (versus the contralateral)
spinal cord and DRG following SNL and the presence of CB₂ mRNA was confirmed in spinal cord microglial cells in culture.¹⁷

The CB₂-specific agonist GW405833, administered chronically, suppressed the development of mechanical allodynia concomitant with suppression of glial activation at the level of the spinal cord.⁸¹ The structurally distinct CB₂-specific agonist, JWH133, also attenuates mechanically-evoked responses of WDR neurons in both naive and spinal nerve ligated rats.⁸⁶ Local injection of JWH133 into the ventroposterolateral nucleus of the thalamus attenuated spontaneous and mechanically-evoked neuronal activity in SNL, but not sham rats, in a CB₂-dependent manner.⁸⁷ Thus, CB₂ receptor activation may exert little functional control under nonpathological conditions. Systemic and spinal administration of the novel CB₂ agonist, A-836339, also attenuates spontaneous and mechanically-evoked neuronal firing of spinal WDR neurons in a CB₂-dependent manner in SNL but not sham rats.⁸⁸ Interestingly, pre-treatment with the CB₁ antagonist, SR141716, enhanced the effects of A-836339 when applied to the L₅ DRG,⁸⁸ indicating that blockade of CB₁ receptors enhanced the antinociceptive effects of a CB₂ agonist, as reported previously.⁸⁹

Two endocannabinoid modulators have been evaluated behaviorally in this model. Compound 17, a novel FAAH inhibitor, reversed mechanical allodynia in SNL rats with the same potency as a 5-fold higher dose of gabapentin.⁹⁰ Additionally, OL135, a compound that accesses the CNS and inhibits FAAH, suppressed mechanical allodynia in a CB₂-dependent manner.⁹¹ Low doses of locally injected URB597 (i.pl.) reduced mechanically-evoked responses of WDR neurons and increased endocannabinoid levels in ipsilateral paw tissue of sham operated rats.⁹² A four-fold higher dose was required for
reduction of mechanically-evoked WDR neuronal responses in SNL rats; these rats showed no corresponding increase in endocannabinoid levels, suggesting that contributions of FAAH to endocannabinoid metabolism may be modified under conditions of neuropathic nociception. The antinociceptive effects of URB597 were blocked by a CB₁-specific antagonist in both sham and SNL rats. In the same study, spinal administration of URB597 was equally efficacious at attenuating mechanically-evoked responses and increasing levels of endogenous cannabinoids in SNL and sham rats and these effects were CB₁-mediated.

Other Nerve Injury Models. Cannabinoids alleviate neuropathic nociception in several other injury models. These studies support a role for CB₁ in the anti-hyperalgesic effects of cannabinoids, although pharmacological specificity has not been consistently assessed in the literature and high doses of cannabinoid agonists can produce motor side-effects which complicate interpretation of behavioral studies. Chronic constriction injury of the infraorbital nerve (CCI-ION) results in thermal hyperalgesia and mechanical allodynia (as measured by head withdrawals) ipsilateral to the site of injury. WIN55,212-2 and HU-210 increased mechanical withdrawal responses and thermal withdrawal latencies on the ipsilateral side of the head in this model. WIN55,212-2 was more efficacious in suppressing mechanical allodynia vs. thermal hyperalgesia in the CCI-ION model. High antihyperalgesic doses of WIN55,212-2 decreased rotarod latencies and body temperature, whereas HU210, at the singular low dose used (10 µg/kg), had no effect on these dependent measures. CB₁ receptor upregulation was observed in both the ipsilateral and contralateral superficial layer of the trigeminal caudal nucleus, and this effect was greater on the ipsilateral side. These and earlier findings from
the same group\textsuperscript{95} indicate that cannabinoids are negative modulators of nociceptive transmission at the superficial layer of the trigeminal caudal subnucleus.

CB\textsubscript{2} receptor immunoreactivity\textsuperscript{96} is increased in the ipsilateral dorsal horn following \textit{L5 spinal nerve transection (L5-SNT)}.\textsuperscript{97} Importantly, co-localization of CB\textsubscript{2} immunoreactivity with markers of microglia and perivascular cells was observed on day 4 post-surgery.\textsuperscript{96} In this study, neither neuronal cells nor astrocytes expressed immunoreactivity for CB\textsubscript{2} receptors.\textsuperscript{96} CP55,940 reversed mechanical allodynia in this model 1 h following a second intrathecal injection, although this dosing paradigm was also associated with motor effects.\textsuperscript{96} Intrathecal JWH015 dose-dependently suppressed behavioral hypersensitivity following a second injection, indicating a cumulative anti-allodynic effect of this drug. Intrathecal JWH015 reduced SNT-induced increases in activated microglia in a CB\textsubscript{2}-dependent manner, further supporting a role for nonneuronal CB\textsubscript{2} receptors in anti-hyperaesthesic effects of CB\textsubscript{2} agonists.\textsuperscript{96}

Two models developed by Walczak and colleagues\textsuperscript{98, 99} involve injuries to the saphenous nerve in rats and mice, respectively. The advantage of injuring the saphenous nerve over other nerves is that the saphenous nerve is an exclusively sensory nerve whereas other nerve injury models typically target nerves that subserve both sensory and motor functions. The first model was produced in rats by \textit{saphenous partial nerve ligation (SPL)}, which involves trapping 30-50\% of the saphenous nerve in a tight ligature.\textsuperscript{98} SPL rats presented with all symptoms except mechanical hyperalgesia (which was present inconsistently throughout testing). WIN55,212-2, administered systemically, suppressed all forms of hyperalgesia and allodynia present.\textsuperscript{98} In rats, SPL increased \textit{\mu}-opioid, CB\textsubscript{1}, and CB\textsubscript{2} receptor protein in ipsilateral hindpaw skin, DRG and lumbar
spinal cord. In a second injury model, *chronic constriction of the saphenous nerve (CCS)* was accomplished by tying two loose ligatures around the saphenous nerve in mice. Systemic WIN55,212-2 suppressed all forms of neuropathic nociception present in this model, including thermal hyperalgesia, cold allodynia, mechanical hyperalgesia and mechanical allodynia. Mu-opioid, CB₁ and CB₂ receptor protein was increased in the ipsilateral spinal cord and hindpaw skin at 7 days post-surgery. Additionally, increased CB₁ receptor protein was observed in contralateral hindpaw skin 7 days post-surgery and increased CB₂ receptor expression was observed in the contralateral spinal cord 1 and 7 days post-surgery. The neurobiological rearrangement of cannabinoid and mu-opioid receptors may contribute to the antinociceptive efficacy of WIN55,212-2 and morphine in this model.

The *spared nerve injury (SNI)* model reliably produces thermal hyperalgesia and mechanical allodynia in studies that tested for both measures. Initial reports of the SNI model indicated the presence of cold allodynia and mechanical hyperalgesia, but none of the papers reviewed here assessed these behaviors in conjunction with cannabinoid treatment. Standard analgesics (e.g. morphine, gabapentin, amitryptiline) are efficacious in treating neuropathic nociception resulting from a crush injury of the sciatic nerve, but showed limited efficacy following SNI. Two mixed cannabinoid CB₁/CB₂ agonists have been tested in this model. Acute WIN55,212-2 suppressed thermal hyperalgesia and mechanical allodynia in both mice lacking CB₁ receptors in primary nociceptors (SNS-CB₁⁻) and their wild-type controls; however differences in the antinociceptive effects of WIN55,212-2 were observed between genotypes, and these effects were greater with mechanical than thermal sensitivity. Comparable responses to WIN55,212-2 were only
observed at doses high enough to induce sedation and rigidity in all mice. SNS-CB$_1$ mice showed exaggerated sensitivity to noxious levels of mechanical stimulation and a cold plate relative to their wild-type counterparts, whereas differential sensitivity was not observed between genotypes with non-noxious levels of mechanical stimulation and noxious levels of thermal stimulation. Thus, CB$_1$ receptors on nociceptors in the periphery account for much of the antinociceptive effects of cannabinoids. A dose-escalation study with BAY 59-3074 in the SNI model indicated that tolerance rapidly develops to side-effects observed following chronic administration (e.g. hypothermia), whereas no loss in analgesic efficacy was observed.

**Spinal cord injury (SCI)** produces mechanical hyperalgesia and allodynia. WIN55,212-2 is the only compound that has been evaluated in the SCI model. Acute WIN55,212-2, administered systemically, suppressed SCI-induced mechanical allodynia in a CB$_1$-dependent manner, although other parameters of neuropathic pain were not assessed. Unlike morphine, chronic administration of WIN55,212-2 reduced mechanical allodynia in the SCI model with no decrease in effectiveness over time.

**Tibial nerve injury (TNI)** is performed by unilaterally axotomizing the tibial branch of the sciatic nerve. Mechanical allodynia and thermal hyperalgesia were present in the initial study describing this technique as well as the study we reviewed. Systemic BAY 59-3074 was shown to attenuate both forms of neuropathic nociception, although pharmacological specificity was not assessed. TNI injury resulted in an upregulation of CB$_1$ receptor mRNA in the contralateral thalamus on day 1 post-surgery, indicating cannabinoid receptor regulation within an important relay nucleus in the ascending pain pathway.
Disease-related Models of Neuropathic Pain. Cannabinoid agonists have been evaluated in animal models of disease-related neuropathic pain, although pharmacological specificity has not been consistently assessed. Here, we review effects of cannabinoids in preclinical models of neuropathic pain induced by diabetes, chemotherapeutic treatment, HIV/antiretroviral treatment, demyelination disorders, multiple sclerosis and post-herpetic neuralgia.

STZ-induced Diabetic Neuropathy. Diabetic neuropathy induced by a single injection of streptozotocin (STZ) resulted in increased sensitivity to noxious and non-noxious levels of mechanical stimulation, and failed to induce thermal hyperalgesia in the studies reviewed here (Table 2.6). None of the studies we reviewed evaluated the presence of cold allodynia. Met-F-AEA, a CB₁-specific agonist based upon the structure of anandamide, the mixed cannabinoid agonist WIN55,212-2 and the CB₂-specific agonist AM1241, administered chronically, suppressed mechanical hyperalgesia associated with STZ-induced diabetic neuropathy. However, mediation by cannabinoid receptors has not been assessed for agonists tested in this model. Daily pre-treatment with indomethacin (COX-1 inhibitor) or L-NOArg (non-selective NOS inhibitor) increased the anti-hyperalgesic actions of low doses of WIN55,212-2, AM1241 and MET-F-AEA in STZ rats to a greater extent than the cannabinoid administered alone, suggesting the presence of antinociceptive synergism between cannabinoid and COX pathways. COX inhibitors may block oxidative metabolism of endocannabinoids, thereby increasing endocannabinoids available to interact with cannabinoid receptors.

Diabetic rats exhibit a decrease in the density of CB₁ receptor protein in DRG. More work is necessary to determine whether this loss of cannabinoid receptors
contributes to the neurodegenerative process in diabetes. Increased levels of endocannabinoids have been found in obese patients suffering from Type II diabetes\textsuperscript{109} and this effect is likely to result from downregulation of FAAH gene expression, an effect which has also been observed in adipocytes sampled from obese women.\textsuperscript{110} Lean males subjected to hyperinsulinemia show a 2-fold increase in FAAH mRNA expression whereas obese males subjected to the same conditions failed to show similar alterations in gene expression.\textsuperscript{111} These findings are suggestive of a negative feedback mechanism that could result in downregulation of the endocannabinoid signaling system. The CB\textsubscript{1} antagonist rimonabant (Acomplia\textregistered) ameliorates insulin resistance and decreases weight gain in patients suffering from metabolic syndromes.\textsuperscript{112} In animal models, rimonabant improves resistance to insulin through pathways that are both dependent and independent of adiponectin, a hormone important for the regulation of glucose and catabolism of fatty acids.\textsuperscript{113} Although adverse side-effects have limited the potential therapeutic efficacy of Acomplia\textregistered, drugs modulating the endocannabinoid system should not be disregarded as targets for potential treatments of diabetes and its associated syndromes. STZ-diabetic mice showed a progressive decline in the radial arm maze and reduced neurological scores, both of which were recovered following treatment with HU-210.\textsuperscript{114} However, these effects were not blocked by a CB\textsubscript{1}-specific agonist. HU-210 did not alter the hyperglycemia index; however, it did normalize cerebral oxidative stress present in diabetic mice.\textsuperscript{114} An increase in the number of apoptotic cells and impaired neurite growth was observed in PC12 cells cultured under hyperglycemic conditions and these effects were effectively treated by HU-210.\textsuperscript{114}
Cannabinoids may show greater therapeutic potential for treating painful diabetic neuropathy compared to opioids. Interestingly, $\Delta^9$-THC exhibited enhanced antinociceptive efficacy in diabetic rats whereas morphine showed reduced antinociceptive efficacy. Furthermore, a non-nociceptive dose of $\Delta^9$-THC, administered in conjunction with morphine, enhanced the antinociceptive properties of morphine in both diabetic and naive mice. Thus, combinations of opioids and cannabinoids may show promise as adjunctive analgesics in humans. Diabetic rats exhibit lower levels of dynorphin and $\beta$-endorphins in cerebrospinal fluid (CSF) relative to non-diabetic rats treated under the same conditions. Administration of $\Delta^9$-THC to diabetic rats restored CSF levels of endogenous dynorphin and leu-enkephalin to levels observed following morphine administration to non-diabetic rats. More work is necessary to understand the mechanism underlying these observations.

Chemotherapy-induced Neuropathy. Cannabinoid modulation of chemotherapy-induced neuropathy has been evaluated with agents from three major classes of chemotherapeutic agents (Table 2.6). A singular study has evaluated cannabinoid modulation of neuropathic nociception induced by cisplatin, a platinum derived agent. WIN55,212-2 prevented the development of mechanical allodynia induced by cisplatin, but failed to produce an anti-emetic benefit in this study. It is possible that the dose of cannabinoid employed, the species used (rat) or toxicity of cisplatin-dosing paradigms may prevent detection of anti-emetic effects in this model. Cannabinoids have been shown to suppress cisplatin-induced emesis in the least shrew.

Paclitaxel has been most frequently studied in the cannabinoid literature with three studies documenting cannabinoid-mediated suppression of paclitaxel-induced
neuropathic nociception. In one study, paclitaxel\textsuperscript{118} produced mechanical allodynia starting on day 5 that continued throughout the timecourse, although thermal hyperalgesia was only present from days 18-21.\textsuperscript{119} WIN55,212-2 suppressed neuropathic nociception in this model but had no effect on body temperature or immobility. WIN55,212-2-induced decreases in spontaneous motor activity were nonetheless observed.\textsuperscript{119} A more recent study using the same paclitaxel dosing paradigm\textsuperscript{118} reported the presence of mechanical allodynia and the absence of thermal hyperalgesia.\textsuperscript{85} Naguib and colleagues\textsuperscript{85} demonstrated that a novel CB\textsubscript{2}-specific agonist, MDA7, suppressed paclitaxel-induced mechanical allodynia, although mediation by CB\textsubscript{2} receptors was not assessed. Using the paclitaxel dosing paradigm described by Flatters and Bennett,\textsuperscript{120} mechanical allodynia, but not thermal hyperalgesia, was observed. In this model, rats showed signs of mechanical allodynia up to 72 days post-paclitaxel.\textsuperscript{89} Systemic administration of either the CB\textsubscript{2} agonist \((R,S)\)-AM1241 or its receptor-active enantiomer \((R)\)-AM1241 produced CB\textsubscript{2}-mediated suppression of paclitaxel-induced mechanical allodynia. \((S)\)-AM1241, the enantiomer exhibiting lower affinity for the CB\textsubscript{2} receptor, failed to produce an anti-allodynic effect.\textsuperscript{89} The novel cannabiliactone, AM1714, also reversed mechanical allodynia associated with paclitaxel treatment in a CB\textsubscript{2}-dependent manner.\textsuperscript{89} Thus, both mixed CB\textsubscript{1}/CB\textsubscript{2} agonists and selective CB\textsubscript{2} agonists suppress paclitaxel-evoked mechanical allodynia.

Cannabinoid modulation of neuropathic nociception has also been evaluated with vincristine, an agent from the vinca-alkaloid class of chemotherapeutic agents. Vincristine produced mechanical allodynia, but not thermal hyperalgesia, in a 10 day injection paradigm\textsuperscript{121}. Systemic and intrathecal, but not intraplantar, WIN55,212-2
suppressed vincristine-induced mechanical allodynia through activation of CB₁ and CB₂ receptors. These findings implicate the spinal cord as an important site of action mediating anti-allodynic effects of cannabinoids. Systemic (R,S)-AM1241 also partially reversed vincristine-induced mechanical allodynia in a CB₂-dependent manner. The anti-allodynic effects of WIN55,212-2 and (R,S)-AM1241 were observed at doses that did not produce intrinsic effects on motor behavior in the bar test. Our studies suggest that clinical trials of cannabinoids for the management of chemotherapy-evoked neuropathy are warranted.

**HIV-associated Sensory Neuropathy.** The mixed cannabinoid agonist WIN55,212-2 is an effective anti-hyperalgesic agent in three distinct animal models of HIV-associated sensory neuropathy (Table 2.6). Rats treated with the antiretroviral agent zalcitabine (ddc) developed mechanical allodynia that persisted up to 43 days post-injection and peaked between days 14 and 32. No hypersensitivity to thermal stimuli or motor deficits was observed following ddc treatment. HIV-1 has indirect interactions with neurons through its binding affinity to the external envelope binding protein gp120; researchers have exploited this mechanism to demonstrate development of peripheral neuropathy in rodents following exposure of the sciatic nerve to the HIV-1 gp120 protein. Perineural HIV-gp120 together with ddc treatment resulted in mechanical allodynia that was greater than either treatment alone; no changes in paw withdrawal latencies to thermal stimuli or motor deficits were reported. Thigmotaxis was present in animals receiving ddc, either alone or in conjunction with HIV-gp120, indicating the presence of anxiety-like behavior in these rats. Rats receiving ddc displayed modest levels of gliosis whereas combined treatment with both HIV-gp120 and ddc increased levels of
Demyelination-induced Neuropathy. WIN55,212-2 has been evaluated in the lysolecithin-induced demyelination model (Table 2.6). Heightened sensitivity to both non-noxious and noxious mechanical stimulation is observed in lysolecithin-treated rats; this hypersensitivity emerged 5 days post-exposure and peaked between 9-15 days post-exposure. Recovery to baseline levels was observed by day 23 post-lysolecithin. WIN55,212-2 attenuated mechanical allodynia and thermal hyperalgesia in this model and remained efficacious for up to one hour post injection. By contrast, DAMGO failed to produce an effect. Notably, the anti-hyperalgesic and anti-allodynic effects of WIN55,212-2 were reversed by a CB1-specific antagonist in both tests.

Multiple Sclerosis-associated Neuropathy. Animal models of multiple sclerosis (MS) have been described, although to our knowledge, no study to date has evaluated cannabinoid-mediated suppression of MS-induced neuropathic nociception. Lynch and colleagues reported the presence of thermal hyperalgesia (tail immersion) and mechanical allodynia in mice that were infected with Theiler’s murine encephalomyelitis virus (TMEV). Interestingly, female mice showed an increased rate of development and greater allodynia than their male counterparts, a finding which mimics the greater
prevalence of neuropathic pain symptoms reported by female MS patients. Cold and mechanical allodynia, but not thermal hyperalgesia, have been reported in a model of autoimmune encephalomyelitis in which mice were immunized with myelin oligodendrocyte glycoprotein (MOG(35-55)); autoimmune encephalomyelitis has been postulated to underlie the development of neuropathic pain in MS. Interestingly, a mouse model of MS (TMEV infection) is also characterized by an upregulation of CB2 receptor mRNA and increases in levels of 2-AG and PEA. Animals treated subchronically with PEA showed improvements in tests of motor performance, measures that were impaired following TMEV infection. Thus, we postulate that cannabinoid CB2 agonists and modulators of endogenous cannabinoids (e.g. MGL inhibitors) would exhibit anti-allodynic efficacy in this model.

Post Herpetic Neuralgia. Cannabinoids and fatty-acid amides suppress neuropathic nociception in an animal model of post herpetic neuralgia (Table 2.6). However, pharmacological specificity has not been consistently assessed in this model. Approximately 50% of rats exposed to the varicella-zoster virus (VZV) developed mechanical allodynia in the ipsilateral paw by 14 days post-infection; no thermal hyperalgesia or cold allodynia was observed. The PEA analogue L-29 suppressed mechanical allodynia in this model with an earlier onset relative to gabapentin. However, neither a CB1- nor CB2-specific antagonist suppressed L-29 mediated suppression of VZV-induced mechanical allodynia. This finding is perhaps unsurprising given that PPAR-α mediates effects of PEA in suppressing neuronal sensitization. However, L-29 nonetheless suppressed neuropathic nociception in the Seltzer model via activation of CB1 and CB2 receptors (see Table 2.4). Systemic WIN55,212-2, administered from days
18-21 post infection, fully reversed mechanical allodynia to baseline levels in this model of post herpetic neuralgia, although pharmacological specificity was not assessed.\textsuperscript{131}

**Cannabinoid Modulation of Neuropathic Pain in Clinical Studies**

Cannabinoids have been evaluated in clinical studies for their suppression of acute, post-operative and neuropathic pain. Based upon our reviews of the literature, cannabinoids exhibit their greatest efficacy when employed for the management of neuropathic pain (Tables 2.7 and 2.8).\textsuperscript{132} There are approximately 460 known chemical constituents in cannabis. Thus, at the outset, it is important to emphasize that smoked cannabis is not the same as oral $\Delta^9$-THC or different mixtures of $\Delta^9$-THC and cannabidiol (e.g. Sativex® and Cannador®). Other drug delivery mechanisms (e.g. oral-mucosal sprays and rectal suppositories containing cannabinoids) have been developed. Evidence to date from clinical studies suggests that these compounds show therapeutic efficacy in suppressing neuropathic pain (Tables 2.7 and 2.8).

Three of the articles reviewed here used smoking as the route of administration, whereas the other thirteen employed oral preparations in the form of pills or oral-mucosal sprays. Side-effects were reported in all studies in a proportion of patients receiving cannabinoid-based medications. The most frequently reported side-effects were dizziness, impairment of balance, feelings of intoxication, dry mouth and dysgeusia (most commonly observed with oral-mucosal sprays), sedation, and hunger. One study reported severe gastrointestinal effects for 10% of patients taking Sativex® versus 0% reporting similar problems in the placebo group.\textsuperscript{133} However, unwanted side-effects, in contrast to analgesic effects, may undergo tolerance.\textsuperscript{134} Side-effects may be minimized using dosing paradigms employing low doses that are only gradually escalated. Below, we review
effects of cannabinoid-based medications in clinical studies employing populations of patients presenting with neuropathic pain. Neuropathic pain induced by HIV infection and/or antiretroviral treatment, multiple sclerosis, brachial plexus avulsion, mixed treatment-resistant neuropathic pain, and others are considered.

HIV-associated neuropathy. Two studies have examined effects of smoked cannabis for the treatment of HIV-associated sensory neuropathy (resulting from HIV infection, dideoxynucleoside antiretroviral therapy, or both) and have reported positive results (Table 2.7). Abrams and colleagues\textsuperscript{135} reported that 52\% of patients (i.e. 13 out of 25 receiving cannabis cigarettes) experienced a greater than 30\% reduction in pain (visual analogue scale daily ratings; VAS). Stimulus-evoked pain testing revealed that the group receiving cannabis experienced a reduction in the area sensitive to mechanical allodynia (with a foam brush or 26g von Frey hair) in the heat and capsaicin sensitization model. Moreover, CD4+, CD8+, and T-cell counts were not negatively impacted by cannabinoid treatment in HIV patients.\textsuperscript{136} In 2009, Ellis and colleagues\textsuperscript{137} reported similar results in a crossover study employing multiple concentrations of $\Delta^9$-THC in cannabis cigarettes administered to patients. Cannabis was superior to placebo in either phase of the crossover as measured with the descriptor differential scale (DDS) or VAS. This study found no changes in heart rate, blood pressure, plasma HIV RNA (viral load; VL), or blood CD4+ lymphocyte counts following cannabis treatment, suggesting that cannabis did not negatively impact the already compromised immune system in these patients. An anonymous cross-sectional questionnaire study revealed that as many as one-third of patients suffering from HIV have used cannabis to treat symptoms.\textsuperscript{138} Patients reported self-dosing with marijuana primarily between 6 PM and 12 AM. Among the symptoms
improved following cannabis were appetite (97% reported improvement), pain (improved in 94% of the patients with pain), nausea (93% reported improvement) and anxiety (93% reported improvement).\textsuperscript{138}

Dronabinol (Marinol\textregistered) is used to counteract AIDS-related wasting and promote appetite in patients suffering from AIDS-related anorexia.\textsuperscript{139} The benefits of $\Delta^9$-THC and nabilone for the treatment of chemotherapy-induced nausea and vomiting have also been validated.\textsuperscript{140, 141} Thus, several features of cannabinoid pharmacology are particularly desirable for an analgesic intervention aimed at managing neuropathic pain in AIDS and cancer patients.

\textit{Multiple Sclerosis-induced Neuropathic Pain.} Several cannabinoid-based medicines have been evaluated in patients suffering from multiple sclerosis (MS)-related neuropathic pain. Cannabinoid-based medications have more frequently been evaluated for efficacy in suppressing MS-related spasticity.\textsuperscript{142} Dronabinol reduced spontaneous pain intensity as measured with a numerical rating scale (NRS) over a treatment period of 3 weeks\textsuperscript{134} and improved overall pain ratings on the category-rating scale over a treatment period of 15 weeks\textsuperscript{143}. Additionally, this drug improved median radiating pain intensity and pressure threshold,\textsuperscript{134} sleep quality, spasms, and spasticity\textsuperscript{143} in MS patients. Cannador\textsuperscript{®} is a medicinal cannabis preparation containing $\Delta^9$-THC and CBD in a 2:1 ratio. Cannabidiol is a natural constituent in cannabis, which has very low affinity for cannabinoid CB$_1$ and CB$_2$ receptors. It may act as a high potency antagonist of cannabinoid agonists and an inverse agonist at CB$_2$ receptors.\textsuperscript{144} CBD may compete with cannabinoid agonists for cannabinoid receptor binding sites, thereby minimizing psychoactivity of drugs that employ a combination of $\Delta^9$-THC and CBD. CBD’s
antinociceptive effects have additionally been attributed to inhibition of anandamide degradation, the compound’s antioxidant properties, or binding to an unknown cannabinoid receptor.\textsuperscript{144} CBD also acts as an agonist at serotonin 5-HT\textsubscript{1a} receptors.\textsuperscript{144} Cannador\textsuperscript{®}, administered over a treatment period of 15 weeks, improved overall pain ratings as well as sleep quality, spasms, and spasticity on category-rating scales in patients suffering from MS-related neuropathic pain.\textsuperscript{143} A one year double-blind, placebo-controlled follow up study in MS patients demonstrated improved symptoms of pain, spasms, spasticity, sleep, shakiness, energy level, and tiredness following administration of either dronabinol or Cannador\textsuperscript{®}.\textsuperscript{145} This study reported that 74\% of the patients in the placebo group, versus 45\% of the patients receiving cannabinoid-based medications, cited a lack of benefit derived from experimental medication as the reason for discontinuation of the trial.\textsuperscript{145} MS patients receiving Sativex\textsuperscript{®} (a medicinal cannabis extract containing approximately a 1:1 ratio of CBD:Δ\textsuperscript{9}-THC, administered as an oral-mucosal spray) reported significant reductions in pain symptoms as measured with the NRS-11 and neuropathic pain scale (NPS) in a 4-week treatment period double-blind, placebo-controlled study.\textsuperscript{146} Ninety-five percent of the patients in the placebo-controlled study chose to enter a two year open-label study with Sativex\textsuperscript{®}.\textsuperscript{147} Fifty-four percent of the patients completed one year and 44\% of patients completed two years of the study. Twenty-five percent withdrew due to adverse events and 95\% experienced one or more adverse events during the course of treatment. The NRS-11, completed at the end of the trial or upon withdrawal, was not different from the earlier randomized study indicating that Sativex\textsuperscript{®} was still suppressing pain. Additionally, patients did not increase the titration of their dose indicating that no tolerance developed to Sativex\textsuperscript{®}. Most doses of
Sativex® were administered between 6 PM and 12 AM demonstrating that pain symptoms may be at their worst during normal sleeping hours for MS patients. A recent meta-analysis examining six studies of cannabinoid-based medications used for the treatment of MS-related neuropathic pain revealed that cannabis preparations were superior to placebo.\textsuperscript{148}

Increased CB\textsubscript{2} immunoreactivity has been reported in spinal cords derived from multiple sclerosis patients.\textsuperscript{149} Here, greater numbers of microglia/macrophage cells expressing CB\textsubscript{2} immunoreactivity were observed relative to controls.\textsuperscript{149} Thus, cannabinoid-based pharmacotherapies consistently show efficacy for suppressing pain due to multiple sclerosis, a disease state associated with an upregulation of CB\textsubscript{2} receptors in microglia.

\textit{Brachial Plexus Avulsion-induced Neuropathy}. A single study has examined patients with neuropathic pain resulting exclusively from a brachial plexus avulsion (Table 2.8). This study\textsuperscript{150} used a three period crossover design with patients self-administering \textit{\Delta}^{9-}\text{THC}, Sativex®, or placebo for 14-20 days per drug. Both \textit{\Delta}^{9}-\text{THC} and Sativex® reduced the primary outcome measure (Box-Scale 11 ordinal rating scale) in patients suffering from brachial plexus avulsion, indicating a reduction in pain symptoms versus placebo. Sleep quality disturbance scores were improved in patients receiving either active drug versus placebo. Eighty percent of the patients chose to enter an open-label study with Sativex® following completion of this randomized study.

CB\textsubscript{2} receptor immunoreactivity has been reported in normal and injured human DRG neurons, brachial plexus nerves, and neuromas as well as peripheral nerve fibers.\textsuperscript{151} However, upregulation of CB\textsubscript{2} receptor immunoreactivity was specifically observed in
injured human nerve specimens and avulsed DRG obtained during surgery for brachial plexus repair. These observations correspond to preclinical observations of cannabinoid receptor upregulation following nerve injury. However, possible changes in CB1 receptor immunoreactivity, were not evaluated in the human tissue, and therefore cannot be excluded.

**Mixed Neuropathic Pain.** Recruitment of a patient population suffering from a specific form of neuropathic pain can be a difficult prospect; therefore several studies include patients in which neuropathic pain is associated with different disease states or injuries (Table 2.8). A 21 patient study reported that ajulemic acid (CT-3) suppressed mixed forms of neuropathic pain, as assessed with the VAS, in the morning (3 hours after drug administration), but not in the afternoon (8 hours following drug administration). Eighteen of those same patients participated in stimulus-evoked pain testing during the study and patients showed a trend towards decreased mechanical allodynia following CT-3 administration. CT-3 binds with high affinity to both CB1 and CB2 receptors and also binds with low affinity to PPARγ receptors. CT-3 has limited CNS availability, which translates into fewer CB1-mediated side-effects.

Smoking cannabis cigarettes also improved spontaneous pain relief and pain unpleasantness VAS ratings in patients suffering from mixed forms of neuropathic pain, but failed to alter stimulus-evoked pain. This study reported that cannabinoids compounded the decreased neurocognitive performance of patients that was present at baseline. Using an “N of 1” preparation, Notcutt and colleagues determined if patients experienced improvements in pain following a 2 week open-label phase with Sativex® prior to initiation of the double-blind, placebo-controlled crossover phase of the study.
Δ⁹-THC and Sativex®, but not placebo or CBD, reduced the VAS rating of the two worst pain symptoms during the crossover phase. Quality of sleep was improved by all cannabinoid based medications and may, therefore, contribute to the therapeutic potential of the cannabinoids. By contrast, opioid analgesics produce deleterious effects on sleep architecture, including reductions in slow wave sleep and promotion of sleep apnea. A similarly structured study reported improved pain ratings (VAS) and spasticity severity following CBD and Δ⁹-THC in patients with mixed neuropathic pain. Δ⁹-THC and Sativex® additionally improved muscle spasms and spasticity severity.

Sativex® improved pain ratings as measured with the NRS in a five-week double-blind, placebo-controlled study performed in patients experiencing unilateral neuropathic pain. In this study, Sativex® reduced mechanical dynamic and punctate allodynia, and improved sleep disturbances. Seventy-one percent of the patients tested chose to continue to the open label study of Sativex® with 63% withdrawing by the end of the study for various reasons. Nabilone (Cesamet®) decreased measures of spasticity-related pain (11-Point Box Test) in patients experiencing chronic upper motor neuron syndrome (UMNS) associated with a number of pain syndromes. In a retrospective review of patient charts at the Pain Center of the McGill University Health Center from 1999-2003, 75% of patients received some benefit from taking nabilone (whether that came in the form of pain relief, improved sleep, decreased nausea or increased appetite).

Two studies have examined the effects of cannabinoid-based medications in patients suffering from spinal cord injuries. An early case study reported pain relief and improvement in spasticity in a patient with a spinal cord injury following oral Δ⁹-THC.
A later study reported that 18% of the patients with spinal cord injuries reported pain relief following treatment with oral dronabinol (mean 31 mg per day), whereas 23% experienced enhancement of pain, resulting in subsequent withdrawal by several patients.\textsuperscript{163} Changes in experimental design after initiation of the study complicate interpretation of these latter findings.\textsuperscript{163}

\textit{Caveats.} We are aware of only two clinical studies that have failed to report efficacy of cannabinoids, relative to placebo, for treatment of mixed neuropathic pain.\textsuperscript{164, 165} Our analysis of the study by Clermont-Gnamien and colleagues\textsuperscript{165} is restricted to information provided in the abstract, published in English. Both of these studies employed eight or fewer subjects and evaluated dronabinol titrated to a dose of 25 mg/day (where tolerated). The mean dose was 16.6 ± 6.5 mg oral dronabinol in one study\textsuperscript{164} and 15 ± 6 mg in the other study.\textsuperscript{165} The two studies associated with negative outcomes for cannabinoids in managing neuropathic pain shared several common features: 1) evaluation of mixed neuropathic pain syndromes known to be refractory to multiple analgesic treatments, 2) evaluation of orally-administered $\Delta^9$-THC (dronabinol) as opposed to mixtures of $\Delta^9$-THC and CBD, or smoked marijuana, 3) small numbers of subjects, and 4) observation of prominent side-effects (e.g. sedation) resulting in high dropout rates. One study reported side-effects that were more prominent in older patients and did not correlate with analgesia.\textsuperscript{164} Of course, one difficulty in evaluating efficacy of analgesics in patients with neuropathic pain refractory to all known treatments is that there is no indication that these patients would respond favorably to any analgesic under the study conditions. In a third study, effects of nabilone were compared with dihydrocodeine in a randomized, crossover double-blind study of three months duration that did not include a
pharmacologically inert placebo condition. In this latter study,\textsuperscript{166} it was concluded that the weak opioid dihydrocodeine was a statistically better treatment for chronic neuropathic pain than nabilone.\textsuperscript{166} Patients in this study exhibited a mean baseline VAS rating of 69.6 mm on a 0-100 mm VAS scale; mean VAS ratings were 59.93 ± 24.42 mm and 58.58 ± 24.08 mm for patients taking nabilone and dihydrocodeine, respectively. However, the authors noted that a small number of subjects responded well to nabilone and side-effects were generally mild and in the expected range.\textsuperscript{166} Benefits of an add-on treatment with nabilone have nonetheless been noted in patients with chronic therapy-resistant pain (observed in causal relationship with a pathological status of the skeletal and locomotor system).\textsuperscript{167} Oral dronabinol produced significant pain relief versus placebo when combined with opioid therapy in both a double-blind, placebo-controlled crossover phase and a subsequent open-label extension.\textsuperscript{168} Patients additionally reported improvements in sleep problems and disturbances while experiencing an increase in sleep adequacy in the open-label phase of the study.\textsuperscript{168} Thus, caution should be exerted prior to concluding that side-effects of cannabinoids seriously limit the therapeutic potential of cannabinoid pharmacotherapies for pain. Combination therapies including a cannabinoid show efficacy for treatment-resistant neuropathic pain and may be employed to limit doses of analgesics or adjuvants associated with adverse side-effects.

\textit{Side-effects.} Diverse neuropathic pain states (characterized as idiopathic, diabetic, immune-mediated, cobalamin-deficiency related, monoclonal gammopathy-related, alcohol abuse-related and other) were recently examined in a prospective evaluation of specific chronic polyneuropathy syndromes and their response to pharmacological therapies.\textsuperscript{169} Intolerable side-effects were observed in all groups of patients receiving
either gabapentinoids, tricyclic antidepressants, anticonvulsants, cannabinoids (nabilone or Sativex®) and topical agents. Notably, the presence of intolerable side-effects was similar amongst the different classes of medications. In this study, most forms of neuropathic pain had similar prevalence rates and responsiveness to the different pharmacotherapies evaluated.

A recent systematic review of adverse effects of medical cannabinoids concluded that most adverse events (96.6%) were not serious and no serious adverse events were related exclusively to cannabinoid administration. Moreover, 99% of serious adverse events from randomized clinical trials were reported in only two trials. Greater numbers of nonserious adverse events were observed following cannabinoid treatment, as expected. Side-effects were equally associated with the different cannabinoid pharmacotherapies; the average rate of nonserious adverse events was higher in patients receiving Sativex® or oral Δ⁹-THC than controls. Thus, the main burden for the clinician is to balance therapeutic efficacy with the risk of intolerable side-effects in the specific patient. High quality trials of long term exposure to cannabinoid based medications, together with careful monitoring of patients, are required to better characterize safety issues related to use of medical cannabinoids.

Conclusions. Cannabis has been used for pain relief for centuries, although the mechanism underlying their analgesic effects has remained poorly understood until the discovery of cannabinoid receptors and their endogenous ligands in the 1990’s. During the last two decades, a large number of research papers have demonstrated the efficacy of cannabinoids and modulators of the endocannabinoid system in suppressing neuropathic pain in animal models. Cannabinoids suppress hyperalgesia and allodynia (i.e.
mechanical allodynia, mechanical hyperalgesia, thermal hyperalgesia and, where evaluated, cold allodynia), induced by diverse neuropathic pain states through CB1 and CB2-specific mechanisms. These studies have elucidated neuronal as well as nonneuronal (i.e. activated microglia) sites of action for cannabinoids in suppressing pathological pain states and documented regulatory changes in cannabinoid receptors and endocannabinoid accumulation in response to peripheral or central nervous system injury. Clinical studies largely reaffirm that cannabinoids show efficacy in suppressing diverse neuropathic pain states in humans. The psychoactive effects of centrally-acting cannabinoid agonists, nonetheless, represent a challenge for pain pharmacotherapies that directly activate CB1 receptors in the brain. However, nonserious adverse events (e.g. dizziness), which pose the major limitation to patient compliance with pharmacotherapy, are not unique to cannabinoids. Approaches that serve to minimize unwanted CNS side-effects (e.g. by combining Δ9-THC with CBD, or by targeting CB2 receptors, peripheral CB1 receptors or the endocannabinoid system) represent an important direction for future research and clinical evaluation. The present review suggests that cannabinoids show promise for treatment of neuropathic pain in humans either alone or as an add-on to other therapeutic agents. Further evaluation of safety profiles associated with long term effects of cannabinoids are, therefore, warranted.
References


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<th>Natural Cannabinoid Ligands and Synthetic Analogues</th>
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<tr>
<td>• Δ9-THC (Dronabinol/Marinol®)</td>
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<tr>
<td>• Cannabidiol (CBD)</td>
<td>• URB602</td>
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<td>• Nabilone (Cesamet®, Δ9-THC analogue)</td>
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## Table 2.2. Antinociceptive Effects of Cannabinoids following Chronic Constriction Injury in Rats

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eCBD, Cannabis Sativa with high CBD content; pCBD, Pure cannabidiol; pTHC, Pure Δ9-tetrahydrocannabinol; SR1, SR141716; SR2, SR144528; ‡Chronic, post-injury; *Only tested in thermal hyperalgesia and mechanical allodynia; †Increased measurements in contralateral paw at dose/s tested
Table 2.3. Antinociceptive Effects of Cannabinoids following Chronic Constriction Injury in Mice

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<th>Compound</th>
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<th>Mechanism</th>
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PEA, Palmitoylethanolamine; SR1, SR141716; SR2, SR144528; ‡Chronic, post-injury; *Only for thermal hyperalgesia and mechanical alldynia, no blockade observed for mechanical hyperalgesia
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Table 2.4. Antinociceptive Effects of Cannabinoids following Partial Sciatic Nerve Ligation (Seltzer Model)
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AEA, Anandamide; 2-AG, 2-arachydonoylglycerol; AJA, Ajulemic Acid; NaGly, N-arachidonoyl glycine; NP, Not Present; PEA, Palmitoylethanolamine; SR1, SR141716; SR2, SR144528; †Increased measurements in contralateral paw at dose/s tested; $Chronic, pre-emptive/post-injury or both; *Post-injury; ‡Pre-emptive and post-injury combined; *Only observed blockade for mechanical allodynia, not thermal hyperalgesia

Tested in rats; Tested in mice
Table 2.5. Antinociceptive Effects of Cannabinoids following Spinal Nerve Ligation (Traditional and Modified)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Route</th>
<th>Thermal</th>
<th>Mechanical Hyperalgesia</th>
<th>Mechanical Allodynia</th>
<th>Mechanism</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAY 59-3074</td>
<td>p.o.</td>
<td>NP</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No (SR1 i.p.)</td>
<td>Yes</td>
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<tr>
<td>CP55,940</td>
<td>i.p.</td>
<td>—</td>
<td>—</td>
<td>Yes</td>
<td>No (CB1+)</td>
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<tr>
<td></td>
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<td>No (CB1-)</td>
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<tr>
<td></td>
<td>i.t.</td>
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<td>—</td>
<td>Yes</td>
<td>No (SR1 i.t.)</td>
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<tr>
<td>Mixed CB1/CB2 agonists</td>
<td></td>
<td></td>
<td></td>
<td>Yes†</td>
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<tr>
<td>WIN55,212-2</td>
<td>i.p.</td>
<td>—</td>
<td>—</td>
<td>Yes (CB1+)</td>
<td>No+ (SR2 i.p.)</td>
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<td>Yes (CB1+)</td>
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<td></td>
<td>i.p.‡</td>
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<td>—</td>
<td>Yes</td>
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<tr>
<td>AM1241</td>
<td>i.p.</td>
<td>—</td>
<td>—</td>
<td>Yes</td>
<td>No (AM251 i.p.)</td>
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<td></td>
<td></td>
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<td>Yes (CB1+)</td>
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<td>Yes (CB1, CB2+)</td>
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<td>Yes (CB1, CB2+)</td>
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<td>Compound 27</td>
<td>i.p.</td>
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<td>Yes</td>
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<td>GW405833</td>
<td>i.p.‡</td>
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<td>L768242</td>
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<td>MDA19</td>
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<tr>
<td>MDA7</td>
<td>i.p.</td>
<td>—</td>
<td>—</td>
<td>Yes</td>
<td>No (AM251 i.p.)</td>
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<td>Endocannabinoid Modulators</td>
<td>Compound 17</td>
<td>i.v.</td>
<td>—</td>
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<td>Yes</td>
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<tr>
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<td>OL135</td>
<td>i.p.</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Yes</td>
<td>No (SR1 i.p.)</td>
</tr>
</tbody>
</table>

NP, Not Present; SR1, SR141716; SR2, SR144528; †Increased measurements in contralateral paw at dose/s tested; ‡Chronic, post-injury; †Increased measurements in contralateral paw at dose/s tested; ‡Chronic, post-injury; †Increased measurements in contralateral paw at dose/s tested

- Tested in rats; — Tested in mice
<table>
<thead>
<tr>
<th>Model</th>
<th>Compound</th>
<th>Route</th>
<th>Thermal</th>
<th>Mechanical Hyperalgesia</th>
<th>Mechanical Alldynia</th>
<th>Mechanism</th>
<th>CB₁</th>
<th>CB₂</th>
<th>Ref</th>
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<tbody>
<tr>
<td>Diabetic Neuropathy</td>
<td>Met-F-AEA</td>
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<td>Yes</td>
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<tr>
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<td></td>
<td>i.p.‡</td>
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<td>Yes</td>
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<td>WIN55,212-2</td>
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<td>Yes</td>
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<tr>
<td>Chemotherapy-induced Neuropathy</td>
<td>WIN55,212-2</td>
<td>i.p.</td>
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<td>Yes</td>
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<td>Yes (SR1 i.p.)</td>
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<td>i.p.‡</td>
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<td>Yes (SR1 i.p.)</td>
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<td>i.pl.</td>
<td>Yes†</td>
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<td>Yes†</td>
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<td>(R,S)-AM1241</td>
<td>i.p.</td>
<td>NP</td>
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<td>Yes</td>
<td>No (SR1 i.p.)</td>
<td>Yes (SR2 i.p.)</td>
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<tr>
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<td>(R)-AM1241</td>
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<td>AM1714</td>
<td>i.p.</td>
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<td>Yes</td>
<td>No (SR1 i.p.)</td>
<td>Yes (SR2 i.p.)</td>
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<tr>
<td>Vincristine</td>
<td>WIN55,212-2</td>
<td>i.p.</td>
<td>NP</td>
<td>—</td>
<td>Yes</td>
<td>Yes (SR1 i.p.)</td>
<td>Yes (SR2 i.p.)</td>
<td>—</td>
<td>122</td>
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<tr>
<td></td>
<td></td>
<td>i.t.</td>
<td>NP</td>
<td>—</td>
<td>Yes</td>
<td>Yes (SR1 i.t.)</td>
<td>Yes (SR2 i.t.)</td>
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<tr>
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<td></td>
<td>i.pl.</td>
<td>NP</td>
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<td>(R,S)-AM1241</td>
<td>i.p.</td>
<td>NP</td>
<td>—</td>
<td>Yes</td>
<td>No (SR1 i.p.)</td>
<td>Yes (SR2 i.p.)</td>
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<tr>
<td>Other</td>
<td>HIV-SN</td>
<td>WIN55,212-2</td>
<td>i.p.‡</td>
<td>NP-heat NP-cold</td>
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<td>Yes*</td>
<td>—</td>
<td>—</td>
<td>124, 123</td>
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<tr>
<td>L-29</td>
<td>i.p.</td>
<td>WIN55,212-2</td>
<td>i.p.</td>
<td>NP-heat NP-cold</td>
<td>—</td>
<td>Yes</td>
<td>Yes (SR1 i.p.)</td>
<td>Yes (SR2 i.p.)</td>
<td>64</td>
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<td>LDPN</td>
<td>WIN55,212-2</td>
<td>i.t.</td>
<td>Yes</td>
<td>—</td>
<td>Yes</td>
<td>Yes (AM251 i.t.)</td>
<td>—</td>
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<td>VZV</td>
<td>L-29</td>
<td>i.p.</td>
<td>NP-heat NP-cold</td>
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<td>Yes</td>
<td>No (SR1 i.p.)</td>
<td>No (SR2 i.p.)</td>
<td>64</td>
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<tr>
<td></td>
<td>WIN55,212-2</td>
<td>i.p.‡</td>
<td>NP-heat NP-cold</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
<td>131</td>
<td></td>
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</tbody>
</table>

ddc, Zalcitabine; HIV-SN, HIV Sensory Neuropathy (includes antiretroviral treatment (ddc), HIV-gp120, and HIV-gp120 + antiretroviral treatment (ddc) models); LDPN = Lysolecithin-induced Demyelination-associated Peripheral Neuropathy of saphenous nerve; NP, Not Present; SR1, SR141716; SR2, SR144528; VZV, Varicella Zoster Virus-induced neuropathy; ‡Chronic, post-injury; §Chronic, pre-emptive and post-injury; †Increased measurements in contralateral paw at dose/s tested; *In antiretroviral (ddc), HIV-gp120, and HIV-gp120 + antiretroviral (ddc) models; ††Only tested in the antiretroviral (ddc) model

- Tested in rats;
- Tested in mice
<table>
<thead>
<tr>
<th>Compound/Route</th>
<th>Primary Outcome Measure</th>
<th>Stimulus Evoked Pain</th>
<th>Secondary Outcome Measures</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-SN</td>
<td>VAS daily pain ratings – 52% reported &gt; 30% reduction in pain</td>
<td>LTS – No Effect Heat and capsaicin sensitization model – Reduced area sensitive to mechanical allodynia</td>
<td>POMS – No Effect</td>
<td>135</td>
</tr>
<tr>
<td>Cannabis cigarettes (3.56% Δ⁹-THC)* Smoking</td>
<td>DDS and VAS pain ratings – 46% reported ≥ 30% reduction in pain</td>
<td>Median radiating pain intensity/pressure pain threshold – Improved Cold and warm sensibility/tactile detection/tactile pain detection/vibration sense/ temporal summation/ mechanical or cold allodynia – No Effect</td>
<td>SF-36 – Improvements in bodily pain and mental health categories</td>
<td>137</td>
</tr>
<tr>
<td>Dronabinol (Marinol®)‡ p.o.</td>
<td>NRS of median spontaneous pain intensity – Reduction from BL on this measure was 20.5% (-0.6 pt.) with Dronabinol vs. placebo</td>
<td>—</td>
<td>—</td>
<td>134</td>
</tr>
<tr>
<td>Sativex®# Oral-Mucosal Spray</td>
<td>*NRS-11 (pain) – -1.25 pt reduction in favor of Sativex®</td>
<td>—</td>
<td>—</td>
<td>146</td>
</tr>
<tr>
<td>Multiple Sclerosis-related Neuropathic Pain</td>
<td>⁶NRS-11 (pain) – No changes in pain scores from randomized 5-wk trial (up to 2 years) – Sativex® still suppressing pain vs. BL</td>
<td>—</td>
<td>44% of patients completed approximately 2 years of open-label study. No increase in titration of dose – No tolerance</td>
<td>147</td>
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<tr>
<td>Condition</td>
<td>Treatment</td>
<td>Improvement/Effect</td>
<td>Rating Scales</td>
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<td>-------------------------------------</td>
<td>-------------------------------------------------------------------------------</td>
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<tr>
<td>Dronabinol (Marinol®)</td>
<td>Ashworth Spasticity Score – No Effect</td>
<td>Improved pain, sleep quality, spasms and spasticity with CBM</td>
<td></td>
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<tr>
<td>Cannador®</td>
<td></td>
<td>10 m walk – Improved with CBM</td>
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<td></td>
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<td>Rivermead Mobility Index/Barthel Index/GHQ-30/UKNDS – No Effect</td>
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<tr>
<td>Dronabinol (Marinol®)</td>
<td>Ashworth Spasticity Score – Improvement following Dronabinol</td>
<td>Category Rating Scales – Improved pain, spasms, spasticity, sleep, shakiness, energy level and tiredness with CBM</td>
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<tr>
<td>Cannador®</td>
<td></td>
<td>Rivermead Mobility Index/Barthel Index/GHQ-30/UKNDS/10 m walk – No Effect</td>
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</tbody>
</table>

BL, Baseline; BSI, Brief Symptom Inventory; CBM, Cannabinoid Based Medicine; DDS, Descriptor Differential Scale; HADS, Hospital Anxiety and Depression Scale; HIV-SN, HIV-associated Sensory Neuropathy; GHQ, General Health Questionnaire; LTS, Long-term Thermal Stimulation; MS, Multiple Sclerosis; NPS, Neuropathic Pain Scale; NRS, Numerical Rating Scale; PGIC, Patient Global Impression of Change; POMS, Profile of Mood States; SF-36, Short Form Health Questionnaire; SIP, Sickness Impact Profile; UKNDS, United Kingdom Neurological Disability Score; VAS, Visual Analogue Scale; VL, Viral Load; *Double-blind, placebo-controlled; ‡Double-blind, placebo-controlled crossover; #Open label extension of randomized double-blind, placebo-controlled study; $Randomized, placebo-controlled; †Double-blind, placebo-controlled 1 year extension.
### Table 2.8. Effects of Cannabinoids in Injury-related and Mixed Neuropathic Pain in Clinical Studies

<table>
<thead>
<tr>
<th>Compound/Route</th>
<th>Primary Outcome Measure</th>
<th>Stimulus Evoked Pain</th>
<th>Secondary Outcome Measures</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brachial Plexus Avulsion</strong></td>
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<tr>
<td>Sativex®/Δ²-THC‡ Oral Mucosal Spray</td>
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<td></td>
<td>Pain Review BS-11/Sleep Quality BS-11/Sleep Disturbances – Improved with CBM GHQ-12 – Improved with Sativex SF-MPQ Pain Rating Index and VAS – Improved with Δ²-THC PDI - No Effect</td>
<td>150</td>
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<tr>
<td><strong>Mixed Neuropathy</strong></td>
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<tr>
<td>Dronabinol (Marinol®)+ p.o.</td>
<td>VAS daily pain ratings – No Effect</td>
<td>Brush-induced mechanical allodynia – No Effect</td>
<td>MPQ/BPI/HADS/Nottingham Health Profile – No Effect</td>
<td>164</td>
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<tr>
<td>Nabilone (Cesamet®)/DHC§ p.o.</td>
<td>VAS daily pain ratings – DHC better than Nabilone</td>
<td></td>
<td>SF-36 – Physical Role improved with Nabilone; Bodily pain improved with DHC</td>
<td>166</td>
</tr>
<tr>
<td>CT-3 (AJA)‡ p.o.</td>
<td>VAS (pain) – CT-3 reduced pain ratings in the morning (3 hrs. post-drug), but not afternoon (8 hrs. post-drug) VRS (pain) – No Effect</td>
<td>Decrease in mechanical hypersensitivity (von Frey) in group receiving AJA prior to placebo (P = 0.052)</td>
<td>TMT; ARCI-M – No effect</td>
<td>152, 153</td>
</tr>
<tr>
<td>Cannabis cigarettes (3.5-7% Δ²-THC)‡ Smoking</td>
<td>Spontaneous Pain Relief VAS - Improved</td>
<td>Mechanical Allodynia (Foam Brush) VAS; Thermal Hyperalgesia VAS – No effect</td>
<td>Pain Unpleasantness VAS/NPS – Improved Degree of Pain Relief PGIC/ Psychoactive effects/Neurocognitive Effects – Greater with Cannabis Mood VAS – No Effect</td>
<td>155</td>
</tr>
<tr>
<td>Δ²-THC/CBD/Sativex®‡ Oral-Mucosal Spray</td>
<td>VAS of 2 worst symptoms – Decrease in symptoms following Δ²-THC and Sativex® relative to placebo</td>
<td></td>
<td>Quality of sleep – Improved with all CBM Duration of sleep – No Effect BDI/GHQ-28– Qualitative improvement in mood following CBM</td>
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</tr>
<tr>
<td>Condition</td>
<td>CBM</td>
<td>Effect</td>
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<tr>
<td>---------------------------------</td>
<td>------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------</td>
<td></td>
<td></td>
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<tr>
<td>VAS Daily Ratings of target symptoms – CBD and Δ⁹-THC improved pain; Δ⁹-THC and Sativex® improved spasms; Δ⁹-THC improved spasticity</td>
<td>—</td>
<td>Numerical Symptom Scale – Spasticity severity improved with all CBM; frequency of muscle spasms improved with Δ⁹-THC and Sativex® VAS daily ratings – Δ⁹-THC improved appetite; Sativex® improved sleep</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unilateral Mixed Neuropathy</td>
<td>Sativex®* Oral-Mucosal Spray</td>
<td>NRS (pain) – -1.48 pt. reduction (22%) in Sativex condition vs. -0.52 pt. (8%) reduction in placebo condition</td>
<td>Mechanical Dynamic Allodynia NRS – Reduction with Sativex® Punctate Mechanical Allodynia – No Effect</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Sleep Disturbances NRS/ NPS/PDI/PGIC (neuropathic pain)/PGIC (pain at allodynic sites) – Improved with Sativex® GHQ-12/BRB-N – No Effect</td>
<td></td>
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<tr>
<td>UMNS</td>
<td>Nabilon (Cesamet®)‡ p.o.</td>
<td>11-Point Box Test of spasticity-related pain – Decreased a median of 2 pts. with Nabilone vs. placebo</td>
<td>Ashworth Score/Rivermead Motor Assessment/Barthel Index – No Effect</td>
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ARCI-M, Addiction Research Center Inventory-Marijuana; BDI, Body Disability Index; BPI, Wisconsin Brief Pain Inventory; BRB-N, Brief Repeatable Battery of Neuropsychological Tests; BS-11, Box Scale; CBM, Cannabinoid Based Medication; DHC, Dihydrocodeine, GHQ, General Health Questionnaire; HADS, Hospital Anxiety and Depression Scale; MPQ, McGill Pain Questionnaire; NPS, Neuropathic Pain Scale; NRS, Numerical Rating Scale; PDI, Pain Disability Index; PGIC, Patient Global Impression of Change; SF-36, Short Form Health Questionnaire; SF-MPQ, Short Form of McGill Questionnaire; TMT, Trial Making Test; UMNS, (chronic) Upper Motor Neuron Syndrome; VAS, Visual Analogue Scale; VRS, Verbal Rating Scale; ‡Double-blind, placebo-controlled crossover; *Open-label, no placebo; $ Double-blind, crossover *Double-blind, placebo-controlled.
CHAPTER 3

ANTINOCICEPTIVE EFFECTS OF RACEMIC AM1241 AND ITS CHIRALLY SYNTHESIZED ENANTIOMERS: LACK OF DEPENDENCE UPON OPIOID RECEPTOR ACTIVATION

Abstract

Cannabinoid CB$_2$ receptors represent a therapeutic target that circumvents unwanted central side-effects (e.g. psychoactivity and/or addiction) associated with activation of CB$_1$ receptors. One of the primary investigative tools used to study functions of the CB$_2$ receptor is the aminoalkylindole (R,S)-AM1241. However, (R,S)-AM1241 has been described as an atypical CB$_2$ agonist which produces antinociception mediated indirectly by opioid receptors. (R,S)-AM1241 and its enantiomers, (R)-AM1241 and (S)-AM1241, were evaluated for antinociception in response to thermal (Hargreaves) and mechanical (von Frey) stimulation. Pharmacological specificity was established using antagonists for CB$_1$ (Rimonabant [SR141716]) and CB$_2$ (SR144528). The opioid antagonist naloxone was administered locally in the paw or systemically to evaluate the contribution of opioid receptors to CB$_2$-mediated antinociception produced by (R,S)-AM1241, (R)-AM1241, and (S)-AM1241. Comparisons were made with the opioid analgesic morphine. (R,S)-AM1241, (R)-AM1241, and (S)-AM1241 (0.033-10 mg/kg i.p.) produced antinociception to thermal, but not mechanical, stimulation of the hindpaw in naive rats. Antinociception produced by (R,S)-AM1241 and (S)-AM1241 exhibited an inverted U-shaped dose response curve. (R)-AM1241 produced greater antinociception than either (S)-AM1241 or (R,S)-AM1241 at the lowest (0.033 and 0.1 mg/kg i.p.) and highest (10 mg/kg i.p.) doses. Similar levels of antinociception were observed at intermediate doses. (R,S)-AM1241, (R)-AM1241, and (S)-AM1241 each produced CB$_2$-mediated antinociception that was blocked by SR144528 but not by Rimonabant. Local and systemic naloxone blocked morphine-induced antinociception but did not block antinoicceptive effects of (R,S)-AM1241, (R)-AM1241 or (S)-AM1241. The antinociceptive effects of the CB$_2$-
selective cannabinoid (R,S)-AM1241, and its enantiomers, (R)-AM1241 and (S)-AM1241 are not dependent upon opioid receptors.

**Key Words:** cannabinoid, CB$_2$, antinociception, endogenous opioid, naloxone, pain
Abbreviations: i.p., intraperitoneal; i.paw, intra-paw
Introduction

*Cannabis sativa* has been used for both medicinal and recreational purposes throughout recorded history. The discovery by Gaoni and Mechoulam (1) of Δ⁹-tetrahydrocannabinol (Δ⁹-THC), the major psychoactive ingredient in marijuana, ushered in a new era of research focused on understanding the functional roles of cannabinoid receptors in the nervous system. The cloning of cannabinoid CB₁ and CB₂ receptors and isolation of their endogenous ligands (endocannabinoids) marked a transition in the cannabinoid field. Cannabinoids could no longer be thought of merely as illicit drugs of abuse, but rather represented pharmacological tools for studying the functional roles of CB₁ and CB₂ receptors in the nervous system. Activation of cannabinoid CB₁ and CB₂ receptors suppresses pathological pain in animal models (for review see 2, 3, 4). CB₁ receptors are localized primarily within the central nervous system (CNS) (5) and are associated with the rewarding aspects of several addictive compounds including nicotine, alcohol, and cocaine (6). Activation of CB₁ receptors produces hypothermia, motor ataxia, catalepsy, and hypoactivity (for review see 7). The discovery of the CB₂ receptor opened the door to exploring the role of this receptor as a therapeutic target for pain and inflammation. CB₂ receptors are localized preferentially, but not exclusively (8, 9), to immune cells in the periphery (10, 11) and are upregulated in the CNS in pathological pain states (12-15). CB₂ agonists lack centrally-mediated side-effects (16, 17), suggesting that they represent a promising therapeutic target for producing antinociception in the absence of unwanted side-effects such as psychoactivity or addiction. Thus, the CB₂ receptor offers the potential to separate analgesic properties of cannabinoids and drug abuse liability. A key pharmacological tool for studying the functional roles of the CB₂
receptor has been the aminoalkylindole \((R,S)\text{-AM1241}\) (for review see 3). Because this compound has been widely used as a research tool, it is important to fully characterize the pharmacological properties of this compound and its two enantiomers \((R)\text{-AM1241}\) and \((S)\text{-AM1241}\).

\((R,S)\text{-AM1241}\), the CB\(_2\) agonist that has most penetrated the literature, has proven an important research tool for investigating CB\(_2\)-mediated antinociception. \((R,S)\text{-AM1241}\) produces antinociception following local (i.paw) and systemic administration in naive rats (16). Behavioral, neurochemical and electrophysiological studies suggest that \((R,S)\text{-AM1241}\) suppresses persistent pain through a CB\(_2\)-specific mechanism (see 3 for review). \((R,S)\text{-AM1241}\) behaves as a CB\(_2\) agonist \textit{in vivo} and a protean agonist \textit{in vitro} (18). In cAMP inhibition assays, \((R,S)\text{- and (R)}\text{-AM1241 are inverse agonists, whereas (S)}\text{-AM1241 is an agonist (19). Antinociception produced by (R,S)\text{-AM1241 has been attributed to an indirect modulation of the endogenous opioid system (16, 20); in naive rats (R,S)\text{-AM1241-induced antinociception is blocked by local injection of naloxone in the paw (20). The report on (R,S)}\text{-AM1241’s purported mechanism of action has motivated testing of novel CB\(_2\) agonists for modulation of the endogenous opioid system. Several compounds have recently been described which differ from (R,S)\text{-AM1241 on this basis (21-23). (S)}\text{-AM1241, which exhibits lower affinity for CB\(_2\) than (R)\text{-AM1241, shows greater efficacy than (R)}\text{-AM1241 in suppressing visceral and inflammatory pain (19). It remains unknown whether preferential efficacy of (S)\text{-AM1241 is observed in naive rats or is attributable to altered CB\(_2\) receptor levels in persistent pain states. Moreover, it remains unclear whether naloxone-sensitivity is a feature of racemic (R,S)\text{-AM1241 or could be restricted to either of its enantiomers.}
We evaluated antinociceptive properties of (R,S)-AM1241 (Kᵢ: CB₁ vs. CB₂: 239.4 nM vs. 3.41 nM) and its enantiomers (R)-AM1241 (Kᵢ: CB₁ vs. CB₂: 139.7 nM vs. 1.4 nM) and (S)-AM1241 (Kᵢ: CB₁ vs. CB₂: 2.03 μM vs. 160.5 nM) (24) in tests of thermal and mechanical sensitivity in naive rats. Pharmacological specificity was evaluated using selective antagonists for CB₁ (Rimonabant [SR141716]), CB₂ (SR144528) and opioid (naloxone) receptors. (R,S)-AM1241, (R)-AM1241 and (S)-AM1241 (Fig 3.1) were evaluated for naloxone sensitivity and compared with morphine.

Materials and Methods

Subjects

Three hundred and sixty adult male Sprague Dawley rats (300-400 g; Harlan, Indianapolis, IN) were used in these experiments. All animals were maintained on a 12 hr light/12 hr dark cycle (7:00 - 19:00) in a temperature controlled facility. Animals were single housed and had access to food and water ad libitum. 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 (25). Animal experiments were conducted in full compliance with local, national, ethical and regulatory principles and local licensing regulations of Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International’s expectations for animal care and use/ethics committees.

Drugs and Chemicals

(R,S)-AM1241, ((R,S)-(2-iodo-5-nitrophenyl)-[1-((1-methyl-piperidin-2-yl)methyl)-1H-indol-3-yl]-methanone), (R)-AM1241, and (S)-AM1241 were synthesized starting from racemic N-methyl-2-hydroxymethyl piperidine which was resolved by
fractional crystallization of the diastereoisomeric dibenzoyltartaric acid salts and this material was used for synthesis of the respective enantiomeric products. The enantiomeric purity of the chiral products was determined using chiral HPLC analysis on CHIRALPAC® AD-H analytical column. Rimonabant (5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide) and SR144528 (5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-N-(1,3,3-rimethylbicyclo[2.2.1]heptan-2-yl)-1H-pyrazole-3-carboxamide) were provided by NIDA. Naloxone hydrochloride dihydrate, morphine sulfate and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO). All drugs delivered intraperitoneally (i.p.) were dissolved in a vehicle of 100% DMSO. This is the same vehicle that has been employed in previous work (16, 20, 26, 27). Cannabinoids were dissolved in a volume of 1 ml/kg bodyweight with the following exceptions. Morphine was dissolved in DMSO and administered subcutaneously (s.c.) in a volume of 1 ml/kg. Thus, the volume of DMSO administered was uniform between animals in all studies involving systemically administered agonists. Naloxone was dissolved in saline and administered locally into the dorsal surface of the paw (intra-paw; 50 µl volume) as described previously (20) or intraperitoneally in a volume of 1 ml/kg.

**General Experimental Methods**

Baseline responses to mechanical stimulation to the hindpaw were evaluated at least 1 hour prior to evaluation of baseline responses to thermal stimulation. In a subset of experiments (approximately 25%), the order of baseline testing was reversed (i.e. baseline responses to thermal stimulation were assessed at least 1 h prior to evaluation of baseline responses to mechanical stimulation). This modification enabled us to confirm
that hypersensitivity to thermal or mechanical stimulation was not produced by the order of testing mechanical and thermal responses (data not shown). Following completion of baseline testing, all rats were returned to their home cages for approximately 2 h prior to administration of drug or vehicle. All studies were conducted by a single experimenter who was blinded to the drug conditions. Animals were randomly assigned to drug or vehicle treatments.

**Assessment of Mechanical Withdrawal Thresholds and Thermal Paw Withdrawal Latencies**

Mechanical withdrawal thresholds were assessed using a digital Electrovonfrey Anesthesiometer (IITC model Alemo 2290-4; Woodland Hills, CA) equipped with a rigid tip. Rats were placed underneath inverted plastic cages and positioned on an elevated mesh platform. Rats were allowed 10-15 min to habituate to the chamber prior to testing. Stimulation was applied to the midplantar region of the hind paw through the floor of a mesh platform. Mechanical stimulation was terminated upon paw withdrawal; consequently, there was no upper threshold limit set for termination of a trial. Mechanical paw withdrawal thresholds are reported as the mean of duplicate determinations averaged across paws.

Paw withdrawal latencies to radiant heat were measured in duplicate for each paw using the Hargreaves test (28) and a commercially available plantar stimulation unit (IITC model 336; Woodland Hills, CA). Rats were placed underneath inverted plastic cages positioned on an elevated glass platform. Rats were allowed 10-15 min to habituate to the chamber prior to testing. Radiant heat was presented to the midplantar region of the hind paw through the floor of the glass platform. The intensity of the heat
Baseline mechanical withdrawal thresholds and thermal paw withdrawal latencies were assessed prior to pharmacological manipulations. Mechanical paw withdrawal thresholds were assessed at 15 min following injection of drug or vehicle. The 15 minute time point was selected because the antinociceptive dose-response profile of (R,S)-AM1241 to thermal stimulation in the Hargreaves test has been previously characterized at this time point following systemic administration (16). Thermal paw withdrawal latencies were subsequently measured in the same animals at 30, 60 and 120 minutes post-injection to assess the time course of CB2 agonist actions.

The antinociceptive effects of aminoalkylindole CB2 agonists were evaluated for responsiveness to mechanical (electrovonfrey) and thermal (in the Hargreaves test) stimulation. Separate groups of animals received either racemic (R,S)-AM1241 (0.033, 0.1, 0.330, 1, 5 or 10 mg/kg i.p.; n = 7-8 per group), chiral (R)-AM1241 (0.033, 0.1, 0.33, 1, 5 or 10 mg/kg i.p.; n = 8 per group), chiral (S)-AM1241 (0.033, 0.1, 0.33, 1, 5 or 10 mg/kg i.p.; n = 8 per group) or vehicle (n = 19). Separate groups received the opioid agonist morphine (2 mg/kg s.c.; n = 8).

To determine pharmacological specificity, either the CB1 antagonist Rimonabant (6 mg/kg i.p.) or the CB2 antagonist SR144528 (6 mg/kg i.p.) was co-administered with either (R,S)-AM1241 (1 mg/kg i.p., n = 7-8 per group), (R)-AM1241 (1 mg/kg i.p., n = 8
per group) or (S)-AM1241 (1 mg/kg i.p., n = 8 per group). Rimonabant (6 mg/kg i.p., n = 8 per group) and SR144528 (6 mg/kg i.p.; n = 8) were administered to separate groups of animals to evaluate possible antagonist-induced changes in basal nociceptive thresholds.

To evaluate whether opioid receptors contributed to the antinociceptive effects of CB₂ agonists from the aminoalkylindole class, (R)-AM1241 (1 mg/kg), (S)-AM1241 (1 mg/kg), (R,S)-AM1241 (1 mg/kg and 0.33 mg/kg i.p.), or morphine (2 mg/kg s.c.) was administered in tandem with a local injection of naloxone in the dorsal surface of the paw (10 μg or 50 μg i.paw; n = 6-8 per group). Additional groups received dorsal paw injections of either naloxone (10 μg or 50 μg i.paw; n = 8 per group) or saline (n = 8). Right or left paw injections were counterbalanced between subjects. Paw withdrawal thresholds and latencies were measured in both the injected and non-injected paw for all animals at baseline and all post-injection time points.

In a separate study, groups of animals received naloxone (10 mg/kg i.p.) 20 min prior to injection of either (R,S)-AM1241 (1 mg/kg i.p.; n = 8), (R)-AM1241 (1 mg/kg i.p.; n = 8), (S)-AM1241 (1 mg/kg i.p.; n = 8), or morphine (2 mg/kg s.c.; n = 8). A separate group of animals received naloxone alone (10 mg/kg i.p.; n = 8).

**Statistical Analyses**

Data were analyzed using analysis of variance (ANOVA) for repeated measures, one-way ANOVA or planned comparison Student t-tests, as appropriate. SPSS 16.0 (SPSS Incorporated, Chicago, IL, USA) statistical software was employed. The Greenhouse-Geisser correction was applied to the interaction term of all repeated factors. Degrees of freedom reported for interaction terms of repeated factors are the uncorrected values. Post hoc comparisons between control groups and other
Experimental groups were performed using the Dunnett test. Post-hoc comparisons between different experimental groups were performed to assess dose-response relationships and pharmacological specificity using the Tukey test. $P \leq 0.05$ was considered statistically significant.

**Results**

**General Results**

Thermal paw withdrawal latencies and mechanical withdrawal thresholds did not differ between right or left paw for any group with the exception of studies in which i.paw injections were administered unilaterally. Therefore, withdrawal thresholds are presented as the mean of duplicate measurements, averaged across paws, in all studies not employing i.paw injections. In all studies, baseline paw withdrawal latencies or mechanical withdrawal thresholds were similar between groups prior to administration of drug or vehicle. Baseline thermal paw withdrawal latencies did not differ between groups; therefore, baselines in the log dose response plots (Fig 3.2) were averaged across all doses of the same drug for statistical analyses. Moreover, thermal paw withdrawal latencies and mechanical withdrawal thresholds did not differ based upon the order of thermal and mechanical testing at baseline; therefore, the two vehicle groups are combined for all studies presented.

$(S)$-AM1241 (10 mg/kg i.p.) induced seizure-like activity (e.g. wet dog shakes, muscle spasms, foaming at the mouth, etc…) in two animals tested. No other animals tested with $(S)$-AM1241 at this or lower doses showed evidence of similar symptoms.

**Responses to Mechanical Stimulation**
Systemic administration of morphine (2 mg/kg s.c.) increased paw withdrawal thresholds to von Frey stimulation relative to baseline pre-injection thresholds (pre-injection vs. post-injection: 65.7 ± 3.1 g vs. 79.0 ± 4.0 g; \(P < 0.05\) planned comparison t-test) (Supplementary Table 3.1). By contrast, neither \((R,S)\)-AM1241 nor \((R)\)-AM1241 nor \((S)\)-AM1241 altered mechanical withdrawal thresholds relative to either baseline or vehicle treatment at the same post-injection time point (see Supplementary Table 3.1). Naloxone treatment completely blocked morphine-induced antinociception to mechanical stimulation (Supplementary Table 3.2). However, naloxone, administered either locally or systemically, did not alter paw withdrawal thresholds when administered either alone or in combination with CB2 specific agonists relative to either baseline (pre-injection) thresholds or vehicle treatment (data not shown). Cannabinoid antagonist co-administration did not alter mechanical withdrawal thresholds in any study (see Supplementary Table 3.2), with one exception. Co-administration of Rimonabant (6 mg/kg i.p.) with \((R,S)\)-AM1241 (1 mg/kg i.p.) increased paw withdrawal thresholds relative to the vehicle condition (pre-injection vs. post-injection: 67.6 ± 4.1 g vs. 107.6 ± 9.5 g; \(F_{9,81} = 2.93, P < 0.01; P < 0.001\) for relevant comparison), all other drug conditions \((P < 0.05\) planned comparison t-tests), and baseline (pre-injection) thresholds \((F_{9,81} = 2.90, P < 0.01; P < 0.01\) planned comparison t-test) (Supplementary Table 3.2).

**The Aminoalkylindole \((R,S)\)-AM1241 and its Enantiomers Produce Antinociception to Thermal but not Mechanical Stimulation**

\((R,S)\)-AM1241 (0.33, 1, and 5 mg/kg i.p.) increased thermal paw withdrawal latencies relative to vehicle treatment at 30 minutes post-injection \((F_{6,59} = 5.71, P < 0.001; P < 0.05\) for each comparison). \((R,S)\)-AM1241 (0.033, 0.33, 1, 5, and 10 mg/kg
i.p.) also increased paw withdrawal latencies relative to baseline at this time point ($F_{6,87} = 13.64, P < 0.001; P < 0.05$ for each comparison; Fig 3.2a). An inverted U-shaped dose response curve was observed at the time point of maximal antinociception (30 min post drug); ($R,S$)-AM1241 (1 mg/kg i.p.) produced greater antinociception than either the two lowest (0.033 and 0.1 mg/kg i.p.) or the highest (10 mg/kg i.p.) doses ($P < 0.05$ for each comparison).

The entire range of doses of ($R$)-AM1241 (0.033, 0.1, 0.33, 1, and 10 mg/kg i.p.) increased thermal paw withdrawal latencies relative to the vehicle condition at 30 minutes post-injection ($F_{6,60} = 8.71, P <0.001; P < 0.001$ for each comparison). All doses of ($R$)-AM1241 also produced antinociception relative to baseline measurements ($F_{6,89} = 24.74, P < 0.001; P < 0.001$ for each comparison, Fig 3.2b).

($S$)-AM1241 (0.1, 0.33, 1, and 5 mg/kg i.p.) increased thermal paw withdrawal latencies relative to vehicle at 30 minutes post-injection ($F_{6,60} = 4.40, P <0.001; P < 0.01$ for each comparison). ($S$)-AM1241 (0.1, 0.33, 1, 5, and 10 mg/kg i.p.) also produced thermal antinociception relative to baseline at this time point ($F_{6,89} = 16.43, P < 0.001; P < 0.05$ for each comparison; Fig 3.2c).

**Comparison of Antinociceptive Effects of Racemic ($R,S$)-AM1241 and its Enantiomers**

Comparisons were made between the antinociceptive effects of racemic ($R,S$)-AM1241 and the enantiomers ($R$)- and ($S$)-AM1241 across the entire dose range. At the time point of maximal antinociception (30 min post-injection), differences in the magnitude of antinociception, relative to baseline, were noted between groups ($F_{17,125} = 2.81, P < 0.001$). Planned comparisons at this time point revealed that the lowest doses of ($R$)-AM1241 (0.033 and 0.1 mg/kg i.p.) produced greater antinociception than either
(S)-AM1241 \((P < 0.05; \text{Fig }3.2d)\) or \((R,S)\)-AM1241 \((P < 0.05)\) at the same doses. The highest dose of \((R)\)-AM1241 \((10 \text{ mg/kg i.p.})\) also produced greater antinociception relative to the same dose of \((R,S)\)-AM1241 \((P < 0.05, \text{planned comparison t-test})\).

Comparisons were subsequently made between the antinociceptive effects of \((R,S)\)-AM1241, \((R)\)-AM1241, and \((S)\)-AM1241, relative to the DMSO control condition, across the full 120 minute time course. The lowest \((0.033 \text{ mg/kg})\), middle \((1 \text{ mg/kg i.p.})\) and highest \((10 \text{ mg/kg i.p.})\) doses were selected for comparison. \((R)\)-AM1241 \((0.033 \text{ mg/kg i.p.})\) produced antinociception relative to all other groups tested at 30 minutes post-injection \((F_{3,39} = 8.89, P < 0.001; P < 0.05 \text{ for each comparison}; \text{Fig }3.3a)\).

Antinociceptive effects of the lowest dose of \((R)\)-AM1241 were notably absent at subsequent time points \((P > 0.25)\). Racemic AM1241 and \((S)\)-AM1241 \((0.033 \text{ mg/kg i.p.})\) failed to produce an antinociceptive effect relative to the DMSO condition at 30 min post-injection \((P > 0.29 \text{ for each comparison})\). Both racemic \((R,S)\)-AM1241 \((1 \text{ mg/kg i.p.})\), and the enantiomers \((R)\)-AM1241 \((1 \text{ mg/kg i.p.})\) and \((S)\)-AM1241 \((1 \text{ mg/kg i.p.})\), produced thermal antinociception in the plantar test at 30 minutes post-injection relative to the DMSO control condition \((F_{3,39} = 15.59, P < 0.001; P < 0.001 \text{ for each comparison}; \text{Fig }3.3b)\). Only \((S)\)-AM1241 \((1 \text{ mg/kg i.p.})\) produced an antinociceptive effect at 60 minutes post-injection \((F_{3,39} = 2.87, P < 0.05; P < 0.05 \text{ for relevant comparison})\).

However, both \((R)\)-AM1241 \((1 \text{ mg/kg i.p.})\) and \((S)\)-AM1241 \((1 \text{ mg/kg i.p.})\) produced antinociception at 120 minutes post-injection \((F_{3,39} = 6.55, P < 0.01; P < 0.05 \text{ for each comparison})\), whereas \((R,S)\)-AM1241 \((1 \text{ mg/kg i.p.})\) failed to do so \((P > 0.26)\). The highest dose of \((R)\)-AM1241 \((10 \text{ mg/kg i.p.})\) also produced antinociception relative to the vehicle condition at 30 minutes post-injection \((F_{3,39} = 5.40, P < 0.01; P < 0.001 \text{ for each comparison})\).
relevant comparison). Antinociceptive effects of \((R)-AM1241\) (10 mg/kg i.p.) were still present at 120 minutes post-injection (60 min: \(F_{3,39} = 5.45, P < 0.01; P < 0.05\) for relevant comparison; 120 min: \(F_{3,39} = 4.368, P < 0.05; P < 0.05\) for relevant comparison; Fig 3.3c). Antinociceptive effects of the highest dose of either \((R,S)-AM1241\) (10 mg/kg i.p.) or \((S)-AM1241\) (10 mg/kg i.p.) were notably absent at all time points \((P > 0.12\) for each comparison).

**Pharmacological Specificity**

Pharmacological specificity was evaluated using doses of \((R,S)-AM1241\), \((R)-AM1241\), and \((S)-AM1241\) that produced maximal antinociception (1 mg/kg i.p.) for all compounds. \((R,S)-AM1241\), \((R)-AM1241\), and \((S)-AM1241\) produced antinociception to thermal stimulation relative to baseline measurements \((P < 0.05)\). As expected, \((R,S)-AM1241\) (1 mg/kg i.p.) produced thermal antinociception in the plantar test that was blocked by SR144528 (6 mg/kg i.p.) but not by Rimonabant (6 mg/kg i.p.) at 30 minutes post-injection \((F_{3,39} = 18.20, P < 0.001; P < 0.01\) for each comparison; Fig 3.4a). Antinociception produced by either \((R)-AM1241\) (1 mg/kg i.p.) \((F_{3,39} = 7.88, P < 0.001; P < 0.05\) for each comparison; Fig 3.4b) or \((S)-AM1241\) (1 mg/kg i.p.) \((F_{3,39} = 6.56, P < 0.01; P < 0.05\) for each comparison; Fig 3.4c) was blocked by SR144528 (6 mg/kg i.p.), but not Rimonabant (6 mg/kg i.p.), at the same time point. Similar effects were observed for \((R)-AM1241\) (1 mg/kg i.p.) at 120 minutes \((F_{3,39} = 7.10, P < 0.01; P < 0.05\) for each comparison; Fig 3.4b) post-injection. However, ANOVA failed to reveal a reliable antinociceptive effect of \((S)-AM1241\) (1 mg/kg i.p.) at 120 min post drug \((P = 0.064)\). Planned comparisons suggested that \((S)-AM1241\) (1 mg/kg i.p.), administered either alone or together with Rimonabant (6 mg/kg i.p.), produced antinociception at this time.
point relative to the vehicle condition ($P < 0.05$ for each planned comparison t-test).

Rimonabant (6 mg/kg i.p.) and SR144528 (6 mg/kg i.p.) did not alter thermal paw withdrawal latencies relative to vehicle at either 30 ($P > 0.66$) or 120 ($P > 0.88$) minutes post-injection (Fig 3.4d).

**Role of Opioid Receptors in Cannabinoid CB2-mediated Antinociception**

To evaluate the contribution of peripheral opioid receptors to AM1241-induced antinociception, we employed a local dose of naloxone validated previously to block the antinociceptive effects of systemic AM1241 (0.1 mg/kg i.p.) in otherwise naive rats (20). Morphine (2 mg/kg s.c.) produced naloxone-sensitive peripheral antinociception in the plantar test at 30 minutes post-injection in our study; this effect was completely blocked by local injection of naloxone (10 $\mu$g i.paw). A peripheral site of action for this blockade was confirmed by the fact that thermal paw withdrawal latencies remained elevated, relative to baseline ($F_{3,37} = 13.17$, $P < 0.001$; Fig 3.5a) and vehicle treatment ($F_{3,37} = 17.67$, $P < 0.001$; $P < 0.01$ for each comparison), in the non-injected paw following systemic morphine administration. Morphine produced antinociception relative to the DMSO condition at 120 minutes post-injection ($F_{3,37} = 5.41$, $P < 0.01$; $P < 0.05$ for relevant comparison; data not shown). However, at this time point locally-injected naloxone was no longer blocking morphine antinociception ($P > 0.98$). Due to lack of efficacy of naloxone blockade at 120 minutes, data presented in Figure 3.5 is restricted to the 30 minute time point. The dose of naloxone (10 $\mu$g i.paw) which completely blocked the antinociceptive effects of morphine (2 mg/kg s.c.) failed to block the antinociceptive effects of either ($R$)-AM1241 (1 mg/kg i.p.) ($F_{3,39} = 17.58$, $P < 0.001$, $P < 0.001$ for each comparison; Fig 3.5b) or ($S$)-AM1241 (1 mg/kg i.p.) ($F_{3,39} = 12.67$, $P < 0.001$; $P < 0.01$.
for each comparison; Fig 3.5c). Moreover, naloxone (10 μg i.p) (F_{3,39} = 5.63, P < 0.01; P < 0.05 for each comparison; Fig 3.5d) and a five-fold higher dose (50 μg i.paw) (F_{3,39} = 11.33, P < 0.01; Fig 3.5e) failed to block the antinociceptive effects of (+,−)-AM1241 (0.33 mg/kg i.p.) relative to vehicle treatment (P < 0.05 for each comparison).

Additionally, naloxone (50 μg i.paw) also failed to block the antinociceptive effects of a higher, more efficacious dose of (+,−)-AM1241 (1 mg/kg i.p.) relative to the vehicle condition (F_{3,39} = 9.33, P < 0.001; P < 0.05 for each comparison; Fig 3.5f). Under these conditions, naloxone (10 μg and 50 μg i.paw) did not alter paw withdrawal latencies in either the injected or non-injected paw relative to animals that received local injections of saline (P > 0.72; data not shown).

Systemic administration of naloxone (10 mg/kg i.p.) blocked thermal antinociception produced by morphine (2 mg/kg s.c.) at 30 minutes post-injection (F_{3,39} = 12.78, P < 0.001; P < 0.001 for each comparison Fig 3.6a), whereas naloxone (10 mg/kg i.p.) alone did not alter paw withdrawal latencies (P > 0.55 for relevant comparison).

Morphine (2 mg/kg s.c.) produced an antinociceptive effect at 120 minutes post-injection relative to either vehicle treatment (F_{3,39} = 3.52, P < 0.05; P < 0.05 for relevant comparison) or baseline pre-injection thresholds (F_{3,39} = 3.47, P < 0.05). However, systemic naloxone (10 mg/kg i.p.) failed to block these observed antinociceptive effects (P > 0.62), suggesting that the duration of action of naloxone blockade was less than 2 h. Data presented in Figure 3.6 is consequently restricted to the 30 minute time point.

Naloxone (10 mg/kg i.p), administered at a dose that completely blocked the antinociceptive effects of morphine (2 mg/kg s.c.) in the same test failed to block thermal antinociception produced by either (+)-AM1241 (1 mg/kg i.p.; F_{2,32} = 17.48, P < 0.001; P
< 0.01 for each comparison; Fig 3.6b), (S)-AM1241 (1 mg/kg i.p.; $F_{2,32} = 10.00, P < 0.01$; $P < 0.01$ for each comparison; Fig 3.6c), or (R,S)-AM1241 (1 mg/kg i.p.; $F_{2,32} = 36.78, P < 0.001$; $P < 0.001$ for each comparison; Fig 3.6d).
Figure 3.1. Chemical structures of the aminoalkylindoles \((R,S)\)-AM1241, \((R)\)-AM1241 and \((S)\)-AM1241.
Figure 3.1. Chemical structures of the aminoalkylindoles (R,S)-AM1241, (R)-AM1241 and (S)-AM1241.
Figure 3.2. Log dose response of (a.) (R,S)-AM1241, (b.) (R)-AM1241 and (c.) (S)-AM1241 shows withdrawal latencies to thermal stimulation in the plantar test at 30 minutes post-injection. Doses are in mg/kg. (d.) Comparison of log dose response plots for (R,S)-AM1241, (R)-AM1241, and (S)-AM1241 at 30 minutes post-injection. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. DMSO control condition, $\perp P < 0.05$, $\perp\perp\perp P < 0.001$ vs. baseline, $^+ P < 0.05$ vs. (R,S)-AM1241 (0.033, 0.1, and 10 mg/kg i.p.), $^XP < 0.05$, vs. (R)-AM1241 (5 mg/kg), $^\# P < 0.05$, $^\#\# P < 0.01$ vs. (S)-AM1241 (0.033 mg/kg i.p.) (ANOVA; Dunnett and Tukey post hoc tests). $^aP < 0.05$, $^aaP < 0.01$ vs. all groups at the same dose, $^bP < 0.05$ vs. (R,S)-AM1241 at the same dose (Student t-test). $N = 7$-19 per group.
Figure 3.2: Log dose response of (R,S)-AM1241, (R)-AM1241 and (S)-AM1241.
Figure 3.3. (a.) $(R)$-AM1241 (0.033 mg/kg i.p.) produced antinociception to thermal stimulation at 30 minutes post-injection. (b.) $(R,S)$-AM1241 (1 mg/kg i.p.) and its enantiomers, $(R)$-AM1241 (1 mg/kg i.p.) and $(S)$-AM1241 (1 mg/kg i.p.) produced thermal antinociception in the plantar test. (c.) $(R)$-AM1241 (10 mg/kg i.p.) produced antinociception, whereas $(R,S)$-AM1241 (10 mg/kg i.p.) and $(S)$-AM1241 (10 mg/kg i.p.) failed to produce an effect. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. DMSO control condition, $^+P < 0.05$ vs. all conditions, $^#P < 0.05$ vs. $(R,S)$-AM1241 (10 mg/kg i.p.) (ANOVA and Dunnett post hoc test). $N = 8-19$ per group.
Figure 3.3: Time course of antinociceptive effects of (R,S)-AM1241, (R)-AM1241 and (S)-AM1241.
Figure 3.4. The CB$_2$ antagonist SR144528 (SR2; 6 mg/kg i.p.) but not the CB$_1$ antagonist Rimonabant (Rim; 6 mg/kg i.p.) blocked the antinociceptive effects of (a.) ($R,S$)-AM1241 (1 mg/kg i.p.) (b.) ($R$)-AM1241 (1 mg/kg i.p.), and (c.) ($S$)-AM1241 (1 mg/kg i.p.). (d.) Rimonabant (Rim; 6 mg/kg i.p.) and SR144528 (6 mg/kg i.p.) produced no changes in paw withdrawal latencies relative to the vehicle condition. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. DMSO control condition, ++$P < 0.01$ vs. ($R,S$)-AM1241 (1) + SR2 (6), ⊥⊥$P < 0.01$, ⊥$P < 0.05$ vs. ($R$)-AM1241 (1) + SR2 (6), #$P < 0.05$ vs. ($S$)-AM1241 (1) + SR2 (6) (ANOVA; Dunnett and Tukey post hoc tests). XX$P < 0.01$, X$P < 0.05$ vs. DMSO control condition, a$P < 0.05$ vs. ($R$)-AM1241 (1) + SR2 (6) (Student t-test). N = 7-19 per group.
Figure 3.4: Pharmacological specificity of antinociception produced by (R,S)-, (R)-, and (S)-AM1241.
Figure 3.5. (a.) Naloxone (10 μg i.paw) blocked the antinociceptive effects of morphine (2 mg/kg s.c.). Ipsi denotes the injected paw and contra denotes the non-injected paw. Naloxone (10 μg i.paw) did not block the antinociceptive effects of (b.) \((R)\)-AM1241 (1 mg/kg i.p.), (c.) \((S)\)-AM1241 (1 mg/kg i.p.), or (d.) \((R,S)\)-AM1241 (0.33 mg/kg i.p.). (e.) Naloxone (50 μg i.paw) did not block the effects of \((R,S)\)-AM1241 (0.33 mg/kg i.p.) or (f.) \((R,S)\)-AM1241 (1 mg/kg i.p.). *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) vs. DMSO control condition, †† \(P < 0.01\) vs. morphine (2) + naloxone (10 μg) – ipsi (ANOVA; Dunnett and Tukey post hoc tests). N = 6-19 per group.
Figure 3.5: Effects of locally injected naloxone on antinociceptive observed following systemic administration of morphine, (R,S)-AM1241, (R)-AM1241, and (S)-AM1241.
Figure 3.6. (a.) Naloxone (10 mg/kg i.p.) blocked the antinociceptive effects of morphine (2 mg/kg s.c.), at a dose that failed to produce an effect when administered alone.

Naloxone (10 mg/kg i.p.) did not block the antinociceptive effects of (b.) (R)-AM1241 (1 mg/kg i.p.), (c.) (S)-AM1241 (1 mg/kg i.p.) or (d.) (R,S)-AM1241 (1 mg/kg i.p.). **$P < 0.01$, ***$P < 0.001$ vs. DMSO control condition. +++$P < 0.001$ vs. all drug groups (ANOVA; Dunnett and Tukey post hoc tests). N = 8-19 per group.
Figure 3.6: Effects of systemic naloxone on antinociceptive observed following systemic administration of morphine, (R,S)-AM1241, (R)-AM1241, and (S)-AM1241.
Table 3.1: Paw withdrawal thresholds (g) in animals that received morphine, racemic AM1241 and its enantiomers

<table>
<thead>
<tr>
<th>Group</th>
<th>Preinjection</th>
<th>Postinjection</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>67.8 ± 3.6</td>
<td>70.9 ± 4.8</td>
</tr>
<tr>
<td>Morphine (2 mg/kg)</td>
<td>65.7 ± 3.1</td>
<td>79.0 ± 4.0+</td>
</tr>
<tr>
<td>(R,S)-AM1241 (0.033 mg/kg)</td>
<td>76.5 ± 3.0</td>
<td>84.3 ± 2.7</td>
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<tr>
<td>(R,S)-AM1241 (0.1 mg/kg)</td>
<td>62.3 ± 5.1</td>
<td>64.7 ± 5.1</td>
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<td>(R,S)-AM1241 (0.33 mg/kg)</td>
<td>67.7 ± 6.6</td>
<td>71.2 ± 8.0</td>
</tr>
<tr>
<td>(R,S)-AM1241 (1 mg/kg)</td>
<td>66.6 ± 6.7</td>
<td>71.8 ± 6.8</td>
</tr>
<tr>
<td>(R,S)-AM1241 (5 mg/kg)</td>
<td>66.6 ± 5.1</td>
<td>65.9 ± 4.1</td>
</tr>
<tr>
<td>(R,S)-AM1241 (10 mg/kg)</td>
<td>76.4 ± 6.1</td>
<td>82.8 ± 9.0</td>
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<tr>
<td>(R)-AM1241 (0.033 mg/kg)</td>
<td>69.2 ± 2.9</td>
<td>80.3 ± 4.2</td>
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<td>(R)-AM1241 (0.1 mg/kg)</td>
<td>68.9 ± 3.7</td>
<td>76.6 ± 7.0</td>
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<td>(R)-AM1241 (0.33 mg/kg)</td>
<td>72.2 ± 5.0</td>
<td>72.1 ± 3.3</td>
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<td>(R)-AM1241 (1 mg/kg)</td>
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<td>(R)-AM1241 (5 mg/kg)</td>
<td>80.7 ± 5.0</td>
<td>69.2 ± 4.7</td>
</tr>
<tr>
<td>(R)-AM1241 (10 mg/kg)</td>
<td>73.3 ± 4.0</td>
<td>80.3 ± 11.0</td>
</tr>
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<td>(S)-AM1241 (0.033 mg/kg)</td>
<td>83.4 ± 3.1</td>
<td>81.1 ± 4.2</td>
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<td>(S)-AM1241 (0.1 mg/kg)</td>
<td>67.7 ± 5.1</td>
<td>70.0 ± 6.0</td>
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<td>(S)-AM1241 (0.33 mg/kg)</td>
<td>70.3 ± 6.2</td>
<td>69.8 ± 5.8</td>
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<td>(S)-AM1241 (1 mg/kg)</td>
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<td>(S)-AM1241 (5 mg/kg)</td>
<td>70.6 ± 3.5</td>
<td>69.9 ± 4.4</td>
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<tr>
<td>(S)-AM1241 (10 mg/kg)</td>
<td>77.8 ± 4.7</td>
<td>85.4 ± 11.8</td>
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Data are mean ± s.e.mean. All drugs were administered intraperitoneally (i.p.). Reported values are 15 minutes postinjection. *P < 0.05 vs. same group preinjection threshold (Student t test).
Table 3.2: Paw withdrawal thresholds (g) in animals undergoing pharmacological specificity testing with morphine, racemic AM1241 and its enantiomers

<table>
<thead>
<tr>
<th>Group</th>
<th>Preinjection</th>
<th>Postinjection</th>
</tr>
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<tbody>
<tr>
<td>DMSO</td>
<td>67.8 ± 3.6</td>
<td>70.9 ± 4.8</td>
</tr>
<tr>
<td>Morphine (2 mg/kg)</td>
<td>65.7 ± 3.1</td>
<td>79.0 ± 4.0⁺</td>
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<tr>
<td>Morphine (2) + Naloxone (10)</td>
<td>76.1 ± 5.9</td>
<td>63.0 ± 9.8</td>
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<tr>
<td>(R,S)-AM1241 (1 mg/kg)</td>
<td>66.6 ± 6.7</td>
<td>71.8 ± 6.8</td>
</tr>
<tr>
<td>(R,S)-AM1241 (1) + SR144528 (6)</td>
<td>73.3 ± 3.0</td>
<td>62.9 ± 4.2</td>
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<tr>
<td>(R,S)-AM1241 (1) + Rimonabant (6)</td>
<td>67.6 ± 4.1</td>
<td>107.6 ± 9.5***⁺⁺⁺†</td>
</tr>
<tr>
<td>(R)-AM1241 (1 mg/kg)</td>
<td>74.1 ± 4.7</td>
<td>73.9 ± 4.8</td>
</tr>
<tr>
<td>(R)-AM1241 (1) + SR144528 (6)</td>
<td>69.7 ± 2.7</td>
<td>68.8 ± 5.1</td>
</tr>
<tr>
<td>(R)-AM1241 (1) + Rimonabant (6)</td>
<td>76.5 ± 5.7</td>
<td>72.2 ± 7.5</td>
</tr>
<tr>
<td>(S)-AM1241 (1 mg/kg)</td>
<td>75.1 ± 7.5</td>
<td>78.5 ± 6.8</td>
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<tr>
<td>(S)-AM1241 (1) + SR144528 (6)</td>
<td>73.8 ± 9.6</td>
<td>65.6 ± 6.5</td>
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<tr>
<td>(S)-AM1241 (1) + Rimonabant (6)</td>
<td>63.4 ± 7.2</td>
<td>81.0 ± 11.5</td>
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<tr>
<td>Naloxone (10 mg/kg)</td>
<td>72.0 ± 5.8</td>
<td>61.4 ± 11.2</td>
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<tr>
<td>Rimonabant (6 mg/kg)</td>
<td>63.3 ± 5.5</td>
<td>75.6 ± 5.7</td>
</tr>
<tr>
<td>SR144528 (6 mg/kg)</td>
<td>70.9 ± 5.1</td>
<td>62.8 ± 5.8</td>
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</table>

Data are mean ± s.e.mean. Doses are in mg/kg. All drugs were administered intraperitoneally (i.p.). Reported values are 15 minutes postinjection. ***P < 0.001 vs. DMSO (ANOVA and Dunnett Post Hoc), **P < 0.01, *P < 0.05 vs. same group preinjection threshold, †P < 0.05 vs. all postinjection drug condition thresholds (Student t test).
Discussion

Racemic AM1241 produces antinociception in the plantar test when administered systemically (16). In our study, (R,S)-AM1241-induced antinociception formed an inverted U-shaped dose response curve at 30 minutes post-injection; lower (0.033 and 0.1 mg/kg i.p.) and higher (10 mg/kg i.p.) doses of the drug were less effective in producing antinociception than a dose of 1 mg/kg i.p. Previous reports of (R,S)-AM1241-induced antinociception (16, 29) did not test higher doses of (R,S)-AM1241 in the plantar test and therefore did not observe this loss of efficacy. However, the inverted U-shaped dose response curve could potentially account for conflicting reports of (R,S)-AM1241’s limited anti-hyperalgesic efficacy (19). Previous work by our lab demonstrated that (R,S)-AM1241 (5 and 10 mg/kg i.p.) was effective at suppressing neuropathic pain induced by administration of the chemotherapeutic agent paclitaxel, whereas a lower dose (1 mg/kg i.p.) failed to produce an effect (30). Thus, it appears that drug efficacy and potency could also be influenced by the receptor state of the animal (i.e. naive vs. neuropathic). As expected, the antinociceptive effects of (R,S)-AM1241 observed in our study were clearly CB2-mediated; these effects were blocked by the CB2 antagonist SR144528 but not by the CB1 antagonist Rimonabant. This observation is consistent with previous demonstrations of CB2-mediated anti-hyperalgesic effects produced by AM1241 in animal models of persistent, inflammatory and neuropathic pain (30-33).

In contrast to the thermal antinociceptive effects of the CB2 agonists observed here in the plantar test, none of the aminoalkylindoles produced an antinociceptive effect to nonnoxious mechanical stimulation, assessed using a highly sensitive electrovonfrey device. This observation is in marked contrast to the opioid analgesic morphine, which
produced reliable, naloxone-sensitive antinociception to mechanical stimulation at the same post-injection time point. Our failure to observe a change in the basal mechanical threshold following administration of either \((R,S)\)-AM1241 or its enantiomers in this test is unlikely to be attributed to selection of an inadequate post-injection time point for evaluation. Malan and colleagues (16) reported robust CB\(_2\)-mediated antinociception to thermal stimulation following systemic administration of \((R,S)\)-AM1241 at 15 min post-injection. However, our results do not preclude the possibility that antinociception could occur to noxious levels of stimulation (e.g. applied with a Randall Selitto device). Moreover, \((R,S)\)-AM1241 does suppress mechanical hypersensitivity to von Frey stimulation under conditions of injury, during which mechanical thresholds are lowered relative to baseline (3). Co-administration of Rimonabant with \((R,S)\)-AM1241 increased mechanical paw withdrawal thresholds. This observation parallels our recent finding of anti-allodynia in paclitaxel-treated animals that received Rimonabant prior to administration of the CB\(_2\) agonist AM1714 (30). Enhanced efficacy of a CB\(_2\) agonist following administration of a CB\(_1\) antagonist has also been reported in a cerebral ischemic injury model (34). These data suggest that blockade of CB\(_1\) receptors with Rimonabant may enhance the tone of the endogenous cannabinoid system, thereby increasing the efficacy of the CB\(_2\) agonist.

Antinociceptive properties of the enantiomers of \((R,S)\)-AM1241 have not previously been evaluated in naive rats. This characterization is important because of the widespread use of AM1241 as a tool to study functional roles of CB\(_2\) receptor activation. Anti-hyperalgesic effects of \((S)\)-AM1241 were previously reported in a visceral and inflammatory pain model (19). In our study, \((S)\)-AM1241 presented a pharmacological
profile which was nearly identical to racemic AM1241. We observed an inverted U-shaped dose response curve following administration of either (S)-AM1241 or (R,S)-AM1241 at the time point of maximal antinociception (30 min). Our data also illustrate that both the lowest (0.033 and 0.10 mg/kg i.p.) and the highest (10 mg/kg i.p.) doses of (R)-AM1241 produced greater antinociception than comparable doses of either (S)-AM1241 or (R,S)-AM1241. At intermediate doses, the compounds produced similar antinociceptive effects. Previous *in vitro* work with the enantiomers noted that (R)- and (R,S)-AM1241 are inverse agonists for rat CB₂ receptors in the cyclase assay, whereas (S)-AM1241 is a full agonist (19). Thus, it is possible that agonist activity in the cyclase assay predicts the antinociceptive efficacy of (S)-AM1241, thereby reconciling the *in vivo* observations with results from *in vitro* receptor binding assays.

Both (R)- and (S)-AM1241 produced thermal antinociception that outlasted that of (R,S)-AM1241 at an identical dose (120 min duration of action as compared to 30 min). This observation may be attributed to the combination of inverse agonist as well as agonist properties of the racemic compound. Differences in metabolic transformation of (R)- and (S)-AM1241 may also contribute to differences in *in vivo* efficacy of these enantiomers. Although (S)-AM1241 was suggested to be the more active enantiomer *in vivo* in suppressing acute visceral and inflammatory pain (19), this observation may be dose-dependent. In a chemotherapy model of neuropathic pain, (R)-AM1241, but not (S)-AM1241, was effective in suppressing neuropathic nociception when a high dose of (R)-AM1241 and (S)-AM1241 were evaluated (30). It is important to note that a high dose of (S)-AM1241 (10 mg/kg i.p.) produced seizure-like effects in two of the eight animals tested in our study, effects not observed with either (R,S)-AM1241 or (R)-
AM1241. \((S)-\text{AM1241 (10 mg/kg i.p.) was previously tested in a chemotherapy model of neuropathic pain and no similar side-effects were observed (30). In addition, (S)-AM1241 (10 mg/kg i.p.) was utilized by Bingham and colleagues (19) in visceral (i.e. paraphenyl quinine writhing test) and inflammatory (i.e. carrageenan) pain models and no similar effects were reported. These latter effects are, therefore, almost certainly due to off-target binding (21).}

To our knowledge, this is the first study to examine naloxone sensitivity of \((R)-\text{and (S)-AM1241, the enantiomers of (R,S)-AM1241. To accomplish this objective, we employed the opioid antagonist, naloxone, administered both locally and systemically. In our study, local and systemic injections of naloxone completely blocked the antinociceptive effects of morphine. Under these conditions, naloxone, administered alone either intra-paw or intraperitoneally, did not alter paw withdrawal latencies or mechanical withdrawal thresholds relative to comparable controls. We evaluated the contribution of peripheral opioid receptors to the antinociception produced by \((R)-\text{and (S)-AM1241 using conditions analogous to those employed by Ibrahim and colleagues (20). Naloxone (10 µg i.paw) was shown previously to block antinociceptive effects of systemic (R,S)-AM1241 (0.1 mg/kg i.p.) in the plantar test (20). However, in our study, this low dose of AM1241 (0.1 mg/kg i.p.) did not produce reliable antinociception relative to vehicle or baseline treatment, so higher doses of racemic and chiral AM1241 (0.33 or 1 mg/kg i.p.) were evaluated for naloxone sensitivity. In our study, locally injected naloxone (10 µg i.paw) completely blocked the antinociceptive effects of systemic morphine in the injected, but not the non-injected paw. However, we were unable to block the antinociceptive effects of either \((R)-\text{AM1241, (S)-AM1241 or (R,S)-}{\text{AM1241.}}\)
AM1241 (1 mg/kg i.p.) with locally administered naloxone (10-50 µg i.paw). The lowest
dose of (R,S)-AM1241 (0.33 mg/kg i.p.) which produced antinociception, relative to the
vehicle condition, in our study was employed as a reference compound in this
experiment. However, antinociception produced by (R,S)-AM1241 was not blocked by
the local dose of naloxone employed by Ibrahim et al. (20), and was also not blocked by a
5-fold higher (50 µg i.paw) dose of naloxone. We observed a similar lack of naloxone-
sensitive blockade of (R,S)-AM1241-induced antinociception with both doses of (R,S)-
AM1241 (0.33 and 1 mg/kg i.p.), suggesting that dose selection is unlikely to account for
these differences. Both our study and that of Ibrahim et al. (20) employed Sprague
Dawley rats and a 100% DMSO vehicle for cannabinoid administration. It is possible
that the naloxone blockade of (R,S)-AM1241-induced antinociception observed by
Ibrahim and colleagues (20) represented a state-dependent or transient phenomenon, that
was no longer present at 30 min post-injection (the earliest time point at which animals
were tested in the plantar test in our study). Differences in animal housing (group
housing vs. single housed), animal handling, stress state of the animals tested, or
endogenous analgesic tone could contribute to differences in naloxone sensitivity of
(R,S)-AM1241-induced antinociception. For example, housing and environmental
factors (e.g. objects in the home cage) can decrease nociception in an inflammatory
model of pain (35) and may differentially alter endogenous analgesic tone. Thus, under
conditions in which endogenous opioid tone is upregulated, a low dose of (R,S)-AM1241
(0.1 mg/kg i.p.) may produce an apparent antinociceptive effect sensitive to blockade by
naloxone (19).
We also evaluated whether systemic administration of naloxone (10 mg/kg i.p.) would block the antinociceptive effects of either (R)-AM1241, (S)-AM1241 or (R,S)-AM1241. The ability of systemic naloxone to block the antinociceptive effect of (R,S)-AM1241 has not previously been evaluated in otherwise naive rats. The dose of naloxone employed here was previously shown to block anti-hyperalgesic effects of (R,S)-AM1241 in a complete Freund’s adjuvant (CFA) model of chronic inflammatory pain (22) as well as the anti-alldynic effects of (R,S)-AM1241 in the spinal nerve ligation model (36). Both of the aforementioned studies employed a high dose of (R,S)-AM1241 (15 mg/kg i.p.). Due to the inverted U-shaped dose-response curve observed for (R,S)-AM1241-induced antinociception, this high dose, in naive rats, might be expected to produce effects comparable to 0.1 or 10 mg/kg i.p. and be less efficacious at inducing antinociception compared to doses of 1 or 5 mg/kg. Moreover, it is also unclear whether this high dose is associated with off-target activity as neither study demonstrated that effects of (R,S)-AM1241 (15 mg/kg i.p.) were CB2-mediated. In our hands, systemic naloxone completely blocked the antinociceptive effects of systemic morphine in the plantar test. However, the same dose of naloxone, administered systemically, failed to block the antinociceptive effects of racemic AM1241, or either of its enantiomers. Our studies suggest that activation of opioid receptors is not sufficient to account for the antinociceptive effects of either (R,S)-AM1241, (R)-AM1241 or (S)-AM1241 in naive animals.

**Conclusion**

The aminoalkylindole (R,S)-AM1241 and its enantiomers (R)-AM1241 and (S)-AM1241 all produce CB2-mediated antinociception that is insensitive to blockade by
naloxone, and consequently, is not dependent upon opioid receptor activation. These observations support the hypothesis that the antinociceptive effects of CB$_2$ agonists do not require opioid receptor activation. Our data suggest that the CB$_2$ receptor remains a promising therapeutic target for the treatment of pain.
References


CHAPTER 4

PHARMACOLOGICAL CHARACTERIZATION OF PUTATIVE CANNABINOID CB₂ AGONISTS FROM THE CANNABILACTONE CLASS: ANTINOCICEPTION WITHOUT CENTRAL-NERVOUS SYSTEM SIDE EFFECTS

Summary

Background and Purpose. Cannabinoid CB2 agonists produce antinociception without central nervous system (CNS) side-effects. This study was designed to pharmacologically characterize the antinociceptive profiles of AM1714 and AM1710, agonists from the cannabiliactone class of cannabinoids.

Experimental Approach. The cannabiliactones were compared with the aminoalkylindole CB2-specific agonist (R,S)-AM1241 in tests of antinociception and CNS activity. Pharmacological specificity was established using CB1 (SR141716) and CB2 (SR144528; AM630) antagonists. CNS side-effects were evaluated in a modified tetrad (tail-flick, rectal temperature, locomotor activity and rota-rod).

Key Results. AM1714 and AM1710 (0.1-10 mg kg\(^{-1}\) i.p.) produced antinociception to thermal but not mechanical stimulation of the hindpaw. Antinociception produced by either (R,S)-AM1241 (1 mg kg\(^{-1}\) i.p.) or the low (0.1 mg kg\(^{-1}\) i.p.) dose of AM1710 was blocked selectively by the CB2 antagonist SR144528. Antinociception produced by AM1714 (5 mg kg\(^{-1}\) i.p.) was blocked by 6 mg kg\(^{-1}\) of either AM630 or SR141716 but not by SR144528 (6 or 10 mg kg\(^{-1}\) i.p.). Antinociceptive effects of AM1710 (5 mg kg\(^{-1}\) i.p.) were blocked by 6 mg kg\(^{-1}\) of either SR144528 or SR141716. Neither AM1714 nor AM1710 produced hypothermia, tail-flick antinociception, or motor ataxia. AM1714 produced hypoactivity at a dose 50-fold higher than the dose that produced maximal antinociception.

Conclusions and Implications. AM1714 and AM1710, CB2-preferring cannabiliactones, produce antinociception in the absence of CNS side-effects. CB1-mediated antinociceptive effects of these compounds may be attributable to peripheral CB1 activity,
off-target effects on fatty-acid amide hydrolase (FAAH), or biologically active metabolites.

**Key Words.** cannabinoid, CB₂, antinociception, tetrad
Abbreviations: FAAH, fatty-acid amide hydrolase; i.p., intraperitoneal
Introduction

Activation of cannabinoid CB2 receptors produces antinociception in animal models of persistent pain (for review see Guindon and Hohmann, 2008). The CB2 receptor represents a promising therapeutic target for the treatment of pathological pain specifically because antinociceptive efficacy is observed in the absence of unwanted central nervous system (CNS) side-effects (Hanus et al., 1999; Malan et al., 2001). Relative to CB1 receptors, a paucity of CB2 receptors is detected in the CNS of naive animals. However, CB2 receptors are upregulated within the CNS in neuropathic pain states (Beltramo et al., 2006; Wotherspoon et al., 2005; Zhang et al., 2003). Upregulation of CB2 receptors may contribute to the efficacy of CB2-specific agonists in treating neuropathic pain (for review see Guindon and Hohmann, 2008). Additional targets for drug development aimed at harnessing the analgesic potential of cannabinoid signaling systems while limiting CNS side-effects have also been described (Anand et al., 2009).

A recently described class of cannabinoids, the cannabilactones, includes the CB2-prefering agonists AM1714 and AM1710 (Khanolkar et al., 2007). Both AM1714 and AM1710 produce antinociception following local (i.paw) administration, suggesting that they produce antinociception, at least in part, through peripheral mechanisms (Khanolkar et al., 2007). We recently demonstrated that AM1714 suppresses neuropathic nociception in a chemotherapy model of peripheral neuropathy through a CB2-specific mechanism (Rahn et al., 2008). However, despite the considerable therapeutic potential of these compounds, antinociceptive effects of the cannabilactones remain relatively uncharacterized. More work is therefore necessary to characterize the in vivo pharmacological profile associated with cannabilactone administration and determine
whether compounds of this class show limited CNS side-effects. It remains unknown whether systemic administration of cannabilactones lack cardinal signs of CB₁ receptor activation (e.g. hypothermia, hypoactivity, motor ataxia) or exhibit an unfavorable CNS profile. This examination is important for validating the therapeutic potential of the cannabilactones for the treatment of pain.

The present studies were conducted to evaluate the antinociceptive properties of the cannabilactones, AM1714 (Kᵢ: CB₁ vs. CB₂: 400 nM vs. 0.8 nM) and AM1710 (Kᵢ: CB₁ vs. CB₂: 360 nM vs. 6.7 nM) (Khanolkar et al., 2007), in tests of thermal (Hargreaves test) and mechanical (von Frey) sensitivity (Fig 4.1). The presence of centrally-mediated side-effects produced by the cannabilactones was evaluated using a modified tetrad (tail-flick, rectal temperature, locomotor activity, rota-rod). Cardinal signs of cannabinoid CB₁ receptor activation include antinociception in the tail-flick test, hypothermia, hypoactivity in the open field test and motor ataxia in the rota-rod test (Martin et al., 1991; Malan et al., 2001). The pharmacological profile of the cannabilactones was compared with the prototypical CB₂-specific agonist (R,S)-AM1241 (Kᵢ: CB₁ vs. CB₂: 239.4 nM vs. 3.41 nM). (R,S)-AM1241 (Fig. 4.1) is a well-characterized CB₂ agonist from the aminoalkylindole class of cannabinoids (Hohmann et al., 2004; Malan et al., 2001; Nackley et al., 2003; Rahn et al., 2007). Pharmacological specificity was determined using selective antagonists for CB₁ (SR141716) and CB₂ (SR144528, AM630). Comparisons were made with appropriate reference compounds (the mixed CB₁/CB₂ agonist WIN55,212-2 and the CB₂ agonist (R,S)-AM1241) tested under identical conditions.
Methods

Subjects

Four hundred and nine adult male Sprague Dawley rats were used in these experiments; two hundred and sixty-four (300-400 g; Harlan, Indianapolis, IN) animals were used in studies of antinociception and one hundred forty-five (250-350 g; Harlan, Indianapolis, IN) animals were used in the tetrad studies. Eighty-nine of the animals in this study are reference control conditions previously published (Rahn et al., 2010). All animals were maintained on a 12 hr light/12 hr dark cycle (7:00 - 19:00; lights on) in a temperature-controlled facility. Animals were single housed and had access to food and water *ad libitum*. 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).

Drugs and Chemicals

AM1714 (1,9-dihydroxy-3-(1′,1′-dimethylheptyl)-6H-benzo[c]chromene-6-one), AM1710 (3-(1′,1′-dimethylheptyl)-1-hydroxy-9-methoxy-6H-benzo[c]chromene-6-one), (R,S)-AM1241 ((R,S)-(2-iodo-5-nitrophenyl)-[1-((1-methyl-piperidin-2-yl)methyl)-1H-indol-3-yl]-methanone), and AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone (Iodopravadoline)) were synthesized in the Makriyannis laboratory by one of the authors (by GT, AZ, and VKV respectively).

SR141716 (5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide) and SR144528 (5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-N-(1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl)-1H-pyrazole-3-carboxamide) were provided by NIDA. WIN55,212-2 ((R)-(+) [2,3-dihydro-5-methyl-3[(4-
morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-1-(naphtalenyl) methanone mesylate salt), and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO). All drugs were dissolved in a vehicle of 100% DMSO and delivered intraperitoneally (i.p.). This is the same vehicle that has been employed in previous work (Ibrahim et al., 2005; Malan et al., 2001; Nackley et al., 2003; Rahn et al., 2008).

Cannabinoids and cannabinoid antagonists were dissolved in a volume of 1 ml kg\(^{-1}\) bodyweight with the following exceptions. Pharmacological specificity of cannabilactone actions was determined by administering antagonists as pre-treatments 20 min prior to the agonist. In these studies, each drug was administered in a volume of 0.5 ml kg\(^{-1}\) to ensure that all studies employed a uniform volume of DMSO.

**General Experimental Methods**

Baseline responses to mechanical stimulation of the hindpaw were evaluated at least 1 h prior to evaluation of baseline responses to thermal stimulation. In a subset of experiments (approximately 25%), the order of baseline testing was reversed (i.e. baseline responses to thermal stimulation were assessed at least 1 h prior to evaluation of baseline responses to mechanical stimulation). This modification enabled us to confirm that hypersensitivity to thermal or mechanical stimulation was not produced by the order of testing mechanical and thermal responses (data not shown). Following completion of baseline testing, all rats were returned to their home cages for approximately 2 h prior to administration of drug or vehicle. This delay was employed to ensure that animals did not develop sensitization to repeated testing.

CNS side-effects were evaluated in two separate groups of animals that comprised the “tetrad testing”. One set of animals was used for tail-flick and rectal temperature
assessment. The second set of animals was used for activity meter and rota-rod testing. Baseline tail-flick latencies were assessed prior to baseline assessments of rectal temperature. Following baseline measurements, animals were returned to their home cages for approximately 2 h prior to drug or vehicle administration. Training for rota-rod took place on the two days preceding the test day. Only animals that met reliability criteria for the rota-rod (i.e. ability to walk on a rotating drum for 30 sec in two separate trials) on the test day received pharmacological treatments. Subjects that failed to meet the rota-rod criteria were subsequently used in the tail flick/rectal temperature or antinociception study after an appropriate delay (i.e. several days). Animals that passed criteria for inclusion in the rota-rod study were returned to their home cages for approximately 2 h prior to drug or vehicle administration. All studies were conducted by a single experimenter who was blinded to the drug condition. Animals were randomly assigned to drug or vehicle conditions.

**Assessment of Mechanical Withdrawal Thresholds and Thermal Paw Withdrawal Latencies**

Mechanical withdrawal thresholds were assessed using a digital Electrovonfrey Anesthesiometer (IITC model Alemo 2290-4; Woodland Hills, CA) equipped with a rigid tip. Rats were placed underneath inverted plastic cages and positioned on an elevated mesh platform. Rats were allowed 10-15 min to habituate to the chamber prior to testing. Stimulation was applied to the midplantar region of the hind paw through the floor of a mesh platform. Mechanical stimulation was terminated upon paw withdrawal; consequently, there was no upper threshold limit set for termination of a trial. Mechanical
Paw withdrawal thresholds are reported as the mean of duplicate determinations averaged across paws.

Paw withdrawal latencies to radiant heat were measured in duplicate for each paw using the Hargreaves test (Hargreaves et al., 1988) and a commercially available plantar stimulation unit (IITC model 336; Woodland Hills, CA). Rats were placed underneath inverted plastic cages positioned on an elevated glass platform. Rats were allowed 10-15 min to habituate to the chamber prior to testing. Radiant heat was presented to the midplantar region of the hind paw through the floor of the glass platform. Stimulation was terminated upon paw withdrawal or after 40 s to prevent tissue damage. Paw withdrawal latencies are reported as the mean of duplicate determinations averaged across paws.

Baseline mechanical withdrawal thresholds and thermal paw withdrawal latencies were assessed prior to pharmacological manipulations. Mechanical paw withdrawal thresholds were re-assessed 15 min following injection of drug or vehicle. Thermal paw withdrawal latencies were measured at 30, 60 and 120 minutes post-injection to assess the time course of CB2 agonist actions.

Antinociception to thermal (in the Hargreaves test) and mechanical (electrovonfrey) stimulation was evaluated in otherwise naive rats. Separate groups of animals received either the racemic (R,S)-AM1241 (0.10, 0.33, 1, 5 and 10 mg kg\(^{-1}\) i.p.; n = 7-8 per group), AM1714 (0.10, 0.33, 1, 5 and 10 mg kg\(^{-1}\) i.p.; n = 8-12 per group), AM1710 (0.10, 0.33, 1, 5 and 10 mg kg\(^{-1}\) i.p.; n = 8-10 per group), or DMSO (n = 19). To determine pharmacological specificity, either the CB1 antagonist SR141716 (6 mg kg\(^{-1}\) i.p.) or the CB2 antagonist SR144528 (6 mg kg\(^{-1}\) i.p.) was co-administered with (R,S)-
AM1241 (1 mg kg\(^{-1}\) i.p., n = 7-8 per group). In separate animals, SR144528 (6 mg kg\(^{-1}\) or 10 mg kg\(^{-1}\) i.p.), SR14176 (6 mg kg\(^{-1}\) i.p.), or AM630 (6 mg kg\(^{-1}\) i.p.) was administered 20 min prior to AM1714 (0.10 mg kg\(^{-1}\) or 5 mg kg\(^{-1}\) i.p.; n = 7-9 per group) or AM1710 (0.10 mg kg\(^{-1}\) or 5 mg kg\(^{-1}\); n = 8-9 per group). SR141716 (6 mg kg\(^{-1}\) i.p., n = 8 per group), SR144528 (6 mg kg\(^{-1}\) i.p.; n = 8) or AM630 (6 mg kg\(^{-1}\) i.p.; n = 7) were administered to separate groups of rats to evaluate possible antagonist-induced changes in basal nociceptive thresholds. Thermal withdrawal latencies were re-determined, in duplicate for each paw, at 30, 60 and 120 min following injection.

**Tetrad Testing**

*Tail-Flick/Rectal Temperature*

A modified tetrad profile was performed to assess CNS side-effects. Tail-flick latency and rectal temperature were assessed in the same animals. Tail-flick (D'Amour *et al.*, 1941) was assessed using a commercially available tail-flick unit (IITC model 336; Woodland Hills, CA). Animals were placed in restraint tubes (IITC model 81; Woodland Hills, CA) and allowed 10 min to habituate prior to testing. Radiant heat was presented to the tail and the latency for the animal to withdraw its tail from the heat source was recorded. Stimulation was terminated when the animal withdrew its tail from the radiant heat source. A cut-off latency of 10 s was employed to prevent tissue damage. Baseline tail-flick latencies are reported as the mean of six tail-flick latencies. Tail-flick latencies were re-determined at 30, 60, and 120 min post-injection and are reported at each time point as the mean of four tail-flick latencies.

Rectal temperature was assessed using a commercially available rectal probe (Physitemp RET-2 rectal probe for rats; Clifton, NJ) and meter (Physitemp Model BAT-
12R; Clifton, NJ). Following assessment of baseline tail-flick latencies, rectal probes, lubricated with Vaseline®, were inserted to a depth of approximately 2.4 cm. Probes were then connected to the meter and body temperature was recorded. Baseline rectal temperature is reported as the mean of four measurements. Rectal temperatures were then determined in duplicate at 35, 65, and 125 min post injection and are reported at each time point as the mean of duplicate determinations, averaged across rats.

To evaluate centrally-mediated antinociception (assessed in the tail-flick test) and hypothermia, separate groups of animals received either (R,S)-AM1241 (1 mg kg⁻¹ i.p.; n = 7), AM1714 (0.10, 5 or 10 mg kg⁻¹ i.p.; n = 6 per group), AM1710 (0.10, 5 or 10 mg kg⁻¹ i.p.; n = 6 per group), DMSO (n = 7), the reference cannabinoid CB₁/CB₂ agonist WIN55,212-2 (5 mg kg⁻¹ i.p.; n = 7) or the CB₁ antagonist SR141716 (6 mg kg⁻¹ i.p.) 20 min prior to the administration of WIN55,212-2 (5 mg kg⁻¹ i.p.; n = 6).

**Activity Meter/Rota-rod**

Locomotor activity and motor ataxia were assessed in the same animals. Distance traveled in an activity meter was assessed by placing rats individually in the center of a polycarbonate activity monitor chamber (Med Associates, St. Albans, VT) measuring 44.5 x 44 x 34 cm housed in a darkened, quiet room. A 25-watt bulb positioned one meter over the chamber provided illumination. Activity was automatically measured by computerized analysis of photobeam interrupts (Med Associates). Total distance (cm) traveled in the arena was used for data analysis. Animals were placed in the activity meter at 20 min post-injection and remained undisturbed in this chamber for 15 min. Following activity meter testing, animals were tested on the rota-rod.
Motor ataxia was assessed using a commercially available rota-rod unit (IITC model 755 RotoRod; Woodland Hills, CA). Animals were required to walk against the motion of a rotating drum increasing in speed from 4 revolutions per min (rpm) to 40 rpm, similar to that described by Fox and colleagues (2001). The descent latency (i.e. the time for an animal to fall off the rotating drum) was recorded (sec). No cut-off latency was employed in the rota-rod test to ensure that detection of subthreshold motor ataxia would not be masked by the cut-off latency employed (Taylor et al., 2003). Rota-rod training took place on the two days prior to the test day. Animals were given a minimum of three practice trials on both training days. Practice trials terminated when the animals fell off of the rotating drum. Training trials in which the animal failed to remain on the rotating drum for a minimum of 10 sec were re-run. On the test day, reliability testing was performed. Animals that could not remain on the rotating drum for 30 seconds in two separate trials failed to meet the criteria (approximately 20%) and were dropped from the experiment. Rota-rod descent latency was calculated after drug administration at 35, 65, and 125 min post injection. Rota-rod latencies at each time point post injection are reported as the mean of two separate rota-rod descent latencies, averaged across rats.

To evaluate possible centrally-mediated side-effects of hypoactivity and motor ataxia, animals received either the DMSO vehicle (n = 8), (R,S)-AM1241 (1 mg kg\(^{-1}\) i.p.; n = 6), AM1714 (0.10 or 5 mg kg\(^{-1}\) i.p.; n = 6-7 per group), AM1710 (0.10, 5 or 10 mg kg\(^{-1}\) i.p.; n = 8-9 per group) or WIN55,212-2 (5 mg kg\(^{-1}\) i.p.; n = 7). To assess pharmacological specificity, separate groups were pre-treated with SR141716 (6 mg kg\(^{-1}\) i.p.) 20 min prior to WIN55,212-2 (5 mg kg\(^{-1}\) i.p.; n = 7), SR141716 (6 mg kg\(^{-1}\) i.p.) 20
min prior to AM1714 (5 mg kg\(^{-1}\) i.p.; n = 9) or AM630 (6 mg kg\(^{-1}\) i.p.) 20 min prior to AM1714 (5 mg kg\(^{-1}\) i.p.; n = 6).

**Statistical Analyses**

Percent change in paw withdrawal latencies from baseline was calculated with the following formula: 
\[
\frac{(\text{Post drug paw withdrawal latency} - \text{baseline})}{\text{baseline}} \times 100.
\]

Antinociception in the tail-flick test was expressed as the percent of maximum possible effect (%MPE), using the formula:

\[
\frac{(\text{Post drug paw withdrawal latency} - \text{baseline})}{(\text{Cut-off value} - \text{baseline})} \times 100
\]

Change in temperature (\(\Delta\) °C) was calculated with the following formula: 
\[
\text{(Post drug temperature} - \text{mean baseline temperature}).
\]

Z-scores were calculated for tetrad animals tested in the activity meter and rota-rod. Four animals with Z-scores of ± 2 standard deviations from the mean in either test were excluded from analysis.

All data was analyzed using analysis of variance (ANOVA) for repeated measures, one-way ANOVA or planned comparison Student t-tests, as appropriate. SPSS 16.0 (SPSS Incorporated, Chicago, IL, USA) was used for statistical analyses. The Greenhouse-Geisser correction was applied to all repeated factors. Post hoc comparisons between control groups and other experimental groups were performed using the Dunnett test. Post-hoc comparisons between different experimental groups were also performed to assess dose-response relationships and pharmacological specificity using the Tukey test.

\(P < 0.05\) was considered statistically significant.
Results

General Results

Thermal paw withdrawal latencies and mechanical paw withdrawal thresholds did not differ between right or left paws for any group. Therefore, withdrawal thresholds in all studies are presented as the mean of duplicate measurements, averaged across paws. Baseline responses (i.e. thermal paw withdrawal latencies or mechanical withdrawal thresholds) were also similar between groups prior to administration of drug or vehicle. Baseline paw withdrawal latencies did not differ between groups in any study; therefore, baselines in the log dose response plot (Fig 4.2) were averaged across all doses of the same drug for statistical analyses. Moreover, paw withdrawal latencies and thresholds did not differ based upon the order of thermal and mechanical testing at baseline; therefore, the two vehicle groups are combined for all studies presented.

One animal that received AM1710 (5 mg kg$^{-1}$ i.p.) died approximately 45 min post injection and was excluded from all analyses. The animal likely died from a misplaced injection as no other animals receiving AM1710 at this, or any other dose tested, showed similar effects or was moribund. Within the tetrad (activity meter/rotarod), two animals from the WIN55,212-2 (5 mg kg$^{-1}$ i.p.) group, one animal from the AM1714 (5) + SR1 (6) and one animal from the AM1710 (10 mg kg$^{-1}$ i.p.) group were excluded from all analyses based on Z-scores.

The Cannabialctones AM1714 and AM1710 Produce Antinociception to Thermal but not Mechanical Stimulation of the Hind Paw

Responses to Mechanical Stimulation
AM1714 (10 mg kg\(^{-1}\) i.p) and AM1710 (1 mg kg\(^{-1}\) i.p) produced modest but reliable increases in mechanical withdrawal thresholds relative to corresponding pre-injection thresholds \((P < 0.05\) planned comparison t-tests; Table 4.1). However, these same doses did not alter post-injection thresholds relative to the vehicle condition. Moreover, antagonist pre-treatment did not alter paw withdrawal thresholds, relative to baseline, in any study (Table 4.2). Paw withdrawal thresholds were not altered by \((R,S)-AM1241\) (data not shown).

**Responses to Thermal Stimulation in the Plantar Test**

The prototypical CB\(_2\) agonist \((R,S)-AM1241\) (0.33, 1, and 5 mg kg\(^{-1}\) i.p.) increased thermal paw withdrawal latencies relative to the vehicle at 30 min post-injection \((F_{5,57} = 7.316, P < 0.001; P < 0.05\) for each comparison), consistent with previous reports. \((R,S)-AM1241\) (0.33, 1, 5, and 10 mg kg\(^{-1}\) i.p.) also increased paw withdrawal latencies relative to baseline at this time point \((F_{5,77} = 15.832, P < 0.001; P < 0.05\) for each comparison; Fig 4.2a). An inverted U-shaped dose response curve was observed at this time point of maximal antinociception (30 min post drug); \((R,S)-AM1241\) (1 mg kg\(^{-1}\) i.p.) produced greater antinociception than either the lower (0.10 mg kg\(^{-1}\) i.p.) or the higher (10 mg kg\(^{-1}\) i.p.) doses \((P < 0.05\) for each comparison). Groups receiving \((R,S)-AM1241\) (1 mg kg\(^{-1}\) i.p.) exhibited the maximal percent change (81.5\%) in paw withdrawal latencies, relative to baseline, at 30 min post injection.

Like the aminoalkylindole \((R,S)-AM1241\), the cannabialactone AM1714 (0.10, 0.33, 1, 5 and 10 mg kg\(^{-1}\) i.p.) increased thermal paw withdrawal latencies at 30 min post injection. This antinociceptive effect was observed relative to either vehicle \((F_{5,66} = 3.924, P < 0.01; P < 0.05\) for each comparison) or baseline paw withdrawal latencies (F
Paw withdrawal latencies were maximally increased in groups receiving AM1714 (0.10, 0.33, 1, 5 and 10 mg kg\(^{-1}\) i.p.) at 30 min post injection; percentage increases, relative to baseline, ranged from 39.3 to 66.8%.

The cannabilactone AM1710 (0.10, 1, 5 and 10 mg kg\(^{-1}\) i.p.), which is structurally related to AM1714, also increased thermal paw withdrawal latencies relative to vehicle at 30 min post drug (F\(_{5,55} = 5.859, P < 0.001; P < 0.05\) for each comparison). All doses of AM1710 also increased paw withdrawal latencies relative to baseline measurements at this time point (F\(_{5,78} = 17.311, P < 0.001; P < 0.05\) for each comparison; Fig 4.2c). Paw withdrawal latencies were maximally increased in groups receiving AM1710 (0.10, 0.33, 1, 5 and 10 mg kg\(^{-1}\) i.p.) at 30 min post injection; percent increases ranged from 31.5 to 64.4%. This drug exhibited a U-shaped dose response with AM1710 (0.33 mg kg\(^{-1}\) i.p.) being the only dose that was not different from vehicle.

**Comparison of Antinociceptive Effects Induced by Cannabilactone and Aminoalkylindole Cannabinoid Agonists**

The doses of AM1714 and AM1710 that produced the greatest antinociceptive effects and longest durations of action (as measured by percent change in paw withdrawal thresholds from baseline measurements) were compared to a well-characterized CB\(_2\) agonist (R,S)-AM1241. (R,S)-AM1241 (1 mg kg\(^{-1}\) i.p.), AM1714 (5 mg kg\(^{-1}\) i.p.), and AM1710 (5 mg kg\(^{-1}\) i.p.) produced robust thermal antinociception (F\(_{3,43} = 16.759, P < 0.001\); Fig 4.3) relative to vehicle (P < 0.01) at 30 min post injection. However, only AM1714 (5 mg kg\(^{-1}\) i.p.) retained antinociceptive efficacy relative to vehicle at 60 min post-injection (P < 0.05 planned comparison t-test); this effect dissipated by 120 min post
drug \((P > 0.10)\). AM1710 (5 mg kg\(^{-1}\) i.p.) increased paw withdrawal latencies relative to vehicle at 120 min post injection \((P < 0.05;\) planned comparison t-test). By contrast, the dose of \((R,S)\)-AM1241 (1 mg kg\(^{-1}\) i.p.) that produced the maximal antinociception, relative to vehicle, at 30 min was no longer active at either 60 or 120 minutes post injection \((P > 0.11)\).

**Pharmacological Specificity**

Consistent with previous reports, the prototypical CB\(_2\) agonist, \((R,S)\)-AM1241 (1 mg kg\(^{-1}\) i.p.) produced CB\(_2\)-mediated thermal antinociception (Malan et al., 2001); this effect was blocked by SR144528 (6 mg kg\(^{-1}\) i.p.) but not by SR141716 (6 mg kg\(^{-1}\) i.p.) at 30 min post-injection \((F_{3,39} = 18.20, P < 0.001; P < 0.01\) for each comparison; Fig 4.4a).

Antinociception produced by the lowest dose of AM1714 (0.10 mg kg\(^{-1}\) i.p.) was completely blocked by either the CB\(_2\) antagonist AM630 (6 mg kg\(^{-1}\) i.p.) \((F_{3,40} = 5.715, P < 0.01)\) or the CB\(_1\) antagonist SR141716 (6 mg kg\(^{-1}\) i.p.) \((F_{3,40} = 5.715, P < 0.01);\) Fig 4.4b) at the time point of maximal antinociception (i.e. 30 minutes post-injection). The antinociceptive effect of AM1714 (0.10 mg kg\(^{-1}\)) was greater than either blockade condition \((P < 0.01\) planned comparison t-tests). Antinociception produced by a fifty-fold higher dose of AM1714 (5 mg kg\(^{-1}\) i.p.) was completely blocked \((F_{5,53} = 2.819, P < 0.05;\) Fig 4.4c) by either the CB\(_2\) antagonist AM630 (6 mg kg\(^{-1}\) i.p.) or the CB\(_1\) antagonist SR141716 (6 mg kg\(^{-1}\) i.p.) \((F_{5,53} = 2.819, P < 0.05;\) Fig 4.4c) at the same time point. By contrast, SR144528 failed to block the antinociceptive effects of AM1714 (5 mg kg\(^{-1}\) i.p.). Post hoc comparisons revealed a reliable antinociceptive effect in AM1714-treated groups pre-treated with the CB\(_2\) antagonist SR144528 (6 and 10 mg kg\(^{-1}\) i.p.) \((P < 0.05\) versus vehicle).
Antinociception produced by the lowest efficacious dose of the structurally related cannabilactone AM1710 (0.10 mg kg\(^{-1}\) i.p.) was selectively blocked by the CB\(_2\) antagonist SR144528 (6 mg kg\(^{-1}\) i.p.) \(F_{3,41} = 3.255, P < 0.05\); Fig 4.4d) but not by the CB\(_1\) antagonist SR141716 (6 mg kg\(^{-1}\) i.p.). AM1710 (0.10 mg kg\(^{-1}\) i.p.) produced antinociception relative to the vehicle condition at 30 min \(P < 0.05\) for comparison) but not at 120 min post injection \(P > 0.08\). By contrast, antinociceptive effects of a higher dose of AM1710 (5 mg kg\(^{-1}\) i.p.) were blocked \(F_{3,40} = 7.450, P < 0.001\); Fig 4.4e) by both SR144528 (6 mg kg\(^{-1}\) i.p.) and SR141716 (6 mg kg\(^{-1}\) i.p.) at 30 min post injection. Antinociceptive effects of AM1710 (5 mg kg\(^{-1}\) i.p.) persisted at 120 minutes post-injection \(P < 0.05\), planned comparison t-test), suggesting that the duration of action of AM1710 (5 mg kg\(^{-1}\) i.p.) outlasted that of AM1710 (0.1 mg kg\(^{-1}\) i.p.), AM1714 (0.1 or 5 mg kg\(^{-1}\) i.p.) or \((R,S)\)-AM1241 (1 mg kg\(^{-1}\) i.p.). Paw withdrawal latencies were similar in groups receiving the vehicle and groups receiving either SR141716 (6 mg kg\(^{-1}\) i.p.) or SR144528 (6 mg kg\(^{-1}\) i.p.) prior to AM1710 (5 mg kg\(^{-1}\) i.p.). None of the antagonists altered thermal paw withdrawal latencies relative to vehicle at 30 \(P > 0.89\) or 120 \(P > 0.36\) minutes post-injection (Fig 4.4f).

**Assessment of CNS Side-Effects: Antinociception in the Tail-flick Test and Hypothermia**

WIN55,212-2 (5 mg kg\(^{-1}\) i.p) produced characteristic CB\(_1\)-mediated antinociception in the tail-flick test that was not produced by either the cannabilactones or \((R,S)\)-AM1241. Tail-flick latencies were elevated in WIN55,212-2-treated groups relative to either vehicle, \((R,S)\)-AM1241 (1 mg kg\(^{-1}\) i.p), all doses of AM1714 \(F_{6,38} = 12.653, P < 0.001; P < 0.05\) for each comparison; Fig 4.5a) and all doses of AM1710
(F_{6,38} = 10.505, P < 0.001; P < 0.05 for each comparison; Fig 4.5b) at all time points post-injection. The CB1 antagonist, SR141716 (6 mg kg\(^{-1}\) i.p), blocked the antinociceptive effects of WIN55,212-2 in the tail-flick test across the entire observation interval (P < 0.05 for each comparison), consistent with mediation by CB1. By contrast, AM1714 (0.1, 5, and 10 mg kg\(^{-1}\) i.p.), AM1710 (0.10, 5, and 10 mg kg\(^{-1}\) i.p.) and (R,S)-AM1241 (1 mg kg\(^{-1}\) i.p) failed to alter tail-flick latencies at any post-injection time point (P > 0.60 for each comparison).

WIN55,212-2 also produced a characteristic CB1-mediated hypothermic effect that was not produced by the cannabilactones or (R,S)-AM1241. WIN55,212-2 (5 mg kg\(^{-1}\) i.p) decreased rectal temperature relative to either vehicle, (R,S)-AM1241 (1 mg kg\(^{-1}\) i.p), all doses of AM1714 (F_{6,44} = 7.542, P < 0.001; P < 0.05 for each comparison; Fig 4.5c) and all doses of AM1710 (F_{6,44} = 8.353, P < 0.001; P < 0.01 for each comparison; Fig 4.5d) at 35 minutes post injection. A hypothermic effect of WIN55,212-2 (5 mg kg\(^{-1}\) i.p) was still apparent, relative to vehicle, at 65 minutes post injection in both studies (F_{6,44} = 3.987, P < 0.01; P < 0.01 for relevant comparison; Fig 4.5c and F_{6,44} = 3.576, P < 0.001, P < 0.01 for relevant comparison; Fig 4.5d). The hypothermic effects of WIN55,212-2 were completely blocked by SR141716 (6 mg kg\(^{-1}\) i.p; P < 0.05 for each comparison), consistent with mediation by CB1. By contrast, AM1714 (0.10, 5, and 10 mg kg\(^{-1}\) i.p.), AM1710 (0.10, 5, and 10 mg kg\(^{-1}\)) and (R,S)-AM1241 did not alter rectal temperature relative to the vehicle condition at any time point (P > 0.08 for each comparison).

**Assessment of CNS Side-effects: Hypoactivity and Motor Ataxia**

WIN55,212-2 produced characteristic CB1-mediated hypoactivity that was not produced by antinociceptive doses of (R,S)-AM1241, AM1714 or AM1710. WIN55,212-
2 (5 mg kg\(^{-1}\) i.p.) decreased distance traveled in the activity meter relative to all other groups (\(F_{3,27} = 12.404, P < 0.001; P < 0.01\) for each comparison in Fig 4.6a; \(F_{5,41} = 14.555, P < 0.001; P < 0.05\) for each comparison in Fig 4.6b; \(F_{4,37} = 9.154, P < 0.001; P < 0.05\) for each comparison in Fig 4.6c). As expected, WIN55,212-2-induced hypoactivity was blocked by the CB\(_1\) antagonist SR141716 (6 mg kg\(^{-1}\) i.p.; \(P < 0.01\) for comparison; Fig. 4.6a). By contrast, \((R,S)\)-AM1241 (1 mg kg\(^{-1}\) i.p.; Fig. 4.6a) did not alter locomotor activity relative to the vehicle condition (\(P > 0.42\)). A dose of AM1714 (0.10 mg kg\(^{-1}\) i.p.) that produced maximal antinociception in the plantar test did not produce hypoactivity (\(P > 0.95\)). However, a fifty-fold higher dose of AM1714 (5 mg kg\(^{-1}\) i.p.) decreased total distance traveled relative to vehicle (\(P < 0.01\)); these effects were blocked by the CB\(_2\) antagonist, AM630 (6 mg kg\(^{-1}\) i.p.) but not by the CB\(_1\) antagonist SR141716 (6 mg kg\(^{-1}\) i.p.; \(P < 0.05\) for relevant comparison; Fig 4.6b). By contrast, AM1710 (0.10, 5 or 10 mg kg\(^{-1}\) i.p.) did not reliably inhibit locomotor activity relative to the vehicle condition at any dose (\(P > 0.11\); Fig 4.6c).

In contrast to the cannabialactones and \((R,S)\)-AM1241 (1 mg kg\(^{-1}\) i.p.), WIN55,212-2 produced CB\(_1\)-mediated motor ataxia in the rota-rod test. WIN55,212-2 (5 mg kg\(^{-1}\) i.p.) impaired the ability of rats to walk on a rotating drum relative to either vehicle or \((R,S)\)-AM1241 (1 mg kg\(^{-1}\) i.p.) (\(F_{3,27} = 9.422, P < 0.001; P < 0.05\) for each comparison; Fig 4.7a). As expected, WIN55,212-2-induced motor ataxia was completely blocked by SR141716 (\(P < 0.001\) for relevant comparison).

WIN55,212-2 also produced motor ataxia relative to the cannabialactones AM1714 (0.10 mg kg\(^{-1}\) i.p.) (\(F_{3,27} = 6.111, P < 0.01; P < 0.05\) for each comparison; Fig 4.7b) and AM1710 (5 and 10 mg kg\(^{-1}\) i.p.) (\(F_{4,37} = 4.790, P < 0.01; P < 0.05\) for relevant
comparison; Fig 4.7c) at 35 min post injection. WIN55,212-2 (5 mg kg\(^{-1}\) i.p.) did not alter rota-rod latencies relative to vehicle at subsequent time points (65 min: \(P > 0.14\) for each comparison and 125 min: \(P > 0.36\) for Fig 4.7a,b,c), suggesting that the antinociceptive effects of WIN55,212-2 outlast the motor ataxic effects of the same doses.

\((R,S)\)-AM1241 (1 mg kg\(^{-1}\) i.p.), AM1714 (0.1 and 5 mg kg\(^{-1}\) i.p.) and AM1710 (0.1, 5, and 10 mg kg\(^{-1}\) i.p.) did not alter rota-rod descent latencies relative to vehicle at any time point \((P > 0.62)\) (Fig. 4.7a-c). Rota-rod latencies were significantly lower in groups receiving WIN55,212-2 relative to groups receiving either AM1714 or AM1710 \((P < 0.05\) for each comparison, planned comparison t-tests).
Figure 4.1. Chemical structures of the aminoalkylindole \((R,S)\)-AM1241, and the cannabiliactones, AM1714 and AM1710.
Figure 4.1: Chemical structures of the aminoalkylindole $(R,S)$-AM1241, and the cannabilaetones, AM1714 and AM1710.
Figure 4.2. Log dose response for (a.) \((R,S)\)-AM1241-, (b.) AM1714- and (c.) AM1710-induced antinociception in the plantar test. Withdrawal latencies to thermal stimulation in the plantar test are shown. BL denotes baseline paw withdrawal latencies observed prior to agonist or vehicle injection. Doses are in mg kg\(^{-1}\). *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) vs. DMSO control condition, †\(P < 0.05\), ††\(P < 0.01\), †††\(P < 0.001\) vs. baseline, ‡\(P < 0.05\) vs. \((R,S)\)-AM1241 (0.1 and 10 mg/kg i.p.) (ANOVA; Dunnett and Tukey post hoc tests). N = 7-19 per group.
Figure 4.2: Log dose response for (R,S)-AM1241-, AM1714- and AM1710-induced antinociception in the plantar test.
Figure 4.3. Comparison of the most effective doses of (R,S)-AM1241, AM1714 and AM1710 observed in the plantar test. **$P < 0.01$, ***$P < 0.001$ vs. DMSO control condition (ANOVA and Dunnett post hoc test). $^\times P < 0.05$ vs. DMSO control condition (Student t-test). $N = 8$-19 per group.
Figure 4.3: Comparison of the most effective doses of (R,S)-AM1241, AM1714 and AM1710 observed in the plantar test.
Figure 4.4. Pharmacological specificity of antinociceptive effects of AM1714, AM1710 and (R,S)-AM1241 in the plantar test. (a.) The CB2 antagonist SR144528 (SR2; 6 mg kg\(^{-1}\) i.p.) but not the CB1 antagonist SR141716 (SR1; 6 mg kg\(^{-1}\) i.p.) blocked the antinociceptive effects of (R,S)-AM1241 (1 mg kg\(^{-1}\) i.p.). (b.) The CB1 antagonist (SR1; 6 mg kg\(^{-1}\) i.p.) and the CB2 antagonist (AM630; 6 mg kg\(^{-1}\) i.p.) blocked the antinociceptive effects of AM1714 (0.10 mg kg\(^{-1}\) i.p.). (c.) The CB1 antagonist SR141716 (SR1; 6 mg kg\(^{-1}\) i.p.) and the CB2 antagonist AM630 (6 mg kg\(^{-1}\) i.p.), but not the CB2 antagonist SR144528 (SR2; 6 and 10 mg kg\(^{-1}\) i.p.), blocked the antinociceptive effects of AM1714 (5 mg kg\(^{-1}\) i.p.). (d.) The CB2 antagonist (SR2; 6 mg kg\(^{-1}\) i.p.), but not the CB1 antagonist (SR1; 6 mg kg\(^{-1}\) i.p.), blocked the antinociceptive effects of AM1710 (0.10 mg kg\(^{-1}\) i.p.). (e.) SR141716 (6 mg kg\(^{-1}\) i.p.) and SR144528 (6 mg kg\(^{-1}\) i.p.) blocked the antinociceptive effects of AM1710 (5 mg kg\(^{-1}\) i.p.). (f.) SR141716 (6 mg kg\(^{-1}\) i.p.), SR144528 (6 mg kg\(^{-1}\) i.p.) and AM630 (6 mg kg\(^{-1}\) i.p.) did not alter paw withdrawal latencies relative to vehicle. \(*P < 0.05, **P < 0.01, ***P < 0.001 vs. DMSO control, \(\dagger\)P < 0.01 vs. (R,S)-AM1241 + SR2, \(\dagger\)P < 0.01 vs. all drug groups, \(\#\)P < 0.05 vs. AM1714 + SR2 (10) (ANOVA; Dunnett and Tukey post hoc tests). \(\alpha\alpha\)P < 0.01 vs. all drug groups, \(\dagger\dagger\)P < 0.05 vs. AM1710 (0.10) + SR1 (6), \(\dagger\dagger\)P < 0.05 vs. AM1710 (5 mg kg\(^{-1}\) i.p.), \(\chi\)P < 0.05 vs. DMSO control (Student t-test). N = 8-19 per group.
Figure 4.4: Pharmacological specificity for antinociception observed in the plantar test following administration of (R,S)-AM1241, AM1714, and AM1710.
Figure 4.5. (a.) Effects of cannabialactone and aminalkylindole cannabinoid agonists on tail-flick antinociception and hypothermia. WIN55,212-2 (5 mg kg\(^{-1}\) i.p.) produced CB\(_1\)-mediated antinociception in the tail-flick test. This effect was blocked by the CB\(_1\) antagonist SR141716 (SR1; 6 mg kg\(^{-1}\) i.p.). Neither \((R,S)\)-AM1241 (1 mg kg\(^{-1}\) i.p.), AM1714 (0.1, 5, and 10 mg kg\(^{-1}\) i.p.), nor (b.) AM1710 (0.1, 5, and 10 mg kg\(^{-1}\) i.p.) produced antinociception in the tail flick test. (c.) WIN55,212-2 (5 mg kg\(^{-1}\) i.p.) decreased rectal temperature relative to baseline through a CB\(_1\) mechanism; this effect was blocked by SR141716 (SR1; 6 mg kg\(^{-1}\) i.p.). Neither \((R,S)\)-AM1241 (1 mg kg\(^{-1}\) i.p.), AM1714 (0.1, 5, and 10 mg kg\(^{-1}\) i.p.), nor (d.) AM1710 (0.1, 5, and 10 mg kg\(^{-1}\) i.p.) altered rectal temperature. **\(P < 0.01\), ***\(P < 0.001\) vs. DMSO control condition, †\(P < 0.05\), ††\(P < 0.01\), †††\(P < 0.001\) vs. all drug conditions, *\(P < 0.05\) vs. AM1714 (0.1 mg kg\(^{-1}\) i.p.), WIN-2 + SR1 and \((R,S)\)-AM1241 (1 mg kg\(^{-1}\) i.p.), ‡\(P < 0.05\) vs. AM1710 (10 mg kg\(^{-1}\) i.p.), WIN-2 + SR1, and \((R,S)\)-AM1241 (1 mg kg\(^{-1}\) i.p.), #\(P < 0.05\) vs. AM1710 (10 mg kg\(^{-1}\) i.p.) (ANOVA; Dunnett and Tukey post hoc tests). N = 6-7 per group.
Figure 4.5: Tail flick latencies (i.e. antinociception) and rectal temperatures (hypothermia) observed following administration of WIN55,212-2, (R,S)-AM1241, AM1714 and AM1710.
Figure 4.6. (a.) Effects of cannabilactone and aminoalkylindole cannabinoid agonists on locomotor activity. WIN55,212-2 (5 mg kg\(^{-1}\) i.p.) reduced total distance traveled (cm) through a CB\(_1\) mechanism. This effect was blocked by the CB\(_1\) antagonist SR141716 (SR1; 6 mg kg\(^{-1}\) i.p.). (\(R,S\))-AM1241 (1 mg kg\(^{-1}\) i.p.) did not alter locomotor activity. (b.) AM1714 (5 mg kg\(^{-1}\) i.p.), but not (0.1 mg kg\(^{-1}\) i.p.) reduced locomotor activity. This effect was blocked by the CB\(_2\) antagonist AM630 (6 mg kg\(^{-1}\) i.p.) but not the CB\(_1\) antagonist SR141716 (6 mg kg\(^{-1}\) i.p.). (c.) AM1710 (0.1, 5, and 10 mg kg\(^{-1}\) i.p.) did not alter locomotor activity. **\(P < 0.01\) ***\(P < 0.001\) vs. DMSO control condition, \(^\dagger\)\(P < 0.05\), \(^\dagger\dagger\)\(P < 0.01\), \(^\dagger\dagger\dagger\)\(P < 0.001\) vs. WIN55,212 (5 mg kg\(^{-1}\) i.p.), \(^\times\times\)\(P < 0.01\) vs. AM1714 (5 mg kg\(^{-1}\) i.p.) and AM1714 (5) + SR1 (6) (ANOVA; Dunnett and Tukey post hoc tests). \(^\#\)\(P < 0.05\) vs. AM1714 (5 mg/kg i.p.) and AM1714 (5) + SR1 (6) (Student t-test). \(N = 6-8\) per group.
Figure 4.6: Total distance traveled (cm) in an activity meter following administration of WIN55,21-2, (R,S)-AM1241, AM1714 and AM1710.
Figure 4.7. (a.) Effects of cannabimimetic and aminoalkylindole cannabinoid agonists on motor ataxia. WIN55,212-2 (5 mg kg\(^{-1}\) i.p.) produced CB\(_1\)-mediated motor ataxia, manifested as a decrease in descent latency (sec) in the rota-rod test. This effect was blocked by SR141716 (SR1; 6 mg kg\(^{-1}\) i.p.). Neither (R,S)-AM1241 (1 mg kg\(^{-1}\) i.p.), (b.) AM1714 (0.1 and 5 mg kg\(^{-1}\) i.p.), nor (c.) AM1710 (0.1, 5, and 10 mg kg\(^{-1}\) i.p.) altered rota-rod latency relative to the vehicle condition. *\(P < 0.05\) vs. DMSO control condition, 
\(\dagger P < 0.05, \ddagger P < 0.01, \ddagger\ddagger P < 0.001\) vs. WIN55,212 (5 mg kg\(^{-1}\) i.p.) (ANOVA; Dunnett and Tukey post hoc tests). \(\times P < 0.05, \times\times P < 0.01\) vs. WIN55,212-2 (Student t-test). \(N = 6-8\) per group.
Figure 4.7: Effects of cannabilactone and aminoalkylindole cannabinoid agonists on motor ataxia as measured by latency on a rotarod.
Table 4.1: Paw withdrawal thresholds (g) to punctuate mechanical stimulation in animals that received the cannabiliactones AM1714 and AM1710

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-injection</th>
<th>Post-Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>67.8 ± 3.6</td>
<td>71.0 ± 4.8</td>
</tr>
<tr>
<td>AM1714 (0.10 mg kg⁻¹)</td>
<td>65.2 ± 3.9</td>
<td>69.7 ± 6.3</td>
</tr>
<tr>
<td>AM1714 (0.33 mg kg⁻¹)</td>
<td>75.4 ± 4.0</td>
<td>72.6 ± 7.8</td>
</tr>
<tr>
<td>AM1714 (1 mg kg⁻¹)</td>
<td>71.6 ± 5.9</td>
<td>66.0 ± 6.0</td>
</tr>
<tr>
<td>AM1714 (5 mg kg⁻¹)</td>
<td>71.2 ± 6.7</td>
<td>81.4 ± 8.1</td>
</tr>
<tr>
<td>AM1714 (10 mg kg⁻¹)</td>
<td>63.8 ± 4.2</td>
<td>77.7 ± 2.7*</td>
</tr>
<tr>
<td>AM1710 (0.10 mg kg⁻¹)</td>
<td>79.1 ± 4.2</td>
<td>74.0 ± 5.9</td>
</tr>
<tr>
<td>AM1710 (0.33 mg kg⁻¹)</td>
<td>66.1 ± 3.1</td>
<td>71.4 ± 4.6</td>
</tr>
<tr>
<td>AM1710 (1 mg kg⁻¹)</td>
<td>63.9 ± 3.6</td>
<td>73.3 ± 2.3*</td>
</tr>
<tr>
<td>AM1710 (5 mg kg⁻¹)</td>
<td>70.2 ± 3.7</td>
<td>64.7 ± 5.3</td>
</tr>
<tr>
<td>AM1710 (10 mg kg⁻¹)</td>
<td>63.9 ± 3.3</td>
<td>68.8 ± 5.5</td>
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</tbody>
</table>

Data are mean ± s.e.mean. * $P < 0.05$ vs. same group pre-injection threshold (Student t-test).
Table 4.2: Paw withdrawal thresholds (g) to punctuate mechanical stimulation in animals undergoing pharmacological specificity testing with AM1714 and AM1710

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-injection</th>
<th>Post-Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>67.8 ± 3.6</td>
<td>71.0 ± 4.7</td>
</tr>
<tr>
<td>AM1714 (0.01 mg kg⁻¹)</td>
<td>65.2 ± 3.9</td>
<td>69.7 ± 6.3</td>
</tr>
<tr>
<td>AM1714 (0.10) + AM630 (6)</td>
<td>83.7 ± 3.2†</td>
<td>86.9 ± 4.9†</td>
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<tr>
<td>AM1714 (0.10) + SR1 (6)</td>
<td>65.8 ± 3.5</td>
<td>70.8 ± 6.4</td>
</tr>
<tr>
<td>AM1714 (5 mg kg⁻¹)</td>
<td>71.2 ± 6.7</td>
<td>81.4 ± 8.1</td>
</tr>
<tr>
<td>AM1714 (5) + SR2 (6)</td>
<td>71.4 ± 4.4</td>
<td>77.4 ± 6.0</td>
</tr>
<tr>
<td>AM1714 (5) + SR2 (10)</td>
<td>69.3 ± 6.8</td>
<td>73.9 ± 3.4</td>
</tr>
<tr>
<td>AM1714 (5) + AM630 (6)</td>
<td>68.7 ± 6.2</td>
<td>66.5 ± 3.4</td>
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<tr>
<td>AM1714 (5) + SR1 (6)</td>
<td>84.9 ± 2.7†</td>
<td>89.7 ± 8.9†</td>
</tr>
<tr>
<td>AM1710 (0.10 mg kg⁻¹)</td>
<td>79.1 ± 4.2</td>
<td>74.0 ± 5.9</td>
</tr>
<tr>
<td>AM1710 (0.10) + SR2 (6)</td>
<td>73.7 ± 5.4</td>
<td>71.6 ± 4.7</td>
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<tr>
<td>AM1710 (0.10) + SR1 (6)</td>
<td>68.0 ± 4.2</td>
<td>69.3 ± 4.7</td>
</tr>
<tr>
<td>AM1710 (5 mg kg⁻¹)</td>
<td>70.2 ± 3.7</td>
<td>64.7 ± 5.3</td>
</tr>
<tr>
<td>AM1710 (5) + SR 2 (6)</td>
<td>81.4 ± 3.7</td>
<td>72.3 ± 3.1</td>
</tr>
<tr>
<td>AM1710 (5) + SR1 (6)</td>
<td>70.5 ± 5.7</td>
<td>64.1 ± 8.5</td>
</tr>
<tr>
<td>SR141716 (6 mg kg⁻¹)</td>
<td>63.3 ± 5.5</td>
<td>75.6 ± 5.7</td>
</tr>
<tr>
<td>SR144528 (6 mg kg⁻¹)</td>
<td>70.9 ± 5.1</td>
<td>62.8 ± 5.8</td>
</tr>
<tr>
<td>AM630 (6 mg kg⁻¹)</td>
<td>64.8 ± 2.3</td>
<td>63.9 ± 4.4</td>
</tr>
</tbody>
</table>

Data are mean ± s.e.mean. Doses are in mg kg⁻¹. SR1 = SR141716; SR2 = SR144528. †P < 0.05 different from DMSO condition (ANOVA and Tukey post hoc test).
Discussion

Novel cannabinoid agonists (AM1714 and AM1710) from the cannibilactone class produce antinociception in the plantar test in the absence of unwanted CNS side-effects. The prototypical aminoalkylindole CB2 agonist \((R,S)\)-AM1241 produced thermal antinociception in the plantar test that was characterized by an inverted U-shaped dose response curve as previously published (Rahn et al., 2010). By contrast, the cannibilactones exhibited fairly linear dose response relationships in their ability to produce antinociception in the same test. The relative lack of dose-dependence observed in the antinociceptive effects of the cannibilactones suggests that dose escalation is unable to further maximize antinociception in the plantar test, at least in naive animals. However, it is important to note that one dose of AM1710 (0.33 mg kg\(^{-1}\) i.p.) did not produce antinociception relative to vehicle, suggestive of a U-shaped dose response curve for AM1710. This U-shaped dose response curve is in contrast to the completely linear dose response curve of AM1714. More work is necessary to determine whether this linear dose response profile reflects a tendency of these ligands or their metabolites to bind irreversibly to CB2 receptors. Our assessments of pharmacological specificity consequently employed pre-treatments with high doses of competitive antagonists, rather than co-administration of antagonists with the agonists.

Both cannibilactone compounds failed to produce antinociception to non-noxious levels of mechanical stimulation relative to vehicle treatment. However, our results do not preclude the possibility that antinociception could occur to noxious levels of stimulation (e.g. applied with a Randall Selitto device). Thus, cannibilactones suppress mechanical allodynia and normalize mechanical withdrawal thresholds (Rahn et al.,
2008) at doses that do not produce antinociception to the same stimulus modality in otherwise naive animals.

AM1710 (5 mg kg\(^{-1}\) i.p.) exhibited a longer duration of action than either its counterpart AM1714 (5 mg kg\(^{-1}\) i.p.) or the aminoalkylindole CB\(_2\) agonist (\(R,S\))-AM1241. In previous work, both AM1714 and AM1710 produced thermal antinociception when administered locally (i.paw) (Khanolkar \textit{et al.}, 2007). However, this is the first study to demonstrate antinociceptive effects of the cannabi lactones following systemic administration in naive animals. In our study, (\(R,S\))-AM1241-induced antinociception was selectively blocked by the CB\(_2\) antagonist SR144528, but not by the CB\(_1\) antagonist SR141716. However, \textit{in vivo} pharmacological specificity of systemically administered cannabi lactones has proven more difficult to interpret. AM1714-induced antinociception was blocked completely by pre-treatment with either the CB\(_1\) antagonist SR141716 or the CB\(_2\) antagonist AM630. However, it was not blocked by a structurally distinct CB\(_2\) antagonist SR144528. Importantly, a low dose of AM1710 (0.1 mg kg\(^{-1}\) i.p.), which produced antinociception comparable to that of AM1710 (5 mg kg\(^{-1}\) i.p.), showed no evidence for mediation by CB\(_1\); antinociception produced by AM1710 (0.1 mg kg\(^{-1}\) i.p.) was insensitive to blockade by SR141716. By contrast, pre-treatment with either SR144528 or SR141716 completely blocked the antinociceptive effects of a higher dose of AM1710 (5 mg kg\(^{-1}\) i.p.). Importantly, neither of these compounds exhibited cardinal signs of CB\(_1\) receptor activation such as antinociception in the tail-flick test, hypothermia or motor ataxia. Thus, any CB\(_1\) activity produced by systemically administered cannabi lactones may be peripheral CB\(_1\) activity or attributable to off-target actions (e.g. low affinity at FAAH) that manipulate endocannabinoid levels capable of binding to CB\(_1\)
receptors. The *in vivo* pharmacology of cannibilactones is more complex than would be expected from the *in vitro* binding affinities (Khanolkar *et al.*, 2007) of these compounds at CB₂ and CB₁ receptors. Differences in the bioactive transformations of the cannibilactones may contribute to the *in vivo* pharmacology of these compounds. Thus, it is noteworthy that AM1710 produced antinociception at 30 and 120 min post injection, but not at 60 min post injection. More work is necessary to identify metabolites of AM1714 and AM1710 and determine whether they are biologically active and brain permeable.

In contrast to our *in vivo* results, *in vitro* binding studies (Khanolkar *et al.*, 2007) suggest that both AM1714 and AM1710 are selective for the CB₂ receptor. Locally administered AM1714 produces thermal antinociception in the plantar test that was blocked by the CB₂-selective antagonist AM630 but not by the CB₁-selective antagonist AM251. Thus, cannibilactone-induced antinociception is mediated, at least in part, by peripheral sites of action. Systemic AM1714 also suppresses chemotherapy-induced neuropathy through a CB₂ specific mechanism; these antihyperalgesic effects were blocked by SR144528, but not by SR141716. In fact, animals receiving SR141716 prior to administration of AM1714 showed enhanced antihyperalgesia (Rahn *et al.*, 2008). Thus, pharmacological specificity of these agonists may differ based upon whether or not these compounds are evaluated systemically or locally in the paw or under conditions (normal vs. neuropathic) in which CB₂ or CB₁ receptors may be upregulated.

Antinociception produced by a high dose (5 mg kg⁻¹ i.p.) of AM1714 or AM1710 was completely blocked by either a CB₂ or a CB₁ antagonist, in the absence of the other drug. Thus, we observed a CB₁ component in the antinociceptive effects of systemically
administered cannabilactones, but no signs of CNS activity in the tetrad. This CB₁ component was not observed in groups that received the aminoalkylindole (R,S)-AM1241 and the same dose of the CB₁ antagonist. This CB₁ component was not observed following local injection of the same compounds into the dorsal surface of the paw (Khanolkar et al., 2007). Biologically active metabolites that bind CB₁ receptors may be generated following systemic but not local doses of the cannibilactones. Consistent with this hypothesis, the same doses of the cannabilactones were largely inactive in the tetrad, which assesses cardinal signs of CB₁ receptor activation. Limited penetration of cannabilactones or their metabolites into the CNS could contribute to the failure of AM1714 and AM1710 to produce hypothermia, motor ataxia or antinociception in the tail-flick test. Moreover, no evidence for a CB₁ component in AM1710-induced antinociception was observed following administration of a lower dose of AM1710 (0.10 mg kg⁻¹ i.p.), which nonetheless produced maximal antinociception in the plantar test. Thus, increasing the dose did not further increase the antinociceptive effects of AM1710 but could presumably increase the amount of AM1710 available for metabolic transformation or off-target actions.

Off-target effects that could result directly in elevations of endocannabinoid levels could potentially contribute to the blockade of cannibilactone-induced antinociception produced by SR141716 in our study. However, this possibility cannot fully account for our data because AM1710, unlike AM1714, lacks affinity at FAAH. The same dose of SR141716 (6 mg kg⁻¹ i.p.) which blocked the antinociceptive effects of AM1710 (5 mg kg⁻¹ i.p.) and AM1714 (0.10 and 5 mg kg⁻¹ i.p.) did not block antinociception produced by either a lower dose of AM1710 (0.10 mg kg⁻¹ i.p.) or (R,S)-AM1241 (1 mg kg⁻¹ i.p.).
Anxiolytic effects of SR141716 (Haller et al., 2002; Rodgers et al., 2003; but see Thiemann et al., 2009) have been observed in CB₁⁻/⁻ knockout mice (Uriguen et al., 2004), suggesting that they may be mediated through a “non-CB₁” site. Thus, it is noteworthy that SR141716 also binds TRPV1 receptors at micromolar concentrations (De Petrocellis et al., 2001). However, a role for TRPV1 in cannabilactone actions is unlikely because activation of TRPV1 produces hypothermia in vivo (Miller et al., 1982) and our studies demonstrate that cannabilactones do not alter body temperature. Blockade of AM1714-induced antinociception by AM630, but not multiple doses of SR144528, is more difficult to explain. To our knowledge, this is the first report of pharmacological blockade produced by AM630 (Kᵢ: 31.2 nM, 165-fold selective over CB₂) which was not produced by SR144528 (Kᵢ: CB₁ vs. CB₂: 0.6 nM vs. 400 nM). More work is necessary to investigate differential blockade of cannabilactones compounds observed with these two well-characterized CB₂-specific antagonists.

This is the first study to assess CNS side-effects in the tetrad produced by the cannabilactones AM1714 and AM1710. Previously, AM1714 (3.3 mg kg⁻¹ i.p.) was tested in the rota-rod where it showed no activity relative to baseline measurements (Khanolkar et al., 2007). Our results indicate that only a high dose of AM1714 (5 mg kg⁻¹ i.p.), fifty-fold higher than the minimal antinociceptive dose identified in our study, produces any “centrally”-mediated effects; however, this observed hypoactivity was blocked by a CB₂ antagonist and not by a CB₁ antagonist. Yao and colleagues (2009) have reported a locomotor deficit following administration of a high dose of a CB₂-specific agonist. However, they were able to reverse this effect with SR141716. One of the features of CB₂ agonists which make them attractive therapeutic targets is their lack
of CNS side-effects. Thus, the CB₂-mediated locomotor effect observed here may reflect a difference in exploratory behavior following AM1714 treatment (e.g. elicited in response to a novel open field) rather than a motor deficit per se. AM1714 did not produce motor ataxia in the rota-rod test. Moreover, the failure of AM1714 to produce motor ataxia cannot be attributed to a subthreshold motor ataxic effect because all animals were allowed to fall off the rota-rod in our study (i.e. a ceiling latency was not employed to prematurely terminate a trial). It is important to emphasize that a fifty-fold lower dose of AM1714 (0.10 mg kg⁻¹ i.p.), which did not alter distance traveled in the activity meter, produced equivalent antinociception in the plantar test. Moreover, no centrally mediated side-effects were observed in animals that received AM1710 (0.1, 5 or 10 mg kg⁻¹ i.p.) or (R,S)-AM1241 (1 mg kg⁻¹ i.p.). Similar results have been reported for (R,S)-AM1241 in a tetrad which did not include tail-flick (Malan et al., 2001). However, higher doses of (R,S)-AM1241 (10 mg kg⁻¹ i.p.) produce modest increases in tail-flick latencies in mice (Ibrahim et al., 2006).

Our results demonstrate that the cannibilactones, AM1714 and AM1710, like the aminoalkylindole (R,S)-AM1241, produce antinociception in the plantar test without altering tail-flick latencies. The plantar test may be more sensitive than the tail-flick test to detection of CB₂-mediated and peripherally-mediated antinociceptive effects (see Guindon and Hohmann, 2008 for review). Our studies suggest that cannibilactones such as AM1710 produce cannabinoid receptor-mediated antinociception at doses that do not produce CNS side-effects typical of CB₁ receptor activation. These observations suggest that the cannibilactones represent a promising class of novel cannabinoid analgesics that lack unwanted CNS side-effects.
References


CHAPTER 5
SELECTIVE ACTIVATION OF CANNABINOID CB₂ RECEPTORS SUPPRESSES NEUROPATHIC NOCICEPTION INDUCED BY TREATMENT WITH THE CHEMOTHERAPEUTIC AGENT PACLITAXEL IN RATS

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Abstract

Activation of cannabinoid CB2 receptors suppresses neuropathic pain induced by traumatic nerve injury. The present studies were conducted to evaluate the efficacy of cannabinoid CB2 receptor activation in suppressing painful peripheral neuropathy evoked by chemotherapeutic treatment with the anti-tumor agent paclitaxel. Rats received paclitaxel (2 mg/kg i.p. per day) on four alternate days to induce mechanical hypersensitivity (mechanical allodynia). Mechanical allodynia was defined as a lowering of the threshold for paw withdrawal to stimulation of the plantar hind paw surface with an electronic von Frey stimulator. Mechanical allodynia developed in paclitaxel-treated animals relative to groups receiving the cremophor: ethanol: saline vehicle at the same times. Two structurally distinct cannabinoid CB2 agonists — the aminoalkylindole (R,S)-AM1241 ((R,S)-(2-iodo-5-nitrophenyl)-[1-((1-methyl-piperidin-2-yl)methyl)-1H-indol-3-yl]-methanone) and the cannabilactone AM1714 (1,9-dihydroxy-3-(1’,1’-dimethylheptyl)-6H-benzo[c]chromene-6-one) — produced a dose-related suppression of established paclitaxel-evoked mechanical allodynia following systemic administration. Pretreatment with the CB2 antagonist SR144528 (5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-N-(1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl)-1H-pyrazole-3-carboxamide), but not the CB1 antagonist SR141716 (5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide), blocked the anti-allodynic effects of both (R,S)-AM1241 and AM1714. Moreover, (R)-AM1241, but not (S)-AM1241, suppressed paclitaxel-evoked mechanical allodynia relative to either vehicle treatment or pre-injection thresholds, consistent with mediation by CB2. Administration of either the CB1 or CB2 antagonist alone failed to alter paclitaxel-evoked...
mechanical allodynia. Moreover, \((R,S)\text{-AM1241}\) did not alter paw withdrawal thresholds in rats that received the cremophor vehicle in lieu of paclitaxel whereas AM1714 induced a modest antinociceptive effect. Our data suggest that cannabinoid CB\(_2\) receptors may be important therapeutic targets for the treatment of chemotherapy-evoked neuropathy.
Introduction

Painful peripheral neuropathy is a well documented side-effect of chemotherapeutic treatment (for review see Polomano and Bennett, 2001; Aley and Levine, 2002). The major classes of antineoplastic agents— the vinca alkaloids (e.g. vincristine), taxane (e.g. paclitaxel) and platinum-derived (e.g. cisplatin) compounds— are associated with the development of dose-limiting neuropathic pain. The chemotherapeutic agent used, dosing schedule, form of cancer, and presence of additional medical complications can impact the occurrence and severity of chemotherapy-induced neuropathy (for review see Cata et al., 2006).

Paclitaxel is commonly used for the treatment of solid tumors, ovarian and breast cancer. Paclitaxel induces antimitotic actions by impeding the cell cycle in the late phases of mitosis, stabilizing microtubule formation, and ultimately inducing apoptosis (Schiff and Horwitz, 1980). Paclitaxel preferentially impairs myelinated Aβ and Aδ fibers which carry sensory information about mechanical stimulation to the central nervous system (CNS) (Dougherty et al., 2004). Paclitaxel-evoked neuropathy is manifested as pain in the distal extremities, forming a glove and stocking pattern (Dougherty et al., 2004). Mitochondrial toxicity is also preferentially localized to long axons innervating distal extremities (Flatters and Bennett, 2006). Thus, effects of paclitaxel are evident in those areas where, due to increased distance of axonal transport and mitochondrial energy demand, disruption in sensation would first be present. Dysfunctional mitochondria could lead to low levels of energy which could potentially impair ion transporters, resulting in spontaneous neuronal firing with no concurrent receptor stimulation (i.e. paraesthesia) (Flatters and Bennett, 2006).
Peripheral neuropathy can limit dosing and duration of chemotherapeutic treatment (Holmes et al., 1991; Rowinsky et al., 1993). Pharmacotherapies for chemotherapy-induced neuropathy are limited because the underlying cellular mechanisms remain incompletely understood. Amytriptyline, gabapentin and opioids are used to treat chemotherapy-induced neuropathy. However, none of these drugs has been shown to completely attenuate neuropathic pain (for review see Lee and Swain, 2006). The absence of approved medications available for preventing or treating this debilitating neuropathy makes the identification of alternative effective analgesics a crucial medical need.

Cannabinoids suppress neuropathic pain induced by traumatic nerve injury, toxic insults and metabolic changes (for review see Hohmann, 2002; Guindon and Hohmann, 2008). Both CB$_1$- (Herzberg et al., 1997; Fox et al., 2001) and CB$_2$- (Ibrahim et al., 2003; Beltramo et al., 2006) specific mechanisms suppress neuropathic nociception evoked by traumatic nerve injury. CB$_1$ receptors are expressed primarily within the CNS (Zimmer et al., 1999). CB$_2$ receptors are expressed primarily, but not exclusively, outside the CNS in cells of the immune system (Munro et al., 1993). CB$_2$ receptors are upregulated in the CNS in neuropathic pain states (Woottherspoon et al., 2005; Beltramo et al., 2006). CB$_2$-selective agonists are not associated with psychoactive and motor effects typical of CB$_1$ receptor activation (Hanus et al., 1999; Malan et al., 2001), making the CB$_2$ receptor an attractive therapeutic target for the treatment of neuropathic pain.

The mixed CB$_1$/CB$_2$ agonist WIN55,212-2 suppresses neuropathic nociception induced by paclitaxel through a CB$_1$-specific mechanism (Pascual et al., 2005). WIN55,212-2 also suppresses vincristine-induced neuropathy through activation of both
CB₁ and CB₂ receptors (Rahn et al., 2007). Activation of CB₂ receptors with \((R,S)\)-AM1241 partially attenuates vincristine-induced neuropathy (Rahn et al., 2007). However, a role for CB₂ receptor activation in suppressing paclitaxel-evoked neuropathy has not been investigated. This investigation is important because distinct mechanisms may underlie development of neuropathic pain induced by different antineoplastic agents (for review see Cata et al., 2006). Neuropathic pain symptoms associated with each chemotherapeutic agent vary and can respond differently to pharmacological treatments (Flatters and Bennett, 2004). We used two structurally distinct CB₂-selective agonists, AM1714 and \((R,S)\)-AM1241 (Fig. 5.1), to evaluate the contribution of CB₂ receptors to cannabinoid modulation of paclitaxel-induced neuropathy. AM1714 is a novel CB₂-selective agonist (\(K_i\): CB₁ vs. CB₂: 400 nM vs. 0.8 nM) from the cannabilactone class of cannabinoids (Khanolkar et al., 2007). AM1714 has recently been shown to induce peripheral antinociception but has not previously been characterized in an animal model of pathological pain. \((R,S)\)-AM1241 is a CB₂-selective agonist from the aminoalkylindole class of cannabinoids. \((R,S)\)-AM1241 behaves as a protean agonist in vitro (Yao et al., 2006) and a CB₂ agonist in vivo (see Guindon and Hohmann, 2008 for review). We also compared the ability of \((R)\)-AM1241 (\(K_i\): CB₁ vs. CB₂: 139.7 nM vs. 1.4 nM), and its less active enantiomer \((S)\)-AM1241 (\(K_i\): CB₁ vs. CB₂: 2029 nM vs. 160.5 nM) (Thakur et al., 2005), to suppress paclitaxel-evoked neuropathy. Pharmacological specificity was evaluated using selective antagonist/inverse agonists for CB₁ (SR141716) and CB₂ (SR144528). Comparisons were made with the prototypical narcotic analgesic morphine.
Methods

Subjects

One hundred and seventy-five adult male Sprague-Dawley rats (301-396g; Harlan, Indianapolis, IN) 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. Bedding containing metabolized paclitaxel was treated as biohazardous waste and disposed of according to the appropriate institutional guidelines.

Drugs and Chemicals

Paclitaxel was obtained from Tecoland (Edison, NJ). \((R,S)\)-AM1241 \((R,S)-(2\text{-iodo-5-nitrophenyl})\text{-[1-((1-methyl-piperidin-2-yl)methyl)-1H-indol-3-yl]-methanone})\), \((R)\)-AM1241, \((S)\)-AM1241, and AM1714 \((1,9\text{-dihydroxy-3-(1',1'-dimethylheptyl)-6H-benzo[c]chromene-6-one})\) were synthesized in the Makriyannis laboratory by one of the authors (by AZ and GT respectively). The \((R)\)- and \((S)\)-enantiomers were prepared by chiral synthesis (by AZ). SR141716 \((5-(4\text{-chlorophenyl})\text{-1-(2,4-dichlorophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide})\) and SR144528 \((5-(4\text{-chloro-3-methylphenyl})\text{-1-(4-methylbenzyl)-N-(1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl)-1H-pyrazole-3-carboxamide})\) were provided by NIDA. Cremophor EL and morphine sulfate were obtained from Sigma Aldrich (St. Louis, MO). Dimethyl Sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA). Paclitaxel was dissolved as previously described (Flatters and Bennett, 2004) and administered in a volume of 1 ml/kg. Briefly, paclitaxel was dissolved in a 1:2 ratio of working stock (1:1 ratio of cremophor EL and
95% ethanol) to saline. All other drugs were dissolved in a vehicle of 100% DMSO for systemic administration and administered in a volume of 1 ml/kg bodyweight.

General Experimental Methods

Baseline withdrawal thresholds to mechanical stimulation of the hind paw were measured on day zero. Rats subsequently received four intraperitoneal (i.p.) injections of either paclitaxel (2 mg/kg/day i.p.) or cremophor: ethanol: saline vehicle (1 ml/kg/day i.p.) on alternate days, immediately following behavioral testing. The injection paradigm consisted of four once-daily injections, administered on days 0, 2, 4, and 6, as described previously (Polomano et al., 2001). Mechanical withdrawal thresholds were measured on days 0, 4, 7, 11, 14, 18, and 21. Behavioral testing was always performed just prior to paclitaxel administration (except for days 2 and 6 on which paw withdrawal thresholds were not assessed). To evaluate the possible resolution of paclitaxel-induced neuropathy, paclitaxel-treated rats were additionally evaluated weekly for the presence of mechanical allodynia for 86 days following the initial injection of paclitaxel in a pilot study. In all studies, the experimenter was blinded to the drug condition. Moreover, a single experimenter tested all animals in any given study.

Assessment of mechanical withdrawal thresholds

Mechanical withdrawal thresholds were assessed using a digital Electrovonfrey Anesthesiometer (IITC model Alemo 2290-4; Woodland Hills, CA) equipped with a rigid tip. Rats were placed underneath inverted plastic cages and positioned on an elevated mesh platform. Rats were allowed to habituate to the chamber for 10 - 15 min prior to testing. Stimulation was applied to the midplantar region of the hind paw through the floor of a mesh platform. Mechanical stimulation was terminated upon paw withdrawal;
consequently, there was no upper threshold limit set for termination of a trial. On the test
day (day 21), baseline mechanical withdrawal thresholds were assessed, and effects of
pharmacological manipulations were subsequently evaluated. Nocifensive responses were
observed in paclitaxel-treated animals at forces (g) that failed to elicit withdrawal
responses prior to chemotherapy treatment. Paclitaxel-induced decreases in mechanical
paw withdrawal thresholds (assessed with the electrovonfrey anesthesiometer) were
therefore defined as mechanical allodynia.

Pre-injection mechanical withdrawal thresholds were measured on day 21 prior to
acute pharmacological manipulations. Paclitaxel-treated animals received systemic
injections of either \((R,S)\)-AM1241 (10 mg/kg i.p.; \(n = 7\)), AM1714 (10 mg/kg i.p.; \(n = 6\))
or DMSO (\(n = 7\)). Mechanical withdrawal thresholds were measured 30, 60, and 90 min
post-injection to assess the time course of CB₂ agonist actions. Subsequent studies
evaluated dose-response and pharmacological specificity by measuring paw withdrawal
thresholds at the time-point of maximal cannabinoid-induced suppression of paclitaxel-
evoked neuropathy (30 min post-injection).

To evaluate dose-response, separate groups of paclitaxel-treated animals received
either the racemate \((R,S)\)-AM1241 (1, 5, or 10 mg/kg i.p.; \(n = 6\)-10 per group), AM1714
(1, 5, or 10 mg/kg i.p.; \(n = 6\) per group) or DMSO (\(n = 11\)). Separate groups of animals
received the enantiomers of \((R,S)\)-AM1241 — \((R)\)-AM1241 (10 mg/kg i.p.; \(n = 6\)), or its
less active enantiomer \((S)\)-AM1241 (10 mg/kg i.p.; \(n = 6\)) — or the opioid agonist
morphine (2 or 4 mg/kg i.p.; \(n = 6\) per group).

To determine pharmacological specificity, separate groups of paclitaxel-treated
rats received \((R,S)\)-AM1241 (10 mg/kg i.p., \(n = 6\)), AM1714 (10 mg/kg i.p., \(n = 6\)),

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SR144528 (10 mg/kg i.p.) administered 20 min prior to either (R,S)-AM1241 (10 mg/kg i.p.; n = 6) or AM1714 (10 mg/kg i.p.; n = 5), SR144528 alone (10 mg/kg i.p.; n = 7) or DMSO (n = 6). In separate groups of animals, SR141716 (10 mg/kg i.p) was administered 20 minutes prior to treatment with either (R,S)-AM1241 (10 mg/kg i.p.; n = 5) or AM1714 (10 mg/kg i.p., n = 8).

Antagonist pre-treatment groups received a double volume of the DMSO vehicle. Paw withdrawal thresholds were therefore compared in animals receiving dual injections of either DMSO or saline to verify that vehicle effects could not account for the pattern of results obtained. Therefore, additional control groups received (i.p.) either saline 20 minutes prior to saline (n = 6) or DMSO 20 minutes prior to DMSO (n = 6). To evaluate possible antinociceptive effects induced by the CB2 agonists, the maximally effective anti-allodynic dose of either AM1714 (10 mg/kg i.p.; n = 6) or (R,S)-AM1241 (10 mg/kg i.p.; n = 6) was additionally administered to cremophor-treated controls. Paw withdrawal thresholds were assessed as described above.

Statistical Analyses

Data were analyzed using analysis of variance (ANOVA) for repeated measures, one-way ANOVA or planned comparison t-tests as appropriate. The Greenhouse-Geissser correction was applied to all repeated factors. Post hoc comparisons between control groups and other experimental groups were performed using the Dunnett test. Post-hoc comparisons between different experimental groups were also performed to assess dose-response relationships and pharmacological specificity using the Tukey test. Post-drug thresholds within a given group were compared with either pre-paclitaxel
(baseline) thresholds or day 21 post-paclitaxel thresholds using paired t-tests. $P < 0.05$ was considered statistically significant.

**Results**

**General Results**

Body weight did not differ between groups prior to the treatment with either paclitaxel or the cremophor: ethanol: saline vehicle. Normal weight gain was observed in groups receiving either the cremophor vehicle or paclitaxel ($F_{2, 213} = 1.3, P > 0.27$, Fig 5.2a). However, one fatality was observed in groups receiving paclitaxel.

In a pilot study conducted to evaluate the resolution of paclitaxel-evoked mechanical allodynia, paw withdrawal thresholds were lower than baseline pre-paclitaxel thresholds beginning on day 7 ($P < 0.05$ planned comparison). Paclitaxel-induced mechanical allodynia was present, relative to baseline, from days 14 – 72 following the initiation of treatment ($P < 0.05$ for all planned comparisons, data not shown). Paw withdrawal thresholds were also similar from day 14 – 72 post-paclitaxel. Therefore, day 21 post-paclitaxel was used to evaluate CB$_2$ agonist actions on paclitaxel-evoked mechanical allodynia in all studies reported herein. Paw withdrawal thresholds did not differ between paclitaxel-treated groups prior to cannabinoid or vehicle treatments on day 21 in any study. By contrast, thermal hyperalgesia was not observed in the present paclitaxel dosing paradigm (data not shown).

Mechanical withdrawal thresholds did not differ between either the right or the left paw for any group on any given day (days 0 – 21); therefore, withdrawal thresholds are presented as the mean of duplicate measurements, averaged across paws. Paw withdrawal thresholds were similar between groups prior to administration of paclitaxel
in any given study. Paclitaxel lowered mechanical paw withdrawal thresholds (i.e. equivalently in each paw) relative to control conditions receiving the cremophor vehicle \((F_{1,115} = 10.140, P < 0.01\); Fig 5.2b). Paclitaxel lowered paw withdrawal thresholds in all studies \((P < 0.001\) in each experiment).

Antagonist pretreatment conditions received dual injections of the DMSO vehicle. Paw withdrawal thresholds were therefore compared in groups receiving DMSO followed by DMSO and saline followed by saline. Post-injection paw withdrawal thresholds did not differ from day 21 pre-injection thresholds in either pretreatment group \((P > 0.54\) for both planned comparison t-tests; Table 5.1). Therefore, the volume of DMSO administered did not alter paclitaxel-evoked paw withdrawal thresholds in our study.

**The CB2 agonists (R,S)-AM1241 and AM1714 Suppress Paclitaxel-evoked Mechanical Allodynia**

In paclitaxel-treated rats, \((R,S)-AM1241 (10 \text{ mg/kg i.p.}) and AM1714 (10 \text{ mg/kg i.p.}) suppressed paclitaxel-induced mechanical allodynia relative to the vehicle condition \((F_{2,16} = 4.05, P < 0.05; P < 0.05\) for each comparison; Fig 5.3). Paclitaxel-induced mechanical allodynia was maximally suppressed by each agonist at 30 minutes post-injection \((F_{2,16} = 5.34, P < 0.05\). At this time point, both \((R,S)-AM1241 and AM1714 normalized thresholds relative to pre-paclitaxel levels \((P < 0.05\) for all comparisons). \((R,S)-AM1241 (10 \text{ mg/kg i.p.; } n = 6) failed to induce an antinociceptive effect in animals that received cremophor: ethanol: saline vehicle in lieu of paclitaxel (Day 21 paw withdrawal threshold (Mean ± SEM) pre-injection vs. post-injection: 42.14 ± 0.36 g vs. 40.93 ± 0.78 g; \(P > 0.32\); planned comparison t-test). However, AM1714 (10 mg/kg i.p. \(n = 6) produced a modest antinociceptive effect (Day 21 paw withdrawal threshold (Mean
± SEM) pre-injection vs. post-injection: 63.21 ± 2.98 g vs. 76.92 ± 4.22 g; \( P < 0.05 \); planned comparison t-test). Moreover, cremophor treatment did not alter day 21 paw withdrawal thresholds relative to day 0 baseline paw withdrawal thresholds in any group. Day 0 baseline paw withdrawal thresholds averaged 46.89 ± 4.23 g and 63.60 ± 4.61 g prior to initiation of cremophor treatment in groups that subsequently received \((R,S)\)-AM1241 and AM1714, respectively on day 21. A lower baseline threshold was observed in the former compared to the latter group (\( P < 0.05 \), t-test). Group differences in baseline paw withdrawal thresholds may reflect individual differences combined with the sensitivity of the electrovonfrey device because each animal’s threshold was highly reliable and reproducible. No differences between day 0 baseline paw withdrawal thresholds were observed for any groups tested by the same experimenter in any given study.

**Effects of \((R,S)\)-AM1241 and its Enantiomers on Paclitaxel-evoked Mechanical Allodynia**

\((R,S)\)-AM1241 increased mechanical withdrawal thresholds in a dose-related fashion relative to the vehicle condition (\( F_{3,29} = 3.31, P < 0.05 \); Fig 5.4a). Both the high (10 mg/kg i.p.) and middle (5 mg/kg i.p.) doses of \((R,S)\)-AM1241 elevated paw withdrawal thresholds relative to vehicle (\( P < 0.05 \) for both comparisons). Effects of the low dose of \((R,S)\)-AM1241 (1 mg/kg i.p.) did not differ from vehicle (\( P > 0.12 \)). Both the high (10 mg/kg i.p.) and the middle (5 mg/kg i.p.) doses of \((R,S)\)-AM1241 also elevated paw withdrawal thresholds relative to pre-injection thresholds determined 21 days following paclitaxel treatment (\( F_{3,29} = 3.54, P < 0.05 \); \( P < 0.05 \)). Neither the low dose of \((R,S)\)-AM1241 (1 mg/kg i.p.) nor DMSO altered paw withdrawal thresholds relative to
pre-injection thresholds assessed on day 21 post-paclitaxel \( (P > 0.10) \). The middle and high doses of \((R,S)\)-AM1241 normalized paw withdrawal thresholds relative to baseline (pre-paclitaxel) thresholds \( (P > 0.16) \), whereas DMSO failed to do so.

\((R)\)-AM1241 increased paw withdrawal thresholds relative to the vehicle condition \( (F_{3,25} = 4.37, P < 0.05, \text{Fig 5.4b}) \) in paclitaxel-treated groups. \((S)\)-AM1241 \((10 \text{ mg/kg i.p.})\) did not significantly elevate paw withdrawal threshold relative to vehicle \( (P > 0.43) \). However, post hoc comparisons failed to reveal differential effects between \((S)\)-AM1241 \((10 \text{ mg/kg i.p.})\) and either \((R,S)\)-AM1241 \((10 \text{ mg/kg i.p.})\) or \((R)\)-AM1241 \((10 \text{ mg/kg i.p.})\) on paw withdrawal thresholds \( (P > 0.24) \). Both \((R)\)-AM1241 \((10 \text{ mg/kg i.p.})\) and \((R,S)\)-AM1241 \((10 \text{ mg/kg i.p.})\) significantly increased paw withdrawal thresholds relative to day 21 pre-injection thresholds \( (P < 0.05) \), whereas \((S)\)-AM1241 failed to do so. \((R,S)\)-AM1241 \((10 \text{ mg/kg i.p.})\) and \((R)\)-AM1241 \((10 \text{ mg/kg i.p.})\) also normalized paw withdrawal thresholds relative to day 0 pre-paclitaxel thresholds \( (F_{3,25} = 3.87, P < 0.05; \text{Fig 5.4b}) \). By contrast, normalization of paw withdrawal thresholds was absent in groups receiving DMSO \( (P < 0.001) \).

**The novel CB₂ agonist AM1714 suppresses paclitaxel-evoked mechanical allodynia**

AM1714 suppressed paclitaxel-induced allodynia in a dose-dependent fashion \( (F_{3,25} = 5.14, P < 0.01, \text{Fig 5.5}) \). All three doses of AM1714 suppressed paclitaxel-evoked mechanical allodynia relative to their vehicle-treated counterparts \( (P < 0.05 \text{ for all comparisons}) \). AM1714 \((1, 5, \text{ and } 10 \text{ mg/kg i.p.})\) also normalized paclitaxel-induced mechanical allodynia relative to pre-paclitaxel baseline thresholds \( (F_{3,25} = 5.63, P < 0.01; P > 0.14 \text{ for all comparisons}; \text{Fig 5.5}) \). The high dose \((10 \text{ mg/kg i.p.}; P < 0.001)\), but not
the middle (5 mg/kg i.p.) or low dose (1 mg/kg i.p.) of AM1714 elevated paw withdrawal thresholds relative to day 21 pre-injection thresholds ($P > 0.23$ for both comparisons).

**Pharmacological Specificity**

Neither the CB$_1$-selective antagonist SR141716 (10 mg/kg i.p.) nor the CB$_2$-selective antagonist SR144528 (10 mg/kg i.p) altered paclitaxel-evoked mechanical allodynia relative to pre-injection thresholds ($P > 0.13$; see Table 5.1). The CB$_2$ antagonist SR144528 blocked the anti-allodynic effects of both $(R,S)$-AM1241 (10 mg/kg i.p.) and AM1714 (10 mg/kg i.p.; $F_{4,23} = 11.155$, $P < 0.001$; $P < 0.01$ for each comparison; Fig 5.6a). Paw withdrawal thresholds in agonist groups pretreated with SR144528 did not differ from the vehicle condition ($P > 0.98$ for each comparison). Post hoc comparisons failed to reveal any differences in the anti-allodynic effects induced by either AM1714 (10 mg/kg i.p.) or $(R,S)$-AM1241 (10 mg/kg i.p.; $P > 0.98$).

SR141716 (10 mg/kg i.p.) failed to block the anti-allodynic effects produced by either $(R,S)$-AM1241 (10 mg/kg i.p.) or AM1714 (10 mg/kg i.p.; $F_{4,31} = 10.788$, $P < 0.001$; Fig 5.6b). Paw withdrawal thresholds in paclitaxel-treated groups receiving DMSO were lower than those observed in groups receiving the CB$_2$ agonists in either the presence or absence of the CB$_1$ antagonist ($P < 0.01$ for each comparison). Paw withdrawal thresholds were similar in groups pretreated with SR141716 to those observed in groups receiving either agonist alone ($P > 0.11$ for each comparison). However, animals receiving SR141716 prior to AM1714 exhibited elevated paw withdrawal thresholds relative to baseline pre-paclitaxel thresholds ($P < 0.01$, planned comparison t-test; Fig. 5.6b). Post drug injection paw withdrawal thresholds were higher
in all groups relative to day 21 pre-injection thresholds with the exception of vehicle \((P < 0.05, \text{planned comparison t-tests})\).

**Effects of Morphine on Paclitaxel-evoked Mechanical Allodynia**

The high dose of morphine (4 mg/kg i.p.) suppressed paclitaxel-induced mechanical allodynia relative to the vehicle condition \((F_{2,20} = 6.023, P < 0.01; P < 0.01\) for relevant comparison; Fig 5.7) and normalized paw withdrawal thresholds relative to pre-paclitaxel baseline thresholds \((P > 0.15)\). The low dose of morphine (2 mg/kg i.p.) failed to alter post-paclitaxel paw withdrawal thresholds.
Figure 5.1. Chemical structures of (R,S)-AM1241, (R)-AM1241, (S)-AM1241 and AM1714.
Figure 5.1: Chemical structures of (R,S)-AM1241, (R)-AM1241, (S)-AM1241 and AM1714.
Figure 5.2. (a.) Weight gain was observed in groups treated with either paclitaxel or cremophor: ethanol: saline vehicle. (b.) Time course of paclitaxel-induced mechanical allodynia, as demonstrated by a lowering of the threshold for paw withdrawal to punctuate mechanical stimulation. Data are Mean ± s.e.mean. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control condition (ANOVA). N = 6-115 per group.
Figure 5.2: Effects of paclitaxel treatment on weight and hypersensitivity to mechanical stimulation (mechanical allodynia).
Figure 5.3. Both cannabinoid CB₂ agonists normalized thresholds relative to pre-paclitaxel levels at 30 minutes post injection. BL denotes baseline (day 0) paw withdrawal thresholds observed prior to paclitaxel treatment. Data are Mean ± s.e.mean. *P < 0.05 vs. all groups (ANOVA and Dunnett’s post hoc test). N = 6-7 per group.
Figure 5.3: Time course for suppression of paclitaxel-induced mechanical allodynia observed following administration of (R,S)-AM1241 and AM1714.
Figure 5.4. (a.) $(R,S)$-AM1241 (1, 5 and 10 mg/kg i.p.) produced a dose-related suppression of paclitaxel-evoked mechanical allodynia. In all panels, Post PTX indicates thresholds observed on day 21 post-paclitaxel. (b.) Both $(R,S)$-AM1241 (10 mg/kg i.p.) and its enantiomer $(R)$-AM1241 (10 mg/kg i.p.) attenuated paclitaxel-evoked mechanical allodynia. $^*P < 0.05$, $^{**}P < 0.01$ vs. control, $^{***}P < 0.001$ vs. baseline (ANOVA, and Dunnett post hoc test), $^{x}P < 0.05$ vs. corresponding group day 21 post-paclitaxel paw withdrawal thresholds, $^{⊥}P < 0.001$ vs. corresponding group baseline pre-paclitaxel paw withdrawal thresholds (t-test). $N = 6-11$ per group.
Figure 5.4: Effects of (R,S)-AM1241, (R)-AM1241, and (S)-AM121 on paclitaxel-induced mechanical allodynia.
Figure 5.5. AM1714 (10 mg/kg) suppressed paclitaxel-induced mechanical allodynia. *$P$ < 0.05, **$P$ < 0.01 different from control, ***$P$ < 0.001 vs. baseline (ANOVA, and Dunnett post hoc test) $\times$ $P$ < 0.05 vs. corresponding group day 21 post-paclitaxel paw withdrawal thresholds, $\dagger$ $P$ < 0.001 vs. corresponding group baseline pre-paclitaxel paw withdrawal thresholds (t-test). N = 6-11 per group.
Figure 5.5: Effects of AM1714 on paclitaxel-induced mechanical allodynia.
Figure 5.6. (a.) The CB$_2$-selective antagonist SR144528 (SR2) blocked the suppression of paclitaxel-evoked mechanical allodynia induced by the CB$_2$ agonists, (R,S)-AM1241 (10 mg/kg i.p.) and AM1714 (10 mg/kg i.p.). (b.) The CB$_1$-selective antagonist SR141716 (SR1) failed to block the anti-allodynic effects of either (R,S)-AM1241 (10 mg/kg i.p.) or AM1714 (10 mg/kg i.p.) in the same model. **$P<0.01$ vs. all groups, $^\dagger P<0.01$ vs. DMSO, AM1714 + SR2 and AM1241 + SR2 (ANOVA, and Dunnett Post Hoc Test). $^\ddagger P<0.001$ vs. corresponding group baseline pre-paclitaxel paw withdrawal thresholds (t-test), $^\times P<0.001$ vs. corresponding group day 21 post-paclitaxel thresholds (t-test). N = 5-11 per group.
Figure 5.6: Pharmacological specificity for suppression of paclitaxel-induced mechanical allodynia observed following administration of \((R,S)\)-AM1241 and AM1714.
Figure 5.7. Morphine (4.0 mg/kg i.p.) blocked mechanical allodynia induced by treatment with paclitaxel. \(***P < 0.001\) vs. baseline, \(P < 0.05\) vs. control (ANOVA and Dunnett Post Hoc Test). \(P < 0.001\) vs. corresponding group baseline pre-paclitaxel paw withdrawal thresholds (t-test). \(N = 6-11\) per group.
Figure 5.7: Effects of morphine on paclitaxel-induced mechanical allodynia.
Table 5.1: Paw withdrawal thresholds (g) in paclitaxel-treated control conditions

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-Paclitaxel</th>
<th>Post-Paclitaxel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 21</td>
</tr>
<tr>
<td></td>
<td>Pre-injection</td>
<td>Pre-injection</td>
</tr>
<tr>
<td>Paclitaxel: DMSO-DMSO</td>
<td>66.915 ± 6.58</td>
<td>46.56 ± 4.23†</td>
</tr>
<tr>
<td>Paclitaxel: Saline-Saline</td>
<td>75.585 ± 6.32</td>
<td>41.20 ± 4.90↑†††</td>
</tr>
<tr>
<td>Paclitaxel: DMSO</td>
<td>63.88 ± 2.74</td>
<td>32.93 ± 2.43↑†††</td>
</tr>
<tr>
<td>Paclitaxel: SR141716</td>
<td>63.59 ± 2.20</td>
<td>37.34 ± 1.77↑†††</td>
</tr>
<tr>
<td>Paclitaxel: SR144528</td>
<td>59.92 ± 4.10</td>
<td>32.64 ± 7.42↑††</td>
</tr>
</tbody>
</table>

Data are mean ± s.e.mean; †††P < 0.001, ††P < 0.01, †P < 0.05 vs. baseline pre-paclitaxel paw withdrawal thresholds for corresponding group, ↓↓↓P < 0.001, ↓↓P < 0.01, ↓P < 0.05 vs. baseline pre-paclitaxel paw withdrawal thresholds for corresponding group (t-test).
Discussion

Two structurally distinct CB2 agonists attenuated mechanical allodynia induced by treatment with the chemotherapeutic agent paclitaxel. Animals receiving paclitaxel remained in relatively good health as evidenced by the observation of normal weight gain during the course of chemotherapy treatment. However, one fatality was observed after two injections of paclitaxel. Paclitaxel-evoked mechanical hypersensitivity cannot be attributed to sensitization to repeated testing; paw withdrawal thresholds were stable in animals receiving the cremophor: ethanol: saline vehicle in lieu of paclitaxel over the same time course. Mechanical allodynia was observed in paclitaxel-treated animals tested weekly up to 3 months after the initiation of chemotherapy treatment in a pilot study. Paw withdrawal thresholds were similarly reduced relative to baseline from day 14 to 72 post-paclitaxel in this study; therefore day 21 was selected for the evaluation of drug effects on paclitaxel-evoked mechanical allodynia. Other studies have similarly reported peaks in neuropathic nociception with the present paclitaxel dosing paradigm from days 16 – 27 post initiation of paclitaxel treatment (Polomano et al., 2001; Flatters and Bennett, 2004). In all subsequent studies, mechanical allodynia developed by day 11 and continued to decrease until the final test day, day 21.

Thermal hyperalgesia was not observed in our study, consistent with previous reports employing the present paclitaxel dosing schedule (Polomano et al., 2001). A CB1-mediated suppression of paclitaxel-induced thermal hyperalgesia has been reported using a cumulative paclitaxel dose of 4 mg/kg (Pascual et al., 2005) compared to our dose of 8 mg/kg. Differences in dosing and timing of paclitaxel injections may account for differences between these studies.
In our study, two structurally distinct cannabinoid CB2 agonists, the aminoalkylindole (R,S)-AM1241 and the cannabilactone AM1714, suppressed paclitaxel-evoked mechanical allodynia through a CB2-specific mechanism. All doses of AM1714 normalized paw withdrawal thresholds relative to pre-paclitaxel levels; however comparisons with day 21 pre-injection thresholds suggest that the high dose (10 mg/kg i.p.) was the most reliably effective dose. The high dose of AM1714 (10 mg/kg i.p.) produced a modest antinociceptive effect in animals treated with the cremophor vehicle in lieu of paclitaxel. By contrast, the high (10 mg/kg i.p.) and middle (5 mg/kg i.p.) but not the low (1 mg/kg i.p.) dose of (R,S)-AM1241 normalized paw withdrawal thresholds to pre-paclitaxel levels without inducing antinociception. Thus, AM1714 but not (R,S)-AM1241 produced antinociception in addition to suppression of allodynia. The mechanisms underlying these differences remain to be explored.

The suppression of paclitaxel-evoked neuropathic nociception induced by AM1241 and AM1714 is likely to be mediated by CB2 receptors. First, multiple CB2 agonists from different chemical classes suppressed paclitaxel-evoked neuropathic nociception. Second, (R)-AM1241, but not (S)-AM1241, suppressed paclitaxel-evoked mechanical allodynia relative to vehicle treatment and pre-injection thresholds, consistent with mediation by CB2. Third, anti-allodynic effects of each agonist were blocked by the CB2 antagonist SR144528. Fourth, the CB1 antagonist SR141716 failed to block the anti-allodynic effects of either (R,S)-AM1241 or AM1714.

In our study, a trend toward enhanced antihyperalgesic efficacy was observed in groups pretreated with SR141716 prior to AM1714. This observation may suggest that blockade of CB1 receptors increases endocannabinoid tone and enhances effects of the
CB₂ agonist (Zhang et al., 2008). Enhancement of CB₂ agonist efficacy by CB₁ receptor blockade was apparent with AM1714, but not (R,S)-AM1241, suggesting possible mechanistic differences between the two agonists. More work is necessary to determine whether (R,S)-AM1241 and AM1714 preferentially activate different signaling pathways or whether off-target effects could contribute to these differences. (R,S)-AM1241, a racemic compound, may exhibit partial agonist properties that counteract this tendency. Putative changes in endocannabinoid tone may be induced by blockade of CB₁ to enhance the anti-allodynic activity of certain CB₂ agonists under conditions in which the balance between CB₁ and CB₂ receptor activation is altered. Blockade of CB₁ may also facilitate interaction of endogenous anandamide with non-CB₁ receptors (e.g. TRPV1) to contribute to the behavioral phenotype. Nonetheless, neither the CB₁ nor the CB₂ antagonist, administered alone, increased paclitaxel-evoked mechanical allodynia. Our data extend previous work documenting that activation of CB₂ suppresses nociception and central sensitization in a variety of tissue and nerve injury models of persistent pain (Ibrahim et al., 2003; Nackley et al., 2003; Beltramo et al., 2006; Jhaveri et al., 2008).

In the present study, we compared the effects of two enantiomers of (R,S)-AM1241 – (R)-AM1241 and (S)-AM1241 – on paclitaxel-evoked mechanical allodynia. (R)-AM1241 binds with 40- (Bingham et al., 2007) to 114- (Thakur et al. 2005) fold higher affinity to CB₂ receptors than (S)-AM1241. This observation is consistent with the ability of (R)-AM1241 to preferentially suppress paclitaxel-evoked mechanical hypersensitivity relative to either vehicle or day 21 pre-injection thresholds. Similar effects were not observed with administration of (S)-AM1241. However, both enantiomers show notable selectivity for CB₂ over CB₁. Thus, it is important to
emphasize that (S)-AM1241 cannot be considered an inactive enantiomer of (R)-AM1241. This property contrasts with that of other aminoalkylindole agonists in which the enantiomer (e.g. WIN55,212-3) of the active compound (WIN55,212-2) fails to bind to cannabinoid receptors. The fact that (S)-AM1241 retains activity at CB2 may account for the efficacy of (S)-AM1241 in models of visceral and inflammatory pain (Bingham et al., 2007) and our failure to differentiate between effects of (R)-AM1241 and (S)-AM1241 in post hoc analyses. Our studies do not preclude the possibility that CB2-mediated anti-allodynic effects of (S)-AM1241 could be detected using a higher dose of (S)-AM1241 or a larger sample size. It is also possible that differences in enantiomer efficacy reflect differences in agonist directed trafficking through different G proteins and signal transduction mechanisms (Shoemaker et al., 2005).

In our study, morphine (4 mg/kg i.p.) suppressed paclitaxel-induced mechanical allodynia and normalized paclitaxel-evoked paw withdrawal thresholds to pre-paclitaxel levels. This same dose was previously reported to be ineffective in suppressing paclitaxel-evoked mechanical hyperalgesia (Flatters and Bennett, 2004). In this latter study, a two-fold higher dose (8 mg/kg i.p.) than that employed here (4 mg/kg i.p.) produced only a 50% reversal of paclitaxel-evoked mechanical allodynia/hyperalgesia whereas the lower dose (4 mg/kg i.p.) was ineffective. A dose of 8 mg/kg also attenuated vincristine-induced mechanical allodynia in our previous work (Rahn et al., 2007). Differences in the dependent measure (i.e. paw withdrawal frequency vs. paw withdrawal threshold in our study), method for assessing mechanical hypersensitivity (i.e. manual von Frey filaments vs. electrovonfrey device in our study) and time of testing (i.e. 1 h vs 30 min post morphine in our study) may account for these differences. Nonetheless,
unwanted side-effects (i.e., sedation, nausea, altered mental status, constipation) remain associated with activation of the opioid system in humans, warranting development and validation of drug targets which lack these unwanted side-effects (Lee et al., 1995).

The mechanism by which paclitaxel induces neuropathic pain symptoms remains unknown. Paclitaxel has been reported to induce neuropathy in the absence of morphological changes in sensory or motor axons in the spinal cord (Polomano et al., 2001). This observation prompted investigations of morphological changes in the periphery. Morphological and immunological changes in sensory nerve fibers have been reported following paclitaxel treatment (Jin et al., 2008). Abnormal calcium homeostasis may also contribute to the development of neuropathic pain symptoms associated with paclitaxel treatment (Siau and Bennett, 2006). Thus, it is noteworthy that blockade of calcium channels is effective in attenuating symptoms of peripheral neuropathy in this model, whereas an NMDA receptor antagonist was without effect (Flatters and Bennett, 2004). A reduction of mechanical hyperalgesia associated with both paclitaxel and vincristine treatment is also observed in TRPV4 knockout mice, suggesting that TRPV4 may also represent a therapeutic target for treatment of chemotherapy-evoked toxic neuropathy (Alessandri-Haber et al., 2008).

More work is necessary to identify the site of action for CB2 agonists in suppressing paclitaxel-evoked neuropathy. Upregulation of the CB2 receptor in the dorsal horn of the spinal cord has been reported after spinal nerve ligation injury or sciatic nerve sectioning in rats (Walczak et al., 2005; Wotherspoon et al., 2005). Moreover, CB2 expression is upregulated in cultured DRG following prior axotomy (Wotherspoon et al., 2005). CB2 receptors have recently been localized within the CNS, specifically on
microglia which are related to macrophages (Cabral et al., 2008). Thus, it is noteworthy that paclitaxel increased the number of macrophages present in both spinal cord and the DRG (Peters et al., 2007). More work is necessary to determine whether CB₂ receptors in the CNS or DRG are upregulated by paclitaxel treatment and contribute to the observed CB₂-mediated suppression of paclitaxel-evoked neuropathy.

The recent observation of increased activation of microglia and astrocytes in paclitaxel-treated rats has led to speculation that these glial cells contribute to chemotherapy-induced neuropathic pain (Ledeboer et al., 2007). Paclitaxel increases levels of activated microglia in lamina III-VI of the spinal cord as well as astrocytes in lamina I-VI of the spinal cord (Peters et al., 2007). Hypertrophy in both glial cell populations is observed following paclitaxel treatment (Peters et al., 2007). Moreover, pharmacologically-induced suppression of glial cells abolished and delayed the incidence of mechanical allodynia in paclitaxel-treated rats (Ledeboer et al., 2007). More work is necessary to determine whether CB₂ agonists suppress paclitaxel-evoked neuropathy by inhibiting microglial activation.
References


CHAPTER 6

SELECTIVE ACTIVATION OF CANNABINOID CB2 RECEPTORS SUPPRESSES NEUROPATHIC NOCICEPTION INDUCED BY TREATMENT WITH THE CHEMOTHERAPEUTIC AGENT PACLITAXEL IN RATS

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Abstract

Acute activation of cannabinoid CB₁ and CB₂ receptors suppresses neuropathic nociception associated with chemotherapeutic treatment. The present study was performed to evaluate the therapeutic efficacy of cannabinoid agonists with distinct mechanisms of action in suppressing the development of chemotherapy-induced neuropathy resulting from paclitaxel treatment in rats. Osmotic minipumps were surgically implanted to achieve prophylactic drug administration both prior to (pre-emptive) and throughout chemotherapeutic treatment. The CB₂-preferring agonist AM1710 suppressed the development of both mechanical and cold allodynia in animals receiving paclitaxel throughout a 30 day time course. Anti-allodynic effects of AM1710 presented in a U-shape dose response curve with both the highest (3.2 mg/kg/day s.c.) and lowest (0.032 mg/kg/day s.c.) doses producing greater effects than the middle dose (0.32 mg/kg/day s.c.) in response to mechanical stimulation. Only the high dose of AM1710 (3.2 mg/kg/day s.c.) showed sustained efficacy in suppressing cold allodynia. Anti-allodynic effects of AM1710 (3.2 mg/kg/day s.c.) observed in response to both mechanical and cold stimulation were mediated by the CB₂ receptor (i.e., blocked by a second pump containing AM630 (3 mg/kg/day s.c.), but not AM251 (3 mg/kg/day s.c.)). The mixed CB₁/CB₂ agonist WIN55,212-2 (0.1 and 0.5 mg/kg/day s.c.) suppressed mechanical and cold allodynia throughout the 30 day time course. The anti-allodynic profile of WIN55,212-2-mediated suppression of mechanical hypersensitivity was primarily dominated by the CB₁ receptor. Concurrent administration of either a CB₁ or CB₂ antagonist was unable to block the anti-allodynic effects of WIN55,212-2 (0.5 mg/kg/day s.c.) elicited by acetone application. The neuropathic pain medication
gabapentin (16 mg/kg/day s.c.) suppressed the development of both mechanical and cold allodynia in groups receiving paclitaxel. Lower doses of gabapentin (4 and 8 mg/kg/day s.c.) were unable to suppress either mechanical or cold allodynia for the duration of the time course. Anti-allodynic effects of both AM1710 and gabapentin were present up to 18 days following the removal of drug (day 39), whereas effects of WIN55,212-2 had a shorter duration (day 33).

**Key Words.** cannabinoid, CB₁, CB₂, chemotherapy, paclitaxel, mechanical allodynia, cold allodynia, osmotic minipump

Abbreviations: DRG; dorsal root ganglion; i.p., intraperitoneal; s.c., subcutaneous
Introduction

Cannabinoids produce behavioral, physiological and/or anatomical changes through activation of CB1 receptors, CB2 receptors, or a combination of both. A CB3 receptor has been proposed, however to date there is controversy about whether or not it has been identified [for review see 1; 2]. The endocannabinoid system is a relatively young neurotransmitter system that has only been investigated in earnest since the discovery of the endogenous ligands (anandamide and 2-arachidonoylglycerol)[3-5] and receptors [6; 7] for this system in the early 1990s.

Cannabinoids have been shown to attenuate or, in some cases, prevent pain associated with a number of conditions including: post-surgical pain [8-10], inflammatory pain [11; 12], organ pain [13-15], and neuropathic pain [for review see 16]. Neuropathic pain is associated with abnormal changes in the peripheral and/or central nervous system that result in non-adaptive pain (i.e. pain that serves no obvious physiological role and can be thought of as dysfunctional). Neuropathic pain is notorious for its unresponsiveness to traditional drug therapies. A form of neuropathic pain that is particularly difficult to treat is the pain resulting from chemotherapeutic treatment, termed chemotherapy-induced neuropathy.

Chemotherapeutic treatment with antineoplastic agents, while in many cases very effective at eliminating harmful malignancies, is also associated with several severe side effects. Although side effects such as emesis, alopecia, and myelosuppression have received the spotlight over the years a new front runner has recently emerged. Neuropathic pain associated with chemotherapeutic treatment is dose-limiting and reported to be one of the major factors influencing a patient’s decision to discontinue
Treatment [17; 18]. Pain resulting from chemotherapeutic treatment is reportedly directly correlated with the cumulative dose of chemotherapeutic agent that a patient receives [19]. Higher cumulative doses are correlated with increased instances of chemotherapy-induced neuropathy. A recent report suggested that cancer patients suffering from chemotherapy-induced neuropathy were more likely to experience other forms of neuropathic pain than their counterparts that did not suffer from this neuropathy [20].

Chemotherapeutic agents are generally divided into three different classes, each of which suppresses tumor growth through different mechanisms. These three major classes are the vinca alkaloids, platinum derived agents, and the taxanes. Taxanes (e.g. paclitaxel, docetaxel) produce antineoplastic effects by stabilizing microtubules through binding to β-tubulin, thereby disrupting normal cell mitosis and triggering the mitochondrial apoptosis pathway [21]. Paclitaxel, originally a derivative of Pacific Yew tree bark that is now produced synthetically, is an agent of choice for the treatment of ovarian, breast, and lung cancers.

Chemotherapy-induced neuropathy resulting from treatment with paclitaxel is poorly treated with currently available drugs. The efficacy of acutely administered cannabinoid agonists activating CB1 [22] and CB2 [23; 24] receptors have been demonstrated in basic research models of chemotherapy-induced neuropathy; however it is unclear whether analgesics activating this system show efficacy in suppressing the development of chemotherapy-induced neuropathy. The purpose of this study was to evaluate the prophylactic treatment of chemotherapy-induced neuropathy with cannabinoid agonists administered prior to, throughout, and following chemotherapeutic treatment.
Methods

Subjects

Two hundred and ten male Sprague-Dawley rats (beginning weight: 300-400g; Harlan, Indianapolis, IN) 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. Animal experiments were conducted in full compliance with local, national, ethical and regulatory principles and local licensing regulations of Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International’s expectations for animal care and use/ethics committees. Animals were allowed a minimum of one week to habituate to the facility prior to beginning of the study. Animals were single housed and maintained in a temperature (range: 70-72°F ± 4°F), humidity (range: 30-70%) controlled facility on a 12 hour light cycle (lights on: 7:00 and lights off: 19:00). Following the initial pilot study (n = 17), all animals with osmotic minipumps were allowed nyla bones in cages due the extended length of the study. Corn cob bedding containing metabolized paclitaxel was treated as chemical hazard waste and disposed of according to the appropriate institutional guidelines.

Drugs and Chemicals

Paclitaxel (Taxol) was obtained from Tecoland (Edison, NJ). Polyethylene Glycol 400 (PEG 400) was purchased from VWR International (West Chester, PA). Acetone was purchased from J.T. Baker (Phillipsburg, NJ). Cremophor EL, Dimethyl Sulfoxide (DMSO), and WIN55,212-2 ((R)-(+)-[2,3-Dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]1,4-benzoazinyl]-(1-naphthalenyl)methanone
mesylate salt) were obtained from Sigma Aldrich (St. Louis, MO). Gabapentin (1-(Aminomethyl)-cyclohexaneacetic acid) was purchased from Spectrum Chemical and Laboratory Products Inc. (New Brunswick, NJ). AM1710 (3-(1’,1’-dimethylheptyl)-1-hydroxy-9-methoxy-6H-benzo[c]chromene-6-one), AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), and AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone (Iodopravadoline)) were synthesized in the Makriyannis laboratory by one of the authors (by GT, VKV, and AZ respectively). Paclitaxel was dissolved as previously described [25] and administered in a volume of 1 ml/kg. Briefly, paclitaxel was dissolved in a 1:2 ratio of working stock (1:1 ratio of cremophor EL and 95% ethanol) to saline. AM1710, WIN55,212-2, AM251, and AM630 were dissolved in a vehicle of 50% DMSO in polyethylene glycol 400. The selected vehicle was the most compatible for dissolving cannabinoids for use in Alzet osmotic minipumps. This vehicle has been used previously with no reported adverse side effects [26-28]. Gabapentin was dissolved in a vehicle of 0.9% sterile saline.

**General Experimental Methods**

All day numbers reference the time *prior to* or following the initiation of chemotherapeutic treatment (i.e. day -6 would correspond to 6 days *prior to* chemotherapeutic treatment, whereas day 6 would be 6 days *post* chemotherapeutic treatment). Animals illustrated in Figure 6.1 (n = 12) did not have osmotic minipumps. These animals were tested concurrently for the development of thermal hyperalgesia and mechanical allodynia resulting from paclitaxel treatment. Baselines for these animals
were taken on day 0 and testing days for von Frey and Hargreaves were alternated to avoid hypersensitization resulting from overstimulation.

All other animals in this study (Figs 6.2 – 6.11) underwent surgical implantation of osmotic minipumps. Baseline withdrawal thresholds to mechanical stimulation of the hind paw were measured on day -8. Baseline withdrawal responses and latencies to cold stimulation (acetone drops) were measured on day -7. On day -6 animals underwent a minor surgical procedure to implant an osmotic minipump (for pharmacological specificity testing two pumps were implanted simultaneously), Alzet model 2ML4 (Cupertino, CA), subcutaneously into the back of the animal. A post-surgery baseline (following pump implantation, but prior to chemotherapeutic treatment) was assessed on days -2 and 0 for mechanical withdrawal thresholds and day -1 for cold allodynia testing. Animals were weighed on all testing and surgical/sacrifice dates. A subset of animals from each group was sacrificed on day 22 for tissue and/or blood collection. All remaining animals underwent surgery to remove the osmotic minipumps and following a short recovery period, the remaining animals were evaluated for responses to mechanical and cold stimulation until day 51.

Doses of drugs were estimated based on the peak performance of the osmotic minipump reported by the manufacturer (2.5 µl/hr) and an average rat weight of 375 grams. A small percentage of animals (4.2%) presented with edema around the pump site (seromas) following surgical implantation of the osmotic minipump. According to Alzet technical support this is a side effect reported in a small percentage of animals that receive osmotic minipumps. Treatment for these animals was supervised by the veterinarian on staff and consisted of fluid draining approximately every 3 days, or as
needed. Six animals (2.8%) had to be re-sutured following surgery. One of the six animals had an infection that was treated from day 16 through day 22 with daily injections of an antibiotic (Enrofloxacin 4.5 mg/ml, 0.4 cc s.c., 2x daily) and sterile water (1 cc s.c., 1x daily) as prescribed by the staff veterinarian. One animal in the paclitaxel-vehicle condition died during its first injection of paclitaxel. Following post-mortem examination it was determined that the death was due to pump malfunction exacerbated by animal restraint during the injection procedure. Since the animal did not undergo chemotherapeutic treatment, the data was excluded from all analyses. One animal in the Taxol-gabapentin (8.0 mg/kg/day s.c.) condition started foaming at the mouth and had severe spasms on day 11 during cold allodynia assessment. Following this episode, the animal was placed back in his cage for approximately 5 minutes where he was given access to food and water, of which he did not partake. No other abnormalities were observed in this animal for the duration of testing on this day or any other testing day. No other animals in this drug group or any other group showed evidence of similar behavior.

All behavioral measurements, surgeries, and chemotherapeutic treatment were performed by a single experimenter. Coded testing sheets were used throughout the study to preserve blinding to the drug conditions. All behavioral testing (von Frey, cold allodynia, thermal hyperalgesia, and activity meter) was done in the presence of a white noise generator to mask extraneous noise.

**Surgical Implantation and Removal of Osmotic Minipumps**

Osmotic minipumps were implanted under isoflurane anesthesia (Isoflo®, Abbott Laboratories, Chicago, IL). A small incision was made between the scapula and a pair of hemostats was used to make a pocket by spreading the connective tissues. The osmotic
minipump was then inserted and the skin incision was closed with sutures. In the instances where two pumps were implanted, the pumps were placed in the same pocket (per the manufacturer’s recommendation). The Alzet model 2ML4 pump has an approximate 2 ml reservoir that releases a preloaded drug or vehicle at a rate of 2.5 ul/hr for up to 28 days. The pump begins to release the preloaded drug approximately 4-6 hours after implantation (once reaching a temperature of 37º C). Osmotic minipumps were weighed before and after being filled with drug or vehicle. The difference of these two values provided an approximation of the pump fill volume. The animals were given three days to recover from the surgery before testing resumed (i.e. the animal was not tested on days -5 through day -3). Animals were either sacrificed or underwent surgery on day 22 to remove pumps; this time point corresponds to the 29th day following pump implantation. At this time point the pump should have released its contents. Following pump removal, the residual volume in the pump was estimated by withdrawing the fluid from the pump reservoir as per the manufacturer’s recommendation. Animals that underwent surgical removal of the osmotic minipumps were allowed three days of recovery (days 23 through 25) prior to the resumption of behavioral testing.

Treatment with the Chemotherapeutic Agent Paclitaxel

Paclitaxel or cremophor vehicle treatment commenced on day 0. Rats received four intraperitoneal (i.p.) injections of either paclitaxel (2 mg/kg/day i.p.) or cremophor: ethanol: saline vehicle (1 ml/kg/day i.p.) on alternate days, immediately following behavioral testing. The injection paradigm consisted of four once-daily injections of paclitaxel (cumulative dose of 8 mg/kg i.p.) or cremophor vehicle, administered on days
0, 2, 4, and 6, as described previously [29]. Behavioral testing was always performed just prior to paclitaxel administration.

**Assessment of Paw Withdrawal Latencies to Thermal Stimulation**

Paw withdrawal latencies to radiant heat were measured in duplicate for each paw using the Hargreaves test [30] and a commercially available plantar stimulation unit (IITC model 336; Woodland Hills, CA). Rats were placed underneath inverted plastic cages positioned on an elevated glass platform. Rats were allowed a minimum of 20 min to habituate to the apparatus prior to testing. Radiant heat was presented to the midplantar region of the hind paw through the floor of the glass platform. The intensity of the heat source was adjusted such that an average baseline latency of approximately 20 sec was achieved [31]. Stimulation was terminated upon paw withdrawal or after 40 s to prevent tissue damage. Two paw withdrawal latencies were measured for each paw. Testing order of the paws was: right, right, left, left. Approximately 4 minute interstimulation intervals were allowed between tests. Thermal withdrawal latencies were evaluated before (day 0) and on days 2, 6, 10, 14 and 18 post-paclitaxel treatment. The same animals were concurrently tested for the development of mechanical allodynia (on days 0, 4, 8, 12, 16 and 20). Baseline responses to mechanical stimulation (methodology is described below in detail) were measured on day 0 before baseline responses to thermal stimulation were measured. A minimum of 1 hour was allowed to elapse between baseline measurements.

**Assessment of Mechanical Withdrawal Thresholds**

Mechanical withdrawal thresholds were assessed using a digital Electrovonfrey Anesthesiometer (IITC model Alemo 2390-5; Woodland Hills, CA) equipped with a rigid tip. Rats were placed underneath inverted plastic cages and positioned on an elevated
mesh platform. Rats were allowed to habituate to the chamber for a minimum of 20 min prior to testing. Stimulation was applied to the midplantar region of the hind paw through the floor of a mesh platform. Mechanical stimulation was terminated upon paw withdrawal; consequently, there was no upper threshold limit set for termination of a trial. Two thresholds were taken for each paw. Testing order of the paws was: right, right, left, left. Approximately 2 minute interstimulation intervals were allowed between tests. Mechanical withdrawal thresholds were measured on days 0, 4, 8, 12, 16 and 20 for animals that did not receive osmotic minipumps (Fig 6.1). Mechanical withdrawal thresholds were measured on days -8, -2, 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 for all animals that received osmotic minipumps. A subset of osmotic minipump animals were tested to day 50; testing for these animals continued with the following schedule: days 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, and 50.

**Assessment of Cold Allodynia**

Cold allodynia was assessed using acetone drops applied to the plantar surface of the hind paw as previously described [32]. Rats were placed underneath inverted plastic cages and positioned on an elevated mesh platform. Rats were allowed to habituate to the chamber for a minimum of 20 min prior to testing. Acetone was loaded into a one cc syringe barrel with no needle tip. Air bubbles were cleared from the syringe prior to acetone application. One drop of acetone (approximately 20 µl) was applied through the mesh platform onto the plantar surface of the hind paw. Care was taken to gently apply the bubble of acetone to the skin on the paw without inducing mechanical stimulation by contact of the syringe barrel with the paw.
Two responses from the animal were recorded. The first was a binary response; presence or absence of paw withdrawal. Paw withdrawal was typically associated with a secondary response from the animal (i.e. rapid flicking of the paw, chattering, urination, biting, and/or licking of the paw). The second response recorded was paw withdrawal latency. In those instances where the paw withdrawal was too rapid to time, an arbitrary value of 0.20 sec was assigned; therefore no paw withdrawal latencies recorded were less than 0.20 sec in duration. Five measurements were taken for each paw. Testing order alternated between paws (i.e. right, left). No cut-off latency was enforced. Approximately 2 min interstimulation intervals were allowed between testing of right and left paws. A minimum interstimulation interval of 5 min was allowed between each pair of paws (right and left) that were tested. Cold allodynia testing took place on days -7, -1, 0, 5, 11, 17 and 21 for all animals with osmotic minipumps. Five days were allowed in between cold allodynia assessments to avoid hypersensitivity associated with excessive testing with one exception. Animals were tested on day 21 as this was the last time point when osmotic minipumps would still be releasing drug (i.e. this was 28 days following implantation of the osmotic minipump). A subset of animals with osmotic minipumps were tested to day 51; testing for these animals continued with the following schedule: days 27, 33, 39, 45, and 51.

Activity Meter Assessment

Seven measures (defined below) were assessed by placing a subset of rats with osmotic minipumps individually in the center of an activity monitor chamber (Coulbourn Instruments, Whitehall, PA) measuring 40.64 x 40.64 x 40.64 cm housed in a darkened room. Red light was used to provide illumination for the experimenter. Tracking beams
were positioned 2.54 cm apart giving 1.27 cm in spatial resolution. Activity was automatically measured by computerized analysis of photobeam interrupts (TruScan 2.0; Coulbourn Instruments, Whitehall, PA). Seven measures were recorded and analyzed:

(1.) **Total distance traveled** in the arena (cm) was defined as the sum of all vectored coordinate changes in the floor plane. (2.) **Rest time** (sec) was defined as the total time less total movement time. (3.) **Time spent in the margins** of the arena (sec) was defined as the total time spent within 2.5-beam margin of the walls. (4.) **Time spent in the center** of the arena (sec) was defined as the total time spent in the center of the arena. (5.) **Stereotypy episodes** were defined as the total number of coordinate changes less than ± 0.999 beam spaces in the floor plane and back to the original point that did not exceed 2 sec apart. Three such movements had to be made before a stereotypy episode started. When the subject moved outside of the region of qualified coordinates or it failed to move for 2 sec the episode terminated. (6.) **Stereotypy time** (sec) was defined as the total time of stereotypic movement episodes as previously defined. (7.) Finally, **entries into the center of the arena** were defined as entries into the arena-center. Animals were allowed a minimum of 15 minutes to habituate to the room prior to being placed in the activity meter. Animals remained undisturbed in the activity meter chamber for 15 min. Nolvasan was used to clean the activity meter after each animal. Activity meter assessment for a subset of animals that received osmotic minipumps took place on days 19 and 31.

**Prophylactic Drug Groups**

Baselines were taken for animals on respective testing days (von Frey: day -8, cold allodynia: day -7) prior to surgical implantation of osmotic minipumps on day -6. Animals were assigned to groups with the use of a random number generator. Animals
assigned to the paclitaxel condition received pumps filled with the CB₂-preferring agonist AM1710 (3.2, 0.32, or 0.032 mg/kg/day s.c., n = 8-14 per group), the mixed CB₁/CB₂ agonist WIN55,212-2 (1, 0.5, or 0.1 mg/kg/day s.c., n = 8-10 per group), the neuropathic pain medication gabapentin (16, 8, or 4 mg/kg/day s.c., n = 8-10 per group), vehicle (50% DMSO in polyethylene glycol 400, n = 14) or saline (n = 4). Animals assigned to the cremophor vehicle control condition received pumps filled with either AM1710 (3.2 mg/kg/day s.c., n = 8), WIN55,212-2 (0.5 mg/kg/day s.c., n = 8), gabapentin (16 mg/kg/day s.c., n = 8), vehicle (50% DMSO in polyethylene glycol 400, n = 10) or saline (n = 4).

Pharmacological specificity in paclitaxel treated animals was assessed with concurrent implantation of two osmotic minipumps. One pump contained an antagonist (AM251 (3 mg/kg/day s.c.) or AM630 (3 mg/kg/day s.c.)) and the other pump contained an agonist (AM1710 (3.2 mg/kg/day s.c., n = 10), WIN55,212-2 (0.5 mg/kg/day s.c., n = 10), or gabapentin (16 mg/kg/day s.c., n = 10)). Separate groups of paclitaxel-treated animals received pumps filled with either AM251 (3 mg/kg/day s.c., n = 8) or AM630 (3 mg/kg/day s.c., n = 8).

Statistical Analyses

Percentage of paw withdrawals following acetone application to the hind paws was calculated using the following formula: ((Total number of paw withdrawals) * 100)/10. Data were analyzed using analysis of variance (ANOVA) for repeated measures or one-way ANOVA. SPSS 18.0 (SPSS Incorporated, Chicago, IL, USA) statistical software was employed. The Greenhouse-Geisser correction was applied to all repeated factors where the epsilon value from Mauchly’s Test of Sphericity was < 0.75 and the
significance level was \( P < 0.05 \). Degrees of freedom reported for interaction terms of repeated factors are the uncorrected values in cases where the Greenhouse-Geisser correction factor was applied. Post hoc comparisons between the primary control group (paclitaxel-vehicle) and other experimental groups were performed using the Dunnett test (2-sided). Post hoc comparisons between different experimental groups were also performed to assess dose-response relationships and pharmacological specificity using the Tukey test. \( P < 0.05 \) was considered statistically significant.

**Results**

**General Results**

Treatment with paclitaxel resulted in only transient changes in sensitivity to thermal stimulation (\( P > 0.14; \) Fig 6.1a). Transient hypoalgesia on day 6 was evident (\( F_{1,10} = 20.745, P < 0.01 \)); however this change was no longer apparent at subsequent time points (\( P > 0.16 \)). The same animals that failed to show hypersensitivity to thermal stimulation (thermal hyperalgesia) developed hypersensitivity to mechanical stimulation (i.e. mechanical allodynia) (\( F_{1,10} = 6.191, P < 0.05; \) Fig 6.1b). As a result of this finding, all animals utilized for the remaining osmotic minipump portion of the study were evaluated for both mechanical and cold allodynia, but not thermal hyperalgesia.

Pump disbursement volume was calculated by subtracting the fill volume from the residual volume in the pump reservoir on day 22. Pump disbursement volume was calculated separately for drugs dissolved in the DMSO (50% DMSO in PEG 400) and saline vehicles. The pump disbursement volume was significantly different between groups that received pumps filled with drugs dissolved in the DMSO vehicle (\( F_{19,180} = 2.213, P < 0.01 \)). A post hoc analysis revealed that the pump disbursement volume for the
Taxol-WIN55,212-2 (1 mg/kg/day s.c.) group was lower than 43% of the animals in groups receiving pumps filled with drugs dissolved in the DMSO vehicle. No other differences were found. The disbursement volume for pumps filled with drugs dissolved in saline did not differ between groups ($P > 0.22$).

Mechanical withdrawal thresholds did not differ between either the right or the left paw on any given day for animals tested up to 20 ($P > 0.45$) and 50 ($P > 0.10$) days post chemotherapy treatment; therefore, withdrawal thresholds are presented as the mean of duplicate measurements, averaged across paws. Two measurements were assessed for cold allodynia, percentage of paw withdrawals and paw withdrawal latencies. Paw withdrawal latencies proved to be a more variable measure of cold allodynia (data not shown), therefore only percentage of paw withdrawals are shown. Percentage of paw withdrawals to cold stimulation did not differ between either the right or the left paw on any given day for animals tested up to 21 and 51 days post chemotherapy, with three exceptions. On days 17 ($F_{1,434} = 5.075$, $P < 0.05$), 21 ($F_{1,434} = 5.922$, $P < 0.05$), and 51 ($F_{1,166} = 5.980$, $P < 0.05$) the number of paw withdrawals in response to topical acetone application on the right hind paw were increased relative to the left hind paw.

Paw withdrawal thresholds to mechanical stimulation, percentage of paw withdrawals following application of acetone to the hind paws, and all measures assessed in the activity meter on day 19 did not differ between paclitaxel animals that received vehicle (50% DMSO in PEG 400; $n = 14$) or saline ($n = 4$). Therefore, these groups were combined for all analyses and are referred to as the Taxol-vehicle group in all figures and text. Similarly, paw withdrawal thresholds, percentage of paw withdrawals following acetone application, and all measures assessed in the activity meter (day 19) did not differ

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between cremophor animals that had pumps containing vehicle (50% DMSO in PEG 400; n = 8) or saline (n = 4). These groups were similarly combined for all analyses and are hereafter referred to as the cremophor-vehicle group. No differences in body weight were noted in animals receiving paclitaxel-vehicle versus their paclitaxel-saline counterparts. Cremophor-saline animals, did however, show evidence of greater weight gain on days 14-21 ($F_{12,204} = 8.455$, $P < 0.001$, $P < 0.05$ for each day) relative to cremophor-vehicle animals.

**Effects of Prophylactic Treatment with AM1710, WIN55,212-2 and Gabapentin on Paclitaxel-evoked Mechanical Allodynia**

No differences in weights were observed for paclitaxel animals receiving osmotic minipumps filled with either AM1710 (3.2, 0.32, and 0.032 mg/kg/day s.c.) ($P > 0.86$; Fig 6.2a) or gabapentin (16, 8, and 4 mg/kg/day s.c.) ($P > 0.73$; Fig 6.2c). Paclitaxel animals receiving osmotic minipumps filled with WIN55,212-2 (0.1 mg/kg/day s.c.) showed greater weight gain over the course of the study relative to other groups ($F_{4,55} = 2.627$, $P < 0.05$; Fig 6.2b).

*Anti-allodynic effects of the CB2-preferring agonist AM1710.* Taxol-vehicle animals developed mechanical allodynia relative to cremophor-vehicle animals on day 2 and this effect persisted until the final day of testing, day 20 ($F_{48,708} = 5.186$, $P < 0.001$; $P < 0.01$ for each comparison; Fig 6.2d). AM1710 (3.2 and 0.032 mg/kg/day s.c.) prevented the development of mechanical allodynia associated with paclitaxel treatment ($F_{4,59} = 41.988$, $P < 0.001$; Fig 6.2d). Mechanical paw withdrawal thresholds of paclitaxel animals receiving AM1710 (3.2 and 0.032 mg/kg/day s.c.) differed from the Taxol-vehicle group beginning on day 4 and this effect was maintained for the duration of the study ($P < 0.05$
for each comparison). AM1710 (3.2 mg/kg/day s.c.) was the most efficacious dose of AM1710 evaluated. Paclitaxel animals receiving AM1710 (3.2 mg/kg/day s.c.) had higher mechanical paw withdrawal thresholds than Taxol animals that received AM1710 (0.32 mg/kg/day s.c.) from days 12-20 ($P < 0.05$ for each comparison). Additionally, paw withdrawal thresholds in Taxol-AM1710 (3.2 mg/kg/day s.c) animals were similar to the cremophor-vehicle group at all time points.

*Anti-allodynic effects of the mixed CB$_1$/CB$_2$ agonist WIN55,212-2.* WIN55,212-2 (0.1 mg/kg/day s.c.) produced an antinociceptive effect *prior to* paclitaxel treatment on day -2 ($P < 0.05$) relative to a vehicle group and the group that received WIN55,212-2 (1.0 mg/kg/day s.c.); this effect however was no longer present by day 0. WIN55,212-2 (0.5 mg/kg/day s.c.) prevented the development of paclitaxel-induced mechanical allodynia ($F_{4,55} = 32.964, P < 0.001$; Fig 6.2e) over the entire time course ($F_{48,660} = 3.880, P < 0.001$). With the exception of a drop in threshold observed for the WIN55,212-2 (0.5 mg/kg/day s.c.) group on day 8, this group’s thresholds differed from the Taxol-vehicle group at all time points ($P < 0.05$ for each comparison). Additionally, paclitaxel animals treated with WIN55,212-2 (0.5 mg/kg/day s.c.) did not have thresholds that differed from the cremophor-vehicle group at any time point. Although WIN55,212-2 (0.5 mg/kg/day s.c.) was the most effective dose tested, WIN55,212-2 (0.1 mg/kg/day s.c.) also suppressed the development of mechanical allodynia in paclitaxel-treated animals up until the final testing day ($P < 0.05$ for each comparison).

*Anti-allodynic effects of the neuropathic pain medication gabapentin.* Gabapentin (16 mg/kg/day s.c.) suppressed the development of paclitaxel-induced mechanical allodynia ($F_{4,53} = 34.755, P < 0.001$) over the entire time course ($F_{48,636} = 5.210, P < 0.001$).
Paclitaxel animals treated with gabapentin (16 mg/kg/day s.c.) exhibited thresholds that
differed from Taxol-vehicle animals at all time points from days 2 – 20 ($P < 0.05$ for each
comparison). Thresholds in the Taxol-gabapentin (16 mg/kg/day s.c.) group only differed
from the cremophor-vehicle group at one time point (day 16; $P < 0.05$). Gabapentin (8
and 4 mg/kg/day s.c.) failed to consistently attenuate paclitaxel-induced mechanical
allodynia, although both doses produced antinociception at several time points.

**Effects of Prophylactic Treatment with AM1710, WIN55,212-2 and Gabapentin on
Paclitaxel-evoked Cold Allodynia**

*Anti-allodynic effects of the CB$_2$-preferring agonist AM1710.* Paclitaxel-induced cold
allodynia (as measured by percentage of paw withdrawals following acetone application
to the hind paws) developed by day 5 and this effect remained highly significant until the
final testing day, day 21 ($F_{20,295} = 6.871, P < 0.001; P < 0.05$ for each comparison; Fig
6.3a). AM1710 (3.2 mg/kg/day s.c.) suppressed the development of paclitaxel-induced
cold allodynia ($F_{4,59} = 14.299, P < 0.001, P < 0.05$ for each comparison). Lower doses of
AM1710 (0.32 and 0.032 mg/kg/day s.c.) suppressed the development of cold allodynia
in paclitaxel-treated rats through day 11 ($P < 0.05$ for each comparison).

*Anti-allodynic effects of the mixed CB$_1$/CB$_2$ agonist WIN55,212-2.* WIN55,212-2 (0.5 and
0.1 mg/kg/day s.c.) prevented the development of cold allodynia in paclitaxel-treated
animals ($F_{4,55} = 11.428, P < 0.001, P < 0.05$ for each comparison; Fig 6.3b) for the
duration of the time course ($F_{20,275} = 7.197, P < 0.001$). WIN55,212-2 (1 mg/kg/day s.c.)
did not fully suppress the development of paclitaxel-induced cold allodynia. However,
animals in this group did show some protection against mechanical allodynia relative to
vehicle treated animals on day 21 ($P < 0.001$).
Anti-allodynic effects of the neuropathic pain medication gabapentin. Gabapentin (16 mg/kg/day s.c.) prevented the development of cold alldynia in animals that received paclitaxel ($F_{4,53} = 14.756, P < 0.001, P < 0.05$ for each comparison; Fig 6.3c) for the duration of the 30 day time course ($F_{20,265} = 6.619, P < 0.001$). Gabapentin (16 mg/kg/day s.c.) was clearly the most efficacious dose of this drug tested throughout the study, however gabapentin (8 mg/kg/day s.c.) attenuated paclitaxel-induced cold alldynia, and this effect was still present by the final day of the study ($P < 0.05$).

**Comparison of AM1710, WIN55,212-2 and Gabapentin in Tests of Mechanical and Cold Alldynia Resulting from Paclitaxel Treatment**

The doses of AM1710 (3.2 mg/kg/day s.c.), WIN55,212-2 (0.5 mg/kg/day s.c.), and gabapentin (16 mg/kg/day s.c.) administered to paclitaxel animals that produced the maximum, sustained anti-allodynic effect in tests of both mechanical and cold alldynia were compared over the 30 day time course. None of the agonists altered weight gain over the course of the study relative to paclitaxel or cremophor animals that received pumps filled with vehicle in lieu of drug ($P > 0.668$; Fig 6.4a). All three drugs suppressed the development of mechanical alldynia in paclitaxel-treated animals ($F_{4,61} = 62.372, P < 0.001; P < 0.05$ for each comparison; Fig 6.4b) over the 30 day time course. ($F_{48,732} = 4.905, P < 0.001$). All agonists had higher paw withdrawal thresholds relative to paclitaxel animals that received vehicle beginning on day 4; this effect lasted until the final test day for all groups ($P < 0.05$ for each comparison). Paclitaxel animals that received pumps containing either WIN55,212-2 (0.5 mg/kg/day s.c.) or gabapentin (16 mg/kg/day s.c.) experienced a drop in thresholds on at least one testing day (days 8: WIN55,212-2 and 16: WIN55,212-2 and gabapentin) relative to cremophor animals that
received vehicle \((P < 0.05\) for each comparison). In contrast, paclitaxel animals that received AM1710 \((3.2 \text{ mg/kg/day s.c.})\) did not experience any changes in threshold relative to the cremophor-vehicle condition. All three agonists suppressed the development of paclitaxel-induced cold allodynia with equal efficacy \((F_{4,61} = 15.976, P < 0.001; P < 0.01\) for each comparison; Fig 6.4c) over the time course \((F_{20,305} = 7.877, P < 0.001)\).

**Pharmacological Specificity**

Paclitaxel-treated animals that received two pumps, one containing AM1710 \((3.2 \text{ mg/kg/day s.c.})\) and one containing AM630 \((3 \text{ mg/kg/day s.c.})\) had increased weight gain relative to cremophor and paclitaxel animals that received vehicle \((F_{4,61} = 2.830, P < 0.05; \text{Fig 6.5a})\) at several time points during the 30 day study \((F_{68, 1037} = 2.227, P < 0.05; P < 0.05\) for each comparison). Animals treated with paclitaxel that received two pumps, one containing WIN55,212-2 \((0.5 \text{ mg/kg/day s.c.})\) in addition to a pump containing either AM630 \((3 \text{ mg/kg/day s.c.})\) or AM251 \((3 \text{ mg/kg/day s.c.})\) showed an increase in weight gain relative to several other groups \((F_{4,57} = 11.643, P < 0.001; P < 0.05\) for each comparison; Fig 6.5b) over the 30 day time course \((F_{68,969} = 5.268, P < 0.001)\). Neither antagonist administered alone to paclitaxel-treated animals altered weight gain over the course of the study relative to vehicle control animals \((P > 0.93; \text{Fig 6.5c})\).

**Mechanical allodynia.**

**Pharmacological specificity of anti-allodynic effects of AM1710.** The anti-allodynic effects observed in paclitaxel-treated animals that received pumps filled with AM1710 \((3.2 \text{ mg/kg/day s.c.})\) were blocked by concurrent administration of the CB\(_2\) antagonist, AM630 \((3 \text{ mg/kg/day s.c.})\), via a second osmotic minipump \((F_{4,61} = 44.885, P < 0.001,\)
Fig 6.5d). AM630 (3 mg/kg/day s.c.) blocked the anti-allodynic effects of AM1710 (3.2 mg/kg/day s.c.) in paclitaxel-treated rats from day 8 through day 20 ($F_{48, 732} = 6.161$, $P < 0.001$, $P < 0.05$ for each comparison). Concomitant treatment with the CB1 antagonist, AM251 (3 mg/kg/day s.c.) produced no changes in the anti-allodynia achieved in response to mechanical stimulation in Taxol-AM1710 (3.2 mg/kg/day s.c.) animals ($P < 0.05$ for each comparison).

**Pharmacological specificity of anti-allodynic effects of WIN55,212-2.** The anti-allodynic profile of WIN55,212-2 (0.5 mg/kg/day s.c.) observed in paclitaxel animals was dominated primarily by activation of the CB1 receptor. Simultaneous release of AM251 (3 mg/kg/day s.c.) and WIN55,212-2 (0.5 mg/kg/day s.c.) in paclitaxel-treated animals suppressed the anti-allodynic effects of WIN55,212-2 (0.5 mg/kg/day s.c.) ($F_{4,57} = 38.335$, $P < 0.001$; Fig 6.5e). AM251 (3 mg/kg/day s.c.) suppressed WIN55,212-2 (0.5 mg/kg/day s.c.)-mediated anti-allodynic effects beginning on day 6 and this suppression lasted throughout the time course of the study ($F_{48, 684} = 4.112$, $P < 0.001$; $P < 0.05$ for each comparison). Paclitaxel animals that received WIN55,212-2 (0.5 mg/kg/day s.c.) in combination with AM630 (3 mg/kg/day) did show some evidence of blockade on several days during the study ($P < 0.05$ for each comparison), however the effect was not consistent.

**Anti-allodynic effects of antagonists administered alone.** Paclitaxel animals that received osmotic minipumps containing either AM630 (3 mg/kg/day s.c.) or AM251 (3 mg/kg/day) developed mechanical allodynia relative to the cremophor-vehicle group ($F_{3,44} = 58.077$, $P < 0.001$, $P < 0.05$ for each comparison; Fig 6.5f) that lasted throughout
the 30 day time course ($F_{36,528} = 6.134$, $P < 0.001$). Paclitaxel animals treated with either antagonist did not differ from the Taxol-vehicle group at any time point.

Cold allodynia.

**Pharmacological specificity of anti-allodynic effects of AM1710.** The protective effects observed following acetone application in paclitaxel-treated animals that received AM1710 (3.2 mg/kg/day) were blocked by administration (via a second pump) of AM630 (3 mg/kg/day s.c.) ($F_{4,61} = 14.178$, $P < 0.001$, Fig 6.6a). The Taxol-AM1710 (3.2) + AM630 (3) group showed increased paw withdrawals relative to the cremophor-vehicle control group on day 11; by day 17 this group showed increased paw withdrawals relative to paclitaxel animals that received AM1710 (3.2 mg/kg/day s.c.) alone, AM1710 (3.2) + AM251 (3), or cremophor-vehicle ($F_{20,305} = 8.201$, $P < 0.001$; $P < 0.05$ for each comparison). Paw withdrawals in response to acetone application were similar in paclitaxel-treated rats that received AM1710 (3.2) + AM251 (3) and AM1710 (3.2 mg/kg/day s.c.) alone.

**Pharmacological specificity of anti-allodynic effects of WIN55,212-2.** The anti-allodynic effects observed in paclitaxel-animals that received WIN55,212-2 (0.5 mg/kg/day s.c.) following topical application of acetone to the hind paws was not blocked by either AM630 (3 mg/kg/day s.c.) or AM251 (3 mg/kg/day s.c.), administered via a second pump ($F_{4,57} = 10.343$, $P < 0.001$; Fig 6.6b) at any time point ($F_{20,285} = 8.415$, $P < 0.001$, $P < 0.05$ for each comparison).

**Anti-allodynic effects of antagonists administered alone.** Paclitaxel animals that received AM630 (3 mg/kg/day s.c.) or AM251 (3 mg/kg/day s.c.) developed cold allodynia ($F_{3,44} = 12.138$, $P < 0.001$; Fig 6.6c) over the time course relative to cremophor-
vehicle control animals ($F_{15,220} = 7.742$, $P < 0.001$, $P < 0.05$ for each comparison).

Paclitaxel treated animals that received pumps containing AM251 (3 mg/kg/day s.c.) showed a decrease in the percentage of paw withdrawals relative to Taxol-vehicle animals on days 11, 17 and 21 ($P < 0.05$ for each comparison); however, the percentage of paw withdrawals on all three days was increased relative to cremophor-vehicle animals ($P < 0.001$ for each comparison).

**Effects of AM1710, WIN55,212-2 and Gabapentin in Tests of Mechanical and Cold Allodynia When Administered to Cremophor Control Animals**

The most efficacious doses of all three agonists were administered to cremophor animals to determine if these drugs were capable of producing antinociceptive effects in animals not experiencing neuropathic nociception. Cremophor animals that received gabapentin (16 mg/kg/day s.c.) showed evidence of increased weight gain ($F_{4,51} = 2.858$, $P < 0.05$; $P < 0.05$ for each comparison; Fig 6.7a) that was no longer present after day 14 ($P > 0.14$). Cremophor animals treated with AM1710 (3.2 mg/kg/day s.c.), WIN55,212-2 (0.5 mg/kg/day s.c.), or gabapentin (6 mg/kg/day s.c.) did not show evidence of antinociception in response to either mechanical ($F_{4,51} = 78.901$, $P < 0.001$; $P < 0.05$ for each comparison; Fig 6.7b) or cold stimulation ($F_{4,51} = 16.278$, $P < 0.001$, $P < 0.05$ for each comparison; Fig 6.7c) relative to cremophor-vehicle animals over the 30 day time course (mechanical stimulation: $F_{48,612} = 6.254$, $P < 0.001$; cold stimulation: $F_{20,255} = 9.215$, $P < 0.001$).

**Effects of AM1710, WIN55,212-2 and Gabapentin on Paclitaxel-induced Mechanical Allodynia Following Drug Removal**
Paclitaxel-vehicle treatment produced mechanical allodynia relative to cremophor-vehicle animals that persisted until the final test day, day 50 ($F_{75,550} = 16.836$, $P < 0.001$, Fig 6.8a). Paclitaxel-treated animals that received osmotic minipumps that dispensed AM1710 (3.2 mg/kg/day s.c.) from days -6 through 22 did not develop mechanical allodynia until after day 38 ($F_{3,22} = 41.754$, $P < 0.001$, $P < 0.05$ for each comparison). Paclitaxel animals that received AM1710 (0.032 mg/kg/day s.c.) until day 22 continued to demonstrate increased paw withdrawal thresholds through day 38 (17 days following drug removal; $P < 0.01$ for each comparison; Fig 6.8a); however after osmotic minipumps were removed there were days where the threshold failed to differ from the paclitaxel-vehicle condition, indicating that mechanical allodynia was beginning to develop (days 28 and 34). WIN55,212-2 (0.5 mg/kg/day s.c.) produced a protective effect against the development of paclitaxel-induced mechanical allodynia ($F_{3,20} = 48.189$, $P < 0.001$; Fig 6.8b) throughout day 32 (11 days following drug removal; $F_{75,500} = 2.218$, $P < 0.01$, $P < 0.05$ for each comparison). The protective effects of WIN55,212-2 (0.1 mg/kg/day s.c.) were not consistent; however during the period of protection provided by WIN55,212-2 (0.5 mg/kg/day s.c.) (days 22-32) this dose of WIN55,212-2 produced an anti-allodynic effect on several testing days. Treatment with gabapentin (16 mg/kg/day s.c.) resulted in protection against the development of paclitaxel-induced mechanical allodynia ($F_{2,17} = 38.836$, $P < 0.001$; Fig 6.8c) up to 15 days following removal of the drug (day 36; $F_{50,425} = 3.288$, $P < 0.001$, $P < 0.05$ for each comparison). Comparisons of the most efficacious doses of each agonist over the 60 day time course indicated that AM1710 (3.2 mg/kg/day s.c.) produced the longest duration (17 days following drug removal) protective effects against the development of paclitaxel-induced
mechanical allodynia ($F_{100,675} = 2.170, P < 0.001, P < 0.05$ for each comparison; Fig 6.8d). Treatment with gabapentin (16 mg/kg/day s.c.) and WIN55,212-2 (0.5 mg/kg/day) each produced protective effects against the development of paclitaxel-induced mechanical allodynia of shorter durations, however of these two drugs WIN55,212-2 (0.5 mg/kg/day s.c.) produced the shortest duration (day 32) and the most variable anti-allodynic effects ($F_{4,27} = 25.445, P < 0.001, P < 0.05$ for each comparison).

**Effects of AM1710, WIN55,212-2 and Gabapentin on Paclitaxel-induced Cold Allodynia Following Drug Removal**

Paclitaxel animals that received osmotic minipumps filled with vehicle showed an increase in percentage of paw withdrawals following topical acetone application until day 45 ($F_{30,220} = 4.709, P < 0.001$; Fig 6.9a). Paclitaxel animals that received pumps containing AM1710 (3.2 mg/kg/day s.c.) from days -6 through 22 did not develop cold allodynia until day 45 ($F_{3,22} = 16.132, P < 0.001; P < 0.05$ for each comparison; Fig 6.9a). The protective effects of AM1710 (3.2 mg/kg/day s.c.) prevented the development of paclitaxel-induced cold allodynia up to 18 days following removal of the drug (day 39; $P < 0.05$ for each comparison). Paclitaxel animals treated with a lower dose of AM1710 (0.032 mg/kg/day s.c.), while lacking the duration of protection observed with the higher dose, did not fully develop cold allodynia until day 39 ($P < 0.05$ for each comparisons), however a decrease in paw withdrawals relative to cremophor-vehicle animals was observed on day 33 ($P < 0.01$). WIN55,212-2 (0.5 and 0.1 mg/kg/day s.c.) treatment in paclitaxel animals suppressed the development of cold allodynia ($F_{3,20} = 12.367, P < 0.001$; Fig 6.9b) up to 12 (day 33) and 18 days (day 39) following drug removal, respectively ($F_{30,200} = 3.784, P < 0.001; P < 0.05$ for each comparison). Prophylactic
treatment with the neuropathic pain medication gabapentin (16 mg/kg/day s.c.) protected against the development of paclitaxel-induced cold allodynia ($F_{2,17} = 21.747; P < 0.001$; Fig 6.9c) up to 18 days following removal of the drug (day 39; $F_{20,170} = 21.747, P < 0.001$). Treatment with either AM1710 (3.2 mg/kg/day s.c.) or gabapentin (16 mg/kg/day s.c.) was equally efficacious in protecting against the development of paclitaxel-induced cold allodynia ($F_{4,27} = 3.174, P < 0.001, P < 0.05$ for each comparison; Fig 6.9d) over the 60 day time course ($F_{40,270} = 3.174, P < 0.001$). Each drug produced a protective effect up to 18 days following drug removal ($P < 0.05$ for each comparison). WIN55,212-2 (0.5 mg/kg/day s.c.) prevented the development of paclitaxel-induced mechanical allodynia up to 12 days following drug removal (day 33) ($P < 0.05$ for each comparison).

**Pharmacological Specificity Following Drug Removal**

**Mechanical allodynia.** AM630 (3 mg/kg/day s.c.) administered from days -6 through 22 blocked the anti-allodynic effects of AM1710 (3.2 mg/kg/day s.c.) in paclitaxel-treated animals ($F_{4,27} = 25.046, P < 0.001$; Fig 6.10a) over 38 days ($F_{100,675} = 2.534, P < 0.001$). Animals in this group continued to show decreased mechanical paw withdrawal thresholds relative to the cremophor-vehicle condition following removal of the drugs (day 22) until the end of the study, day 50 ($P < 0.05$ for each comparison). In contrast, paclitaxel animals that received prophylactic treatment with AM1710 (3.2 mg/kg/day s.c.) + AM251 (3 mg/kg/day s.c.) did not develop mechanical allodynia until day 40, consistent with paclitaxel animals that received AM1710 (3.2 mg/kg/day s.c.) alone ($P < 0.05$ for each comparison). AM251 (3 mg/kg/day s.c.) administered from days -6 through day 22 suppressed the anti-alldynic effects observed with WIN55,212-2 (0.5 mg/kg/day s.c.) in paclitaxel-treated animals ($F_{4,27} = 41.884, P < 0.001, P < 0.05$ for each comparison).
comparison; Fig 6.10b) over 32 days. 

$F_{100,675} = 2.234, P < 0.001$. Animals in this group continued to show decreased paw withdrawal thresholds relative to cremophor-vehicle animals following surgical removal of the osmotic minipumps until the end of the study ($P < 0.05$ for each comparison). Animals in the Taxol-WIN55,212-2 (0.5) + AM630 (3) group did not fully develop mechanical allodynia until day 34 ($P < 0.05$ for each comparison), consistent with paclitaxel animals previously treated with WIN55,212-2 (0.5 mg/kg/day s.c.) alone. However, this group differed from the cremophor-vehicle group at several time points following removal of the osmotic minipumps until the cessation of anti-allodynic effects (day 34) ($P < 0.05$ for each comparison).

**Cold allodynia.** AM630 (3 mg/kg/day s.c.) administered from days -6 through 22 blocked the anti-allodynic effects of AM1710 (3.2 mg/kg/day s.c.) in paclitaxel-treated animals ($F_{4,27} = 12.388, P < 0.001, P < 0.05$ for each comparison; Fig 6.11a) over 39 days ($F_{40,270} = 3.687, P < 0.001$). Animals in this group continued to show decreased paw withdrawals following topical acetone application relative to cremophor-vehicle animals following removal of the drugs (day 22) until the end of the study (day 50) ($P < 0.05$ for each comparison). Following removal of osmotic minipumps, animals in the Taxol-AM1710 (3.2) + AM251 (3) group failed to develop cold allodynia until day 45 ($P < 0.05$ for each comparison), consistent with protective effects observed in the Taxol-AM1710 (3.2 mg/kg/day s.c.) group. Neither AM630 (3 mg/kg/day s.c.) nor AM251 (3 mg/kg/day s.c.), administered from day -6 through day 22, blocked the anti-allodynic effects of WIN55,212-2 (0.5 mg/kg/day s.c.) ($F_{4,27} = 8.965, P < 0.001, P < 0.05$ for each comparison; Fig 6.11b). Both groups showed anti-allodynic effects up to day 39 relative to the cremophor-vehicle group (18 days following drug removal). ($F_{40,270} = 3.677, P <$
The anti-allodynic effects observed in the WIN55,212-2 blockade conditions relative to the Taxol-vehicle group were slightly longer in duration than the protective effects observed with WIN55,212-2 (0.5 mg/kg/day s.c.) administered alone.

**Activity Meter**

*Day 19.* Seven measures were assessed in the activity meter: total distance traveled (cm), rest time (sec), time spent in the margins of the arena (sec), time spent in the center of the arena (sec), stereotypy episodes, stereotypy time (sec), and number of entries into the center of the arena. Table 6.1 displays the means for total distance traveled (cm). All other measures that were significant are reported in text, but data are not presented in tabular form. Total distance traveled did not differ between either paclitaxel or cremophor animals that received osmotic minipumps filled with vehicle ($P > 0.11$; Table 6.1). Paclitaxel and cremophor animals treated with vehicle did not differ on any other measure ($P > 0.14$ for all measures).

*AM1710 groups.* Total distance traveled was not different between paclitaxel animals that received AM1710 alone or in combination with either antagonist relative to paclitaxel or cremophor treated animals that received vehicle in lieu of drug ($P > 0.13$; Table 6.1). Paclitaxel animals that received AM1710 (3.2) + AM630 (3) did show evidence of decreased margin time ($296.3 \pm 25.4$ sec (Mean ± SEM)) and increased center time ($603.7 \pm 25.4$ sec (Mean ± SEM)) relative to paclitaxel- (margin time: 431.1 ± 22.3 sec; center time: 427.4 ± 33.7 sec (Mean ± SEM)) and cremophor-treated animals (margin time: 427.4 ± 33.7 sec; center time: 472.6 ± 33.7 sec (Mean ± SEM)) that received vehicle ($F_{5,52} = 2.557, P < 0.05; P < 0.05$ for each comparison). Paclitaxel
animals that received AM1710 (0.032 mg/kg/day s.c.) had increased center entries relative to paclitaxel animals that received AM1710 (3.2) + AM630 (3) (303.0 ± 18.0 vs. 227.4 ± 13.9 (Mean ± SEM)). No other recorded measures were significant ($P > 0.12$ for each measure).

**WIN55,212-2 groups.** Total distance traveled recorded for animals in the Taxol-WIN55,212-2 (0.5) + AM630 (3) group was increased relative to cremophor-vehicle animals ($F_{5,54} = 2.951, P < 0.05; P < 0.05$ for relevant comparison; Table 6.1). No other differences in distance traveled for paclitaxel animals that received WIN55,212-2 alone, or in combination with antagonists were observed. The same group showed increased evidence of stereotypy episodes ($F_{5,54} = 2.497, P < 0.05; P < 0.05$ for relevant comparison) relative to cremophor-vehicle treated animals (Taxol-WIN55,212-2 (0.5) + AM630 (3): 352.5 ± 6.9 vs. cremophor-vehicle: 315.6 ± 10.8 (Mean ± SEM)), but not increased stereotypy time ($P > 0.79$). The Taxol-WIN55,212-2 (0.5) + AM630 (3) (431.6 ± 11.4 sec (Mean ± SEM)) group also showed evidence of decreased rest time ($F_{5,54} = 3.574, P < 0.01; P < 0.01$ for relevant comparison) relative to cremophor-vehicle animals (497.5 ± 13.6 sec (Mean ± SEM)). No other recorded measures were significant ($P > 0.26$ for each measure).

**Gabapentin groups.** Total distance traveled for animals in the Taxol-gabapentin (16 mg/kg/day s.c.) group was increased relative to the cremophor-vehicle condition ($F_{2,29} = 3.608, P < 0.05$; Table 6.1). No other recorded measures were significant for paclitaxel animals that received pumps filled with gabapentin (16 mg/kg/day s.c.) relative to cremophor- or paclitaxel-vehicle animals ($P > 0.05$ for each measure).
Antagonist groups. Antagonist administration to paclitaxel animals did not alter distance traveled (Table 6.1) or any other measure assessed relative to cremophor- or paclitaxel-treated animals that received vehicle ($P > 0.31$ for each measure).

Day 31. No difference in distance traveled or any other measure was observed in animals treated with either paclitaxel or cremophor that received osmotic minipumps filled with vehicle ($P > 0.19$ for each measure).

AM1710 groups. Paclitaxel animals that had received AM1710 (3.2 or 0.032 mg/kg/day s.c.) from days -6 through 22 alone, or in combination with either antagonist (AM630 (3 mg/kg/day s.c.) or AM251 (3 mg/kg/day s.c.)), did not differ from paclitaxel- or cremophor-vehicle animals on any recorded measure in the activity meter on day 31 ($P > 0.19$ for each measure; Table 6.2).

WIN55,212-2 groups. Paclitaxel animals that had previously received WIN55,212-2 (0.5 mg/kg/day s.c.) in combination with AM630 (3 mg/kg/day s.c.) showed increased distance traveled relative to animals that had previously been treated with WIN55,212-2 (0.5 mg/kg/day s.c.) alone ($F_{5,30} = 2.769, P < 0.05$; $P < 0.05$ for relevant comparison; Table 6.2). Animals treated prophylactically with WIN55,212-2 (0.5 mg/kg/day s.c.) in combination with AM251 (3 mg/kg/day s.c.) had increased entries into the center of the arena ($288.7 ± 11.5$ (Mean ± SEM)) relative to cremophor animals that received vehicle ($205.4 ± 14.8$ (Mean ± SEM)) ($F_{5,30} = 2.935, P < 0.05$; $P < 0.05$ for relevant comparison). Interestingly, no changes in center time were observed for these groups ($P > 0.71$). No other measure recorded measures were significant ($P > .08$ for each measure).

Gabapentin groups. Paclitaxel-treated animals that had received prophylactic treatment with gabapentin (16 mg/kg/day s.c.) did not differ from paclitaxel- or cremophor-treated
vehicle controls on any recorded measure in the activity meter on day 31 ($P > 0.27$ for each measure; Table 6.2).
Figure 6.1. (a.) Paclitaxel treatment produced transient thermal hypoalgesia, however no long term changes in thermal paw withdrawal latencies were noted throughout the time course. (b.) The same animals which failed to demonstrate long term changes in response to thermal stimulation, developed mechanical allodynia. Inj indicates days when injections of paclitaxel or cremophor vehicle occurred. Taxol indicates paclitaxel. *$P < 0.05$, **$P < 0.01$ vs. Cremophor Vehicle, (ANOVA). N = 6 per group.
Figure 6.1: Effects of paclitaxel on thermal hyperalgesia and mechanical allodynia.
Figure 6.2. (a.) Treatment with AM1710 did not alter weight gain. (b.) Change in body weight was increased following treatment with WIN55,212-2 (0.1 mg/kg/day s.c.). (c.) Gabapentin did not alter weight gain relative to control conditions. (d.) The CB2-preferring agonist AM1710 suppressed paclitaxel-induced mechanical allodynia. Treatment with AM1710 resulted in a U-shaped dose response curve with the highest (3.2 mg/kg/day s.c.) and lowest (0.032 mg/kg/day s.c.) doses producing sustained effects; in contrast, anti-allodynic effects of the middle dose (0.32 mg/kg/day s.c.) were no longer observed at the end of the time course. (e.) WIN55,212-2 dose-dependently suppressed paclitaxel-induced mechanical allodynia. Lower doses (0.5 and 0.1 mg/kg/day s.c.) were more effective than the highest (1.0 mg/kg/day s.c.) dose of WIN55,212-2 tested. (f.) Gabapentin (16 mg/kg/day s.c.) suppressed paclitaxel-induced mechanical allodynia relative to the control group that received vehicle in lieu of drug. Lower doses (8 and 4 mg/kg/day s.c.) of this drug were no longer efficacious at the end of the time course. *P < 0.05, **P < 0.01, ***P < 0.001 vs. Cremophor-Vehicle, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. Taxol-Vehicle, ^P < 0.05 vs. Taxol-Agonist (high dose), †P < 0.05, ††P < 0.01, †††P < 0.001 vs. Taxol-Agonist (middle dose), §P < 0.05, vs. Taxol-Agonist (low dose), βP < 0.05, ββP < 0.01, βββP < 0.001 Taxol-Agonist (middle and low doses) vs. Taxol-Vehicle, ′P < 0.05 Taxol-Agonist (high dose) vs. Taxol-Vehicle, ′′P < 0.01, ′′′P < 0.001 Taxol-Agonist (high and low doses) vs. Taxol-Vehicle, ″P < 0.05 Taxol-Agonist (all doses) vs. Taxol-Vehicle, †P < 0.05 Taxol-Agonist (high and middle doses) vs. Taxol-Vehicle, §P < 0.05 Taxol-Agonist (high and middle doses) vs. Taxol-Vehicle, φφP < 0.01 vs. Taxol-Agonist (middle and low doses) and vehicle. The first drug listed indicates whether the animal received cremophor or paclitaxel (Taxol) treatment. The second drug indicates what the osmotic minipump was filled with. Day numbers
reference days post chemotherapeutic treatment (i.e., negative days indicate days prior to chemotherapeutic treatment). Surgery indicates the day (Day -6) on which osmotic minipumps were implanted subcutaneously on the back of the animal. (ANOVA; Dunnett and Tukey post hoc tests). N = 8-18 per group.
Figure 6.2: Effects of prophylactic AM1710, WIN55,212-2, and gabapentin on weight and paclitaxel-induced mechanical allodynia.
Figure 6.3. (a.) AM1710 (3.2 mg/kg/day s.c.) (b.) WIN55,212-2 (0.5 and 0.1 mg/kg/day s.c.) and (c.) gabapentin (16 mg/kg/day s.c.) suppressed cold allodynia associated with paclitaxel treatment as measured by percentage of paw withdrawals to topical acetone application over the 28 day time course. Anti-allodynic effects of all 3 drugs were sustained for all time points tested post-paclitaxel treatment. *P < 0.05, **P < 0.01, ***P < 0.001 vs. Cremophor-Vehicle, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. Taxol-Vehicle, ++P < 0.01 vs. Taxol-Agonist (middle dose), $$$P < 0.01, vs. Taxol-Agonist (low dose), αP < 0.05 Taxol-Agonist (all doses) vs. Taxol-Vehicle, βP < 0.05 Taxol-Agonist (middle and low doses) vs. Taxol-Vehicle, φP < 0.05, φφφP < 0.001 vs. Taxol-Agonist (middle and low doses) (ANOVA; Dunnett and Tukey post hoc tests). N = 8-18 per group.
Figure 6.3: Effects of prophylactic AM1710, WIN55,212-2, and gabapentin on paclitaxel-induced cold allodynia.
Figure 6.4. (a.) Paclitaxel animals treated with AM1710 (3.2 mg/kg/day s.c.), WIN55,212-2 (0.5 mg/kg/day s.c.) or gabapentin (16 mg/kg/day s.c.) did not show evidence of altered weight gain relative to paclitaxel and cremophor animals that received treatment with vehicle. The CB2-preferring agonist, AM1710 (3.2 mg/kg/day s.c.), the mixed cannabinoid CB1/CB2 agonist WIN55,212-2 (0.5 mg/kg/day s.c.) and the gabapentin (16 mg/kg/day s.c.) suppressed the development of both (b.) mechanical and (c.) cold allodynia associated with paclitaxel treatment. *P < 0.05, **P < 0.01, ***P <0.001 vs. Cremophor-Vehicle, #P < 0.05, ##P <0.01, ###P <0.001 Taxol-AM1710 (3.2 mg/kg/day s.c.), Taxol-WIN55,212-2 (0.5 mg/kg/day s.c.), and Taxol-Gabapentin (16 mg/kg/day s.c.) vs. Taxol-Vehicle, +P < 0.05 Taxol-Gabapentin (16 mg/kg/day s.c.) and Taxol-WIN55,212-2 (0.5 mg/kg/day s.c.) vs. Taxol-Vehicle (ANOVA; Dunnett and Tukey post hoc tests). N = 10-18 per group.
Figure 6.4: Comparison of weights and anti-allodynia observed in paclitaxel-treated animals following prophylactic treatment with AM1710, WIN55,212-2, or gabapentin.
Figure 6.5. Implantation of two osmotic minipumps increased weight gain (and fluid retention) in animals receiving pumps filled with either antagonist (AM630 or AM251) in addition to (a.) AM1710 (3.2 mg/kg/day s.c.) or (b.) WIN55,212-2 (0.5 mg/kg/day s.c.) (c.) No changes in weight were observed in animals receiving single pumps containing either antagonist alone. (d.) The suppression of paclitaxel-induced mechanical allodynia observed with AM1710 (3.2 mg/kg/day s.c.) was mediated by the CB$_2$ receptor (i.e. blocked by a second pump filled with AM630 (3 mg/kg/day s.c.), but not a pump filled with AM251 (3 mg/kg/day s.c.)). (e.) The anti-allodynic profile of WIN55,212-2 was primarily dominated by the CB$_1$ receptor; however the CB$_2$ receptor was marginally involved. (f.) Neither the CB$_1$ antagonist, AM251 (3 mg/kg/day s.c.), nor the CB$_2$ antagonist AM630 (3 mg/kg/day s.c.) produced an effect on paw withdrawal thresholds to mechanical stimulation in animals treated with paclitaxel. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. Cremophor-Vehicle, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. Taxol-Vehicle, + $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$ vs. Taxol-Agonist, $^aP < 0.05$ Taxol-Agonist + AM630 (3) and Taxol-Agonist + AM251 (3) vs. Cremophor-Vehicle and Taxol-Vehicle, $^bP < 0.05$, $^bbP < 0.01$, $^bbbP < 0.001$ Taxol-Agonist + AM630 (3) and Taxol-Agonist + AM251 (3) vs. Cremophor-Vehicle, Taxol-Vehicle and Taxol-Agonist, $^xP < 0.05$, $^xxP < 0.01$, $^xxxP < 0.001$ Taxol-Agonist and Taxol-Agonist + AM251 (3) vs. Taxol-Vehicle, $^tP < 0.05$ vs. Taxol-Agonist, Taxol-Agonist + AM630 (3), and Cremophor-Vehicle, $^φP < 0.05$, $^φφP < 0.01$, $^φφφP < 0.001$ Taxol-Agonist + AM630 (3) and Taxol-Agonist + AM251 (3) vs. Taxol-Agonist, $^⊥P < 0.05$, $^⊥⊥P < 0.01$, $^⊥⊥⊥P < 0.001$ vs. Taxol-Agonist, Taxol-Agonist + AM251 (3), and Cremophor-Vehicle. $^^P < 0.05$, $^_^P < 0.01$, $^_^_^P < 0.001$ vs. Taxol-Agonist, Taxol-Agonist + AM251 (3), and Cremophor-Vehicle.
mg/kg/day s.c.) and Taxol-AM251 (3 mg/kg/day s.c.) vs. Cremophor-Vehicle. Doses are in mg/kg/day s.c. (ANOVA; Dunnett and Tukey post hoc tests). N = 10-18 per group.
Figure 6.5: Change in weights and pharmacological specificity in a test of mechanical allodynia observed in paclitaxel-treated animals that received prophylactic treatment with AM1710 and WIN55,212-2 in combination with cannabinoid antagonists.
Figure 6.6. (a.) AM630 (3 mg/kg/day s.c.), but not AM251 (3 mg/kg/day s.c.) blocked the anti-allodynic effects of AM1710 (3.2 mg/kg/day s.c.) in a behavioral test of cold allodynia. (b.) Neither AM630 (3 mg/kg/day s.c.) nor AM251 (3 mg/kg/day s.c.) consistently blocked the anti-allodynic effects of WIN55,212-2 (0.5 mg/kg/day s.c.) following topical application of acetone to the hind paws. (c.) Neither antagonist alone altered the percentage of paw withdrawals following acetone application in animals that received treatment with paclitaxel. *P < 0.05, **P < 0.01, ***P < 0.001 vs. Cremophor-Vehicle, †P < 0.05, ‡P < 0.01, §§P < 0.001 vs. Taxol-Vehicle, □□P < 0.01, □□□P < 0.001 vs. Taxol-Agonist and Taxol-Agonist + AM251 (3) vs. Taxol-Vehicle, ‡‡‡‡P < 0.01 vs. Taxol-Agonist + AM630 (3), ††P < 0.05, †††P < 0.001 vs. Taxol-Agonist, Taxol-Agonist + AM251 (3) and Cremophor-Vehicle, ‡‡P < 0.05, ‡‡‡P < 0.001 Taxol-Agonist, Taxol-Agonist + AM251 (3), Taxol-Agonist + AM630 (3) and vs. Taxol-Vehicle, ‡P < 0.05 vs. Taxol-AM630 (3 mg/kg/day s.c.) and Taxol-AM251 (3 mg/kg/day s.c.) vs. Taxol-Vehicle and Cremophor-Vehicle. Doses are in mg/kg/day s.c. (ANOVA; Dunnett and Tukey post hoc tests). N = 10-18 per group.
Figure 6.6: Pharmacological specificity of paclitaxel-induced cold allodynia in animals that received AM1710 and WIN55,212-2 in combination with cannabinoid antagonists.
Figure 6.7. (a.) Cremophor treated animals receiving gabapentin (16 mg/kg/day s.c.) had increased weight gain early during treatment that was no longer present after Day 14. Cremophor animals receiving osmotic minipumps containing AM1710 (3.2 mg/kg/day s.c.), WIN55,212-2 (0.5 mg/kg/day s.c.) or gabapentin (16 mg/kg/day s.c.) did not have altered (b.) paw withdrawal thresholds to mechanical stimulation or (c.) paw withdrawals following acetone application relative to comparable cremophor controls receiving vehicle. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. Cremophor-Vehicle, #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ Cremophor-AM1710 (3.2 mg/kg/day s.c.), Cremophor-WIN55,212-2 (0.5 mg/kg/day s.c.), Cremophor-Gabapentin (16 mg/kg/day s.c.) vs. Taxol-Vehicle, †$P < 0.05$, ‡$P < 0.01$ Cremophor-Gabapentin (16 mg/kg/day s.c.) vs. Taxol-Vehicle, §$P < 0.05$ Cremophor-Gabapentin (16) and Cremophor-WIN55,212-2 (0.5 mg/kg/day s.c.) vs. Taxol-Vehicle, ^$P < 0.05$ Cremophor-Gabapentin (16 mg/kg/day s.c.) and Cremophor-AM1710 (3.2 mg/kg/day s.c.) vs. Taxol-Vehicle (ANOVA; Dunnett and Tukey post hoc tests). N = 8-18 per group.
Figure 6.7: Effects of AM1710, WIN55,212-2 and gabapentin administered to cremophor control animals on weight, mechanical and cold allodynia.
Figure 6.8. (a.) AM1710 (3.2 mg/kg/day s.c.) suppressed hypersensitivity to mechanical stimulation up to Day 38 (17 days following the removal of drug), whereas a lower dose (0.032 mg/kg/day s.c.) was effective until Day 36. (b.) WIN55,212-2 (0.5 mg/kg/day s.c.) suppressed paclitaxel-induced mechanical allodynia only 11 days (Day 32) following the removal of drug. (c.) Gabapentin suppressed the development of mechanical allodynia in rats receiving paclitaxel up to 15 days (Day 36) following the removal of the drug. (d.) The anti-allodynic effects observed in paclitaxel-treated rats receiving pumps filled with either AM1710 (3.2 mg/kg/day s.c.) or gabapentin (16 mg/kg/day s.c.) produced longer duration protective effects against the development of paclitaxel-induced mechanical allodynia relative to rats treated with WIN55,212-2 (0.5 mg/kg/day s.c.). *P < 0.05, **P < 0.01, ***P < 0.001 vs. Cremophor-Vehicle, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. Taxol-Vehicle, †P < 0.05, ††P < 0.01, †††P < 0.001 Taxol-Agonist (both doses) vs. Taxol-Vehicle, ^P < 0.05 vs. all groups, §P < 0.05, §§§P < 0.001 Taxol-Agonist (both doses) vs. Cremophor-Vehicle, ‡P < 0.05 Taxol-WIN55,212-2 (0.5 mg/kg/day s.c.) vs. Cremophor-Vehicle, †‡P < 0.05, ††‡P < 0.01, †††‡P < 0.001 Taxol-AM1710 (3.2 mg/kg/day s.c.), Taxol-WIN55,212-2 (0.5 mg/kg/day s.c.), and Taxol-Gabapentin (16 mg/kg/day s.c.) vs. TAXol-Vehicle, †§P < 0.05, †§§P < 0.01 Taxol-Gabapentin (16 mg/kg/day s.c.) and Taxol-AM1710 (3.2 mg/kg/day s.c.) vs. Taxol-Vehicle, ††P < 0.05 Taxol-AM1710 (3.2 mg/kg/day s.c.) and Taxol-WIN55,212-2 (0.5 mg/kg/day s.c.) vs. Taxol-Vehicle, δP < 0.05 Taxol-WIN55,212-2 (0.5 mg/kg/day s.c.) and Taxol-AM1710 (3.2 mg/kg/day s.c.) vs. Taxol-Vehicle, νP < 0.05, ωP < 0.01 Taxol-All agonists vs. Cremophor-Vehicle (ANOVA; Dunnett and Tukey post hoc tests). N = 4-8 per group. Source: Rahn et al., (in preparation).
a

- Cremophor-Vehicle
- Taxol-Vehicle
- Taxol-AM1710 (3.2 mg/kg/day s.c.)
- Taxol-AM1710 (0.032 mg/kg/day s.c.)

b

- Cremophor-Vehicle
- Taxol-Vehicle
- Taxol-WIN55,212-2 (0.5 mg/kg/day s.c.)
- Taxol-WIN55,212-2 (0.1 mg/kg/day s.c.)
Figure 6.8: Long-term effects of AM1710, WIN55,212-2 and gabapentin on paclitaxel-induced mechanical allodynia in animals following removal of osmotic minipumps.
Figure 6.9. (a.) AM1710 (3.2 mg/kg/day s.c.) suppressed cold allodynia in animals receiving paclitaxel up to day 39 (18 days following the removal of drug). (b.) WIN55,212-2 (0.5 and 0.1 mg/kg/day s.c.) suppressed paclitaxel-induced cold allodynia only 12 days (Day 33) following the removal of drug. (c.) Gabapentin suppressed the development of mechanical allodynia in rats receiving paclitaxel up to 18 days (Day 36) following the removal of the drug. (d.) The anti-allodynic effects observed in paclitaxel-treated rats receiving pumps filled with either AM1710 (3.2 mg/kg/day s.c.) or gabapentin (16 mg/kg/day s.c.) produced longer duration protective effects against the development of paclitaxel-induced cold allodynia relative to rats treated with WIN55,212-2 (0.5 mg/kg/day s.c.). *P < 0.05, **P < 0.01, ***P < 0.001 vs. Cremophor-Vehicle †P < 0.05, ‡P < 0.01, §§P < 0.001 vs. Taxol-Vehicle ‡P < 0.05 vs. Taxol-AM1710 (0.032 mg/kg/day s.c.) ⊥P < 0.05, ⊥⊥P < 0.01 Taxol-Agonist (both doses) vs. Taxol-Vehicle, φP < 0.05, φφP < 0.01 Taxol-AM1710 (3.2 mg/kg/day s.c.), Taxol-WIN55,212-2 (0.5 mg/kg/day s.c.) and Taxol-Gabapentin (16 mg/kg/day s.c.) vs. Taxol-Vehicle, ⌧P < 0.05 Taxol-AM1710 (3.2 mg/kg/day s.c.) and Taxol-Gabapentin (16 mg/kg/day s.c.) vs. Taxol-Vehicle (ANOVA; Dunnett and Tukey post hoc tests). N = 4-8 per group.
**A**

- Cremophor-Vehicle
- Taxol-Vehicle
- Taxol-AM1710 (3.2 mg/kg/day s.c.)
- Taxol-AM1710 (0.032 mg/kg/day s.c.)

**B**

- Cremophor-Vehicle
- Taxol-Vehicle
- Taxol-WIN55,212-2 (0.5 mg/kg/day s.c.)
- Taxol-WIN55,212-2 (0.1 mg/kg/day s.c.)

**C**

- Cremophor-Vehicle
- Taxol-Vehicle
- Taxol-Gabapentin (16 mg/kg/day s.c.)
Figure 6.9: Long-term effects of AM1710, WIN55,212-2 and gabapentin on paclitaxel-induced cold allodynia in animals following removal of osmotic minipumps.
Figure 6.10. (a.) AM1710 (3.2 mg/kg/day s.c.) suppressed hypersensitivity to mechanical stimulation up to Day 38 (17 days following the removal of drug). The effects of AM1710 were mediated by the CB$_2$ receptor (i.e. blocked by a second pump filled with AM630 (3 mg/kg/day s.c.) but not a pump filled with AM251 (3 mg/kg/day s.c.)). (b.) WIN55,212-2 (0.5 mg/kg/day s.c.) suppressed paclitaxel-induced mechanical allodynia up to Day 32 (11 days following removal of the osmotic minipump). Effects of this drug were relatively weak and blockade of this effect was not fully achieved with either receptor antagonist, however anti-allodynic protective effects did appear to be primarily mediated by CB$_1$ receptor activation. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. Cremophor-Vehicle, $#P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs. Taxol-Vehicle, $^\wedge P < 0.05$

Taxol-Agonist + AM251 (3) vs. Taxol-Vehicle, $^xP < 0.05$, $^{xx}P < 0.01$, $^{xxx}P < 0.001$

Taxol-Agonist and Taxol-Agonist + AM251 (3) vs. Taxol-Vehicle, $\perp P < 0.05$, $\perp\perp P < 0.01$, $\perp\perp\perp P < 0.001$ vs. Taxol-Agonist, Taxol-Agonist + AM251 (3) and Cremophor-Vehicle, $^\alpha P < 0.05$ Taxol-Agonist + AM630 (3) vs. Cremophor-Vehicle, $^\beta P < 0.05$, $^{\beta\beta}P < 0.01$ Taxol-Agonist, Taxol-Agonist + AM251 (3), and Taxol-Agonist + AM630 (3) vs. Cremophor-Vehicle, $^{\phi\phi}P < 0.01$ Taxol-Agonist + AM251 (3), and Taxol-Agonist + AM630 (3) vs. Taxol-Agonist and Cremophor-Vehicle, $^+P < 0.05$ vs. Taxol-Agonist, $^\gamma P < 0.01$ Taxol-Agonist + AM251 (3) and Taxol-Agonist + AM630 (3) vs. Cremophor-Vehicle. Doses are in mg/kg/day s.c. (ANOVA; Dunnett and Tukey post hoc tests). N = 6-8 per group.
Figure 6.10: Pharmacological specificity of long-term effects observed with AM1710 and WIN55,212-2 on paclitaxel-induced mechanical allodynia in animals following removal of osmotic minipumps.
Figure 6.11. (a.) Anti-allodynia observed in Taxol-AM1710 (3.2 mg/kg/day s.c.) animals up to 18 days following removal of the drug were mediated by activation of the CB2 receptor. (b.) Protection against the development of cold allodynia in paclitaxel-treated rats receiving WIN55,212-2 was not mediated by activation of either the CB1 or the CB2 receptor. *P < 0.05, **P < 0.01, ***P < 0.001 vs. Cremophor-Vehicle, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. Taxol-Vehicle, +P < 0.05, ++P < 0.01 vs. Taxol-Agonist + AM630 (3), \*P < 0.05, \*\*P < 0.01 Taxol-Agonist and Taxol-Agonist + AM251 (3) vs. Taxol-Vehicle, \#\#P < 0.05 vs. Taxol-Agonist, Taxol-Agonist + AM251 (3) and Cremophor-Vehicle \#\#\#P < 0.001 Taxol-Agonist, Taxol-Agonist + AM251 (3) and Taxol-Agonist + AM630 (3) vs. Cremophor-Vehicle, ^\^P < 0.01 Taxol-Agonist and Taxol-Agonist + AM630 (3) vs. Taxol-Vehicle, °P < 0.05 Taxol-Agonist + AM630 (3) and Taxol-Agonist + AM251 (3) vs. Cremophor-Vehicle. All doses are in mg/kg/day s.c. (ANOVA; Dunnett and Tukey post hoc tests). N = 6-8 per group.
Figure 6.11: Pharmacological specificity of long-term effects observed with AM1710 and WIN55,212-2 on paclitaxel-induced cold allodynia in animals in animals following removal of osmotic minipumps.
Table 6.1: Total distance traveled (cm) in a 15 min activity meter session on day 19

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Distance Traveled</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxol-Vehicle</td>
<td>9108.7 ± 390.5</td>
<td>8</td>
</tr>
<tr>
<td>Cremophor-Vehicle</td>
<td>8186.0 ± 355.6</td>
<td>14</td>
</tr>
<tr>
<td>Taxol-AM1710 (3.2 mg/kg/day s.c.)</td>
<td>8637.4 ± 412.4</td>
<td>6</td>
</tr>
<tr>
<td>Taxol-AM1710 (0.032 mg/kg/day s.c.)</td>
<td>9482.2 ± 419.9</td>
<td>10</td>
</tr>
<tr>
<td>Taxol-AM1710 (3.2) + AM630 (3)</td>
<td>8562.2 ± 364.2</td>
<td>10</td>
</tr>
<tr>
<td>Taxol-AM1710 (3.2) + AM251 (3)</td>
<td>8378.1 ± 300.7</td>
<td>10</td>
</tr>
<tr>
<td>Taxol-WIN55,212-2 (0.5 mg/kg/day s.c.)</td>
<td>9100.4 ± 312.7</td>
<td>10</td>
</tr>
<tr>
<td>Taxol-WIN55,212-2 (0.1 mg/kg/day s.c.)</td>
<td>9652.2 ± 396.2</td>
<td>8</td>
</tr>
<tr>
<td>Taxol-WIN55,212-2 (0.5) + AM630 (3)</td>
<td>9827.7 ± 333.9*</td>
<td>10</td>
</tr>
<tr>
<td>Taxol-WIN55,212-2 (0.5) + AM251 (3)</td>
<td>8739.3 ± 413.4</td>
<td>10</td>
</tr>
<tr>
<td>Taxol-Gabapentin (16 mg/kg/day s.c.)</td>
<td>9494.2 ± 365.3*</td>
<td>10</td>
</tr>
<tr>
<td>Taxol-AM630 (3 mg/kg/day s.c.)</td>
<td>8016.6 ± 482.3</td>
<td>8</td>
</tr>
<tr>
<td>Taxol-AM251 (3 mg/kg/day s.c.)</td>
<td>8442.1 ± 420.1</td>
<td>8</td>
</tr>
</tbody>
</table>

Data are mean ± s.e.mean. Statistical comparisons are denoted with line divisions. All divisions within the table were compared against the cremophor- and paclitaxel-vehicle control animals. Doses are in mg/kg/day s.c. *P < 0.05 vs Cremophor-Vehicle (ANOVA, Tukey Post Hoc).
Table 6.2: Total distance traveled (cm) in a 15 min activity meter session on day 31

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Distance Traveled</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxol-Vehicle</td>
<td>8369.0 ± 161.7</td>
<td>6</td>
</tr>
<tr>
<td>Cremophor-Vehicle</td>
<td>7843.0 ± 275.2</td>
<td>8</td>
</tr>
<tr>
<td>Taxol-AM1710 (3.2 mg/kg/day s.c.)</td>
<td>7976.1 ± 469.5</td>
<td>6</td>
</tr>
<tr>
<td>Taxol-AM1710 (0.032 mg/kg/day s.c.)</td>
<td>8263.9 ± 423.6</td>
<td>6</td>
</tr>
<tr>
<td>Taxol-AM1710 (3.2) + AM630 (3)</td>
<td>7577.3 ± 499.3</td>
<td>6</td>
</tr>
<tr>
<td>Taxol-AM1710 (3.2) + AM251 (3)</td>
<td>7269.8 ± 238.3</td>
<td>6</td>
</tr>
<tr>
<td>Taxol-WIN55,212-2 (0.5 mg/kg/day s.c.)</td>
<td>7679.7 ± 249.0</td>
<td>6</td>
</tr>
<tr>
<td>Taxol-WIN55,212-2 (0.1 mg/kg/day s.c.)</td>
<td>8986.9 ± 1023.7</td>
<td>4</td>
</tr>
<tr>
<td>Taxol-WIN55,212-2 (0.5) + AM630 (3)</td>
<td>94067.0 ± 273.4*</td>
<td>6</td>
</tr>
<tr>
<td>Taxol-WIN55,212-2 (0.5) + AM251 (3)</td>
<td>8699.2 ± 381.1</td>
<td>6</td>
</tr>
<tr>
<td>Taxol-Gabapentin (16 mg/kg/day s.c.)</td>
<td>7888.1 ± 384.3</td>
<td>6</td>
</tr>
</tbody>
</table>

Data are mean ± s.e.mean. Statistical comparisons are denoted with line divisions. All divisions within the table were compared against the cremophor- and paclitaxel-vehicle control animals. Doses are in mg/kg/day s.c. *P < 0.05 vs. Taxol-WIN55,212-2 (0.5 mg/kg/day s.c.) (ANOVA, Tukey Post Hoc).
Discussion

Paclitaxel treatment [29] resulted in the development of mechanical allodynia, but not thermal hyperalgesia. Thermal hyperalgesia has previously been reported in a model of paclitaxel-induced neuropathic nociception [29]; however it appears to be dose dependent. In the same study, paclitaxel treatment (cumulative dose of 8 mg/kg i.p.) resulted in robust cold allodynia (percentage of paw withdrawals in response to topical acetone) that lasted through the final test day, day 28 [29]. Collectively, the previous reported findings and our own observations prompted us to employ the present experimental design to evaluate prophylactic analgesics in paclitaxel and cremophor vehicle animals in tests of mechanical and cold allodynia.

Animals remained in relatively good health throughout the course of the study. No changes in body weight were observed between animals that received cremophor-vehicle and paclitaxel-vehicle. The one fatality in the study was the result of an osmotic minipump that malfunctioned and was not associated with paclitaxel treatment. Prophylactic administration of the CB2 agonist, AM1710, the mixed cannabinoid CB1/CB2 agonist, WIN55,212-2, and the neuropathic pain medication, gabapentin, suppressed the development of both mechanical and cold allodynia associated with paclitaxel treatment. Paw withdrawal latency in response to topical acetone application is a reported measure of cold alldynia [33-35]; however we found this measure variable and consequently reported only percentage of paw withdrawals. The most efficacious doses of AM1710, WIN55,212-2 and gabapentin did not induce antinociception when administered to cremophor control animals.
Activity meter assessments, both during prophylactic drug treatment (day 19) and following drug removal (day 31), failed to yield major differences in total distance traveled or any other recorded measure. Paclitaxel rats receiving agonists administered alone or in combination with antagonists did not show hypoactivity, a sign of CNS depression. Our findings also failed to yield thigmotactic behavior in paclitaxel-treated rats relative to cremophor vehicle rats, an effect that has previously been reported in a model of human immunodeficiency virus (HIV) associated sensory neuropathy [36; 37]. Thigmotaxis associated with HIV anti-retroviral treatment was successfully attenuated with either a single administration of gabapentin (30 mg/kg i.p.) or morphine (2.5 mg/kg i.p.). Both drugs were as efficacious as diazepam (1 mg/kg i.p.), demonstrating the anxiolytic effects of both analgesics [38]. The lack of thigmotaxis in our study may be due to differences in the neuropathic pain model or equipment. Our study utilized an activity meter (40.64 cm x 40.64 cm) that may not be as sensitive as an open field (1 m x 1 m) in detecting thigmotaxis.

**Prophylactic Administration of the CB₂-preferring agonist AM1710 Suppresses Mechanical and Cold Allodynia Associated with Paclitaxel Treatment**

Several cannabinoid CB₂ agonists have been tested for amelioration of established paclitaxel-induced neuropathic nociception [23; 24]; however this is the first study to examine the effects of prophylactic administration on the development of paclitaxel-induced neuropathic nociception. The novel cannabiliactone AM1710 has not previously been tested in the paclitaxel model, however work from our lab demonstrated that the structurally similar cannabiliactone AM1714 suppressed paclitaxel-induced mechanical allodynia following acute administration on day 21 [24]. Prophylactic treatment with
AM1710 resulted in suppression of paclitaxel-induced mechanical and cold allodynia that presented in a U-shaped dose response curve, with a high (3.2 mg/kg/day s.c.) and low (0.032 mg/kg/day s.c.) dose showing greater efficacy than a middle dose (0.32 mg/kg/day s.c.). A similar dose response curve was obtained in a test of thermal antinociception (plantar test) with naive animals (Rahn et al., unpublished data). The protective effects of prophylactic AM1710 (3.2 mg/kg/day s.c.) lasted 17-18 days following drug removal for tests of mechanical and cold alldynia, respectively. Effects of AM1710 (3.2 mg/kg/day s.c.) in tests of cold and mechanical alldynia associated with paclitaxel treatment were clearly mediated by activation of the CB2 receptor.

**Prophylactic Administration of the Mixed Cannabinoid CB1/CB2 agonist WIN55,212-2 Suppresses Mechanical and Cold Alldynia Associated with Paclitaxel Treatment**

WIN55,212-2 has previously been shown to ameliorate established paclitaxel-induced mechanical alldynia [22]; however this is the first study to examine prophylactic administration of this potent mixed cannabinoid agonist in a model of paclitaxel-induced neuropathic nociception. Paclitaxel animals treated with WIN55,212-2 (0.1 mg/kg/day s.c.) showed evidence of increased weight gain throughout the course of the study relative to several other groups. No other paclitaxel group that received an agonist alone showed similar changes in weight. Although food intake was not assessed in this study, it has been suggested that CB1 receptor activation can result in orexigenic effects [for review see 39]. This effect of WIN55,212-2 (0.1 mg/kg/day s.c.) in paclitaxel-treated animals is interesting given that higher doses (1-2 mg/kg i.p.) were not able to attenuate anorexia or weight loss in animals treated with the platinum-derived chemotherapeutic agent, cisplatin [40]. It is possible that while higher doses of
WIN55,212-2 are effective at suppressing neuropathic nociception, a lower dose is required to increase feeding behavior.

The highest dose of WIN55,212-2 (1.0 mg/kg/day s.c.) was the least efficacious dose in suppressing paclitaxel-evoked allodynia. However, this group received less of the preloaded drug via the osmotic minipump than nearly half of the other animals receiving drugs in the same vehicle. It is possible that the drug was at the maximum level of solubility and, over time, fell out of solution and clogged the flow moderator thereby reducing the overall release of the drug. WIN55,212-2 (0.5 and 0.1 mg/kg/day s.c.) suppressed the development of paclitaxel-induced mechanical and cold allodynia throughout the 30 day time course. The protective effects of both doses consistently lasted up to 11 (mechanical allodynia) and 12 (cold allodynia) days following drug removal.

Increased weight gain was observed in paclitaxel animals that received 2 pumps, one containing WIN55,212-2 (0.5 mg/kg/day s.c.) and the other containing an antagonist (AM630 (3 mg/kg/day s.c.) or AM251 (3 mg/kg/day s.c.)). The weight of the second pump (approximately 8 grams) and fluid retention in the subcutaneous pocket where pumps were inserted (as noted by the experimenter upon pump removal) cannot fully account for observed changes.

The anti-allodynic effects of WIN55,212-2 (0.5 mg/kg/day s.c.) observed in response to mechanical stimulation were dominated by activation of the CB₁ receptor. Blockade of the CB₂ receptor with AM630 (3 mg/kg/day s.c.) did not eliminate the WIN55,212-2 (0.5 mg/kg/day s.c.)-mediated suppression of paclitaxel-induced mechanical allodynia; however intermittent blockade by AM630 indicates some
contribution of the CB2 receptor. Interestingly, blockade of the anti-allodynic effects of WIN55,212-2 (0.5 mg/kg/day s.c.) in response to topical acetone application was not achieved with either antagonist. Blockade of both cannabinoid CB1 and CB2 receptors may be required to prevent the anti-allodynic effects of this drug. There were limitations to the solubility of these compounds which prohibited co-administration of both antagonists in one pump and implantation of three pumps is not possible. We could find only one report of WIN55,212-2-induced suppression of cold allodynia in a neuropathic pain model (spinal nerve ligation) where pharmacological specificity was assessed. In this study, the anti-allodynic effects of WIN55,212-2 (2.5 mg/kg i.p.) were blocked by a CB1 antagonist (SR141716a), but not a CB2 antagonist (SR144528) [41]. Few studies have examined cannabinoid-mediated modulation of cold allodynia in a neuropathic pain model and more work is necessary to determine the functional contribution of each receptor to the suppression of this form of nociception.

**Proposed Mechanisms of Action for Cannabinoid-mediated Suppression of Paclitaxel-induced Neuropathic Nociception**

Paclitaxel treatment is associated with atypical (swollen and vacuolated) mitochondria [42]. Paclitaxel may increase the release of intracellular calcium via opening of the mitochondrial permeability transition pore (mPTP) [43; 44]. This release is purported to underlie the neuronal hyperexcitability observed following treatment with paclitaxel [45]. THC has been shown to decrease mitochondrial membrane potential in vitro in a manner that was suppressed by cyclosporine A, suggesting the effect was mediated by mPTP [46]. The phytocannabinoid, cannabidiol, has recently been shown to prevent calcium oscillations associated with inducible cell hyperexcitability via
modulation of the mitochondrial Na⁺/Ca²⁺-exchanger; however mPTP modulation was not reported to underlie this observed effect [47]. The presence of free radicals (associated with increased Ca²⁺) can also induce the mPTP to open; thus explaining the effectiveness of Phenyl N-tert-butylnitrone (PBN), a free radical scavenger, in suppressing paclitaxel-induced mechanical allodynia [48]. WIN55,212-2 has been shown to inhibit the production of free radicals in interleukin (IL)-1beta-stimulated human fetal astrocytes [49]. More work is necessary to determine if paclitaxel-induced alterations in mitochondria, free radical production, and subsequent dysregulation of intracellular calcium associated with the opening of mPTPs may be potential mechanisms of action for cannabinoid-mediated suppression of paclitaxel-induced neuropathic nociception.

**Pro-apoptotic Effects of Cannabinoids**

Cannabinoids have been shown to inhibit cancer cell growth in several cell lines [50]. A recent study found that an anaplastic thyroid carcinoma cell line subjected to interleukin (IL)-12 gene transfer had increased CB₂ mRNA expression. Activation of this receptor via the CB₂-specific agonist, JWH133, resulted in increased apoptosis of tumor cells. Overexpression of the CB₂ receptor in this cell line was also associated with enhanced cytotoxicity of paclitaxel [51]. The pro-apoptotic actions of cannabinoids associated with CB₂ receptor activation have been shown to work through activation of p38 mitogen-activated protein kinases (MAPK) in leukemia cells [52], activation of this same pathway is critical for paclitaxel-induced apoptosis [53]. Paclitaxel and anandamide have also recently been shown to function synergistically to induce apoptosis in a gastric cell cancer line that only expressed the CB₁ receptor [54]. The combination of cannabinoid agonists and chemotherapeutic treatment with paclitaxel in an *in vivo* tumor
model might provide evidence of cannabinoid-mediated apoptotic effects and suppression of neuropathic nociception.

Prophylactic Administration of Gabapentin Suppresses Mechanical and Cold Allodynia Associated with Paclitaxel Treatment

Gabapentin (16 mg/kg/day s.c.) administered to paclitaxel-treated rats suppressed the development of both mechanical and cold allodynia during the 30 day time course. Lower doses of gabapentin (8 and 4 mg/kg/day s.c.) failed to produce sustained anti-allodynic effects. Following drug removal, gabapentin (16 mg/kg/day s.c.) produced a protective effect for up to 15 to 18 days against the development of paclitaxel-induced mechanical and cold allodynia, respectively.

Proposed Mechanisms of Action for Gabapentin-mediated Suppression of Paclitaxel-induced Neuropathic Nociception

Previous work in a model of paclitaxel-induced mechanical allodynia indicated that a single injection of gabapentin (100 mg/kg i.p.) produced no change in paw withdrawal thresholds; multiple injections were required before alterations were observed [55]. This report is in contrast to a study that found acute administration of gabapentin (10 and 30 mg/kg i.p.) attenuated established paclitaxel-induced mechanical allodynia (4 mg/kg single injection) in mice [56]. It was also recently reported that oral (30 and 100 mg/kg) and intrathecal (30 and 100 µg) administration of gabapentin attenuated established paclitaxel-induced mechanical allodynia (5 mg/kg single injection) in mice [57]. Differences in species (mice vs. rats) or paclitaxel dosing paradigm may account for these discrepancies. We did not test the anti-allodynic efficacy of acute gabapentin in our study.
Gabapentin binds to $\alpha_{2}\delta$-1 subunits of neuronal voltage gated calcium channels. Paclitaxel treatment increases protein [55] and mRNA [57] expression of the $\alpha_{2}\delta$-1 subunit in the dorsal spinal cord but not the dorsal root ganglia (DRG). Increased $\alpha_{2}\delta$-1 protein expression in the dorsal spinal cord was normalized following repeated injections of gabapentin [55]. Gauchan and colleagues [57] reported that changes in the mRNA expression of the $\alpha_{2}\delta$-1 subunit were dependent on the chemotherapeutic agent utilized. Vincristine was not associated with any changes in mRNA expression of the $\alpha_{2}\delta$-1 subunit, whereas oxaliplatin increased mRNA expression in the DRG, but not the dorsal spinal cord.

Gabapentin was shown to be ineffective in a recent Phase III clinical trial for patients suffering from chemotherapy-induced neuropathy. All patients experienced improvement in symptoms regardless of stage in the crossover trial. It is important to note however, that although groups were stratified based on chemotherapeutic agent, trial groups were not analyzed separately by this measure [58]. The differential changes in expression of the $\alpha_{2}\delta$-1 subunit may account for negative effects of gabapentin in a clinical trial that failed to separate patients by chemotherapeutic agent utilized.

Prophylactic treatment has been tested as a preventive strategy for paclitaxel-induced neuropathic nociception with several different drugs [for review see 59]. Here we demonstrate that cannabinoid agonists with different mechanisms of action, and the neuropathic pain medication, gabapentin, prevent the development of paclitaxel-induced neuropathic nociception during treatment and up to two weeks following drug removal. More work is necessary to determine the mechanisms of action for both cannabinoid- and gabapentin-mediated suppression of paclitaxel-induced neuropathy.
References


[51]Shi Y, Zou M, Baitei EY, Alzahrani AS, Parhar RS, Al-Makhalafi Z, Al-Mohanna FA. Cannabinoid 2 receptor induction by IL-12 and its potential as a therapeutic


CHAPTER 7
GENERAL DISCUSSION

The studies presented herein demonstrate that exogenous cannabinoid CB$_2$ receptor agonists are potent analgesics. Cannabinoid CB$_2$ receptor activation lacks unwanted side effects associated with more traditional pharmacotherapies for neuropathic pain, making this receptor a desirable target for analgesic development.

The antinociceptive profiles observed in the plantar test for the aminoalkylindole, (R,S)-AM1241, and its enantiomers, as well as the cannabilactones, AM1714 and AM1710, demonstrate the analgesic efficacy of these CB$_2$-preferring compounds. It was previously reported that the antinociception observed following administration of (R,S)-AM1241 was attributable to modulation of the endogenous opioid system. However, the data presented here clearly demonstrate that the antinociceptive profile of (R,S)-AM1241 is not associated with activation of the endogenous opioid system. Furthermore, antinociception observed with both (R)- and (S)-AM1241 was not associated with activation of the endogenous opioid system. This finding alters the classification of (R,S)-AM1241 as an atypical cannabinoid CB$_2$ agonist, and negates the need to test novel CB$_2$ agonists for modulation of the endogenous opioid system in vivo. The cannabilactones, AM1714 and AM1710, produce antinociception in the plantar test that was associated with activation of both the CB$_1$ and CB$_2$ receptor, depending upon the dose. This is surprising based on both the in vitro binding data for these compounds, as well as the fact that the data presented demonstrate that antinociceptive doses of these drugs lack the
hallmark central nervous system side effects (assessed in the tetrad) associated with activation of the CB$_1$ receptor.

Cannabinoid CB$_2$ agonists from both the aminoalkylindole and cannabilactone classes suppressed established neuropathic nociception induced by treatment with the chemotherapeutic agent paclitaxel through CB$_2$-specific mechanisms. Both ($R,S$)-AM1241, ($R$)-AM1241, and AM1714 ameliorated established paclitaxel-induced mechanical allodynia following acute administration. Prophylactic administration of AM1710, as well as the mixed cannabinoid CB$_1$/CB$_2$ agonist WIN55,212-2, suppressed the development of paclitaxel-induced neuropathy throughout a 30 day time course. Anti-allodynic effects of AM1710 were clearly mediated by the CB$_2$ receptor, whereas similar effects observed with WIN55,212-2 were dominated by activation of the CB$_1$ receptor. Paclitaxel animals that received AM1710 did not develop allodynia until 18 days following removal of the drug, whereas WIN55,212-2 produced a shorter duration protective effect (12 days following drug removal).

The findings of these studies collectively demonstrate the untapped potential of cannabinoid CB$_2$ agonists for the management of diverse forms of neuropathic pain. Neuropathic pain is notoriously difficult to treat and a singular approach is not successful for most patients. One drug may be efficacious for a certain percentage of the population, but not another. This is not terribly surprising considering that researchers are not even certain that they have isolated all of the physiological alterations and aberrations responsible for the induction and maintenance of neuropathic pain. It is important that researchers continue to develop new drugs with novel targets for the treatment of neuropathies. The cannabinoid system has the potential to help meet the need for novel
analgesic development; specifically the CB₂ receptor represents a viable target for future analgesic development in clinical settings.