THE INTERACTION BETWEEN OPIOIDS AND CANNABINOIDS

IN THE RAT PERIAQUEDUCTAL GRAY

By

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THE INTERACTION BETWEEN OPIOIDS AND CANNABINOIDS
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Abstract

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Opioids and cannabinoids are well known for their analgesic properties. Both classes of
drugs exert their effects, in part, by activating a descending pain modulatory pathway
that includes the midbrain periaqueductal gray (PAG) and the rostral ventromedial
medulla (RVM). Individually, the opioid and cannabinoid systems have been well
characterized in this pathway. Cannabinoid/opioid interactions however, remain poorly
understood. The aim of these studies was to characterize the interaction between
opioids and cannabinoids in the descending pain pathway. Behavioral studies
demonstrate that repeated morphine microinjections in the rat PAG enhance
subsequent cannabinoid antinociception, and vice versa. Furthermore, microinjections
of cannabinoids into the PAG also attenuate the development of morphine tolerance.
However, when cannabinoids are repeatedly injected into the RVM, they cause
neurotoxic lesions. Nonetheless, acute co-administration of morphine and cannabinoids
in the RVM produces greater antinociception than when either drug is administered
alone, which is consistent with earlier reports of antinociceptive synergy between the
drugs. The subsequent anatomical and electrophysiological studies were conducted in
order to determine the neural mechanisms underlying the bi-directional antinociceptive enhancement between cannabinoids and opioids. Confocal and electron microscopy revealed that CB1 expression in the PAG is largely somatodendritic, however CB1-labeled axons and axon terminals are widespread. CB1 and mu-opioid receptors colocalized on 32% of PAG neurons, and a subset of mu-opioid-labeled cells received appositions from (presumed presynaptic) CB1 profiles. Thus, interactions between opioids and cannabinoids are likely to occur within the same neuron, but synaptic adaptations could also occur where CB1 terminals synapse onto mu-opioid cells.

Synaptic adaptations were next examined using the in vitro whole-cell patch clamp method. Previous research has shown that both cannabinoids and opioids can reduce GABAergic transmission in the PAG, and the ability of opioids to reduce GABAergic transmission is diminished in morphine tolerant animals. The present study demonstrates that chronic morphine has no effect on cannabinoid inhibition of GABAergic transmission. These results indicate a lack of cellular cross-tolerance between opioids and cannabinoids, which correlates with antinociceptive enhancement between the drugs. The results of these studies support the therapeutic potential of combined opioid/cannabinoid administration for pain relief.
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CHAPTER ONE: INTRODUCTION

BACKGROUND AND SIGNIFICANCE

Millions of people in the United States are affected by acute and chronic pain every year. Pain-related healthcare costs, lost productivity, and lost income account for an economic burden of more than 100 billion dollars annually in the US (National Center for Health Statistics, 2006). Millions of Americans manage their pain with prescription medications, however 60% of patients taking opioids for pain relief still experience daily breakthrough pain that severely impacts their quality of life (American Pain Foundation, 2006). Despite an array of pain therapies available to the modern clinician, often those living in the most pain are inadequately treated. For example, 50 to 75% of all terminal cancer patients die in moderate to severe pain, and more than 50% of hospitalized patients experience pain in the last days of their lives (Weiss et al., 2001). These inadequacies highlight a clear need for effective treatments for the millions of people living in pain.

Opioids and cannabinoids have been used for their analgesic properties for thousands of years. Despite the considerable side effects associated with opioids, including the development of tolerance, they remain the most effective treatment for severe pain. The side effects of cannabinoids are less serious than those of opioids, but their antinociceptive efficacy is also much less than opioids (Hohmann et al., 1999c, Frank et
Co-administration of these drugs leads to potentiated antinociception (Cichewicz and McCarthy, 2003, Tham et al., 2005, Roberts et al., 2006), making opioid/cannabinoid co-administration an attractive treatment for severe or chronic pain. Interactions between cannabinoids and opioids raise questions about the antinociceptive effects of opioids and cannabinoids, the brain regions, and the neural mechanisms underlying these interactions.

Both classes of drugs exert their effects, in part, through the periaqueductal gray (PAG) (Tsou and Jang, 1964, Jensen and Yaksh, 1986, Martin et al., 1995, Lichtman et al., 1996, Morgan et al., 1998). Interestingly, the PAG is also involved in the development of tolerance to morphine’s antinociceptive effect (Jacquet and Lajtha, 1974, Siuciak and Advokat, 1987, Tortorici et al., 1999, Morgan et al., 2006a). The PAG may also mediate the attenuation of morphine tolerance after pretreatment with cannabinoids (Wilson et al., 2008). The role of the descending pain pathway in the development and/or attenuation of tolerance between opioids and cannabinoids is unclear, however the therapeutic potential of this effect warrants further study of these phenomenon.

**OPIOIDS**

Opioid antinociception

The three types of opioid receptors distributed throughout the central nervous system are the mu, delta, and kappa receptors. The endogenous ligands that bind to these G-
protein coupled receptors are the endorphins, enkephalins, dynorphins, and endomorphins. Most analgesia-producing drugs, such as morphine and hydrocodone, exert their effects primarily through the mu receptor (Mansour et al., 1995). These drugs produce analgesia (or antinociception), in part, by acting on a descending modulatory system that projects from the periaqueductal gray (PAG, Figure 1) to the rostral ventromedial medulla (RVM), and finally to the dorsal horn in the spinal cord, where pain signals from the periphery are inhibited (Basbaum and Fields, 1984). Microinjection of morphine directly into the PAG is sufficient to produce antinociception throughout the body (Tsou and Jang, 1964, Jacquet and Lajtha, 1974, Jensen and Yaksh, 1986, Morgan et al., 1998). In addition, blockade of opioid receptors in the PAG inhibits the antinociceptive effect of peripherally administered morphine (Zambotti et al., 1982, Randich et al., 1992, Lane et al., 2005, Bernal et al., 2007).

Physiological and anatomical studies have demonstrated glutamatergic and GABAergic neurons projecting from the PAG to the RVM (Kalyuzhny and Wessendorf, 1998, Morgan et al., 2008, Drew et al., 2009, Maione et al., 2009). RVM cells, which project to the dorsal horn of the spinal cord, fall into three categories: ON, OFF, and NEUTRAL cells (Fields et al., 1983). ON and OFF cells facilitate descending pain signals, either by firing rapidly at the onset of nocifensive behavior (ON-cells), or by entering a period of silence (OFF-cells; Fields et al., 1983). Activation of OFF-cells by any means, including morphine administration, results in antinociception (Barbaro et al., 1986, Fang et al.,
The disinhibition of PAG neurons projecting to OFF-cells in the RVM is better characterized than other PAG-RVM connections. The mechanism of disinhibition involves a decrease in GABAergic release by PAG interneurons (Behbehani et al., 1990, Vaughan et al., 1997, Vaughan et al., 2000), thus resulting in activation of PAG output neurons onto OFF-cells in the RVM (Heinricher et al., 1994), which in turn inhibit nociceptive signals in the dorsal horn.

Opioid tolerance in the PAG

The PAG is not only a vital component in the mediation of antinociception, but it also plays a significant role in the development of tolerance to the antinociceptive effect of opioids. Repeated microinjection of morphine into the PAG produces tolerance (Jacquet and Lajtha, 1974, Siuciak and Advokat, 1987, Tortorici et al., 1999, Morgan et al., 2006b), and tolerance to systemic morphine administration is attenuated by blockade of opioid receptors in the ventrolateral PAG (Lane et al., 2005). The precise mechanisms by which the PAG mediates the development of tolerance are somewhat unclear, however it is likely that opioid-sensitive GABAergic neurons are involved (Morgan et al., 2003). GABAergic neurons harvested from morphine-tolerant rats show an upregulation of adenylyl cyclase (Ingram et al., 1998, Garzon et al., 2005). Whether this or other mechanisms contribute to tolerance is unknown. Thus, there is a clear need for novel therapies that limit the development of opioid tolerance and simultaneously provide effective pain management.
CANNABINOIDS

One possible method for improving the treatment of pain is to combine opioids with other analgesic drugs such as cannabinoids. There are two well-characterized cannabinoid receptors, CB1 and CB2, and recent evidence has suggested the presence of a third cannabinoid-like receptor (Begg et al., 2005, Kreitzer and Stella, 2009). The CB1 receptor is largely found in the central nervous system (CNS), while CB2 expression is widespread in the periphery; however, CB1 and CB2 receptors are both found throughout the CNS and periphery (Pettit et al., 1998, Hohmann and Herkenham, 1999, Adami et al., 2002, Gong et al., 2006, Onaivi et al., 2006). The endogenous ligands for these receptors are anandamide, 2-arachidonoyl glycerol, 2-arachidonyl glyceryl ether, N-arachidonoyl-dopamine, and virdhamine. With the exception of anandamide, knowledge of the physiological action and relevance of the endogenous cannabinoids is somewhat limited. The most well known exogenous cannabinoid is delta-9 tetrahydrocannabinol (THC), the primary psychoactive component in botanical cannabis. THC and its analogues produce antinociception, which has led to their use in analgesic therapy, albeit controversially in the United States due to the classification of cannabinoids as Schedule I drugs with no accepted medicinal purpose (Walker et al., 1999, Walker et al., 2001, Corey, 2005).
Cannabinoid antinociception

Cannabinoids have been shown to produce antinociception via a variety of administration routes, including oral, subcutaneous, intrathecal, intraperitoneal, intracerebral, and topical (Bloom and Dewey, 1978, Lichtman et al., 1996, Manzanares et al., 1998, Cichewicz et al., 1999, Hohmann et al., 1999c, a, Hohmann, 2002, Dogrul et al., 2003). Although cannabinoids act at spinal sites to produce antinociception (Yaksh, 1981, Smith and Martin, 1992, Welch et al., 1995), these effects can be attenuated by spinal transection, which suggests supraspinal antinociceptive mechanisms (Lichtman and Martin, 1991, Hohmann et al., 1999b). Additional evidence suggests that cannabinoids appear to inhibit pain, in part, by activating the same descending modulatory system as opioids (Meng et al., 1998). Both CB1 and CB2 receptors are found in the PAG (Herkenham et al., 1991, Maileux and Vanderhaeghen, 1992, Tsou et al., 1998, Cristino et al., 2006, Gong et al., 2006), and microinjection of cannabinoids into the PAG produces antinociception (Martin et al., 1995, Lichtman et al., 1996, Martin et al., 1998, Meng and Johansen, 2004). Despite activation of the same descending modulatory pathway, it is clear that opioids and cannabinoids produce antinociception through separate (although possibly interrelated) mechanisms (Bloom and Dewey, 1978, Welch, 1993, Meng et al., 1998, Massi et al., 2001). For example, naloxone, an opioid receptor antagonist, is unable to block cannabinoid-induced antinociception (Welch, 1993), however it is somewhat effective in preventing cannabinoid-induced hypothermia (Bloom and Dewey, 1978).
OPIOID/CANNABINOID INTERACTIONS

The interaction between opioids and cannabinoids is complex and has interesting implications for clinical applications. It is well established that co-administration of a wide variety of cannabinoids and opioids produces greater antinociception than either drug administered alone (Smith et al., 1998, Cichewicz and McCarthy, 2003, Tham et al., 2005, Roberts et al., 2006) and that cannabinoids enhance the antinociception produced by opioids (Smith et al., 1998, Cichewicz et al., 1999, Yesilyurt et al., 2003, Cichewicz et al., 2005). In addition to the effects of cannabinoids on antinociception, they have a significant impact on the development of opioid tolerance. While some earlier studies show the development of cross-tolerance between the drugs (Hine, 1985, Thorat and Bhargava, 1994, Basilico et al., 1999) more recent data have shown that cross-tolerance does not develop (Mao et al., 2000, Yesilyurt and Dogrul, 2004). In fact, evidence to the contrary indicates that cannabinoid co-administration or pre-treatment attenuates the development of opioid tolerance (Cichewicz and Welch, 2003, Smith et al., 2007, Wilson et al., 2008). In addition, cannabinoid antinociception is enhanced in morphine-tolerant animals compared with morphine-naïve animals (Rubino et al., 1997, Cichewicz and Welch, 2003). Taken together, these data suggest that a treatment protocol that combines cannabinoids and opioids may prove to be clinically beneficial by enhancing antinociception while simultaneously reducing tolerance. The role of the PAG in this behavioral interaction is addressed in Chapter 2, and expanded to include the role of the RVM in Chapter 3.
The neuroanatomical, cellular, and molecular mechanisms by which the cannabinoid and opioid systems interact to produce enhanced antinociception are not fully understood. One of the earliest characterizations of the interaction between these systems was the discovery that acutely administered cannabinoid agonists induce the release of specific endogenous opioids. For example, the cannabinoid agonists CP55,940 and THC induce the release of dynorphin B in the rat spinal cord (Pugh et al., 1997, Houder et al., 2000), and THC also induces the release of dynorphin A and leucine enkephalin (Mason et al., 1999). Data from the PAG showing an increase in pro-enkephalin mRNA in the PAG after cannabinoid administration (Manzanares et al., 1998) are consistent with these findings.

Another possible explanation for the enhanced antinociception and reduced tolerance produced by the combination of opioids and cannabinoids may be related to the neuroanatomy of the PAG. Some reports indicate that cannabinoids are more potent when microinjected into the dorsal PAG compared with the ventrolateral PAG (Martin et al., 1995, Finn et al., 2003). Conflicting results (Lichtman et al., 1996) may be attributable to differences in experimental protocol such as the particular CB1 agonist administered. While the ventrolateral PAG is necessary and sufficient for the development of morphine tolerance, the dorsal PAG is resistant to morphine tolerance (Tortorici et al., 1999). Furthermore, the dorsolateral PAG is also involved in mediating
stress-induced analgesia (Hohmann et al., 2005). Clearly there are functional
differences in both the cannabinoid and opioid systems within sub-regions of the PAG
that may have anatomical underpinnings. The extent to which these anatomical systems
may overlap, oppose, or influence one another is unknown, and will be addressed in
Chapter 4.

Physiologically, opioids and cannabinoids have distinct, albeit overlapping mechanisms,
which could contribute to their interactive effects on tolerance. In intact slice
preparations, cannabinoids and opioids can inhibit evoked IPSPs and EPSPs (Vaughan
and Christie, 1997, Vaughan et al., 2000). However, unlike mu-opioids, cannabinoids
have no effect on inwardly-rectifying K+ or Ca2+ conductances in isolated PAG cells
(Osborne et al., 1996, Connor and Christie, 1998, Vaughan et al., 2000). These findings
indicate that while opioids are able to directly trigger post-synaptic events, cannabinoids
exert their effects through pre-synaptic inhibition. Although opioid-induced GABAergic
transmission is altered in morphine-tolerant tissue (Ingram et al., 1998, Fyfe et al.,
2010), the effects of cannabinoids on neurons from morphine tolerant animals have not
been studied. It is also unknown how PAG neurons respond to in vitro opioids under
cannabinoid-tolerant conditions. Despite distinct electrophysiological mechanisms, it is
possible that pre-synaptic cannabinoid inhibition and additional opioid hyperpolarization
(Osborne et al., 1996) converge on the same PAG neurons to produce enhanced
antinociception (Figure 2). Chapter 5 begins to address these gaps in the knowledge
base by using in vitro electrophysiology techniques to study the effects of cannabinoids on GABAergic transmission in morphine-tolerant tissue.

Cannabinoids and opioids could also have a direct receptor-receptor interaction that could underlie nocifensive behavior. At some reward-related CNS sites, chronic WIN 55,212-2 exposure causes upregulation of mu-opioid receptors, and chronic morphine induces upregulation of CB1 receptors (Lim et al., 2005, Fattore et al., 2007). Similar opioid-induced upregulation of CB1 and CB2 receptors is seen in the dorsal horn of the spinal cord (Lim et al., 2005). Chronic opioid administration causes a decrease in the expression of all three types of opioid receptors (Tao et al., 1988, Yoburn et al., 1990, Bernstein and Welch, 1998, Trapaidze et al., 2000). However, this effect is reversible by co-administration of THC and morphine (Cichewicz et al., 2001). These studies indicate a clear, bi-directional linkage between the mu-opioid and CB1 receptors under drug-tolerant conditions. However, changes in receptor density have not been thoroughly studied in the rat PAG, and what little evidence there is suggests that the PAG is different from reward structures. Specifically, tolerance to the cannabinoid agonist CP-55,940 is associated with an increase in mu opioid receptor density, but morphine tolerance does not have any effect on the density of CB1 receptors (Vigano et al., 2005). These contrasting results (lack of bi-directionality in the PAG) could indicate region-specific, species-specific, or agonist-specific effects. Chapter 4 addresses these
issues, using immunocytochemical techniques to examine CB1 receptor expression after chronic drug exposure.

Alternatively, enhanced antinociceptive behavior could be mediated by converging molecular mechanisms in PAG neurons (that is, when CB1 and MOR are co-localized). CB1 and mu-opioid receptors are both G-protein coupled receptors that interact primarily with G\textsubscript{i} and G\textsubscript{o} proteins (Connor and Christie, 1999, Howlett et al., 2002). Both opioids and cannabinoids can inhibit adenylyl cyclase, resulting in decreased production and intracellular accumulation of cAMP (Howlett, 1985, Childers et al., 1992, Vigano et al., 2005). Importantly, the upregulation of adenylyl cyclase is also associated with morphine tolerance (Ingram et al., 1998, Bohn et al., 2000). Furthermore, mitogen-activated protein kinase (MAPK) pathways are modulated by both CB1 and mu-opioid receptor activation (Howlett, 2005, Asensio et al., 2006). Because extracellular-related kinase (ERK)1/2 is involved in morphine tolerance in the PAG (Macey et al., 2009), this intracellular signaling cascade could be a crucial convergence point at which cannabinoids and opioids produce enhanced antinociception and attenuated tolerance in the PAG. It is important to note that much of these data were collected from regions outside the PAG. Considering this brain region’s important role in the development of tolerance and the modulation of pain, it is critical to fully characterize opioid/cannabinoid molecular interactions in PAG neurons. Many of the hypothesized cellular and molecular interactions between cannabinoids and opioids depend upon their co-localization within
the same cell. Chapter 4 will address this issue by examining mu-opioid peptide (MOP) and CB1 receptors in the PAG using confocal and electron microscopy.
**Figure 1:** Descending pain modulatory pathway projecting from the periaqueductal gray (PAG), to the rostral ventromedial medulla (RVM) and to the dorsal horn in the spinal cord. Images modified from the rat brain atlas of Paxinos and Watson (2005), with the permission of the publisher.
**Figure 2**: Hypothetical model of convergence of CB1 and mu-opioid signaling in GABAergic PAG terminals. CB1 activation may inhibit GABA release by activating potassium channels, inhibiting voltage-dependent calcium channels, or directly interfering with vesicular release. Additional hyperpolarization via mu-opioid receptor (MOR)-mediated potassium conductance could underlie enhanced antinociception in GABAergic terminals.
REFERENCES


CHAPTER TWO: Repeated Cannabinoid Injections Into The Rat Periaqueductal Gray Enhance Subsequent Morphine Antinociception

Wilson, A.R., Maher, L., & Morgan M.M.

ATTRIBUTIONS

The following chapter consists of a manuscript that was published in the journal *Neuropharmacology* in 2008. The article has been reproduced here with the permission of the publisher. The contributions of the authors are as follows:

Wilson, A.R.: Responsible for experimental conception, data collection, data analysis and interpretation, statistical analyses, construction of figures, first draft of manuscript, and final draft of manuscript

Maher, L.: Technical assistance in behavioral data collection, and preliminary data analysis

Morgan, M.M.: Supervision, facilities and materials, and manuscript revisions
Repeated cannabinoid injections into the rat periaqueductal gray enhance subsequent morphine antinociception

Adrienne R. Wilson, Lauren Maher, Michael M. Morgan

1. Introduction

Opiates such as morphine are the most effective treatment for severe pain. Morphine produces antinociception in part by activating the descending pain modulation system that projects from the periaqueductal gray (PAG) to the rostral ventromedial medulla (RVM) to the dorsal horn of the spinal cord (Basbaum and Fields, 1984). Morphine microinjection into the PAG is sufficient to produce antinociception (Jensen and Yaksh, 1986; Morgan et al., 1988), and blocking opioids in the PAG is sufficient to attenuate the antinociceptive effect of systemic morphine administration (Bernal et al., 2007; Lane et al., 2005; Randich et al., 1992; Zambotti et al., 1982).

The ventrolateral region of the PAG has been shown to play an important role in the development of tolerance to the antinociceptive effects of morphine. Tolerance develops to repeated microinjections of morphine into the ventrolateral PAG (Jacquet and Lajtha, 1974; Morgan et al., 2006a; Sicciak and Advokat, 1987; Tortorici et al., 1999) and blocking opioid receptors in the ventrolateral PAG attenuates tolerance to systemic morphine administration (Lane et al., 2015).

One way to limit the development of tolerance is to limit the duration of drug administration (Suzuki et al., 1983). This can be accomplished while maintaining pain treatment by alternating administration of different drugs. Given that microinjection of cannabinoid agonists into the PAG produces antinociception (Lichtman et al., 1990; Martin et al., 1995, 1998; Meng and Johansen, 2004; Welch and Stevens, 1992), alternating administration of a cannabinoid agonist and morphine could maintain the potency of both drugs. Of course, this is possible only if cross-tolerance does not develop from cannabinoids to morphine. The synergistic antinociceptive produced by systemic administration of cannabinoids and morphine suggests that these drugs produce antinociception through different mechanisms (Cichewicz and McCarthy, 2003; Roberts et al., 2006; Tham et al., 2005). The lack of cross-tolerance between opioids and cannabinoids after intrathecal or systemic administration (Mao et al., 2000; Yesilyurt and Dogru, 2004) is consistent with this hypothesis. The objective of the present study was to test this hypothesis in the PAG by examining changes in...
morphine potency following repeated microinjections of the cannabinoid receptor agonist HU-210.

2. Methods

2.1. Subjects

Male Sprague-Dawley rats (212–461 g; Harlan, Kez, N. J.) were anesthetized with equithesin (60 mg/kg, i.p.) and stereotaxically implanted with a 23-gauge (0.92 mm) stainless steel guide cannula aimed at the ventrolateral PAG (5 mm long, AP = 2.3 mm; ML = −0.6 mm; DV = −4.0 mm from lambda). The guide cannula was held in place with dental cement affixed to two screws in the skull. Following surgery, a removable stainless steel stylet was inserted into the guide cannula, and the rat was housed individually with food and water available ad libitum. Lights were maintained on a reverse 12 h light/dark cycle so that all injections and testing occurred during the animals' active phase, rather than during the light, inactive phase.

Rats were handled daily prior to and following surgery. Testing began one week following surgery. Animals were not re-used for subsequent experiments, nor were they pretreated with more than one drug or drug combination. Experiments were conducted in accordance with the animal care and use guidelines of the Institutional Animal Care and Use Committee at Washington State University approved this research.

2.2. Microinjections

The day before the first microinjection, an 11 mm injection cannula was inserted into the guide cannula without administration of drugs. This procedure reduces artifacts on the test day resulting from mechanical damage to neurons and habituates rats to the injection procedure.

Microinjections were administered through a 31-gauge (0.22 mm), 11 mm long injection cannula inserted into and extending 2 mm beyond the tip of the 9 mm guide cannula. The injection cannula was connected to a 1 μl syringe (Hamilton Co., Reno, NV) with PE20 tubing filled with sterile water. All microinjections were administered in a volume of 0.4 μl over 40 s while the rat was gently restrained. The injection cannula remained in place an additional 20 s to minimize backflow of the drug up the cannula tract. Following the injection, the stylet was reinserted into the guide cannula and the rat was returned to its home cage.

Although repeated microinjections may cause some localized damage, this is inevitable in any microinjection study. Microinjections administered outside of the target area were used as a control for the effects of repeated microinjections.

2.3. Drugs

Drug doses and concentrations were selected based on previous studies (Finn et al., 2003; Morgan et al., 2005). For pretreatment procedures, morphine (a gift from the National Institute on Drug Abuse), the cannabinoid receptor agonist HU-210 (Tocris, St. Louis, MO), and the cannabinoid receptor antagonist AM-251 (Tocris) were dissolved in 0.9% DMSO and saline. The control groups received microinjections of vehicle (60% DMSO in saline). Morphine was dissolved in saline for the cumulative dose assessment of morphine potency (Morgan et al., 2006). Rats receiving morphine and HU-210 were received drugs in a single injection. Each rat was used for only one experiment, and all rats in each experiment received the same number of microinjections, regardless of treatment. In Experiments 1 and 5, animals were given four injections of 0.4 μl. In Experiments 2, 3, and 4, animals were given four injections of 0.4 μl during pretreatment, and five subsequent 0.4 μl injections to assess morphine dose–response characteristics. For the HU-210 cumulative dosing procedure, actual doses injected were 3.2, 2.4, 1.6, and 0.8 μg, resulting in cumulative quartile log doses of 3.2, 5.6, 10.0, and 16.8 μg for the morphine cumulative dosing procedure, actual doses injected were 1.0, 0.8, 1.4, 2.4, and 4.4 μg, resulting in cumulative quartile log doses of 1.0, 3.2, 5.6, and 10.0 μg.

2.4. Behavioural tests

Nocturnal activity was assessed using the hot plate test because this test can be used repeatedly and is sensitive to morphine antinociception (Morgan et al., 2006b). The hot plate test measures the latency for a rat to lick a hind paw when placed on a 52.5 °C surface. The rat was immediately removed from the hot plate following a response, or after 40 s if no response occurred. Hot plate calibration at this temperature produced baseline latencies of 9–14 s. Given that microinjection of morphine into the ventrolateral PAG causes circling or immobility (Morgan et al., 1998), locomotion was assessed by placing the rat in an open field (1 × 0.6 m) and counting the number of squares (15 × 15 cm) entered by the forepaw in 30 s. The experimenter conducting the behavioral tests was blind to the pretreatment condition of the animals.

2.5. Experiment 1: acute HU-210 PAG microinjections

The antinociceptive effect of microinjecting cumulative doses of HU-210 into the ventrolateral PAG was assessed (N = 10). Microinjections were administered every 20 min in cumulative quartile log doses of 3.2, 5.6, 10.0, and 18.0 μg (4 μl). In six rats, the highest injection administered was 14.4 μg, not 18.0 μg. Following baseline measurements, hot plate and open field tests were conducted 15 min after each microinjection of HU-210.

2.6. Experiment 2: morphine/HU-210 cross-tolerance

Rats were injected with morphine (5 μg/0.4 μl, N = 8), HU-210 (5 μg/0.4 μl, N = 6), 60% DMSO vehicle (N = 6), or a morphine/HU-210 combination (N = 7) twice a day (09:30 and 16:00) for two days (Trials 1–4). Thirty minutes after the first injection on Day 1, nociception was assessed using the hot plate test. No testing was conducted following injections on Trials 2–4 to limit changes in latency caused by repeated testing (Gamble and Milne, 1993; Lane et al., 2004).

On Day 3, tolerance to morphine was assessed using cumulative dose microinjections (1.8, 3.2, 5.6, and 10 μg/0.4 μl) into the ventrolateral PAG at 20 min intervals (Morgan et al., 2006a). Nociception and open field activity were assessed 15 min after each microinjection. This procedure was applied consistently in all subsequent experiments where the cumulative morphine dosing procedure was used.

2.7. Experiment 3: cannabinoid antagonist

This experiment was conducted to determine whether the effect of HU-210 on morphine antinociception was mediated by cannabinoid receptors. Rats were injected intraperitoneally with either the cannabinoid antagonist AM-251 (1 μg/kg, N = 14) or an equivalent volume of 60% DMSO vehicle (N = 8), HU-210 (5 μg/0.4 μl), or a morphine/HU-210 combination (N = 7) 15 min later. This procedure was repeated twice a day for two days (Trials 1–4). Nociception was assessed using the hot plate test 30 min after microinjection of HU-210 on Trial 1. No testing was conducted on Trials 2–4. On Day 3 (Trial 3) morphine antinociception was assessed using the cumulative microinjection procedure described in Experiment 2.

2.8. Experiment 4: duration of HU-210 induced enhancement

The objective of this experiment was to determine the duration of changes produced by repeated microinjection of HU-210 into the ventrolateral PAG. The experimental procedure was identical to Experiment 2 except that rats were tested on Day 3 instead of Day 2. Rats were pretreated with ventrolateral PAG microinjections of morphine (N = 8), HU-210 (N = 5), 60% DMSO vehicle (N = 7), or a morphine/HU-210 combination (N = 5) twice a day for two days (Trials 1–4). No drug administration or behavioral testing was conducted on Days 1–7.

2.9. Experiment 5: acute HU-210 and morphine interaction

If the effects of HU-210 pretreatment on morphine antinociception are caused by residual HU-210 in the PAG, then acute microinjection of HU-210 into the ventrolateral PAG should have similar effects. Cumulative dose microinjections of morphine into the ventrolateral PAG were performed as described in Experiment 2 (1.0, 1.8, 3.2, 5.6, and 10 μg/0.4 μl). Half of the animals (N = 8/group) received 5 μg HU-210 dissolved in the first morphine microinjection (1.0 μg/0.4 μl). For the first injection, 60% DMSO in both groups of animals, with subsequent morphine doses dissolved in saline. Injections were 20 min apart and nociception and open field activity were assessed 15 min after each injection.

2.10. Histology

Rats were euthanized following testing by administering a lethal dose of Halothane. The injection site was marked by microinjecting Cresyl Violet (0.2 μl) into the PAG, and the brain was removed and placed in formalin (10%). At least 2 days later the brain was sectioned coronally (100 μm) and the location of the injection site identified using the atlas of Paxinos and Watson (2005).

2.11. Data analysis

Dose–response curves were generated for hot plate data using nonlinear regression (Graph Pad Prism). The half-maximal effective dose (ED50) was calculated for each condition (Tallarida, 2000). Analysis of variance (ANOVA) was used to assess changes in hot plate and open field activity (SPSS). Dunnett's values calculated from dose–response curves (GraphPad and ANOVA (Tallarida, 2000) were used to assess changes in potency. Post hoc comparisons were made with 95% confidence intervals. Statistical significance was defined as a probability of less than 0.05.

3. Results

Data were derived from rats with microinjection placements in or along the border of the ventrolateral PAG (Fig. 1). Placements outside the ventrolateral PAG were used as negative controls, allowing for comparisons to on-site data.
3.1. Experiment 1: acute HU-210 PAG microinjections

Microinjection of HU-210 (3.2, 5.6, 10.0, and 18.0 μg/0.4 μL) into the ventrolateral PAG caused a small increase in hot plate latency. Microinjection of the highest dose of HU-210 (14.4 or 18.0 μg/0.4 μL) into the ventrolateral PAG resulted in a 59% increase in hot plate latency compared to baseline (9.5 ± 0.8 vs. 15.1 ± 1.4 s; F(4,45) = 4.7, p < 0.05). This increase is modest compared to morphine antinociception (Morgan et al., 2006a), but comparable to the antinociception reported in previous PAG microinjection studies (Finn et al., 2003; Lichtman et al., 1996). Also in concordance with previous reports, there was no dose-dependent effect of HU-210 on locomotor behavior (Finn et al., 2003). Mean open field activity ranged from 13 to 25 squares entered following administration of the various HU-210 doses.

3.2. Experiment 2: morphine/HU-210 cross-tolerance

Microinjection of HU-210 (5 μg/0.4 μL) into the ventrolateral PAG did not cause a statistically significant increase in hot plate latency compared to vehicle treated rats on Trial 1 (F(1,23) = 3.1, p > 0.05). In contrast, microinjection of morphine (5 μg/0.4 μL) into the ventrolateral PAG caused a significant increase in hot plate latency to 33.1 ± 4.0 s compared to 14.9 ± 0.9 s for vehicle treated controls (F(1,23) = 42.3, p < 0.05). The morphine/HU-210 group showed the greatest increase in hot plate latency (38.9 ± 1.1 s), although this antinociception did not differ significantly from rats treated with morphine alone.

Mean locomotor activity on Trial 1 did not differ between morphine (20.8 ± 6.5 squares entered), HU-210 (21.7 ± 2.4), or vehicle-pretreated animals (27.0 ± 1.8; F(3,23) = 2.1, p > 0.05). Combined administration of HU-210 and morphine produced the greatest decrease in locomotor activity compared to vehicle-injected animals (9.9 ± 5.1), but this effect also failed to reach statistical significance (Tukey’s, p = 0.10).

Tolerance and cross-tolerance to ventrolateral PAG morphine microinjection were assessed on Trial 5. Rats pretreated with morphine showed a rightward shift in the morphine dose–response curve, as would be expected with the development of tolerance (F(3,127) = 4.1, p < 0.05, Fig. 2A). The D50 value for rats pretreated with vehicle was outside the 95% confidence interval of the rats pretreated with HU-210, indicating that HU-210-pretreated rats had greater morphine antinociception on Trial 5 compared to vehicle-pretreated rats (Fig. 2A, Table 1). HU-210 microinjections outside the vPAG (N = 4) did not display this enhanced morphine antinociception (mean hot plate latencies ranged from 12.6 to 20.7 s following the cumulative morphine microinjection procedure).

Co-administration of HU-210 and morphine on Trials 1–4 blocked tolerance to morphine (Fig. 2B). The D50 value for the combined morphine/HU-210 group value did not differ significantly from the vehicle-pretreated group when tested with morphine on Trial 5 (Table 1), despite showing maximal antinociception on Trial 1.

Microinjection of morphine into the ventrolateral PAG caused circling in some rats (N = 9), immobility in a few (N = 4) and no effect in others (N = 14), as previously reported (Morgan et al., 1998). These locomotor effects occurred equally in morphine-treated and in HU-210/morphine-treated animals. Previous reports of explosive flight reactions to dorsal–lateral PAG morphine injections (Morgan et al., 1998) were not seen in this study, as both on and off-site injections were outside of the dorsal–lateral PAG. There was no significant difference in locomotor activity between any of the groups at any dose (F(2,22) = 0.3, p = 0.83). Morphine administration produced a dose-dependent decrease in locomotor activity in all four groups (Fig. 3).

3.3. Experiment 3: cannabinoid antagonism

In this experiment, we assessed morphine antinociception after pretreatment with systemic AM-251 (cannabinoid antagonist) and intracranial HU-210. Mean baseline hot plate latencies were similar in rats pretreated with HU-210 (15.6 ± 1.3 s) and HU-210/AM-251 (14.8 ± 1.0 s). Pretreatment with AM-251 blocked the leftward shift in the morphine dose–response curve produced by HU-210 pretreatment (F(1,106) = 3.95, p < 0.05, Fig. 4). The morphine D50 for animals pretreated with HU-210 was 1.53 μg (C.I. = 0.70–2.35), whereas the D50 for animals pretreated with i.p. AM-251 prior to microinjection of HU-210 was 4.32 μg (C.I. = 1.87–6.77).

3.4. Experiment 4: duration of HU-210-induced enhancement

The longevity of the HU-210 enhancement of morphine antinociception was assessed by testing rats 6 days after termination of HU-210 pretreatment (on Day 8). Baseline hot plate latencies on Day 8 did not differ between the four pretreatment groups (ranged from 12.2 to 15.9 s). Repeated administration of morphine on Trials 1–4 produced tolerance to morphine on Day 8, as the morphine D50 value fell outside of the vehicle confidence interval (Table 1). The D50 values for the other pretreatment groups fell within the
Fig. 2. Morphine antinociceptive potency following pretreatment with HU-210 and/or morphine. (A) Repeated microinjection of morphine caused a rightward shift in the dose–response curve for morphine antinociception as expected with the development of tolerance. Morphine potency was greatest in rats pretreated with HU-210 on Trials 1–4 as shown by the leftward shift in the morphine dose–response curve. (B) Co-administration of morphine and HU-210 on Trials 1–4 attenuated the development of tolerance when morphine was microinjected on Day 3. The dose–response curve for the vehicle group has been shown in both panels A and B to allow for visual comparison of shifts relative to vehicle. Average baseline latencies were 13.9 (morphine), 14.5 (HU-210), 13.5 (HU-210/morphine) and 13.9 (vehicle) seconds. See Table 1 for $D_{50}$ values. Fitted lines visually indicate trends and cannot be considered perfect fits to the data.

vehicle confidence interval (Table 1; $F_{3,107} = 0.6$, $p > 0.05$). Unlike Experiment 2, morphine potency in rats pretreated with HU-210 alone did not differ from vehicle-pretreated animals (Fig. 5).

3.5. Experiment 5: acute HU-210 and acute morphine

The enhanced morphine antinociception produced by HU-210 pretreatment could be caused by residual HU-210 in the PAG. To test this hypothesis, the acute effect of HU-210 microinjection on morphine antinociception was assessed. HU-210 (5 µg) was administered with the first of 5 cumulative morphine microinjections. Co-administration of HU-210 and morphine caused a reduction of maximal antinociception compared to animals microinjected with morphine alone (Fig. 6). This difference was statistically significant as revealed by a one-tailed t-test ($t_{15} = 1.77, p < 0.05$).

4. Discussion

These experiments show that co-administration of HU-210 and morphine prevented the development of tolerance to morphine’s antinociceptive effect, and of greatest interest, HU-210 pretreatment enhanced subsequent morphine antinociception. This enhancement of antinociception was prevented by AM-251 administration, and the enhancement also disappeared when animals were tested 6 days after HU-210 pretreatment. In addition, this enhanced antinociception is not caused by residual HU-210 in

Table 1

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Day 3 $D_{50}$ (µg)</th>
<th>95% CI</th>
<th>Day 8 $D_{50}$ (µg)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HU-210</td>
<td>1.77</td>
<td>0.55–2.99</td>
<td>5.82</td>
<td>2.11–9.53</td>
</tr>
<tr>
<td>Morphine/HU</td>
<td>2.54</td>
<td>0.60–5.11</td>
<td>5.61</td>
<td>2.27–12.95</td>
</tr>
<tr>
<td>Morphine</td>
<td>6.78</td>
<td>4.88–8.69</td>
<td>6.09</td>
<td>3.85–10.0</td>
</tr>
<tr>
<td>Vehicle</td>
<td>3.82</td>
<td>1.03–6.81</td>
<td>4.18</td>
<td>1.80–6.56</td>
</tr>
</tbody>
</table>

*Confidence interval.
the PAG because acute co-administration of HU-210 and morphine diminished morphine’s antinociceptive effect.

Although opioids and cannabinoids activate the same descending antinociceptive modulatory system (Meng et al., 1998), evidence suggests they trigger this system through separate and distinct mechanisms. First, when co-administered systemically, delta-9-tetrahydrocannabinol (THC) and morphine produce synergistic antinociception (Cichewicz and McCarthy, 2003; Tham et al., 2005). Second, administration of the opioid receptor antagonist naloxone blocks the antinociceptive effect of morphine, but not that of cannabinoids (Bloom and Dewey, 1978; Massi et al., 2001; Meng et al., 1988; Welch, 1993). Third, unlike μ-opioids, the cannabinoid receptor agonist WIN55,212-2 has no effect on isolated PAG cells, yet in intact slice preparations WIN55,212-2 can inhibit evoked IPSPs and EPSPs (Vaughan et al., 2000). This difference indicates that while opioids can directly trigger postsynaptic events, cannabinoids modulate neural activity through presynaptic inhibition. The lack of cross-tolerance between HU-210 and morphine repeated here is consistent with these differences.

The use of marijuana as a therapeutic pain management tool has generated a great deal of publicity and controversy. THC, the primary psychoactive compound in marijuana, has been found to be effective in pain management (Corey, 2005; Walker et al., 1999; 2001). Cannabinoid antinociception can be produced in rats through a wide range of administration techniques, including oral, subcutaneous, intrathecal, intraperitoneal, intracerebral and topical (Bloom and Dewey, 1978; Cichewicz et al., 1999; Dogrul et al., 2003; Hoffmann, 2002; Hoffmann et al., 1998a,b; Lichtman et al., 1996; Manzanares et al., 1998; Welch and Stevens, 1992). The PAG also appears to contribute to the antinociceptive effects of cannabinoids. CB1 and CB2 receptors are localized in the PAG (Cristino et al., 2006; Gong et al., 2006; Mailleux and Vanderhaeghen, 1992; Tsou et al., 1998), and microinjection of cannabinoids into the PAG produces antinociception (Lichtman et al., 1996; Martin et al., 1995, 1998; Meng and Johansen, 2004; Welch and Stevens, 1992). In addition, microinjection of cannabinoid receptor agonists into the PAG attenuates stress-induced antinociception (Hoffmann et al., 2005).

In the current study, microinjection of HU-210 into the ventrolateral PAG caused a small increase in hot plate latency compared to that produced by microinjection of morphine. These data are consistent with previous research showing that HU-210 is more effective in producing antinociception when injected into the dorsal, compared to the ventral PAG (Martin et al., 1995). Our finding that acute co-administration of HU-210 and morphine into the PAG attenuates morphine antinociception may suggest that opioids and cannabinoids could have opposing effects within the PAG.

The PAG not only plays a significant role in opioid-mediated antinociception, but it also contributes to the development of tolerance to morphine’s antinociceptive effect. Repeated microinjection of morphine directly into the ventrolateral PAG is sufficient to produce tolerance to the drug’s antinociceptive effects (Morgan et al., 2006a; Tortorici et al., 1999), a finding confirmed here. Moreover, inactivation of the opioid receptors in the PAG attenuates the development of tolerance to systemic morphine administration (Lane et al., 2005). These findings indicate that the PAG is both sufficient and necessary for the development of tolerance to morphine. This tolerance appears to be mediated by opioid sensitive GABAergic neurons because direct repeated activation of ventrolateral PAG output neurons does not produce tolerance (Morgan et al., 2003). In vitro electrophysiological recordings from PAG neurons derived from rats pretreated with morphine show an upregulation of adenyl cyclase in these GABAergic neurons (Garzon et al., 2005; Ingram et al., 1998). In the present study, PAG-mediated morphine tolerance was prevented by pretreatment with the cannabinoid agonist HU-210. The morphine antinociception produced in animals pretreated with
HU-210/morphine was no different than the antinoceptive produced in vehicle-pretreated controls. This reversal of tolerance is surprising because combined microinjection of HU-210 and morphine produced the greatest antinoicceptive on Trial 1. This phenomenon is further substantiated by recent findings demonstrating similar attenuation of morphine tolerance by THC (Smith et al., 2007).

The cannabinoid antagonist AM-251 blocked HU-210’s enhancement of antinoception, indicating that HU-210 enhances morphine antinoicceptive via the CB1 receptor, rather than a non-specific action of microinjecting HU-210 into the PAG. The CB1 antagonist was administered systemically, and could therefore act at many sites in addition to the PAG. However, because morphine and HU-210 were administered directly into the PAG, it is unlikely that any site outside of the PAG-mediated this effect. It is also unlikely that residual cannabinoids cause the increased antinoicceptive we observe on Day 3, because this would require residual HU-210 to remain in the brain 16 h after the last microinjection. Moreover, acute HU-210 administration attenuated, rather than enhanced morphine-induced antinoicception in Experiment 5.

The effect of HU-210 on antinoicception is no longer present by Day 6. The mechanism by which cannabinoid pretreatment enhances antinoicception in the short term (i.e., 16 h after administration), but not in the long term is not clear. However, this loss of effect is consistent with the loss of tolerance to PAG morphine antinoicception that occurs between one and two weeks after morphine administration (Morgan et al., 2005). The exact time course of HU-210 enhancement of morphine antinoicception is unknown.

In contrast to our data from the ventrolateral PAG, blockade of the cannabinoid system in the spinal cord has been shown to prevent the development of tolerance to morphine (Trang et al., 2007). This difference between the spinal cord and the ventrolateral PAG could be caused by different experimental procedures or differences in the mechanism for tolerance at the two sites. Previous studies examining tolerance suggest that the mechanisms for tolerance differ between the ventrolateral PAG and spinal cord. NMDA receptor antagonists disrupt morphine tolerance when applied at the spinal level (Triulzi and Abel, 1991), but not when microinjected into the PAG (Morgan et al., personal observation).

Intrathecal co-administration of the mu-opioid receptor agonist DAMGO and morphine prevents morphine tolerance (He et al., 2002). In the PAG however, co-administration not only results in tolerance to DAMGO’s antinoicceptive effect, but DAMGO enhances tolerance to morphine (Meyer et al., 2007). Taken together, these results indicate that morphine tolerance differs at the spinal and supraspinal levels.

Despite the differences in tolerance between the spinal cord and the PAG, it is noteworthy and clinically relevant that systemic administration of opioids and cannabinoids shows antinoicceptive enhancement in a manner similar to that found in the present study. That is, morphine antinoicception is greater in animals exposed to cannabinoids (Cichewicz et al., 1999, 2005; Smith et al., 1998; Veseljurt et al., 2002). Our data demonstrate the enhancement of morphine antinoicception following HU-210 pretreatment, but not when the drugs are co-administered acutely. In addition, cannabinoid antinoicception is greater in morphine-tolerant animals than in morphine-naive animals (Cichewicz and Welch, 2003; Rubin et al., 1997; Smith et al., 2007; Viganò et al., 2005).

Thus, it appears that antinoicceptive enhancement between cannabinoids and opioids is bidirectional. This bidirectionality of enhanced antinoicception may be partially explained by the reciprocal upregulation of CB1 and mu-opioid receptors after chronic exposure to opioids or cannabinoids, respectively (Fattore et al., 2007). Although the PAG was not examined specifically, this receptor upregulation occurs in a wide range of brain areas and includes enhanced receptor efficacy. Similar changes appear to occur in the PAG as indicated by an increase in mu-opioid receptor binding and proenkephalin mRNA following systemic administration of the cannabinoid agonist CP-55,940 (Manzanares et al., 1998; Viganò et al., 2005).

Despite these findings, interactions between systemically administered cannabinoids and opioids have been shown to vary widely. Some studies show cross-tolerance between cannabinoids and opioids (Massi et al., 2001; Thorat and Bhargava, 1994), while other studies failed to find such an effect (Valverde et al., 2001; Veseljurt and Dogrun, 2004). Such differences might be attributed to the different methods of administration utilized (e.g., topical vs. intraperitoneal) or differences between cannabinoid agonists. Conversely, and consistent with our results, some investigators show that co-administration of a cannabinoid and morphine blocks the development of tolerance to morphine (Cichewicz and McCarthy, 2003). Also, as mentioned previously, cannabinoid antinoicception is enhanced in morphine-tolerant animals (Cichewicz and Welch, 2003; Rubin et al., 1997; Viganò et al., 2005).

Administering low morphine doses for short periods of time may attenuate the negative attributes associated with morphine use. The side effects that accompany cannabis and other cannabinoid therapies, such as nausea and drowsiness, are less severe than that produced by opioids, yet the analgesia they provide is minimal compared to opioids. Modern clinicians face the challenge of finding a treatment protocol that leads to maximal pain relief and limited side effects. The present data showed no enhancement of motor inhibition produced by morphine in rats pretreated with HU-210, suggesting that HU-210 enhancement of morphine may be specific to antinoicception.

Acknowledgements

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References

CHAPTER THREE: Repeated Morphine Microinjection into the Rat Periaqueductal Gray Enhances subsequent Cannabinoid Antinociception

ABSTRACT

Opioids and cannabinoids interact to produce antinociception that is greater than when either drug is administered alone. Recent experimental data has suggested that antinociceptive enhancement between opioids and cannabinoids could be mediated by the midbrain periaqueductal gray (PAG). This study tested the hypothesis that the PAG mediates bi-directional antinociceptive enhancement between opioids and cannabinoids. Repeated morphine administration significantly enhanced subsequent HU-210 antinociception compared to vehicle-pretreated animals. These results implicate the PAG as a critical brain region involved in the bi-directional enhancement of antinociception between opioids and cannabinoids.
INTRODUCTION

Opioids and cannabinoids have been shown to interact in several ways. Some studies have demonstrated that co-administration of the drugs produces antinociceptive synergy (Cichewicz and McCarthy, 2003, Tham et al., 2005, Roberts et al., 2006). Also, cannabinoids have been shown to enhance the antinociceptive effect of opioids (Smith et al., 1998a, Cichewicz et al., 1999b, Yesilyurt et al., 2003, Cichewicz et al., 2005). This antinociceptive enhancement is bi-directional, given that morphine pretreatment can enhance the effect of subsequent cannabinoid administration. This enhancement can occur either during co-administration, when THC is administered on the 7th day of morphine pellet implantation (Rubino et al., 1997), or after pretreatment, when THC is given 12 hours after the last of a series of escalating morphine doses (Cichewicz and Welch, 2003). Furthermore, morphine tolerance can be attenuated by the co-administration of cannabinoids (Smith et al., 2007).

Both opioids and cannabinoids produce antinociception, in part, by activation a descending pain modulatory pathway that originates in the midbrain periaqueductal gray (PAG). Injections of either cannabinoids or morphine into the PAG produce widespread antinociception (Morgan and Whitney, 2000, Finn et al., 2003, Meng and Johansen, 2004, Morgan et al., 2006). Moreover, repeated cannabinoid administration enhances subsequent morphine antinociception and attenuates the development of morphine tolerance (Wilson et al., 2008). These results suggest that the bi-directional
enhancement of antinociception between opioids and cannabinoids may be mediated by the PAG, however the effects of repeated opioid administration on subsequent cannabinoid antinociception have not been investigated. This study was conducted to test the hypothesis that repeated morphine microinjections into the PAG enhance the antinociceptive effect of subsequent cannabinoid administration.

MATERIALS AND METHODS

Subjects

Male Sprague-Dawley rats (245-281 g; Harlan, Kent WA) were anesthetized with equithesin (60 mg/kg, i.p.) and stereotaxically implanted with a 23 gauge (.573 mm) stainless steel guide cannula aimed at the ventrolateral PAG (9 mm long, AP +2.3 mm; ML -0.6 mm; DV -4.6 mm from lambda). The guide cannula was held in place with dental cement affixed to two screws in the skull. Following surgery, a removable stainless steel stylet was inserted into the guide cannula, and the rat was housed individually with food and water available ad libitum. Lights were maintained on a reverse 12 h light/dark cycle so that all injections and testing occurred during the animals’ active phase, rather than during the light, inactive phase.

Rats were handled daily prior to and following surgery. Testing began one week following surgery. Experiments were conducted in accordance with the animal care and use guidelines of the International Association for the Study of Pain. The Institutional
Animal Care and Use Committee at Washington State University approved this research.

**Microinjections**

The day before the first microinjection, an 11 mm injection cannula was inserted into the guide cannula without administration of drugs. This procedure reduces artifacts on the test day resulting from mechanical damage to neurons and habituates rats to the injection procedure.

Microinjections were administered through a 31 gauge (.226 mm), 11 mm long injection cannula inserted into and extending 2 mm beyond the tip of the 9 mm guide cannula. The injection cannula was connected to a 1 µl syringe (Hamilton Co., Reno, NV) with PE20 tubing filled with sterile water. All microinjections were administered in a volume of 0.4 µL over 40 s while the rat was gently restrained. The injection cannula remained in place an additional 20 s to minimize backflow of the drug up the cannula tract. Following the injection, the stylet was reinserted into the guide cannula and the rat was returned to its home cage.

**Drugs**

Drug doses and concentrations were selected based on previous studies (Finn et al., 2003, Morgan et al., 2005b). For pretreatment procedures, morphine sulfate (a gift from
the National Institute on Drug Abuse) was dissolved in saline. The control group received microinjections of saline. The CB1 receptor agonist HU-210 was dissolved in 1:1:8 (ethanol: Tween 80: saline) for the cumulative dose assessment of HU-210 potency. For the HU-210 cumulative dosing procedure, rats received two injections of 5 µg, followed by one injection of 10 µg, resulting in cumulative doses of 5, 10, and 20 µg. Although cumulative dosing protocols in our laboratory are typically on a quarter-log scale (Morgan et al., 2005a, Wilson et al., 2008), cannabinoid insolubility prevented the administration of doses higher than 10 µg/0.4 µL.

**Behavioral Tests**

Nociception was assessed using the hot plate test. The hot plate test measures the latency for a rat to lick a hind paw when placed on a 52.5°C surface. The rat was immediately removed from the hot plate following a response, or after 50 s if no response occurred. Hot plate calibration at this temperature produced baseline latencies of 15 to 17 s. The experimenter conducting the behavioral tests was blind to the pretreatment condition of the animals.

**Experimental Design**

Pretreatment vIPAG microinjections of morphine (5 µg, N = 9) or saline (N = 7) were administered twice daily (0900 and 1600 h) for two days (Trials 1-4). This repeated morphine administration protocol reliably produces morphine tolerance in our laboratory.
(Morgan et al., 2005a, Fossum et al., 2008, Wilson et al., 2008). On the third day (Trial 5), cannabinoid potency was assessed by microinjecting cumulative doses of HU-210 (5, 10, and 20 µg) into the ventrolateral PAG. Microinjections were administered every 20 min. Following baseline measurements, the hot plate test was conducted 15 min after each microinjection of HU-210. Statistical comparisons of drug effects were made using Student’s t-test (StatPlus), and differences were considered significant if $p < 0.05$.

**Histology**

Rats were euthanized following testing by administering a lethal dose of Halothane. The brain was removed and placed in formalin (10%). At least 2 days later the brain was sectioned coronally (100 µm) and the location of the injection site (scar tissue from the repeated insertion of the microinjector) was identified using the atlas of Paxinos and Watson (Paxinos and Watson, 2005).

**RESULTS**

Microinjection of 5 µg morphine on Trial 1 caused complete antinociception in 8 out of 9 rats (hot plate latencies of 50 s). Baseline hot plate latencies on Trial 5 were the same for the morphine and saline-pretreated groups ($t(14) = 2.14, p = 0.34$). In control rats, the highest injection of HU-210 produced an average hot plate latency of 22.3 s (20.9% of the maximum possible effect). The highest PAG microinjection of HU-210 produced greater antinociception in morphine-pretreated rats than in saline-pretreated rats ($t(14) =$
1.76, $p = 0.04$, Figure 1), which is consistent with previous reports of enhanced cannabinoid antinociception during opioid tolerance (Cichewicz and Welch, 2003).

**DISCUSSION**

Opioids and cannabinoids enhance antinociception in a bi-directional manner. That is, morphine pretreatment enhances subsequent cannabinoid antinociception (Rubino et al., 1997, Cichewicz and Welch, 2003), and cannabinoid pretreatment enhances subsequent morphine antinociception (Smith et al., 1998b, Cichewicz et al., 1999a). The results of this study substantiate these findings, given the morphine pretreatment enhancement of HU-210 microinjections in the PAG. Furthermore, microinjection of cannabinoids in the PAG also enhances the antinociceptive effect of subsequent morphine administration (Wilson et al., 2008). Taken together, these results suggest that bi-directional enhancement of antinociception between opioids and cannabinoids is mediated by the PAG. Investigation of the underlying mechanisms in this brain region is warranted, given the therapeutically beneficial implications of cannabinoid/opioid interactions.
**FIGURES**

![Graph](image)

**Figure 1:** Morphine pretreatment enhances the antinociceptive effect of HU-210 administration 24 hours later. The cumulative HU-210 dose of 20 µg produced greater antinociception in morphine-pretreated rats than in vehicle-pretreated rats. Asterisk indicates difference greater than control group, with a p value < 0.05. Error bars represent standard error of the mean (SEM).
REFERENCES


CHAPTER FOUR: Repeated Cannabinoid Microinjections in the Rat Rostral Ventromedial Medulla Produce Neurotoxic Lesions

ABSTRACT

Cannabinoids and opioids produce analgesia, in part, by activating a descending pain pathway that includes the midbrain periaqueductal gray (PAG) and the rostral ventromedial medulla (RVM). Microinjection of cannabinoids or opioids into these brain regions produces antinociception, and pretreatment with cannabinoids in the PAG attenuates the development of morphine tolerance. When the drugs are co-administered systemically, the antinociceptive effects are greater than when the drugs are given individually. The results of this study are consistent with these previous findings, and demonstrate that acute co-administration of cannabinoids and opioids in the RVM produced greater antinociception than when the drugs were administered alone. Repeated administration of cannabinoids had no effect on subsequent morphine potency, and repeated co-administration of cannabinoids and morphine appeared to cause cross-tolerance to subsequent morphine injections. However, repeated CB1 agonist injections caused lesions in the RVM, which may have interfered with the development of tolerance and/or nociceptive signaling. The importance of characterizing cannabinoid/opioid interactions in the descending pain pathway is thus complicated by the susceptibility of the RVM to cannabinoid toxicity.
INTRODUCTION

Opioids and cannabinoids produce antinociception in part by activating a descending pain modulatory pathway that originates in the periaqueductal gray (PAG), and projects to the spinal cord via the rostral ventromedial medulla (RVM). Electrical stimulation of either the RVM or PAG produces systemic antinociception (Cannon et al., 1982). Endogenous opioids are synthesized in neurons throughout the PAG and RVM (Hill et al., 1983), and the localization of both opioid and cannabinoid receptors in these brain regions has been demonstrated using a variety of anatomical techniques (Herkenham et al., 1991, Mailleux and Vanderhaeghen, 1992, Kalyuzhny et al., 1996, Tsou et al., 1998, Wang and Wessendorf, 1999, Commons et al., 2000). Microinjection of cannabinoids or opioids into either the PAG or RVM produces antinociception (Morgan and Whitney, 2000, Finn et al., 2003, Meng and Johansen, 2004, Morgan et al., 2006a), and morphine activates PAG output neurons that project directly to the RVM (Loyd et al., 2007). Furthermore, cannabinoid microinjections in the PAG enhance the antinociception produced by subsequent morphine administration (Wilson et al., 2008).

Despite being part of the same descending nociceptive system and sharing several nociceptive mechanisms, the PAG and the RVM appear to differ in regard to the development of tolerance. Tolerance develops rapidly after repeated microinjections of morphine into the ventrolateral PAG (Morgan et al., 2006a) but the RVM is resistant to the development of morphine tolerance (Morgan et al., 2005a). The PAG also
demonstrates attenuated morphine tolerance when the animal receives repeated cannabinoid microinjections (Wilson et al., 2008). However, the effects of cannabinoids on morphine tolerance have not been examined in the RVM.

The purpose of this study was to test the hypothesis that cannabinoid enhancement of morphine antinociception and attenuation of tolerance (Wilson et al. 2008) is a phenomenon specific to the PAG. Because the PAG and RVM have differential sensitivity to morphine tolerance (Morgan et al., 2005a), we did not expect to see cannabinoid-induced morphine sensitization in the RVM.

MATERIALS AND METHODS

Subjects

Male Sprague-Dawley rats (210-321 g; Harlan, Kent WA) were anesthetized with equithesin (60 mg/kg, i.p.) and stereotaxically implanted with a 23-gauge (.573 mm) stainless steel guide cannula aimed at the rostroventromedial medulla (12 mm long, AP -2.2 mm; ML 0.0 mm; DV -8.8 mm from lambda). The guide cannula was held in place with dental cement affixed to two screws in the skull. Following surgery, a removable stainless steel stylet was inserted into the guide cannula, and the rat was housed individually, on a reverse 12 h light/dark cycle, with food and water available ad libitum.
Rats were handled daily prior to surgery, and were given one week to recover from surgery before behavioral testing commenced. Animals were not re-used for subsequent experiments, nor were they pre-treated with more than one drug or drug combination. Experiments were conducted in accordance with the animal care and use guidelines of the International Association for the Study of Pain. The Institutional Animal Care and Use Committee at Washington State University approved this research.

Microinjections

The day before the first microinjection, a 14 mm injection cannula was inserted into the guide cannula without administration of drugs. This procedure reduces confounds on the test day resulting from mechanical damage to neurons, and habituates rats to the injection procedure.

Microinjections were administered through a 31 gauge (.226 mm), 14 mm long injection cannula inserted into and extending 2 mm beyond the tip of the 12 mm guide cannula. The injection cannula was connected to a 1 μL syringe (Hamilton Co., Reno, NV) with PE20 tubing filled with sterile water. All microinjections were administered in a volume of 0.5 μL over 50 s while the rat was gently restrained. The injection cannula remained in place an additional 20 s to minimize backflow of the drug up the cannula tract. Following the injection, the stylet was reinserted into the guide cannula and the rat was returned to its home cage.
Drugs

Drug doses and concentrations were selected based on previous studies (Finn et al., 2003, Morgan et al., 2005b). For pretreatment procedures, morphine sulfate (a gift from the National Institute on Drug Abuse), the cannabinoid receptor agonists HU-210 (Tocris, St. Louis, MO), WIN 55,212-2 (Cayman Chemical, Ann Arbor, MI) and the cannabinoid receptor antagonist AM-251 (Tocris) were dissolved in Tween 80, ethanol, and saline in a 1:1:8 ratio. The control groups received microinjections of vehicle (Tween 80: ethanol: saline). Morphine was dissolved in saline for the cumulative dose assessment of morphine potency (Morgan et al. 2006). Rats receiving morphine and HU-210 received both drugs in a single injection. Each rat was used for only one experiment, and all rats in each experiment received the same number of microinjections, regardless of treatment. Animals were given four injections of 0.5 µL during pre-treatment, and five subsequent 0.4 µL injections to assess morphine dose response characteristics. For the morphine cumulative dosing procedure, actual doses injected were 1.0, 0.8, 1.4, 2.4, and 4.4 µg, resulting in cumulative quarter log doses of 1.0, 1.8, 3.2, 5.6, and 10 µg.

Behavioral Tests

Nociception was assessed using the hot plate test. The hot plate test measures the latency for a rat to lick a hind paw when placed on a 52.5°C surface. The rat was immediately removed from the hot plate following a response, or after 50 s if no
response occurred. At this temperature the hot plate produced average baseline latencies of 14 to 20 s.

Experimental paradigm

Rats were injected with WIN 55,212-2 (5 μg/0.5 μL, N = 21), HU-210 (5 μg/0.5 μL, N = 16), 1:1:8 vehicle (N = 37), morphine (5 μg/0.5 μL, N = 17), or a morphine/CB1 agonist combination (HU-210, N = 7; WIN 55,212-2, N = 14). Injections were given twice a day (0930 & 1600) for two days (Trials 1 - 4). Rats receiving combined opioid/cannabinoid injections received both drugs in a single injection of 0.5 μL. All drugs were mixed and injected in a vehicle of Tween 80, ethanol, and saline (1:1:8). Thirty minutes after the first injection on Day 1, nociception was assessed using the hot plate test. No testing was conducted following injections on Trials 2-4 to limit changes in latency caused by repeated testing (Gamble and Milne, 1989, Lane et al., 2004).

On Day 3 (Trial 5), tolerance to morphine was assessed using cumulative dose microinjections (1.0, 1.8, 3.2, 5.6, and 10 μg/0.4 μL) into the RVM at 20 min intervals (Morgan et al., 2006b). Nociception was assessed 15 min after each microinjection. Because there were no significant differences between HU-210 and WIN effects on antinociception or tolerance, these groups were combined for final analysis. Data from 3 rats was excluded from final analysis because the baseline hot plate latency on Day 3
was at least 2 standard deviations higher than the mean hot plate latency for the rat's pretreatment condition.

Histology

Rats were euthanized following testing by administering a lethal dose of Halothane, and the brain was removed and placed in formalin (10%). At least 2 days later the brain was sectioned coronally (50 μm), and slices were mounted on gelatin-coated slides. After serial dehydration in increasing concentrations of ethanol and glacial acetic acid, slides were stained using cresyl violet acetate. Histoclear (Electron Microscopy Sciences, Hatfield, PA) was used as a clearing agent before coverslipping slides with Permount (Fisher Chemical, Fairlawn, NJ). Slides were viewed under an upright light microscope, and the location of the injection site was identified using the atlas of (Paxinos and Watson, 2005). Data from 43 animals were not included in final analysis because the injection site was either outside the RVM, or not visible under a light microscope.

Data Analysis

Dose-response curves were generated for hot plate data using nonlinear regression (Graph Pad Prism). The half-maximal effective dose ($D_{50}$) was calculated for each condition (Tallarida, 2000). Analysis of variance (Kazakov et al.) was used to assess changes in hot plate and open field activity (SPSS). $D_{50}$ values calculated from dose response curves (GraphPad) and ANOVA (Tallarida, 2000) were used to assess
changes in potency. Post hoc comparisons were made with 95% confidence intervals. Statistical significance was defined as a probability of less than .05. For histology experiments, Image J (NIH) was used to measure the size of lesions in the RVM (in mm²), and ANOVA (SPSS) was used to assess lesion size.

RESULTS

Repeated microinjection of the cannabinoid agonists produced profound catalepsy, illness, and lethargy in many animals, which interfered with nocifensive behavior and led to the removal of many animals from the study. In total, 59% of the animals that were pretreated with cannabinoids were removed from the study before they were tested on Trial 5 (in some cases after a single pretreatment injection, Table 1). Histological examination of the microinjection sites in cannabinoid-pretreated animals revealed significantly larger lesions than the scar tissue from the injection procedure visible in vehicle-pretreated animals (Table 2, \( t(9) = 1.83, p < .05 \), Figure 1).

The following data were collected from animals that subsisted to Trial 5 without behavioral confounds (WIN 55,212-2 (N = 6), HU-210 (N = 4), 1:1:8 vehicle (N = 7), morphine (N = 6), morphine+HU-210 (N = 2), morphine+WIN 55,212-2 (N = 3)). Acute microinjections of either 5 \( \mu \)g CB1 agonist or morphine were insufficient to produce an antinociceptive effect that was greater than vehicle alone. However, rats that received the combination CB1 agonist/morphine injection demonstrated significant
antinociception (Figure 2). Thus, the antinociceptive effect of co-administration of CB1 agonists and morphine was greater than that of injecting either drug alone (F(3,22) = 9.5, p < 0.05).

Mean baseline hot plate latencies on Trial 5 were relatively high in animals that were pre-treated with cannabinoids (vehicle = 14.3, morphine = 15.6, CB1 = 20.6, CB1+Morphine = 14.5). Repeated microinjections of morphine caused a rightward shift in the morphine dose response curve compared with the vehicle group as expected with the development of tolerance (Figure 3). Pre-treatment with CB1 agonists had no effect on morphine potency when rats were tested on Trial 5, as the ED$_{50}$ for CB1-pretreated animals was nearly identical to vehicle-pretreated animals (Table 3). There was a significant rightward shift in the morphine dose-response curve in animals that were pretreated with CB1 agonists in combination with morphine (Figure 3). However, the negative side effects of the cannabinoids may have interfered with or falsely inflated ED$_{50}$ values on Trial 5.

**DISCUSSION**

Despite a significant shift in the morphine dose response curve on Trial 5, it is difficult to support the assertion that repeated co-administration of CB1 agonists and morphine induced cross-tolerance to morphine, given the substantial CB1 agonist-induced lesions in the RVM. CB1 activation has been shown to reduce cell proliferation, and initiate cell
death signaling cascades via mitogen activated protein kinases (MAPK, (Mukhopadhyay et al., 2010, Yang et al., 2010). The cannabinoid system can also stimulate the release of tumor necrosis factor-alpha, which can also trigger apoptotic cell death (De Laurentiis et al., 2010). Although cell death effects have not specifically been examined in the PAG or RVM, the differential activation of these pathways in these two brain regions could account for microinjection-induced lesions in the RVM, but not the PAG (Wilson et al., 2008). The large lesions resulting from microinjection of CB1 agonists in this study could most certainly interfere with nociceptive signaling and cellular adaptations that underlie the development of tolerance. CB1 agonist-induced lesions could also interfere with nociceptive thresholds or nocifensive responses, considering the relatively high baseline hot plate latencies on Trial 5, compared to the morphine and vehicle groups. Combined, these factors make it difficult to accurately evaluate cannabinoid pretreatment effects on morphine tolerance on Trial 5.

Although 5 μg CB1 agonist or morphine were sub-analgesic when administered individually, acute RVM microinjection of combined CB1 agonists/morphine produced significant antinociception. These results are consistent with previous reports demonstrating that the antinociceptive effect of co-administered opioids and cannabinoids is greater than the effect of administering either drug alone (Cichewicz and McCarthy, 2003, Tham et al., 2005, Roberts et al., 2006). These results provide
further support for the involvement of the descending pain pathway in combined opioid/cannabinoid therapy.

Repeated microinjections of cannabinoids had no effect on morphine antinociceptive tolerance on Trial 5. These results differ from the effects of cannabinoid pretreatment in the PAG and periphery, which sensitize the morphine antinociceptive response (Smith et al., 2007, Wilson et al., 2008). The failure of cannabinoids to attenuate the development of morphine tolerance in the RVM is not entirely surprising, given that the PAG and RVM have different mechanisms underlying the development of tolerance (Morgan et al., 2005a). Thus, cannabinoid attenuation of morphine tolerance appears to be mediated largely by the PAG. Alternatively, the inability of cannabinoids to attenuate morphine tolerance in the RVM could be explained by CB1-induced lesions.

The results of this study provide new insight into the acute effects of co-administered opioids and cannabinoids in the descending pain pathway. However, the susceptibility of the RVM to CB1 agonist-induced lesions highlights the challenges of studying the effects of repeated cannabinoid exposure on morphine tolerance.

**Acknowledgements**

I would like to thank Melissa Herschbach and Edvinas Pocius for their technical assistance in conducting behavioral experiments and preliminary data analysis.
**Figure 1:** Micrographs demonstrating CB1 agonist-induced lesions in the RVM.

Repeated administration of HU-210 caused the largest lesions in the RVM, regardless of whether the drug was injected alone or in combination with morphine.
Figure 2: Effects of acutely injected CB1 agonists, morphine, and CB1 agonists combined with morphine into the RVM. Hot plate latencies for acutely microinjected CB1 agonists or morphine were not significantly different from vehicle. Microinjection of CB1 agonists combined with morphine resulted in significant antinociception that was greater than when either drug was administered alone. Asterisk indicates $p$ value of 0.05, difference from vehicle.
Figure 3: Dose-response analysis of morphine tolerance on Trial 5. Morphine pretreatment caused a rightward shift in the dose response curve, as did the CB1+morphine combination pretreatment. Repeated microinjections of CB1 agonists had no effect on morphine potency on Trial 5.
TABLES

Table 1
Comparison of rats excluded from study (Trial 5) because of catalepsy or illness

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Total # of Rats</th>
<th># of Rats excluded</th>
<th>% excluded</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIN 55,212-2</td>
<td>21</td>
<td>10</td>
<td>48%</td>
</tr>
<tr>
<td>WIN+Morphine</td>
<td>14</td>
<td>8</td>
<td>57%</td>
</tr>
<tr>
<td>HU-210</td>
<td>16</td>
<td>11</td>
<td>69%</td>
</tr>
<tr>
<td>HU-210+Morphine</td>
<td>7</td>
<td>5</td>
<td>71%</td>
</tr>
<tr>
<td><strong>Total CB1</strong></td>
<td><strong>58</strong></td>
<td><strong>34</strong></td>
<td><strong>59%</strong></td>
</tr>
<tr>
<td>Morphine</td>
<td>17</td>
<td>1</td>
<td>6%</td>
</tr>
<tr>
<td>Vehicle</td>
<td>37</td>
<td>3</td>
<td>8%</td>
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</table>

Table 2
Comparison of CB1 agonist-induced lesions in the RVM

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Avg. Lesion Size (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIN 55,212-2</td>
<td>0.91</td>
</tr>
<tr>
<td>HU-210</td>
<td>1.08</td>
</tr>
<tr>
<td>HU-210+Morphine</td>
<td>1.24</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.35</td>
</tr>
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</table>
Table 3
Comparison of morphine ED\textsubscript{50} values on Trial 5

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>ED\textsubscript{50}</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB1</td>
<td>3.99</td>
<td>2.51 – 5.46</td>
</tr>
<tr>
<td>CB1+Morphine</td>
<td>12.02</td>
<td>5.64 – 18.40</td>
</tr>
<tr>
<td>Morphine</td>
<td>6.98</td>
<td>5.46 – 8.50</td>
</tr>
<tr>
<td>Vehicle</td>
<td>4.06</td>
<td>2.80 – 5.32</td>
</tr>
</tbody>
</table>
REFERENCES


CHAPTER FIVE: Distribution of CB1 Cannabinoid Receptors and Their Relationship with Mu-Opioid Receptors in the Rat Periaqueductal Gray


ATTRIBUTIONS

This chapter consists of a manuscript in preparation for publication. The contributions of each author are as follows:

Wilson, A.R.: Responsible for experimental conception, data collection, data analysis and interpretation, first draft of manuscript, and final draft of manuscript

Hegarty, D.M.: Training and assistance with data collection and analysis (including statistical analysis), constructing figures, and editing manuscript

Morgan, M.M.: Supervision and manuscript revisions

Aicher, S.A.: Supervision, facilities and materials, and manuscript revisions
CHAPTER FIVE: Distribution of CB1 Cannabinoid Receptors and Their Relationship with Mu-Opioid Receptors in the Rat Periaqueductal Gray


ABSTRACT

The periaqueductal gray (PAG) is part of a descending pain modulatory system that, when activated, produces widespread and profound antinociception. Microinjection of either opioids or cannabinoids into the PAG elicits antinociception. Moreover, microinjection of the cannabinoid 1 (CB1) receptor agonist HU-210 into the PAG enhances the antinociceptive effect of subsequent morphine injections, indicating a direct relationship between these two systems. The objective of this study was to characterize the distribution of CB1 receptors in the dorsolateral and ventrolateral PAG in relationship to mu-opioid peptide (MOP) receptors. Immunocytochemical analysis revealed extensive and diffuse CB1 labeling in the PAG, 60% of which was found in somatodendritic profiles. CB1 and MOP receptor immunolabeling was co-localized in 32% of fluorescent-Nissl stained cells that were analyzed. Eight percent (8%) of PAG neurons that were MOPr-immunoreactive received CB1 appositions, and ultrastructural analysis confirmed the presence CB1-immunoreactive axon terminals in the PAG. These results indicate that behavioral interactions between cannabinoids and opioids may be the result of cellular adaptations within PAG neurons co-expressing CB1 and
MOP receptors, and that synaptic adaptations could also partially contribute to cannabinoid-induced enhancement of morphine antinociception.
INTRODUCTION

Opioids and cannabinoids have been shown to have analgesic properties. Although the analgesic efficacy of cannabinoids is less substantial than opioids, recent experimental data suggests that opioids and cannabinoids may interact in therapeutically beneficial ways. Systemic co-administration of cannabinoid and opioid agonists results in synergistic antinociception (Cichewicz and McCarthy, 2003, Tham et al., 2005, Roberts et al., 2006, Smith et al., 2007), and systemic pre-treatment or co-administration of cannabinoids attenuates the development of morphine tolerance (Cichewicz and Welch, 2003, Smith et al., 2007, Wilson et al., 2008).

A number of findings indicate that the periaqueductal gray (PAG) contributes to the enhanced antinociception produced by co-administration of opioids and cannabinoids. Microinjection of either cannabinoids or opioids into the PAG produces antinociception (Jensen and Yaksh, 1986, Martin et al., 1995, Lichtman et al., 1996, Morgan et al., 1998, Finn et al., 2003), and microinjection of a CB1 agonist into the ventrolateral PAG enhances subsequent morphine antinociception (Wilson et al., 2008). These effects could be produced by co-localization of receptors on the same neurons. Autoradiography, in situ hybridization, and immunohistochemistry studies confirm CB1 and mu-opioid peptide receptor (MOPr) expression in the PAG (Herkenham et al., 1991, Mailleux and Vanderhaeghen, 1992, Kalyuzhny et al., 1996, Tsou et al., 1998, Commons et al., 2000). The CB1 and MOP receptors are each found on
somatodendritic profiles in the PAG (Tsou et al., 1998, Commons et al., 2000). In the dorsal horn of the spinal cord, both receptor types co-localize on dendrites (Salio et al., 2001, Salio et al., 2002b). However, to our knowledge, CB1/MOR co-localization has not been demonstrated in PAG neurons. One objective of this manuscript is to determine whether a similar somatodendritic co-localization pattern occurs in the PAG.

Alternatively, cannabinoid/opioid interactions could also occur between neurons where the receptors are not co-localized on the same cell. Endocannabinoids are known as retrograde messengers, with CB1 receptors located on presynaptic terminals (Wilson and Nicoll, 2001, Alger, 2002, Wilson and Nicoll, 2002). Thus, CB1 terminals projecting onto MOPr-expressing cells could be alternative sites of opioid/cannabinoid interaction. One goal of this study is to determine whether or not this type of morphology exists in the PAG.

CB1 agonists produce greater antinociception when injected into the dorsolateral PAG compared to the ventrolateral PAG (Martin et al., 1995), whereas the antinociception produced by microinjection of morphine into the dorsal and lateral regions of the PAG is associated with severe flight responses (Morgan et al., 1998). Furthermore, the dorsolateral PAG is specialized to produce cannabinoid-mediated stress-induced analgesia (Hohmann et al., 2005). These functional differences in sub-regions of the PAG could have anatomical underpinnings, which this study will elucidate by comparing the distribution of CB1 and MOP receptors in these regions.
Previous reports have shown that chronic drug administration can induce changes in the expression of the CB1 and MOP receptors in several sites in the central nervous system. In reward-related brain areas, there is a reciprocal up-regulation of CB1 and MOP receptors after chronic drug exposure (Fattore et al., 2007). Similarly, chronic morphine administration increases the expression of both CB1 and CB2 receptors in the dorsal horn of the spinal cord (Lim et al., 2005, Fattore et al., 2007). These effects have not been studied in the PAG, and the final objective of this study is to determine if changes in receptor expression underlie sensitized morphine responses after chronic cannabinoid administration.

The behavioral and anatomical data cited above suggest that CB1 and MOP receptors have direct interactions in the PAG. The current study was undertaken to characterize CB1 distribution in the PAG, and test the following hypotheses: 1) Are CB1 and MOP receptors co-localized on the same neurons? 2) Do CB1 presynaptic terminals make contact with MOP-r expressing cells? 3) Are there differences in the amount or pattern of CB1 receptor labeling in the dorsolateral compared to the ventrolateral PAG? and 4) Does chronic drug administration cause an up-regulation of receptor expression in the PAG?
MATERIALS AND METHODS

Subjects

Experiments were performed in male Sprague-Dawley rats (220-280g, Harlan, Indianapolis, IN), housed three to a cage. Animals were kept on a reverse light-dark cycle with food and water available ad libitum. Experiments were conducted in accordance with the care and use guidelines of the International Association for the Study of Pain. The Institutional Care and Use Committee of Washington State University approved this research.

Perfusion and tissue preparation for immunocytochemistry

Rats were given a lethal dose of sodium pentobarbital (150 mg/kg, i.p.), and perfused transcardially through the ascending aorta with 10 mL of heparinized saline (1000 U/mL), followed by 50 mL of 3.8% acrolein in 2% paraformaldehyde, followed by 200 mL of 2% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) (Aicher et al., 2003, Hegarty et al., 2010). Immediately following perfusion, brains were removed, and blocks of tissue containing the PAG were placed in fixative for 30 min and then transferred to 0.1 M PB. Blocks of tissue were sectioned coronally on a vibrating microtome (Leica Microsystems, Inc., Buffalo Grove, IL) at 40 µm. Prior to immunocytochemical processing, free-floating sections were placed in 1% NaBH₄ (Sigma-Aldrich; St. Louis, MO) for 30 min to bind remaining free aldehydes and increase the antigenicity of acrolein-perfused tissues (Hegarty et al., 2010).
Experiment 1: Distribution of CB1 and MOP receptors in the PAG

Dual-label immunofluorescent labeling

Dual-label immunofluorescent studies were performed as previously described (Hegarty et al., 2010). Tissue sections from 4 naïve rats were incubated in 0.5% bovine serum albumin (BSA; Sigma) in 0.1 M Tris-buffered saline (TS) for 30 minutes to reduce nonspecific binding followed by incubation in a primary antibody cocktail made in 0.1% BSA and 0.25% Triton X-100 (Sigma) in 0.1 M TS for 2 nights at 4°C with continuous agitation. The primary antibody cocktail was made up of a polyclonal rabbit anti-CB1 antibody (1:1000; gift from Dr. Ken Mackie), directed against a synthetic peptide of the last 73 amino acid residues (residues 401-473) of the intracellular C-terminus of the rat CB1 receptor (Hajos et al., 2000, Wager-Miller et al., 2002, Suarez et al., 2010), and a guinea pig anti-Opioid Receptor, m (MOR) antibody (1:5000; Millipore, Billerica, MA), directed against a synthetic peptide of amino acid residues 384-398 of the C-terminus of the rat MOPr (manufacturer’s technical specifications). Tissue sections were rinsed and then incubated in a cocktail of fluorescent secondary antibodies made in 0.1% BSA in 0.1 M TS for 2 hours at room temperature protected from light. In order to visualize the CB1r, a donkey anti-rabbit antibody conjugated to Alexa Fluor 488 (1:800; Invitrogen, Carlsbad, CA) was used. We used a donkey anti-guinea pig antibody conjugated with Cy5 (Jackson ImmunoResearch, West Grove, PA) to visualize the MOR primary antibody. This combination of secondary antibodies was chosen to ensure that the
fluorophores would be spectrally distinct. Tissue sections were rinsed again and then incubated in NeuroTrace® 530/615 red fluorescent Nissl stain (NT, 1:250; Invitrogen) made in 0.1 M PB for 20 minutes light-protected at room temperature according to manufacturer’s instructions. After profuse rinsing in 0.1 M PB, sections were mounted on gelatin-coated slides, coverslipped with ProLong Gold™ antifade reagent (Invitrogen), sealed and stored at -20°C to preserve labeling.

Antisera specificity

Previous studies have demonstrated that the rabbit anti-CB1 antibody recognizes its antigen in rat cerebellum tissue using Western blotting (Suarez et al., 2008) and that CB1 labeling is absent in cerebellar and hippocampal tissue from CB1 receptor knockout (KO) mice (Suarez et al., 2008, Suarez et al., 2010). CB1 immunoreactivity was abolished by incubating drug-naïve rat PAG tissue in a cocktail that contained the CB1 antibody and the antigenic peptide corresponding to the last 73 amino acid residues (residues 401-473) of the intracellular C-terminus of the rat CB1 receptor. Preadsorption of the guinea pig anti-MOR primary antibody with the control peptide corresponding to C-terminus residues 384-398 of the rat MOPr abolished MOR immunoreactivity in the PAG, compared to sections in which standard immunocytochemistry was performed (Figure 1).
The specificity of the secondary antibodies was confirmed by omitting the primary antibody or by incubating the tissue with one primary antibody (e.g. rabbit anti-CB1) followed by the wrong secondary antibody (Cy5-conjugated donkey anti-guinea pig). There was no immunolabeling in any of the mismatch combinations tested.

Confocal microscopy and data analysis
Fluorescent labeling in PAG sections was viewed using a Zeiss LSM 510 META confocal microscope outfitted with an Argon/2 laser and 2 HeNe lasers (Carl Zeiss MicroImaging, Thornwood, NY). One PAG slice per animal was assessed for the presence of CB1 and MOP receptor labeling and NT staining. Anatomical landmarks such as the size and shape of the cerebral aqueduct were used to select slices that were approximately -7.6 mm from bregma. The ventrolateral and dorsolateral regions of PAG from each rat were individually imaged with a Plan-Neofluar 40x / 1.3 NA oil immersion objective using a single pass, multi-tracking format to minimize overlap. In each region, Z-stacks were bounded by the vertical extent of antibody labeling and NT staining. Zeiss ZEN software was used to assign color profiles to fluorescence images and analysis (see below). Confocal micrographs used for publication are projections of one or several optical sections and adjusted for optimal brightness and contrast using Zeiss ZEN software.
Images from four animals were analyzed to determine the distribution of CB1 and MOR immunoreactivity in the rat ventrolateral and dorsolateral PAG. First, with the channels used to detect CB1 and MOR immunofluorescent labeling turned off, cells stained with NT were identified and numbered. Cells were considered to be stained with NT if the entire cell body and nucleus were visible in at least two consecutive optical sections. A total of 421 (217 ventrolateral, 204 dorsolateral) NT cells were examined in the PAG. After identifying NT-stained cells, the channels used to detect CB1 and MOR immunolabeling were turned on individually and each cell was analyzed for colocalization with CB1 or MOR labeling or for appositions with CB1 or MOR-labeled varicosities. A cell was scored as CB1-immunoreactive (-ir) or MOR-ir if labeling was present within the boundaries of the NT-stained cell, in at least two consecutive optical sections. NT-stained cells that were CB1-ir or MOR-ir or that coexpressed CB1 and MOR were further analyzed for appositions with CB1-ir and MOR-ir axonal varicosities. Varicosities were defined as approximately circular profiles along an axonal fiber that were at least twice the diameter of that fiber (Bailey and Ribeiro-da-Silva, 2006). If a varicosity was directly adjacent to an NT-stained cell in two consecutive optical sections, it was considered an apposition. Analysis of the CB1 and MOR immunoreactivity content of NT-stained cells as well as the appositional analysis was assessed by two independent and blinded observers.
Immunogold labeling for electron microscopy

Tissue sections to be used for electron microscopic analysis of CB1r distribution in PAG were processed using colloidal gold immunocytochemical methods similar to those described previously (Aicher et al., 1997, Hegarty et al., 2007). The freeze-thaw method in which the PAG tissue sections were briefly immersed into liquid chlorodifluoromethane (Freon) followed by liquid nitrogen was used to increase antibody penetration of the tissue. Tissue sections were then blocked in 0.5% BSA in 0.1 M TS followed by incubation in the primary antibody solution consisting of the polyclonal rabbit anti-CB1 antibody (1:250) in 0.1% BSA in 0.1 M TS for 2 nights 4°C with continuous agitation. Following rinses, tissue sections were incubated in colloidal gold-conjugated goat anti-rabbit secondary antibody (1:50; Electron Microscopy Sciences (EMS), Hatfield, PA) for 2 hours at room temperature. Tissue sections were then rinsed and fixed in 2% electron microscopy (EM) grade glutaraldehyde. Silver intensification of the gold particles was performed using the Silver Enhancement Kit for Light and Electron Microscopy (Ted Pella, Redding, CA). Tissue sections were then osmicated in 1% osmium tetroxide for 15 min, rinsed in 0.1 M PB, dehydrated through ethanols and propylene oxide and then incubated overnight in a 1:1 mixture of propylene oxide and EMBed 812 (EMS). The following day, tissue sections were incubated in EMBed 812 for 2 hours and then embedded between two sheets of Aclar flurohalocarbon plastic film (Ted Pella). The embedded tissue was placed in a 60°C oven for 2 nights. Regions of ventrolateral and dorsolateral PAG that contained CB1 labeling were glued onto Beem
capsules (Ted Pella) and sectioned on an ultramicrotome (Leica Microsystems, Inc.) at 75 nm. Ultrathin sections were then collected onto 400 mesh copper grids (EMS) and counterstained with uranyl acetate and lead citrate.

Electron microscopy and data analysis

Ultrastructural analysis was conducted on plastic-embedded sections of dorsolateral and ventrolateral PAG from three animals. Electron microscopic image collection was performed on a Tecnai 12 electron microscope (FEI, Hillsboro, OR) interfaced to a digital camera and associated software (Advanced Microscopy Techniques, Danvers, MA). Ultrathin sections were examined and chosen based on the optimal preservation of morphological details and maximal detection of the immunogold labeling (Peters, 1991). Sections were selected from an area just below the surface of the tissue, at the tissue/plastic interface, where the penetration of the antibody was optimal in order to avoid underdetection of the immunogold antigen (Chan et al., 1990, Aicher et al., 2003). Images were assessed for: (1) the type of profile (i.e., perikarya, dendrites, terminals, axons or glia) labeled with immunogold, (2) the minimum cross-sectional diameter of immunogold-labeled profiles and (3) the type of synaptic input to labeled perikarya or dendrites (symmetric or asymmetric) or the type of synapse formed by labeled terminals (Peters, 1991). In order for a profile to be considered positive for immunogold labeling, two or more gold particles had to be present in perikarya, dendrites, terminals and glia. In small, unmyelinated axons, the criterion was at least one gold particle associated with
the plasmalemmal surface (Aicher et al., 2003, Hegarty et al., 2007). Immunogold-labeled profiles were further analyzed for the distribution of gold particles on the plasmalemmal surface compared to intracellular membrane sites (Drake et al., 2005). All micrographs analyzed were initially calibrated to their respective scale bars.

**Experiment 2: Effects of chronic drug administration on CB1 receptor expression**

Chronic drug administration

Rats were weighed and injected (s.c.) twice daily for three days, with either the cannabinoid receptor agonist Δ(9)-tetrahydrocannabinol (THC) (a gift from the National Institute on Drug Abuse (NIDA), 10 mg/kg, N = 3), morphine sulfate, (NIDA, N = 3) or an equivalent volume of vehicle (N = 3). All drugs were dissolved in a vehicle of 50% ethanol, 50% dimethyl sulfoxide (DMSO). Nociception was assessed using the hot plate test. The hot plate test measured the latency for the animal to lick a hind paw when placed on a 52.5°C surface. The rat was immediately removed from the apparatus following a response or after 40 seconds if no response occurred. Hot plate calibration at this temperature produced baseline latencies of 8.7 to 11.4 seconds. Baseline latencies were obtained immediately before the animal received its first injection of THC or morphine sulfate on Day 1. Tolerance to the antinociceptive effect of THC and morphine was assessed 30 minutes after the final injection on Day 2.
Light Microscopy

Immunocytochemistry for light microscopic peroxidase detection of the CB1 receptor in the PAG was performed as previously described (Hegarty et al., 2007). Following incubation in 0.5% BSA in 0.1 M TS for 30 minutes, PAG sections were incubated in the rabbit anti-CB1 (1:1000) primary antibody with 0.1% BSA, 0.25% Triton X-100 (Sigma) in 0.1 M TS for 2 nights at 4°C with continuous agitation. Bound CB1 primary antibody was visualized by incubating the PAG tissue sections in biotinylated goat anti-rabbit IgG secondary antibody (1:400, Vector Laboratories, Burlingame, CA) followed by incubation in Avidin-Biotin (Elite Vectastain ABC kit; Vector Laboratories) and diaminobenzidine-hydrogen peroxide (DAB-H2O2) solutions. Trios of animals were processed through identical immunocytochemical conditions to ensure comparable labeling conditions. Tissue punctures through the ventral edge of vibratome sections were used to distinguish drug pretreatment conditions, and tissue sections from the trio of animals were combined during all antibody incubations. Tissue sections were mounted on slides, serially dehydrated in increasing ethanol concentrations and xylenes, and coverslipped using DPX mounting medium (Sigma). One section per animal at the midpoint of PAG (approximately -7.6 mm from bregma, N = 3 rats in each condition) was identified based on the size and shape of the cerebral aqueduct, and images of both the dorsolateral and ventrolateral PAG were taken from each section using an Olympus BX51 microscope equipped with a DP71 camera (Olympus America, Center Valley, PA) and associated software. MetaMorph imaging software (Molecular
Devices Inc., Sunnyvale, CA) was used to measure the density of peroxidase labeling in each image. Briefly, a blinded observer drew four identical regions of interest (ROI) onto each image: one background ROI in the cerebral aqueduct and three ROIs in the PAG. The density of peroxidase labeling from the background ROI was subtracted from the average density from the three ROIs drawn on the PAG. ROIs of the same dimensions were used for each image and they were situated in similar areas in the fields of view.

Statistical analyses
In confocal experiments, t-test (SigmaStat, Systat Software, Inc., San Jose, CA) was used to compare the number of CB1/MOR colocalizations and appositions between the dorsolateral and ventrolateral PAG. In chronic drug treatment experiments, ANOVA (SigmaStat) was used to assess compare changes in drug potency between the first and last injections. An ANOVA was also run on the raw densities (SigmaStat) to analyze drug-induced changes in CB1r density.

RESULTS

Experiment 1: Distribution of CB1 and Mu-opioid receptors in the PAG

CB1 immunolabeling in the PAG

CB1 immunoreactivity in the ventrolateral and dorsolateral PAG is diffuse and extensive (Fig 1). CB1 immunofluorescence appears to be a mix of somatodendritic and
presynaptic structures. The majority (62%) of Neurotrace (NT)-stained cell bodies analyzed co-localize with CB1 immunofluorescence (N = 261, Fig. 2), however there is also a great deal of CB1 immunoreactivity outside of cell bodies (Fig 2), which is consistent with the axonal and astrocytic labeling seen in other CNS sites (Tsou et al., 1998, Salio et al., 2002a, Navarrete and Araque, 2008). A subset of NT-stained cells also received appositions from CB1-immunoreactive (-ir) structures, including CB1-ir cells (N = 21, 5%, Fig 2) and cells that were not CB1-ir (N = 5, 1%).

Electron microscopy was used to more clearly determine the sub-cellular distribution of CB1-ir in the PAG. Immunogold labeling was diffuse in both the ventrolateral and dorsolateral PAG, and 100% of discernable cell bodies contained CB1-immunogold particles. There were no significant differences between the dorsolateral and ventrolateral PAG in either the pattern (number of labeled profiles; t(2) = 4.3, \( p = 0.07 \)) or amount of gold labeling (t(2) = 4.3, \( p = 0.29 \)). Consistent with the immunofluorescence experiments, immunogold labeling was found in somatodendritic and presynaptic structures, as well as in glia (Figure 3). Of the 558 CB1-gold labeled profiles examined, 60% were postsynaptic, 26% were presynaptic, and 14% were glial, which is consistent with the large proportion of somatodendritic CB1-ir seen in other CNS sites (Scavone et al., 2010). In CB1-ir axon terminals, 77% of the gold particles were cytoplasmic, while the remaining 23% were plasmalemmal, which is consistent with CB1-ir in the dorsal horn of the spinal cord (Salio et al., 2001). The majority (83%) of
CB1-ir axon terminals with distinguishable synaptic specializations formed symmetrical synapses (Figure 3).

Relationship of CB1 receptors and Mu-opioid receptors in the PAG

Figure 4 demonstrates the mostly somatodendritic pattern of MOR immunoreactivity in the PAG. Of all NT-stained cells that were examined, 28% were immunoreactive for MOR alone (N = 116), while 32% of all NT-stained cells contained both MOR and CB1 immunolabeling (N = 134, Figure 4). A subset of MOR-ir cells received appositions from CB1-ir profiles, which are presumed to be presynaptic elements such as axon terminals (N = 35, 8%). Many NT-stained cells in the PAG that received CB1 appositions were cells that were immunoreactive for both CB1 and MOR (N = 20, 33%, Figure 4). It should also be noted that 44 of the 421 NT-stained cells examined (10%) were not immunoreactive for either MOR or CB1.

PAG subregional MOR/CB1 expression

Table 2 demonstrates the results of CB1 and MOR labeling in the dorsolateral and ventrolateral PAG. Immunolabeling was highly consistent between regions and among animals. The dorsolateral and ventrolateral PAG were similar in the number of cells expressing both CB1 and MOR immunoreactivity (t(6) = 2.45, p = 0.79), and CB1 appositions onto all NT-stained cells t(14) = 2.14, p = 0.61).
Experiment 2: Effects of chronic drug administration on CB1 receptor expression

Previous studies have demonstrated that chronic opioid exposure can enhance CB1 receptor density in reward-related brain regions (Fattore et al., 2007), however this effect has never been examined in the PAG. Antinociceptive tests confirmed that chronic THC or morphine sulfate administration caused tolerance to the antinociceptive effect of both drugs, as measured by the hot plate (Table 1, F(1,6) = 22.69, p = 0.003). PAG tissue from these animals was assessed for changes in the density of the CB1 immunolabeling. Neither chronic morphine nor THC treatment had a significant effect on the density of CB1 immunolabeling in either the ventrolateral or dorsolateral PAG (F(2,8) = 0.53, p = 0.62) Figure 5).

DISCUSSION

CB1 receptor expression is diffuse and extensive in both the dorsolateral and ventrolateral sub-regions of the PAG, with the majority of CB1 immunoreactivity in somatodendritic profiles. CB1 and MOP receptors were frequently co-localized in the PAG, suggesting that intracellular adaptations in these cells may underlie the behavioral interactions of opioids and cannabinoids. We also found that CB1-ir profiles (likely to be presynaptic) were often apposed to cells expressing MOP receptors, and electron microscope studies confirmed the presence of CB1 axon terminals in the PAG. Finally, chronic drug treatment had no effect on the amount of receptor immunolabeling in either the dorsolateral or ventrolateral PAG.
The CB1 receptor is one of the most ubiquitously expressed G-protein coupled receptors in the central nervous system (CNS), and the results of this study confirm this widespread distribution. Our results were consistent with previous reports that demonstrated somatodendritic (Salio et al., 2002b), axonal/terminal (Tsou et al., 1998), and astrocytic (Salio et al., 2002a, Navarrete and Araque, 2008) CB1 immunolabeling in the CNS. Although cannabinoids are well known as retrograde messengers (Ohno-Shosaku et al., 2001, Hohmann et al., 2005), the large number of somatodendritic profiles we observed suggests that the CB1 receptor may have additional functions in the PAG.

Cannabinoid/opioid interaction has been demonstrated in various behavioral responses and across many administration modalities (Ayhan et al., 1979, Simoneau et al., 2001, Massi et al., 2003, Cichewicz et al., 2005). Systemically co-administered opioids and cannabinoids produce potentiated antinociception (Smith et al., 1998, Cichewicz and McCarthy, 2003). In the PAG, co-administration of cannabinoids and opioids does not produce acute antinociceptive synergy, however, pre-treatment microinjections of cannabinoids create a sensitized morphine antinociceptive response and attenuates the development of morphine tolerance (Wilson et al., 2008). These results suggest that cellular or synaptic adaptations occur within the PAG after chronic drug treatment.
This study is the first to demonstrate the high degree of co-localization of the CB1 and MOP receptors in a brain area that is critically involved in descending pain modulation and opioid tolerance. These results are consistent with previous findings that CB1 and MOP receptors co-localize in somatodendritic profiles in the dorsal horn of the spinal cord (Salio et al., 2001). The co-localization of these receptors indicates that behavioral enhancement of antinociception may be at least partially explained by intracellular signaling changes within these neurons. Because low-dose combination of opioids and cannabinoids does not alter receptor-mediated G-protein activation (Smith et al., 2007), antinociceptive enhancement between opioids and cannabinoids is more likely to occur as a result of altered downstream signaling such as cAMP or mitogen-activated protein kinases (MAPK) (Childers et al., 1992, Macey et al., 2009, Howlett et al., 2010).

Our results also demonstrate the presynaptic localization of CB1 receptors on axons and axon terminals in the dorsolateral and ventrolateral PAG, which is consistent with previous reports of CB1 expression (Tsou et al., 1998, Salio et al., 2001, Scavone et al., 2010). Furthermore, our results show that some MOR-ir cells receive appositions from CB1-ir profiles, which are presumed to be presynaptic. Networks consisting of MOPr-expressing cells receiving inputs from CB1-expressing axon terminals could thus undergo synaptic adaptations that underlie behavioral interactions between cannabinoids and opioids after chronic drug treatment. GABAergic disinhibition by cannabinoids and opioids in the PAG underlies antinociception (Depaulis et al., 1987,
Osborne et al., 1996, Vaughan and Christie, 1997), and the majority of CB1-ir terminals in this study formed symmetrical (inhibitory) synapses. Taken together, these results suggest that CB1/MOR appositions are highly likely to occur in GABAergic networks within the PAG. Dual-label electron microscopic studies of these presynaptic CB1r- and postsynaptic MOPr-expressing sites will provide a more precise measure of the relative role these networks play in behavioral cannabinoid/opioid interactions.

Given that the dorsolateral PAG is involved in cannabinoid-mediated stress-induced antinociception (Hohmann et al., 2005) and produces better cannabinoid antinociception than the vlPAG (Lichtman et al., 1996), sub-regional differences in the pattern of MOR and CB1 immunoreactivity would be expected. There was a surprising degree of consistency of co-localization and appositions between the dorsolateral and ventrolateral PAG, as well as the amount and pattern of CB1-immunolabeling at the ultrastructural level. Thus, PAG sub-regional differences in antinociceptive potency (Martin et al., 1995) and flight responses (Morgan et al., 1998) seem unrelated to the pattern or extent of CB1 receptor expression in the PAG. The differential behavior driven by these two sub-regions is more likely the result of differential outputs from these regions.

In the spinal cord and reward-related brain regions, chronic opioid exposure increases the expression of the CB1 receptor (Lim et al., 2005, Fattore et al., 2007). In the current
study, neither chronic opioid nor cannabinoid exposure had any effect on the density of CB1 expression in the dorsolateral or ventrolateral PAG. These results suggest that the up-regulation of CB1 receptors after chronic morphine exposure may be region-specific, or dependent upon the drug administration method. Thus, antinociceptive enhancement or tolerance prevention between cannabinoids and opioids seems unrelated to receptor density.

Cannabinoids and opioids interact in a number of ways that could be therapeutically beneficial. The results of this study confirm the co-localization of the CB1r and MOPr in the PAG, which is critically involved in the development of opioid tolerance. The potential for cannabinoids to enhance morphine antinociception and attenuate morphine tolerance warrants the investigation of these mechanisms at the cellular and molecular level.

Acknowledgments

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antibody. The authors would also like to thank Sam Hermes and James Huang for their technical assistance.
Figure 1: By light microscopy, diffuse CB1 labeling is found in ventrolateral and dorsolateral PAG. The black boxes delineate the areas of the ventrolateral and dorsolateral PAG that were imaged for confocal and electron microscopic analysis. The representative diagram of the PAG is modified from the digital atlas of Paxinos and Watson [Paxinos and Watson, 2005] and is reproduced here with permission from the publisher. Scale bar = 2mm
Figure 2: Confocal micrographs show immunofluorescent labeling of CB1 (A, green) and NeuroTrace staining (B, red) of cells in the ventrolateral PAG. C: An overlay image shows CB1-labeled cells receiving appositions from CB1-labeled varicosities (arrows). Scale bar = 10µm for panels A – C.
Figure 3: Electron micrograph demonstrates presynaptic as well as axon terminal CB1-gold immunolabeling. “T” indicates a CB1-gold labeled cell forming a symmetrical synapse (arrow) onto a CB1-labeled dendrite (“D”). Scale bar = 1 μm.
Figure 4. Confocal micrographs of CB1 (A, green), NeuroTrace (B, red) and MOR (C, magenta) in PAG. D: The overlay image shows a NeuroTrace-stained cell that is CB1- and MOR-ir and is receiving appositions from CB1-labeled varicosities. Scale bar = 5µm for panels A – D.
Figure 5. Density of CB1 immunoperoxidase labeling in ventrolateral (vl) and dorsolateral (dl) PAG from animals chronically treated with morphine or THC. The densities of CB1 labeling in morphine- and THC-treated rats are expressed as a percent of the density measured in vehicle control rats (dotted line, 100%). Chronic morphine or THC treatment had no significant effect on the density of CB1 immunoperoxidase labeling in either the vlPAG or dlPAG.
TABLES

Table 1
Antinociceptive tolerance to repeated THC (10mg/kg) and morphine (5mg/kg) injections
______________________________________________________________________
Average hot plate latencies (s)
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Table 2
MOR1 and CB1 distribution in the dorsolateral and ventrolateral PAG
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CHAPTER SIX: Lack of cellular cross-tolerance between opioids and cannabinoids in the rat periaqueductal gray

ABSTRACT

Cannabinoids and opioids produce antinociception, in part, by influencing GABAergic synaptic transmission in the descending pain pathway, particularly in the midbrain periaqueductal gray (PAG). Because chronic opioids and cannabinoids interact on the behavioral level to enhance antinociception and attenuate tolerance, it is plausible that these effects are mediated by altered GABAergic transmission in the PAG. Using in vitro electrophysiology techniques, this study tested the hypotheses that opioid tolerance improves cannabinoid antinociception as a result of altered GABAergic transmission in the PAG. In slices from untreated animals WIN 55,212-2 reduced the amplitude of electrically evoked inhibitory postsynaptic currents (eIPSCs) with an IC$_{50}$ of approximately 30 nM. The inhibition of eIPSCs produced by WIN 55,212-2 (30 nM) was similar in morphine and vehicle-pretreated animals. These results indicate a lack of cellular cross-tolerance between opioids and cannabinoids at the level of GABAergic presynaptic terminals in the PAG.
INTRODUCTION

Opioids and cannabinoids are well known for their antinociceptive properties. Both classes of drugs produce antinociception, in part, by activating a descending pain modulatory system that originates in the midbrain periaqueductal gray (PAG). Opioids can directly hyperpolarize PAG cells by activating an inwardly-rectifying K⁺ conductance (Chieng and Christie, 1994a), however their antinociceptive effects have also been linked to presynaptic mechanisms. GABAergic interneurons in the PAG exhibit a tonic level of firing that inhibits output neurons projecting to the rostral ventromedial medulla (Basbaum and Fields, 1984, Fields et al., 1991). Opioids inhibit GABA release in these interneurons by activating voltage-sensitive potassium channels (Kᵥ) on pre-synaptic nerve terminals (Chieng and Christie, 1994b, Vaughan and Christie, 1997, Vaughan et al., 1997). The disinhibition of GABAergic output neurons is a critical component of morphine antinociception in the PAG (Moreau and Fields, 1986, Vaughan and Christie, 1997), and the desensitization of Kᵥ channels is associated with the development of morphine tolerance (Christie et al., 1987). Although the precise mechanisms of opioid tolerance are elusive, recent experimental data have shown that cannabinoid pretreatment can attenuate the development of opioid tolerance, both systemically and when the drugs are microinjected into the PAG (Smith et al., 2007, Wilson et al., 2008). Furthermore, cannabinoid antinociception is enhanced in morphine-tolerant animals compared with morphine-naïve animals (Chapter 3, Rubino et al., 1997, Cichewicz and Welch, 2003). This bi-directional relationship between cannabinoids and opioids is
potentially of therapeutic value, and the mechanisms underlying this interaction could provide insight on the mechanisms of morphine tolerance.

Previous research has shown that in vitro superfusion of either cannabinoids or opioids decreases the amplitude of electrically evoked GABAergic inhibitory postsynaptic currents (eIPSCs) in PAG neurons (Vaughan and Christie, 1997, Vaughan et al., 2000, Fyfe et al., 2010). Furthermore, prolonged opioid exposure attenuates the amplitude of DAMGO-induced inhibition of evoked IPSCs, which suggests that evoked IPSC attenuation is a cellular correlate of behavioral antinociceptive tolerance (Fyfe et al., 2010). The effects of in vitro cannabinoids in morphine tolerant tissue have not been investigated. The purpose of this study was to test the hypothesis that opioid enhancement of cannabinoid antinociception is a result of altered GABAergic transmission in the PAG. Given the bi-directional antinociceptive enhancement between opioids and cannabinoids, it would be expected that cannabinoid-induced eIPSC amplitude would be greater in opioid-tolerant neurons than in saline-pretreated neurons.

**MATERIALS AND METHODS**

*Morphine tolerance induction*

For in vitro experiments, 16- to 25-d-old male and female Sprague-Dawley rats were used. Some of these animals were injected twice daily for two days with either 5 mg/kg s.c. morphine sulfate (N = 8, National Measurement Institute, Sydney, Australia) or an
equivalent volume of saline vehicle (N = 9). Rats were returned to their home cage after each injection, and were used for \textit{in vitro} experiments the day after the final pre-treatment injection. All animal protocols were approved by the Royal North Shore Hospital institutional Animal Care and Ethics committee.

\textit{Slice preparation}

Rats (12-36 d old) were deeply anesthetized with isoflurane and decapitated. Brains were quickly removed and submerged in ice-cold artificial cerebrospinal fluid (ACSF, in mM; 126 NaCl, 2.5 KCl, 1.4 NaH$_2$PO$_4$, 1.2MgCl$_2$, 2.4CaCl$_2$, 11 glucose, and 25 NaHCO$_3$). Coronal midbrain sections (300 $\mu$m) that contained the PAG were cut using a vibratome (VT1000S, Leica Microsystems) in ice-cold ACSF. PAG slices were maintained at 34°C, submerged in ACSF and equilibrated with 95% O$_2$ and 5% CO$_2$. Slices were then individually transferred to the recording chamber and superfused continuously (1.8 mL/min) with 34°C ACSF.

\textit{Electrophysiology}

PAG neurons were visualized using infrared Nomarski optics on an upright microscope (BX51WI, Olympus, Sydney, Australia). Data were collected using an Axopatch 700B amplifier (Molecular Devices) with an internal solution containing (in mM): 140 CsCl, 10 EGTA, 5 HEPES, 2 CaCl$_2$, 2 MgATP, and 3 QX-314 (pH 7.3; osmolarity, 270 –290 mOsm/L). Series resistance (< 20 MΩ) was compensated by 80% and continuously
monitored during experiments. Neurons were voltage-clamped at -70 mV, and postsynaptic currents were evoked every 12 s using a bipolar tungsten-stimulating electrode (tip separation 100 µm) placed immediately inside the outer boundary of the vlPAG (50–200 µm from the recording electrode). The stimulation strength (1–70 V, 40 – 400 µs) was adjusted until consistent postsynaptic currents were obtained (between 500 and 2000 pA). Drugs were applied by bath superfusion, in the presence of the non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX) (5 µM) and the glycine receptor antagonist strychnine (5 µM), to ensure isolation of GABA<sub>Δ</sub>-mediated evoked inhibitory postsynaptic currents (eIPSCs, Vaughan and Christie, 1997). These eIPSC were abolished by the GABA<sub>Δ</sub> antagonists SR95531 (Gabazine 10 µM, Figure 2, N = 43) and bicuculine (100 µM, Figure 3a, b, N = 14).

Because a CsCl-based internal solution was used in these experiments, the intracellular/extracellular chloride concentrations were approximately equimolar, resulting in inward eIPSCs currents. IPSCs were filtered (2 kHz low-pass filter) and sampled (10 kHz) for on-line and later off-line analysis (Axograph X; Axograph Scientific Software). All numerical data are expressed as mean ± SEM. Statistical comparisons of drug effects were made using Student’s t test (StatPlus), and differences were considered significant if <i>p</i> < 0.05.
Drug solutions

DAMGO, CNQX and strychnine hydrochloride were obtained from Sigma (Sydney, Australia); 1-(2,4-dichlorophenyl)- 5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1 H-pyrazole-3-carboxamide (AM251), (-)-bicuculline methiodide, and SR95531 (Gabazine) from Ascent Scientific (Bristol, UK); naloxone hydrochloride and WIN 55,212-2 from Tocris Bioscience (Bristol, UK); QX-314 bromide from Alomone Laboratories. Stock solutions of drugs were made in distilled water, except bicuculine (added directly to ACSF), AM251, and WIN 55,212-2 (in DMSO), then diluted to working concentrations using ACSF immediately before use and applied by superfusion.

RESULTS

First, the effect of WIN 55,212-2 on GABAergic synaptic transmission was examined in drug-naïve vlPAG neurons. Whole-cell voltage clamp recordings were made from neurons of various size and morphology throughout the vlPAG (N = 33). Superfusion of the pan-cannabinoid receptor agonist WIN 55,212-2 (10 nM – 3 µM) produced a reduction in the amplitude of eIPSCs which was reversed following addition of the CB1 receptor antagonist AM251 (3 µM, Figure 1a,b). The WIN 55,212-2 induced reduction in eIPSC amplitude was concentration-dependent, with an IC_{50} of 27 nM (90 % confidence interval 6 – 69 nM) and a Hill-slope of -1.6 ± 0.6 (Figure 1c). At a concentration of 30 nM, WIN 55,212-2 reduced the eIPSC amplitude to 73% ± 7% of pre-drug levels (N = 6).
Previous research has shown that the ability of opioids to attenuate eIPSC amplitude is reduced in morphine-tolerant PAG neurons (Fyfe et al., 2010). In the current experiment however, superfusion of DAMGO (100 nM) reduced eIPSC amplitude to a similar extent in neurons from morphine-pretreated rats (60% ± 9% of pre-DAMGO) compared to vehicle-pretreated rats (52% ± 7% of pre-DAMGO; t(9) = 2.26, p = 0.53, Figure 2a, b, 3). These results indicate a lack of cross-tolerance between morphine and DAMGO.

To assess effect of chronic morphine treatment on cannabinoid attenuation of eIPSCs, an EC\textsubscript{50} concentration of WIN 55,212-2 (30 nM) was superfused onto slices from vehicle- and morphine-pretreated rats (Figure 2c, d). WIN 55,212-2 reduced the eIPSC amplitude to a similar extent in PAG neurons from vehicle-pretreated (65% ± 5% of pre-WIN55,212-2) and morphine-pretreated groups (67% ± 7% of pre-WIN55,212-2; t(8) = 1.86, p = 0.42, Figure 3).

**DISCUSSION**

The results of this study support previous research that demonstrates WIN 55,212-2 to inhibition of GABAergic transmission in the PAG, via CB1 receptors (Vaughan et al., 2000). The similarity of WIN-induced effects between morphine-pretreated and vehicle-pretreated cells would seem to indicate that cellular cross-tolerance does not develop between opioids and cannabinoids in the vlPAG. However, morphine also failed to
produce cross-tolerance to DAMGO, which is contrary to previous findings (Fyfe et al., 2010).

Previous studies have suggested that cannabinoids and opioids produce antinociception, in part, by inhibiting GABAergic transmission in the descending pain pathway (Heinricher et al., 1991, Vaughan and Christie, 1997, Vaughan et al., 1999, Vaughan et al., 2000). This study supports these findings, given that WIN 55,212-2 was able to decrease the amplitude of electrically evoked IPSCs from neurons in the PAG. This effect was mediated by the CB1 receptor, because eIPSC inhibition was reversed by addition of the CB1 receptor antagonist AM251. These results also provide a functional role for the expression of CB1 receptors in the PAG observed in anatomical studies (Herkenham et al., 1991, Mailleux and Vanderhaeghen, 1992, Tsou et al., 1998).

The lack of cellular cross-tolerance between morphine and DAMGO in this study is surprising, given the similarity between the morphine tolerance protocols of the current and previous studies (Fyfe et al., 2010). Nonetheless, opioid inhibition of eIPSCs is sensitive to the pretreatment protocol, due to the withdrawal-induced increase of cAMP (Ingram et al., 1998). The opposing effects of tolerance and withdrawal observed in slice preparation are also seen at the postsynaptic level for opioids (Bagley et al., 2005). Thus, slight differences in morphine pretreatment protocols could have significant
effects on subsequent in vitro opioid effects. The lack of cross-tolerance to DAMGO in the current study may also be related to differences in the animals used in these studies, which were conducted in different countries.

Despite the lack of cross tolerance to DAMGO, the failure of chronic morphine treatment to reduce WIN-induced effects on GABAergic transmission in PAG neurons could indicate a lack of cellular enhancement or cross-tolerance between morphine and WIN 55,212-2. A lack of cellular cross-tolerance would be expected, given the aforementioned ability of morphine pretreatment to enhance cannabinoid antinociception (i.e., behavioral cross-sensitization as opposed to cross-tolerance). Alternatively, because cannabinoids can also reduce glutamate transmission in the PAG (Vaughan et al., 2000), it is possible that morphine tolerance may have greater effects on WIN-induced glutamate transmission than GABA transmission. However, the effect of cannabinoids on glutamate transmission in morphine-tolerant tissue has not been examined.

The bi-directional antinociceptive enhancement between opioids and cannabinoids has beneficial therapeutic potential in human pain management. Determining the cellular correlates of behavioral cross-sensitization after chronic drug treatment is a critical step in utilizing combined cannabinoid/opioid paradigms in therapeutic pain management, and thus warrants further investigation. These effects are particularly important to study.
in the PAG because of the critical role this brain region plays in the development of
opioid tolerance.

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**Figure 1**: WIN 55,212-2 decreases eIPSC amplitude in a concentration-dependent manner. **A**: Timecourse of evoked IPSC amplitudes during superfusion of WIN 55, 212-2 (WIN, 30 nM and 1 µM), the CB1 receptor antagonist AM251 (3 µM), and Gabazine (10 µM). Each point is the mean of two consecutive evoked IPSCs. **B**: Averaged traces
of eIPSCs before (control) and during WIN 55,212-2, AM251, and Gabazine superfusion. The 30 nM concentration inhibited eIPSC amplitude by 73% ± 7% of control C: Concentration-response analysis for WIN 55,212-2-mediated inhibition of eIPSC amplitude in drug-naïve PAG neurons resulted in an IC50 of 27 nM (N = 33, 3-6 cells per concentration: 1 nM, - 3 µM).
Figure 2: Morphine tolerance has no effect on the magnitude of DAMGO or WIN 55,212-2-mediated inhibition of eIPSC amplitude. **A-B:** Averaged traces of eIPSCs before (control) and during DAMGO (100 nM), naloxone (1 µM) and bicuculine (100 µM) superfusion. **C-D:** Averaged traces of eIPSCs before (control) and during WIN 55,212-2
(30 nM, AM251 (3 μM), and Gabazine (10 μM) superfusion, in neurons from vehicle-pretreated rats (A, C) and morphine-pretreated rats (B, D).
**Figure 3:** Repeated morphine administration has no effect on the magnitude of DAMGO or WIN 55,212-2-mediated inhibition of eIPSC amplitude. Bar graph indicates the mean amplitude of eIPSCs recorded from vIPAG neurons in either vehicle (open bars) or morphine-pretreated rats (black bars), expressed as a percentage of the pre-drug control (% Pre) level. There was no statistically significant difference in mean eIPSC amplitude between morphine and vehicle-pretreated cells, for either DAMGO or WIN 55,212-2 experiments.
REFERENCES


CHAPTER SEVEN: GENERAL DISCUSSION

Both opioids and cannabinoids exert their effects, in part, by activating a descending pain modulatory pathway that originates in the PAG, projects to the RVM, and on to the dorsal horn of the spinal cord. The opioid and cannabinoid systems share many characteristics and mechanisms. For instance, the expression of CB1 and mu-opioid receptors has been well characterized in the descending pain pathway (Herkenham et al., 1991, Malleux and Vanderhaeghen, 1992, Kalyuzhny et al., 1996, Tsou et al., 1998, Wang and Wessendorf, 1999, Commons et al., 2000). Also, microinjections of either drug in the descending pain pathway produce antinociception (Morgan and Whitney, 2000, Finn et al., 2003, Meng and Johansen, 2004, Morgan et al., 2006). It is also well established that GABAergic disinhibition plays a major role in both cannabinoid and opioid descending pain modulation (Moreau and Fields, 1986, Vaughan and Christie, 1997, Vaughan et al., 2000).

However, cannabinoid/opioid interactions in descending pain modulation remain poorly understood. The aim of the studies described in Chapters 2 -5 was to characterize the interaction between opioids and cannabinoids in the descending pain modulatory pathway. This included analysis at the levels of behavioral pharmacology, anatomy, and physiology. The behavioral studies described here demonstrate that repeated morphine microinjections in the rat PAG enhance subsequent cannabinoid antinociception, and
vice versa (Wilson et al., 2008). This bi-directional enhancement between the drugs could be of great therapeutic value for patients undergoing opioid pharmacotherapy. Furthermore, microinjections of cannabinoids into the PAG also attenuate the development of morphine tolerance (Wilson et al., 2008). This is a key finding that highlights a mechanism by which opioid tolerance can be mitigated. Cannabinoid attenuation of morphine tolerance could reduce the need for dose escalation that is common with the development of opioid tolerance. Dose reduction, in turn, may also reduce additional negative side effects of opioids, including nausea, constipation, and abuse potential.

However, when cannabinoids are repeatedly injected into the RVM, they cause neurotoxic lesions. This finding was surprising, given the aforementioned effects of microinjecting cannabinoids into the PAG. Although previous studies have demonstrated the ability of cannabinoids to activate intracellular cell-death signaling cascades (De Laurentiis et al., 2010, Mukhopadhyay et al., 2010, Yang et al., 2010), none of these studies have included the PAG or RVM. Considering 1) the important role that these brain regions play in descending pain modulation and the development of tolerance, and 2) the therapeutic potential of cannabinoids, it would be critical to determine the cell-death risks associated with cannabinoid receptor activation. Nonetheless, acute (as opposed to repeated) co-administration of morphine and cannabinoids in the RVM produces greater antinociception than when either drug is
administered alone, which is consistent with earlier reports of antinociceptive synergy between the drugs (Smith et al., 1998, Cichewicz and McCarthy, 2003, Tham et al., 2005, Roberts et al., 2006).

The neural mechanisms underlying the bi-directional antinociceptive enhancement between cannabinoids and opioids remain poorly understood. An understanding of the morphology of the CB1 and mu-opioid receptors would provide a significant contribution to characterizing the interaction of these systems. Although the mu-opioid receptor has been well studied at the ultrastructural level in the PAG (Commons et al., 2000), the ultrastructural distribution of the CB1 receptor has not. In the current work, confocal and electron microscopy revealed that CB1 expression in the PAG is largely somatodendritic, which is surprising given the well-established role of endocannabinoids as retrograde messengers (Kreitzer and Regehr, 2001, Maejima et al., 2001, Wilson and Nicoll, 2001). This finding suggests that the CB1 receptor may have additional roles in the PAG. For instance, given their ability to activate Ca\(^{2+}\) and K\(^{+}\) conductances (Howlett et al., 2010), cannabinoids could function as overall modulators of neuronal excitability.

Many cellular and molecular convergence points between the opioid and cannabinoid systems depend upon their co-localization in the same cell (Macey et al., 2009, Howlett et al., 2010). The studies in Chapter 4 are the first to demonstrate CB1 and mu-opioid
receptor co-localization in this region. CB1 and mu-opioid receptors co-localized on 32% of PAG neurons, which indicates that interactions between opioids and cannabinoids could occur within the same neuron. Because the G-protein activation and extracellular related kinase (ERK) cascades are similar in opioid and cannabinoid receptor activation (Howlett et al., 2010, Macey et al., 2010, Parolaro et al., 2010), the results of this study provide an anatomical substrate at which these molecular interactions could occur. A subset of mu-opioid-labeled cells received appositions from (presumed presynaptic) CB1 receptor profiles, and ultrastructural analysis confirmed the presence of CB1-labeled axon terminals in the PAG. Thus, synaptic adaptations after chronic drug treatment could occur where CB1 terminals synapse onto mu-opioid cells (see below).

These findings are in line with previous studies demonstrating the role of pre-synaptic CB1 receptors in neurotransmitter release and retrograde signaling (Vaughan et al., 2000, Kreitzer and Regehr, 2001, Maejima et al., 2001, Wilson and Nicoll, 2001).

Synaptic adaptations after chronic drug treatment were next examined using the in vitro whole-cell patch clamp method. Previous research has shown that both cannabinoids and opioids can reduce GABAergic transmission in the PAG (Chieng and Christie, 1994, Vaughan et al., 1997, Vaughan et al., 2000, Vaughan et al., 2003), and that the ability of opioids to reduce GABAergic transmission is diminished in morphine tolerant animals (Fyfe et al., 2010). The experiments in Chapter 5 however, are the first to examine the effects of in vitro cannabinoids in morphine-tolerant tissue. The results of this study
demonstrate that chronic morphine has no effect on cannabinoid inhibition of GABAergic transmission. These results indicate a lack of cellular enhancement or cross-tolerance between opioids and cannabinoids even though antinociceptive enhancement is evident between the drugs. Alternatively, GABA signaling may not underlie the behavioral interactions between opioids and cannabinoids, thus highlighting the need to examine changes in glutamatergic transmission after chronic drug exposure. However, because this study also failed to find cross-tolerance between morphine and DAMGO in the same animals, these conclusions remain speculative.

The studies described in this body of work have significantly contributed to the knowledge base of cannabinoid/opioid interactions. They have provided evidence for a therapeutically advantageous drug interaction, in a brain region that is critical for pain modulation and opioid tolerance. Furthermore, they have provided an anatomical substrate for molecular interactions between CB1 and mu-opioid receptor signaling. These studies also have highlighted a need to distinguish the potentially neurotoxic effects of cannabinoids from their therapeutic effects. Finally, these studies have laid the groundwork for the synaptic adaptations that occur after chronic drug treatment. Future studies will include an examination of the molecular signaling events underlying the bi-directional enhancement of antinociception between opioids and cannabinoids. These adaptations will be further characterized in GABAergic networks, and expanded to include glutamatergic networks as well.
REFERENCES


