Modulation of the Endocannabinoid System Attenuates Naloxone-Precipitated Morphine Withdrawal-Induced Place Aversions in Acutely Dependent Rats

by

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ABSTRACT

MODULATION OF THE ENDOCANNABINOID SYSTEM ATTENUATES NALOXONE-PRECIPTATED MORPHINE WITHDRAWAL-INDUCED PLACE AVERSIONS IN ACUTELY DEPENDENT RATS

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Modulation of the endocannabinoid system is effective in reducing somatic symptoms of opioid withdrawal, however, much less is known regarding its ability to reduce affective opioid withdrawal. Given that the brain regions mediating somatic and affective opioid withdrawal are dissociable, this dissertation aimed to evaluate the ability of CB1 receptor modulation to reduce affective opioid withdrawal in acutely dependent Sprague Dawley rats.

Affective opioid withdrawal can be quantified using a naloxone-precipitated morphine withdrawal (MWD) induced conditioned place aversion (CPA). Rats are made acutely dependent with a single high dose of morphine and withdrawal is precipitated 24 hours later with naloxone. Using this paradigm, the ability of systemically administered CB1 receptor antagonists/neutral antagonists (AM251, AM4113, AM6527) and inhibitors of endocannabinoid hydrolysis (fatty acid amide hydrolase, FAAH; monoacylglycerol lipase, MAGL) were evaluated to prevent the establishment of the MWD CPA. All CB1 receptor antagonists tested and the MAGL inhibitor (elevates 2-arachidonoylglycerol; 2-AG), MJN110, interfered with the MWD CPA. The two FAAH inhibitors tested (elevates anandamide; AEA), PF3845 and URB597, were without significant effect.
An evaluation of the brain regions mediating the systemic effects of these compounds revealed a double dissociation of CB₁ receptor antagonism and agonism to reduce establishment of the MWD CPA in the extended amygdala and associated regions. Specifically, the CB₁ receptor antagonist, AM251, interfered with the CPA when microinfused into the central nucleus of the amygdala (CeA) and the bed nucleus of the stria terminalis (BNST), whereas the MAGL inhibitor, MJN110, interfered with the CPA when microinfused into the basolateral amygdala (BLA) and the interoceptive insular cortex (IC).

The ability of endocannabinoid modulation to reduce affective opioid withdrawal is discussed. Ultimately, the findings presented suggest that modulation of the endocannabinoid system may have therapeutic potential in the treatment of affective opioid withdrawal.
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CHAPTER 1

General Introduction

Opiate addiction is a chronic brain disorder characterized by plastic neurobiological changes that result in compulsive drug use, recurrent drug seeking and taking despite negative consequences, and a loss of control in limiting drug intake (Camí & Farré, 2003). In 2010, it was estimated that 15.5 million people worldwide met criteria for opioid dependence (Degenhardt et al., 2014). With the recent ‘opioid epidemic’ due to the introduction and campaign for the pharmaceutical opioid analgesic OxyContin in 1995, there has been a reported 900% increase in the number of individuals seeking opioid addiction treatment services (Kolodny et al., 2015). Even so, treatment for opiate addiction has poor outcomes and success rates (Bart, 2012) and often requires long-term maintenance therapy with synthetic opioids which themselves possess abuse potential and only mask the underlying problem (Stotts, Dodrill, & Kosten, 2009). Owing to the high abuse liability of opioids and the lack of effectiveness of current pharmacological treatments in the management of dependence, a better understanding of the neurobiology of opioid addiction and the exploration of new treatment options is warranted.

Due to the ability of all drugs of abuse to increase extracellular dopamine, the first theories of addiction focused on elucidating the role of critical dopaminergic synapses within the central nervous system. The culmination of this research has led to the description of an important mesocorticolimbic reward circuit, a neural network interconnecting dopaminergic neurons in the ventral tegmental area (VTA) to cortical regions (including the prefrontal cortex [PFC], orbitofrontal cortex [OFC] and anterior cingulate) and limbic structures (including the nucleus accumbens [NAcc], ventral pallidum [VP], hippocampus and amygdala) (Feltenstein & See, 2009). Today, the progression and intricacies of drug addiction can be attributed to
neurochemical alterations occurring within this reward circuit and numerous studies have pointed to the involvement of dopamine, glutamate, GABA, noradrenaline, serotonin and various neuropeptides as contributing to the addictive process (Bardo, 1998; Hyman, Malenka, & Nestler, 2006; Koob et al., 2004; Ross & Peselow, 2009). Recently, of growing interest, is the involvement of the endocannabinoid system in the etiology and maintenance of drug addiction (Serrano & Parsons, 2011). In particular, a large body of evidence reveals a significant cannabinoid-opioid interaction in mediating opiate dependence. Consequently, an elucidation of the interaction between these two systems and the contribution of the endocannabinoid system in ameliorating opiate addiction will be the focus of the current dissertation.

**Opiate Addiction: A Multi-Motivational Disorder**

Opiate addiction has been characterized as a motivational disorder involving both impulsive and compulsive features (Goodman, 2008). The impulsivity of drug addiction may better be described as positive reinforcement wherein the rewarding properties of the drug increase its frequency of use, whereas the compulsivity of drug addiction may better be described as negative reinforcement wherein the aversive properties associated with drug abstinence lead to an increase in drug use (Koob & Le Moal, 2001). An individual may typically begin using a drug for medical purposes or to experience its acute positive rewarding and euphoric properties. Subsequent drug use then develops as a user impulsively seeks the drug to produce pleasure and gratification (Wise, 1980). As neurobiological changes occur in the nervous system in an attempt to counteract the drug’s perturbations and maintain a stable hedonic state, the user enters into an allostatic state (Koob & Le Moal, 1997) where tolerance to the drug’s positive reinforcing effects builds and the user begins to self-administer increasingly larger doses in order to achieve the same gratifying effects (Feltenstein & See, 2009). During this
time, drug use shifts from a predominantly impulsive disorder to one involving compulsivity motivated by negative reinforcement (Bechara, Nader, & Van Der Kooy, 1998; Goodman, 2008). Specifically, due to the neurobiological adaptations which occurred to counterbalance drug use, drug abstinence now results in an aversive physical and affective withdrawal state reflective of the body’s inability to maintain stability without drug usage (Goodman, 2008; Koob & Le Moal, 1997). The addict now also seeks the drug in an attempt to avoid and alleviate such aversive effects (Camí & Farré, 2003). In addition to the primary positive and negative reinforcement that contributes to the process of drug addiction, secondary positive and negative reinforcement also develops through classical conditioning (Koob & Le Moal, 2001). Secondary reinforcement occurs when a neutral stimulus is repeatedly paired with the primary reinforcer such that exposure/avoidance of the once neutral stimulus, now the conditioned stimulus, strengthens the occurrence of drug use in much the same way as the primary positive/negative reinforcer, respectively. For example, secondary negative reinforcement occurs through the removal of the conditioned negative reinforcing effects associated with conditioned withdrawal (Koob & Le Moal, 2001).

The plastic neurobiological changes that occur in the central nervous system during drug use in an attempt to maintain homeostasis are suspected to underlie the persistent nature of addiction (De Vries & Shippenberg, 2002). In particular, it has been suggested that the conditioned reinforcing effects seen in drug addiction may play a role in drug relapse (Koob & Le Moal, 2001). The craving experienced by addicts which promotes drug relapse even long after drug withdrawal has ceased has been shown to be induced by re-exposure to the drug, drug-associated cues, or stress (Shaham, Shalev, Lu, De Wit, & Stewart, 2003). This is consistent with
Pavlov’s theory of classical conditioning in which reinstatement of a learned behavior occurs following a priming presentation of a reinforcing stimulus (Shaham et al., 2003).

Although the process of drug addiction is far more complex than outlined above, many theorists agree that addiction is a multi-motivational disorder with impulsive drug reward and compulsive alleviation of drug withdrawal as primary reinforcers and secondary reinforcers contributing to drug relapse.

**The Endogenous Opioid System and Addiction**

The endogenous opioid receptors belong to the superfamily of seven transmembrane domain receptors and include the three well characterized receptor subtypes μ, δ, and κ. These receptors are bound by the endogenously produced peptides endorphin, enkephalin and dynorphin, which are derived from the precursors proopiomelanocortin (POMC), proenkephalin (PENK) and prodynorphin (PDYN), respectively (Trigo, Martin-García, Berrendero, Robledo, & Maldonado, 2010). Endorphins bind to μ and δ receptors with comparable affinity, while enkephalins preferentially bind to δ receptors and dynorphins to κ receptors (Koneru, Satyanarayana, & Rizman, 2009). More recently, an additional opioid receptor, the ORL 1 receptor, and its putative endogenous ligand, nociceptin/orphanin FQ (OFQ), have been identified (Koneru, Satyanarayana, & Rizman, 2009). Activation of these receptors leads to an inhibition of cAMP production and voltage gated Ca\(^{2+}\) channels, and stimulation of potassium channels and the MAP kinase cascade, resulting in a reduction of neurotransmitter activity and release (Trigo et al., 2010). Opioid receptors and their peptides are found throughout the central and peripheral nervous system including brain reward centers such as the NAcc, VTA, PFC, hypothalamus and the extended amygdala, where they modulate natural reward processes.
However, when exogenous opiates such as morphine are administered, the delicate balance becomes disrupted and leads to the aberrant behavior known as addiction.

Most commonly abused opiates, including morphine and heroin, are agonists of the μ opioid receptor. Indeed, activation of the μ-receptor has been linked to the high abuse liability of opioids (Devine & Wise, 1994) and is responsible for mediating the rewarding effects of morphine (Matthes et al., 1996). Within the mesocorticolimbic reward system, exogenous opioids facilitate DA release by acting on μ-opioid receptors in the NAcc and VTA. In the VTA, this is accomplished through presynaptic inhibition of GABA release onto DA neurons projecting to the NAcc (Johnson & North, 1992). This mechanism of action parallels that produced by cannabinoids within the VTA and may be one method by which these systems interact in reward processes.

The Endogenous Cannabinoid System and Addiction

The endocannabinoid system has recently been identified as one of the main neuromodulators of the central nervous system (López-Moreno, González-Cuevas, Moreno, & Navarro, 2008). It is composed of the well characterized CB1 receptor, found abundantly in the central nervous system (CNS) as well as in some peripheral tissues, and the CB2 receptor, located mainly in peripheral immune cells but also more recently identified in regions of the CNS (Maldonado, Valverde, & Berrendero, 2006). These receptors are bound by the endogenous cannabinoid agonists, anandamide (AEA) and 2-arachidonoylglycerol (2AG), which are degraded by the enzymes, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively (Palmer, Thakur, & Makriyannis, 2002). Endocannabinoids mediate their effects through retrograde signaling mechanisms whereby endocannabinoids released from a depolarized postsynaptic neuron travel in a retrograde manner to the presynaptic neuron where
binding to the receptor inhibits subsequent release of a variety of excitatory or inhibitory neurotransmitters. As such, the endocannabinoid system is involved in the regulation of many behavioral processes including reward (Mechoulam & Parker, 2013).

The endocannabinoid system has been implicated as serving a regulatory role in mediating reward processes (Gardner, 2005). This is not surprising since CB₁ receptors are found abundantly throughout the reward circuit (Maldonado et al., 2006). Indeed, endocannabinoids are thought to mediate the activation of dopaminergic neurons in the mesocorticolimbic pathway indirectly by modulating glutamatergic and GABAergic input into the VTA and glutamatergic activation in the NAcc (Maldonado et al., 2006). Analogous to the dysregulation of extracellular dopamine seen in drug addiction, chronic drug use has also been associated with a dysregulation of extracellular AEA and 2AG, including altered binding and functionality of the CB₁ receptor in many areas of the reward circuit (Fattore, Fadda, & Fratta, 2007).

Additionally, endocannabinoid activity is known to play a role in mediating short and long-term synaptic plasticity in a number of brain regions associated with the reward circuit including the VTA, NAcc and the PFC, as well as the amygdala and hippocampus which are thought to be important in reward associated learning and memory (Maldonado et al., 2006; Sidhpura & Parsons, 2011). Evidence suggests that many drugs of abuse cause a dysregulation of endocannabinoid mediated plasticity in many of these regions which is thought to contribute to the aberrant compulsive behavior seen in drug dependence and relapse (Sidhpura & Parsons, 2011).
**Cannabinoid and Opioid Interaction**

Having noted the endocannabinoid and opioid systems’ modulatory role in regulating reward processes, substantial evidence has implicated these systems as having reciprocal interactions within this pathway. For the purpose of this thesis, a review of the cross-modulatory effects of these systems on drug addiction will be described with a particular emphasis on dependence and reinstatement.

**Somatic Withdrawal.** Drug dependence can be described as the need to use a drug, with drug abstinence leading to withdrawal (Venkatesan & Suresh, 2008). In animals, the intensity of somatic withdrawal is quantified by scoring the presence or severity of several physical signs for 10-30 minutes immediately following precipitated withdrawal, or every 6-9 hours for several days following spontaneous withdrawal (Maldonado, Stinus, & Koob, 1996). To facilitate the quantification of the withdrawal syndrome, Gellert and Holtzman (1978) developed a weighted scale consisting of graded symptoms including percentage of weight loss, number of escape jumps, number of wet dog shakes, number of abdominal constrictions, and checked signs including diarrhea, facial fasciculations/teeth chattering, swallowing, salivation, chromodacryorrhea, ptosis, abnormal posture, erection/ejaculation/genital grooming, and irritability. In assessing the ability of pharmacological cannabinoid manipulations to alleviate the intensity of somatic withdrawal, most studies use a variation of this scale to determine whether individual signs or a global rating of withdrawal has significantly decreased.

Beginning with an evaluation of the ability of the opioid and cannabinoid systems to mutually precipitate somatic withdrawal, a study by Hirschhorn and Rosecrans (1974) found that rats chronically treated with delta-9 tetrahydrocannabinol ($\Delta^9$-THC; 4 mg/kg) showed symptoms of opiate withdrawal when given acute administration of the opioid antagonist naloxone (1
mg/kg), a finding which was later replicated in 1977 by Kaymakçalan, Ayhan, and Tulunay. Several decades later, these findings were paralleled with the results from a study by Navarro et al. (1998) reporting that acute administration of the cannabinoid antagonist SR141716A was able to precipitate withdrawal symptoms in morphine-dependent rats.

Moreover, evidence for the ability of the cannabinoid and opioid system to reciprocally attenuate somatic withdrawal has also been noted. Specifically, in 1975, Hine, Torrelio, and Gershon reported that acute administration of high doses of $\Delta^9$-THC (5 mg/kg and 10 mg/kg) attenuated naloxone-precipitated withdrawal symptoms in methadone dependent rats, a finding which was soon replicated in morphine-dependent mice (Bhargava, 1976a,b). The above findings were later extended by a number of studies reporting the ability of exogenously administered AEA (5mg/kg), 2AG (10 μg/ mouse), and the anandamide transport inhibitor AM404 (2 and 10 mg/kg), as being additionally effective in attenuating opioid withdrawal symptoms (Del Arco et al., 2002; Vela, Ruiz-Gayo, & Fuentes, 1995; Yamaguchi et al., 2001). More recently, Lichtman et al. (2001) confirmed the bidirectional relationship of these systems in a study demonstrating morphine’s ability to dose-dependently attenuate SR 141716A (10 mg/kg) precipitated withdrawal in $\Delta^9$-THC -dependent mice.

In addition to the above, a very recent study by Ramesh et al. (2011) reported that maximal inhibition of the endocannabinoid hydrolytic enzymes, FAAH and MAGL, has also been found to attenuate some symptoms of precipitated opioid withdrawal (jumping, paw tremors, but not diarrhea or weight loss) in mice in a CB$_1$ dependent manner. This finding is especially important since endocannabinoids, being produced ‘on demand’, have a greater specificity of action than exogenously administered cannabinoids and, therefore, would have less adverse effects as a treatment option for opiate addiction.
Although contradictory to the above, studies using knockout (KO) mice have provided additional support of a cross-dependence between these two systems. A study by Ledent et al. (1999) investigating the relevance of CB₁ receptors in opiate dependence reported that both the reinforcing properties of morphine and the severity of naloxone-precipitated morphine withdrawal were significantly reduced in CB₁ receptor KO mice, a finding that was later supported in a similar study by Lichtman et al. (2001). Related studies have also suggested a role for the opioid system in cannabinoid dependence. A study by Valverde, Maldonado, Valjent, Zimmer, and Zimmer (2000) revealed that SR141716A-precipitated withdrawal in Δ⁹-THC dependent mice is significantly reduced in pre-proenkephalin KO mice. Furthermore, μ-opioid receptor KO mice and double μ- and δ-opioid receptor KO mice that have been made Δ⁹-THC dependent show attenuated withdrawal symptoms (Castane, Robledo, Matifas, Kieffer, & Maldonado, 2003; Lichtman et al., 2001).

In agreement with the mutant mice KO studies, chronic (5 days) pharmacological blockade, but not acute blockade, of the CB₁ receptor with SR141716A (5 mg/kg) during the concurrent development of morphine dependence significantly attenuated naloxone-precipitated withdrawal behaviors (digging, teeth chattering, penile licking, diarrhea) while having no effect on morphine analgesia (Mas-Nieto et al., 2001; Rubino, Massi, Vigano, Fuzio, & Parolaro, 2000).

**Affective Withdrawal.** Although an investigation of the role of the opioid system in affective cannabinoid withdrawal is largely absent, a small literature does exist on the role of the cannabinoid system in affective opioid withdrawal. In evaluating the role of the cannabinoid system in affective opioid withdrawal, the conditioned place aversion (CPA) paradigm and the operant responding of food paradigm have been employed. The CPA paradigm typically
involves pairing naloxone-precipitated morphine withdrawal (in acutely or chronically dependent animals) with a specific environmental context, such that, upon re-exposure to this context in a drug-free state, animals will preferentially avoid the withdrawal paired context verses a context that was previously paired with a placebo saline injection (Sanchis-Segura and Spanagel, 2006). In the operant responding for food paradigm, a pharmacological treatment is deemed effective in reducing affective withdrawal if it is able to suppress a withdrawal-induced reduction in operant responding for food (Maldonado et al., 1996).

To date, manipulations of the cannabinoid system on affective opioid withdrawal have focused exclusively on antagonism of the cannabinoid system, reporting largely inconsistent findings. Indeed, the first study published reported the inability of the μ-opioid receptor antagonist, naloxone, to precipitate a morphine withdrawal-induced place aversion in morphine dependent CB₁ receptor KO mice (Ledent et al., 1999). Conversely, a similar experiment conducted only a year later with CB₁ mutant mice found no effect on the ability of naloxone-precipitated withdrawal to produce a place aversion (Martin, Ledent, Parmentier, Maldonado, & Valverde, 2000). Finally, acute pharmacological blockade of the CB₁ receptor with SR141716A was actually found to precipitate withdrawal in abstinent morphine dependent rats as indicated by its ability to produce a CPA and reduce operant responding for food (Navarro et al., 2001). Evidently, additional experiments will be required to elucidate the role of the cannabinoid system in affective opioid withdrawal.

It has been clearly demonstrated in the literature that a cross-dependence between the opioid and cannabinoid system exists, however, evidence regarding the direction (agonism or antagonism) by which the endocannabinoid system is capable of attenuating opioid dependence has been contradictory and remains to be elucidated (a review of all studies has been presented in
Moreover, research regarding the ability of the endocannabinoid system to attenuate the aversive affective motivational state of dependence is lacking even though it is an important component of dependence that needs to be addressed.

**Reinstatement.** Analogous to the cross-dependence noted, the cannabinoid and opioid system have also demonstrated a cross-modulatory effect on drug reinstatement. Reinstatement can be described as resumption of extinguished drug-seeking behavior following a priming dose of the drug, re-exposure to drug-associated cues, or stress (Shaham et al., 2003). Specifically, the cannabinoid agonists HU-210 (20 µg/kg), WIN 55,212-2 (0.15 and 0.3 mg/kg) and CP55, 940 (0.05 and 0.1 mg/kg), but not Δ⁹-THC (0.1 and 1 mg/kg), have been found to reinstate heroin-seeking following extinction training (De Vries, Homberg, Binnekade, Raasø, & Schoffelmeer, 2003; Fattore, Spano, Cossu, Deiana, & Fratta, 2003). A reciprocal effect was later confirmed in a study by Spano et al. (2004) which demonstrated that heroin (0.5 mg/kg) is also able to reinstate extinguished drug-seeking for the cannabinoid agonist WIN 55,212-2.

Blockade of drug-seeking behavior by inverse agonists/antagonists of these opposing systems has also been found. Indeed, in the same study, Spano et al. (2004) additionally noted the ability of naloxone (1 mg/kg) to block reinstatement of cannabinoid-seeking with a priming dose of the cannabinoid agonist WIN 55,212-2 (0.25 mg/kg). Correspondingly, SR141716A has been shown to dose-dependently (0.3 – 3 mg/kg) block reinstatement of extinguished heroin-seeking in rats following a priming dose of heroin (Fattore et al., 2003) and heroin associated cues (De Vries et al., 2003). Moreover, SR141716A has been implicated not only in preventing the reinstatement of heroin drug-seeking, but has also been shown effective in attenuating the reinstatement of many other drugs of abuse and non-drug food rewards (Sidhpura & Parsons, 2011).
As described above, the ability of a cannabinoid antagonist/inverse agonist to prevent positive motivational reinstatement of opiate drug-seeking has been well established, however, addiction is a disorder involving both positive and negative reinforcement. As a result, studies investigating the ability of a cannabinoid antagonist/inverse agonist to prevent negative motivational reinstatement are warranted.

**Potential mechanisms of interaction.** The primary receptors attributed to mediating the cannabinoid-opioid interaction are the μ-opioid receptor and the CB1 cannabinoid receptor. Although CB1 receptors are predominantly presynaptic, while μ-opioid receptors can be found both presynaptically and postsynaptically, activation of either receptor leads to the similar inhibition of a variety of neurotransmitter release (Häring et al., 2015; Howlett et al., 2002; Illés, 1989; Schlicker & Kathmann, 2001). Indeed, opioids and cannabinoids have been found to similarly increase dopamine release in the NAcc by presynaptically inhibiting GABA release onto dopaminergic neurons located in the VTA (Maldonado et al., 2006; Trigo et al., 2010). These receptors have also been found to be similarly expressed in the brain reward circuit (Solinas, Yasar, & Goldberg, 2007) and their functionality and density reciprocally altered through changes in activity. Specifically, chronic heroin administration results in increased CB1 receptor density and function in the reward pathway whereas decreased CB1 receptor function has been reported in μ-opioid receptor KO mice (Berrendero, Mendizabal, Murtra, Kieffer, & Maldonado, 2003; Fattore, Viganò, et al., 2007; Gonzalez et al., 2003). Similarly, chronic cannabinoid administration leads to increased μ-opioid receptor density and functionality in many areas of the reward circuit (Fattore, Viganò, et al., 2007). Additionally, co-localization of CB1 and μ-opioid receptor mRNA has also been observed in brain regions relevant for withdrawal such as the NAcc and the central nucleus of the amygdala (Navarro et al., 1998).
The exact neurobiological mechanisms through which these reciprocal effects are mediated remain to be elucidated yet several possibilities have been noted. First, cannabinoid and opioid receptor activation has been found to increase endogenous opioid peptide and endocannabinoid release, respectively (Serrano & Parsons, 2011). It is also possible that these effects may be mediated through a convergence or divergence of neurotransmitter release or signal transduction mechanisms (Fattore et al., 2005). Indeed, chronic treatment of the opioid and CB₁ receptors with agonists such as nociceptin/OFQ peptide and WIN55, 212–2, respectively, produced superactivation of adenylyl cyclase activity through G<sub>i/o</sub>-coupled receptor mechanisms (Chan & Wong, 1999; Rhee, Nevo, & Avidor-Reiss, 2000). As well, both cannabinoid and opioid withdrawal has been associated with compensatory increases in cAMP activity, albeit in different brain regions (Maldonado et al., 2006). Finally, although endogenous opioid peptides and endocannabinoid ligands do not act directly on cannabinoid and opioid receptors, respectively, some aspects of the cannabinoid-opioid interaction may be mediated through direct mechanisms. Specifically, a recent study by Seely et al., (2012) reported that AM-251 and rimonabant bind with mid-nanomolar affinity to µ-opioid receptors, suggesting that these cannabinoid antagonists/inverse agonists may be acting as direct antagonists at this receptor when high doses are employed (Seely et al., 2012). As well, CB₁ and µ-opioid receptor have been found to allosterically interact in the NAcc core causing non-additive and synergistic effects on glutamate and GABA release, respectively. These effects were reversed by their respective antagonists, but antagonist co-administration blocked these antagonistic effects, suggesting the potential for G-protein coupled heterodimeric receptor complexes (Schoffelmeer et al., 2006).
The Role of Aversive Motivational Dependence & Reinstatement in Addiction

As reviewed above, the interaction of these systems on the manifestation of somatic signs of physical dependence and the positive motivational component of reinstatement has been well studied and suggests that manipulation of the endocannabinoid system may be effective in treating opiate addiction. Despite these findings, the question as to whether the endocannabinoid system is effective in modulating the aversive, affective motivational component of dependence and reinstatement has yet to be addressed. As indicated by the American Psychiatric Association (1994) somatic signs of withdrawal represent only a subset of the motivational symptoms present in drug addiction. In fact, it has even been suggested that the somatic symptoms of dependence may be largely irrelevant to motivated drug use since addicts often relapse after somatic signs have dissipated (Koob & Le Moal, 1997). As a result, determining the endocannabinoid system’s efficacy in interfering with the establishment and reinstatement of the aversive, affective motivational component of opiate dependence is necessary in order to consider its true potential in the treatment of opiate addiction.

Animal Model for Assessing Aversive Motivational Dependence & Reinstatement

The place-conditioning paradigm represents a well-established animal model capable of assessing the affective motivational properties of rewarding/aversive drugs (Sanchis-Segura & Spanagel, 2006). As briefly mentioned, this paradigm utilizes a two-compartment tactile conditioning box where, on separate occasions, one compartment is paired with saline while the other is paired with a rewarding/aversive drug. The affective motivational properties of the rewarding/aversive drug are determined by re-exposing the animal to both compartments in a
drug-free test and measuring their approach/avoidance of the rewarding/aversive drug-paired compartment.

A study by Parker, Cyr, Santi, and Burton (2002) investigating the negative motivational effect of morphine withdrawal have shown that when a single dose of morphine is administered 24 hr prior to naloxone, acute naloxone administration produces a robust place avoidance after only one conditioning cycle. Furthermore, this study employed control groups to investigate the effects of naloxone administration alone (i.e. saline-saline, morphine-saline, saline-naloxone, morphine-naloxone) and found that naloxone was incapable of producing a place aversion following a single conditioning cycle. Moreover, when two conditioning trials were used, the place aversion induced with morphine pretreatment prior to naloxone was significantly stronger than the place aversion induced by naloxone alone (Parker & Joshi, 1998). These findings suggest that a single morphine pretreatment is capable of inducing a state of acute opioid dependence. Although morphine is no longer in the system 24 hr post morphine administration, morphine pretreatment enhances the level of constitutively active µ-opioid receptors thereby rendering the inverse agonist properties of naloxone more effective (Shoblock & Maidment, 2006). Importantly, this same enhancement of constitutively active µ-opioid receptors is also what is thought to underlie the development of dependence through chronic opioid exposure (Liu & Prather, 2001). Even though only a single dose of morphine is required to achieve acute dependence, the behavioral signs seen in acute and chronic opioid withdrawal are qualitatively similar suggesting that the neural pathways and cellular adaptations occurring in acute and chronic opioid dependence are similar (Azar, Jones, & Schulteis, 2003). This paradigm of naloxone-precipitated morphine withdrawal-induced conditioned place aversion (NPMW-CPA)
therefore makes it possible to test the potential of different pharmacological treatments to modulate the negative motivational state seen in opiate addiction.

In addition, NPMW-CPA has been found to be a more sensitive index of opiate withdrawal when compared to both suppression of operant responding (SOR) and somatic signs of withdrawal since the potency of naloxone required to produce CPA is significantly less than that to produce SOR and somatic indices (Azar et al., 2003). Medications such as buprenorphine, which are currently used in the long-term treatment of opiate addiction to alleviate the aversive effects of withdrawal, have also been found effective in preventing NPMW-CPA providing predictive validity for this model to discriminate against medication for the treatment of the aversive motivational state seen in opiate dependence (Stinus, Cador, Zorrilla, & Koob, 2005).

Lastly, the place conditioning paradigm is also capable of detecting the aversive motivational state of opiate addiction that mediates reinstatement. Indeed, a study by Li et al. (2007) demonstrated that a CPA can be reliably reinstated in extinguished rats that are given a NPMW drug prime, providing support for conditioned aversion as a secondary negative reinforcer in opiate addiction.

**Mechanisms Underlying Aversive Motivational Dependence & Reinstatement**

As mentioned, dysregulation of the mesocorticolimbic pathway is commonly observed in drug addiction and the aversive motivational component of drug withdrawal is thought to represent opponent-process-like changes in this system (Koob & Le Moal, 2001). Consequently, it is likely that many of the neurobiological substrates involved in mediating the primary rewarding properties of drugs of abuse are also involved in mediating the negative motivational effects of drug withdrawal, an occurrence described as a within systems neuroadaptation (Koob,
2009b). Alternatively, recent neuroanatomical evidence has also described the role for a separate entity located within the basal forebrain known as the extended amygdala. This entity comprising the NAcc shell, the central nucleus of the amygdala (CeA) and the bed nucleus of the stria terminalis (BNST) has been described as the anti-reward circuit and represents a brain stress system thought to limit reward (Koob, 2009b). Changes within this system have been termed a between systems neuroadaptation as they are secondary to the primary rewarding properties of drugs, however, it has also been argued that this entity is also involved in mediating primary reward (Koob & Le Moal, 2006). As a result, a discussion of the neurochemical alterations occurring in both brain systems as it relates to opioid use and their relative roles in mediating aversive motivational dependence and reinstatement follows. The ability for cannabinoid manipulation to attenuate aversive motivational withdrawal and reinstatement may suggest interference in the processes described.

The mesocorticolimbic reward circuit. Within the mesocorticolimbic reward pathway, opioid use results in membrane hyperpolarization and neuronal inhibition of many structures projecting to the VTA; this includes inhibition of GABA input from the NAcc and VP, and inhibition of glutamate from the PFC and amygdala (De Vries & Shippenberg, 2002). Mu-opioid agonists have also been found to activate VTA dopamine neurons and increase dopamine transmission in the NAcc through GABA inhibition (Johnson & North, 1992). This increase in extracellular dopamine leads to changes in GABA, dynorphin, and enkephalin transmission in the VTA, VP, and NAcc itself (De Vries & Shippenberg, 2002). Termination of opioid use, or administration of a competitive opioid antagonist, leads to opponent process changes in the aforementioned circuitry and consequently, is associated with a decrease of extracellular dopamine in the NAcc (Pothos, Rada, Mark, & Hoebel, 1991). This decrease in dopamine has
been attributed to mediating withdrawal since dopamine D2 receptor agonists to this region have been found to block somatic signs of precipitated opioid withdrawal (Harris & Aston-Jones, 1994). However, support for this dopamine dependent hypothesis is inconsistent as others have failed to alter symptoms of somatic and motivational withdrawal with dopaminergic lesions of the NAcc (Caillé et al., 2003).

Within the mesocorticolimbic reward circuit, the NAcc and the amygdala have been identified as primary structures in mediating affective motivational withdrawal. Indeed, injections of hydrophilic opioid antagonists into these brain regions suppressed operant responding for opioids in dependent rats (Koob, Wall, & Bloom, 1989) and produced a place aversion in opioid dependent rats (Stinus, Le Moal, & Koob, 1990). When investigating the ability of intracerebral injections of methylnaloxonium to produce a place aversion, the NAcc also proved to be the most sensitive site of action (Stinus et al., 1990). Additionally, the NAcc and basolateral amygdala have been identified as having primary roles in drug and cue induced reinstatement of cocaine drug-seeking, respectively, (Koob & Le Moal, 2006) and it is possible that the same regions mediate aversive reinstatement for opioids as well. Specifically, regions such as the dorsal PFC, NAcc and VP have been identified as critical for cocaine-primed reinstatement (McFarland & Kalivas, 2001), while inactivation of the basolateral amygdala (BLA), and dorsal medial PFC blocked cue-induced reinstatement (Shaham et al., 2003).

**The extended amygdala.** Some of the first studies to implicate the extended amygdala as integral to affective opioid withdrawal examined the expression of c-Fos protein, a measure of neuronal activation, in different brain regions during the expression of withdrawal. These studies found the greatest increase of c-Fos expression to be localized to regions of the extended amygdala (Frenois, Cador, Caille, Stinus, & Le Moine, 2002; Gracy, Dankiewicz, & Koob,
Affective opioid withdrawal within the extended amygdala is hypothesized to be driven by increases in corticotropin-releasing factor (CRF) and norepinephrine. Blockade of CRF within the CeA in particular, has been found to block establishment of a CPA produced by opioid withdrawal (Heinrichs, Menzaghi, Schulteis, Koob, & Stinus, 1995). The CeA has also been identified as a major source of CRF within the BNST (Sakanaka, Shibasaki, & Lederis, 1986). Inactivation or blockade of norepinephrine within the BNST or CeA has also proven to be effective in preventing the aversive state of opioid withdrawal as indicated by morphine withdrawal-induced CPAs (Delfs, Zhu, Druhan, & Aston-Jones, 2000; Watanabe et al., 2003). The BNST and CeA have also been identified as critical in mediating stress-induced (footshock) reinstatement of heroin-seeking (Shaham et al., 2003). Again, CRF and norepinephrine seem to mediate this effect since CRF$_1$ antagonists (Lu, Liu, & Ceng, 2001; Shaham, Erb, Leung, Buczek, & Stewart, 1998) and $\alpha_2$ adrenergic agonists (Erb et al., 2000; Highfield, Yap, Grimm, Shalev, & Shaham, 2001; Shaham, Highfield, Delfs, Leung, & Stewart, 2000) successfully interfered with stress-induced reinstatement of heroin-seeking.

As mentioned, cannabinoid manipulations resulting in neurochemical alterations in any of the brain regions outlined above may help attenuate the aversive motivational affect seen in opiate addiction. Indeed, cannabinoid expression has been found through the mesocorticolimbic dopamine system and the extended amygdala (Koob & Le Moal, 2006).
The Present Experiments

A thorough investigation of the cannabinoid system’s effects on the positive motivational state of opiate reward and reinstatement, as well its effects on the somatic symptoms of withdrawal, is present in the literature, however research investigating its effects on the negative motivational state of opiate addiction is lacking. Furthermore, the literature regarding the involvement of the cannabinoid system in attenuating opioid dependence has been contradictory. As a result, the present experiments attempt to elucidate the neurobiological mechanisms underlying the cannabinoid-opioid interaction and the contribution of modulating the cannabinoid system to ameliorate the aversive, affective motivational state of opiate dependence and reinstatement.

Chapter 2 investigates a series of systemic experiments aimed at determining the contribution of endocannabinoid enhancement and blockade on the aversive motivational state of opioid withdrawal and reinstatement. The first experiment examines the ability of the FAAH inhibitors, URB597 and PF3845, to interfere with the establishment of a NPMW-CPA. As described, evidence of the ability of FAAH inhibitors to attenuate somatic symptoms of opiate withdrawal (but not diarrhea) has been demonstrated (Ramesh et al., 2011). FAAH inhibitors have also been shown to lack rewarding or aversive properties on their own in the place conditioning (Gobbi et al., 2005) or self-administration paradigm (Justinova et al., 2008) reducing their liability for abuse or promoting negative affect. Consequently, if elevation of endogenous levels of anandamide is found to be effective in attenuating the aversive motivational state of opiate dependence, FAAH inhibitors may have potential in the treatment of opiate addiction with the added benefit of minor adverse effects (in comparison to exogenously administered cannabinoid agonists) due to their specificity of action.
The second experiment in Chapter 2 investigates the ability of acute AM251 treatment, a CB₁ receptor antagonist, to prevent establishment of a NPMW-CPA. This experiment, in combination with the first, attempts to elucidate the contradictory findings regarding the ability of CB₁ agonists and antagonists to mutually attenuate opioid withdrawal. As described, CB₁ receptor KO mice displayed reduced withdrawal symptoms following naloxone-precipitated morphine withdrawal. Furthermore, chronic blockade of the CB₁ receptor with SR141716A was additionally effective in attenuating opioid withdrawal. Although acute blockade of the CB₁ receptor was unsuccessful in attenuating somatic symptoms of NPMW (Rubino et al., 2000), an investigation of acute blockade on establishment will be conducted in light of evidence implicating the CPA procedure as a more sensitive index of opioid withdrawal than somatic symptoms (Azar et al., 2003). In addition, there is contradictory evidence implicating acute SR141716A treatment as being effective in attenuating certain symptoms of somatic withdrawal such as ptosis and jumping (Trang, Ma, Chabot, Quirion, & Jhamandas, 2006). However, although CB₁ antagonists show potential for preventing motivational withdrawal, CB₁ antagonists/inverse agonists such as AM251 have been found to have adverse clinical side effects which limit their therapeutic application. Indeed, SR141716A was withdrawn from the European market as an anti-obesity medicine following reports of its ability to induce nausea, emesis, anxiety and depressed mood (Bergman et al., 2008; Christensen, Kristensen, Bartels, Bliddal, & Astrup, 2007; Sink et al., 2010). Following its removal, it was speculated that these adverse effects may be attributed solely to the inverse agonist properties of the drug which act to suppress basal CB₁ receptor signaling levels (Bergman et al., 2008). In support of this, studies investigating the pharmacological effects of a newly developed ‘neutral’ CB₁ antagonist, known as AM4113, have found it to be effective in reducing food intake without the aforementioned
adverse physical and psychiatric consequences (Bergman et al., 2008; Sink et al., 2008). Additionally, AM4113 does not elevate cAMP levels as does AM251 and rimonabant, indicating that it does not act as a CB₁ inverse agonist (Sink et al., 2010). As a result, the ability of a neutral CB₁ antagonist to block the aversive and affective motivational state of opiate addiction was also investigated in order to evaluate the potential clinical implications of the study. While CB₁ antagonists analogous to AM251 have been shown to have aversive effects when used chronically, the dose employed in the current studies has not been found to produce a CPP or CPA when administered alone (Chaperon, Soubrié, Puech, & Thiébot, 1998).

Finding AM251 and AM4113 were successful in interfering with the establishment of a NPMW-CPA, the third study of the series in Chapter 2 sought to establish if their effectiveness extended to aversive reinstatement. As indicated, CB₁ inverse agonists/antagonists have been found to be effective in attenuating the positive reinforcing effects mediating reinstatement of opioid drug-seeking (De Vries et al., 2003; Fattore et al., 2003). Since it is possible that the same brain regions that mediate positive reinstatement also mediate negative reinstatement, an evaluation of the former is warranted.

The final study to conclude Chapter 2 elaborated on the results of AM251 and AM4113 to interfere with the establishment of the NPMW-CPA. While this study suggested that neutral CB₁ antagonists could have therapeutic potential in reducing aversive withdrawal, AM4113’s poor bioavailability would limit its treatment. Consequently, we tested the ability of an orally bioavailable neutral antagonist, AM6527, to replicate our previous findings.

Chapter 3 of the dissertation further investigated the ability of enhanced endocannabinoid tone to reduce affective withdrawal by evaluating the ability of a MAGL inhibitor to prevent the
establishment of a naloxone-precipitated morphine withdrawal-induced conditioned place aversion. While the experiments from Chapter 2 suggest FAAH inhibitors are ineffective in reducing affective withdrawal, an investigation into the effects of a MAGL inhibitor was warranted since MAGL inhibitors have been shown to be more effective than FAAH inhibitors in attenuating somatic symptoms of morphine withdrawal (Ramesh et al., 2011). Finding the MAGL inhibitor (MJN110) was capable of interfering with the place aversion, the remainder of the chapter attempts to identify the brain regions through which the MAGL inhibitor and CB₁ antagonists are mediating their effects. As described earlier, the extended amygdala represents a circuit of brain regions attributed to mediating affective withdrawal, and the CeA in particular, represents a region critical for the establishment of a NPMW-CPA (Frenois, Cador, Caille, Stinus, & Le Moine, 2002; Frenois, Stinus, Di Blasi, Cador, & Le Moine, 2005; Gracy, Dankiewicz, & Koob, 2001; Ishida et al., 2008; Jin et al., 2004, 2005; Watanabe et al., 2002; Xu et al., 2012). As a result, we explored whether delivery of AM251 or MJN110, directly to the CeA and associated brain regions (basolateral amygdala [BLA] and interoceptive insular cortex [IC]), could replicate our systemic findings and interfere with affective withdrawal as indicated by the place aversion paradigm.

Discovering an intriguing dissociation between the brain regions mediating the effects of the MAGL inhibitor (BLA, interoceptive IC) and the CB₁ receptor antagonist (CeA) on affective morphine withdrawal, Chapter 4 extends these findings by investigating the effectiveness of MJN110 and AM251 in an extended amygdala region with connectivity to each of the three brain regions examined. Indeed, the bed nucleus of the stria terminalis (BNST) has also been shown to play a role in the establishment of a naloxone-precipitated conditioned place aversion (Aston-Jones, Delfs, Druhan, & Zhu, 1999; Frenois et al., 2002) and receives GABAergic
connections from the CeA (Krettek & Price, 1978; Weller & Smith, 1982), and glutamatergic projections from the BLA and the interoceptive IC (Dong, Petrovich, & Swanson, 2001; McDonald, Shammah-Lagnado, Shi, & Davis, 1999). As hypothesized, endocannabinoid modulation within the BNST did interfere with the establishment of a naloxone-precipitated conditioned place aversion, with antagonism of the CB1 receptor, and not inhibition of MAGL activity, proving to have similar efficacy as when delivered to the CeA.

Ultimately, the research presented in this dissertation aims to contribute to the growing body of knowledge regarding the role of the endocannabinoid system in opioid addiction to allow for the proper evaluation of targeting this system as a treatment option.
Table 1. Effect of different cannabinoid compounds on spontaneous or precipitated somatic and affective opioid withdrawal. Dosing reflects whether compounds were given acutely prior to withdrawal, pre-chronic (chronically prior to morphine administration) or co-chronic (concurrently with morphine during the development of dependence). *Italicized rows* indicate experiments tested on acutely dependent animals. † quasi morphine withdrawal, ‡ spontaneous withdrawal. Adapted from Wills & Parker (2016).

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**Notes:**
- **AM404** and **AM251** are acute inhibitors.
- **FAAH inhibitors** include PF-3845, URB-597, and SA-57.
- **MAGL inhibitor** is JZL-184.
- **Dual FAAH/MAGL Inhibitors** include SA-57, JZL-184 + PF-3845.
- **CB1 Antagonists** include SR141716A and AM251.
- **Spontaneous Agonists** include Δ9 THC, Nabilone, Nantradol, and Levonantradol.

**Effects:**
- **AM404** and **AM251** are acute inhibitors.
- **FAAH inhibitors** reduce the effect of Δ9 THC.
- **MAGL inhibitor** reduces the effect of Δ9 THC.
- **Dual FAAH/MAGL Inhibitors** reduce the effect of Δ9 THC.
- **CB1 Antagonists** reduce the effect of Δ9 THC.
- **Spontaneous Agonists** include Δ9 THC, Nabilone, Nantradol, and Levonantradol.
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<td>JZL-184 + PF-3845</td>
<td>acute</td>
<td>reduced</td>
<td>mice</td>
<td>Ramesh et al. (2013)</td>
</tr>
<tr>
<td>CB₁ antagonist</td>
<td>SR141716A</td>
<td>acute</td>
<td>failed to precipitate</td>
<td>mice</td>
<td>Lichtman et al. (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>precipitated</td>
<td>rats</td>
<td>Navarro et al. (1998)</td>
</tr>
</tbody>
</table>
Table 2. List of cannabinoid compounds used in dissertation

<table>
<thead>
<tr>
<th>Compound Type</th>
<th>Compound Names</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB₁ receptor antagonist/inverse agonist</td>
<td>AM251</td>
<td>Blocks receptor activation; suppresses constitutive activity</td>
</tr>
<tr>
<td>Neutral CB₁ receptor antagonist</td>
<td>AM4113 AM6527 (orally bioavailable)</td>
<td>Blocks receptor activation</td>
</tr>
<tr>
<td>Fatty acid amide hydrolase (FAAH) inhibitor</td>
<td>URB597 PF3845 PF3845</td>
<td>Elevates endogenous levels of CB₁ receptor ligand anandamide (AEA)</td>
</tr>
<tr>
<td>Monoacylglycerol lipase (MAGL) inhibitor</td>
<td>MJN110</td>
<td>Elevates endogenous levels of CB₁ receptor ligand 2-arachidonoylglycerol (2-AG)</td>
</tr>
</tbody>
</table>
CB₁ Antagonism: Interference with affective properties of acute naloxone-precipitated morphine withdrawal in rats

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Rationale. Modulation of the endocannabinoid system has been found to interfere with opiate withdrawal. The potential of activation and blockade of the endocannabinoid system to prevent the aversive-affective state of naloxone-precipitated morphine withdrawal (MWD) was investigated in a one-trial conditioned place aversion (CPA) paradigm. Objective. CPA provides a sensitive measure of the motivational effects of acute MWD. The potential of the fatty acid amide hydrolase (FAAH) inhibitors, URB597 and PF3845, the CB₁ antagonist/inverse agonist, AM251, and the neutral CB₁ antagonists, AM4113 and AM6527 (oral), to interfere with establishment of a MWD induced CPA was investigated. As well, the potential of AM251 and AM4113 to interfere with reinstatement of a previously established MWD-induced CPA was investigated. Materials and Methods. Using a one-trial place conditioning paradigm, rats were administered naloxone (1 mg/kg, sc) 24 hr after receiving a high dose of morphine (20 mg/kg, sc) and were placed on the conditioning floor. To determine the effect of each pretreatment drug on the establishment of the MWD-induced CPA, URB597 (0.3 mg/kg ip), PF3845 (10 mg/kg, ip), AM251 (1 or 2.5 mg/kg ip), AM4113 (1 or 2.5 mg/kg ip) and AM6527 (5 mg/kg oral) were administered prior to conditioning. Results. AM251 (2.5, but not 1 mg/k), AM4113 and AM6527, but not URB597 or PF3845, interfered with the establishment of the MWD-induced CPA. AM251 and AM4113 did not prevent reinstatement of the CPA. Conclusions. Neutral antagonism of the CB₁ receptor reduces the aversive affective properties of morphine withdrawal.

Keywords: Conditioning, Addiction, Dependence, Aversion, URB597, AM251, AM4113, FAAH, CB₁ antagonist, naloxone, morphine withdrawal
Introduction

Of growing interest has been the involvement of the endocannabinoid system in the etiology and maintenance of opiate addiction. Considerable evidence suggests that CB₁ receptor antagonists/inverse agonists (SR141716 or AM251) attenuate the rewarding effects of opioids in the self-administration (Caillé & Parsons, 2003; De Vries et al., 2003; Navarro et al., 2001; Solinas, Panlilio, Antoniou, Pappas, & Goldberg, 2003) and place conditioning paradigms (Chaperon et al., 1998; Mas-Nieto et al., 2001; Navarro et al., 2001; Singh, Verty, McGregor, & Mallet, 2004). Furthermore, SR1741716 dose-dependently (0.3 – 3 mg/kg) blocks reinstatement of previously extinguished heroin-seeking in rats following a priming dose of heroin (Fattore et al., 2003) and heroin associated cues (De Vries et al., 2003). Consequently, it has been suggested that pharmacological blockade of the CB₁ receptor with antagonists/inverse agonists may also have therapeutic value in the treatment of opiate addiction.

However, opiate addiction is a disorder characterized by both positive and negative reinforcement exerted through physiological and psychological processes (Koob & Le Moal, 2001) and the role of manipulations of the endocannabinoid system on the aversive effects of morphine withdrawal (MWD) have been less well investigated. The intensity of naloxone-precipitated MWD symptoms are significantly reduced in CB₁ receptor knockout mice (Ledent et al., 1999; Lichtman et al., 2001). In agreement, chronic pharmacological blockade, of the CB₁ receptor with SR141716 during the concurrent development of morphine dependence reduced naloxone-precipitated MWD while having no effect on morphine analgesia (Mas-Nieto et al., 2001; Rubino et al., 2000). Somewhat paradoxically, however, THC (Bhargava, 1976a, 1976b), anandamide (Vela, Ruiz-Gayo, & Fuentes, 1995), and 2-AG (Yamaguchi et al., 2001) have also been reported to reduce naloxone-precipitated MWD in opiate-dependent rodents. Most recently, Ramesh et al. (2011) reported that elevation of anandamide, by the fatty acid amide
hydrolase (FAAH) inhibitor, PF3845 (10 mg/kg, ip), attenuated symptoms of naloxone-
precipitated MWD such as jumps and paw flutters (but not diarrhea or weight loss) in mice. All
effects were reversed by SR141716. Therefore, the potential of CB₁ agonists versus antagonists
to reverse naloxone- precipitated MWD effects is somewhat controversial.

An alternative to the investigation of the effect of endocannabinoid manipulations on
somatic symptoms of MWD is the investigation of the affective motivational symptoms of opiate
withdrawal using the place conditioning paradigm. MWD symptoms may be precipitated by an
opioid antagonist after 1-2 experiences with a high dose of morphine in humans (Heishman,
Stitzer, Bigelow, & Liebson, 1990; June, Stitzer, & Cone, 1995) or in rats (McDonald, Parker, &
Siegel, 1997; Meyer & Sparber, 1977; Parker et al., 2002; Parker & Joshi, 1998). Indeed, when
rats are injected with a single high dose of morphine (20 mg/kg subcutaneous [sc]), but not
saline, 24-48 hours prior to naloxone (1 mg/kg, sc), they display a robust conditioned place
aversion (CPA; Parker et al., 2002). That is, although naloxone alone did not produce a CPA,
naloxone 24-48 hr after a prime with a high dose of morphine did produce a strong CPA. The
CPA produced by acute naloxone precipitated MWD is highly resistant to extinction (Manwell et
al., 2009; McCallum, Limebeer, & Parker, 2010), but once extinguished the CPA can be
reinstitated by priming with naloxone-precipitated morphine withdrawal (Li et al., 2007;
McCallum et al., 2010). Therefore, the paradigm lends itself to the investigation of the effects of
manipulations of the endocannabinoid system on the establishment and reinstatement of the
aversive effects of MWD.

Because of the controversial role of cannabinoid agonists and/or antagonists in the relief
of somatic symptoms of naloxone-precipitated opiate withdrawal, the following experiments
examined the potential of the FAAH inhibitors, URB597 and PF3845, and CB₁ antagonists to
prevent the establishment and reinstatement of a place aversion. First, we evaluated the potential of the FAAH inhibitors URB597 and PF3845, which neither produce place conditioning (Gobbi et al., 2005) nor increase the reinforcing effects of the opiate heroin (Solinas et al., 2005), to interfere with the affectively aversive effects of naloxone-precipitated MWD. Since FAAH inhibition failed to interfere with the CPA, the potential of the CB₁ inverse agonist/antagonist AM251 to interfere with a single cycle morphine withdrawal CPA was evaluated. Since CB₁ inverse agonists/antagonists (SR141716 and AM251) have been shown to have adverse clinical side effects of nausea, anxiety and depression (Bergman et al., 2008; Christensen et al., 2007; Sink et al., 2010), the neutral CB₁ receptor antagonist, AM4113, was also assessed for its potential to interfere with the establishment of a CPA produced by naloxone-precipitated MWD. Finding both AM251 and AM4113 were successful in preventing establishment of the CPA, their potential to interfere with the reinstatement of an extinguished CPA by a naloxone-precipitated morphine prime was investigated, but such an effect was not revealed. To extend the clinical relevance of the findings, an orally bioavailable CB₁ neutral antagonist, AM6527, was evaluated for its potential to prevent establishment of a naloxone-precipitated MWD-induced CPA.

Materials and methods

Subjects

Subjects were 207 male Sprague-Dawley rats. Animals were housed individually in opaque shoebox cages with food and water *ad libitum*. They were maintained on a 12hr/12hr reverse light/dark schedule (lights off at 7 a.m.) with experiments being conducted during the dark cycle. The colony room in which the rats were held was kept at an ambient temperature of 21 degrees Celsius. All animal procedures were approved by the Animal Care Committee of the University of Guelph and adhere to the guidelines of the Canadian Council of Animal Care.
Drugs

Morphine and naloxone were prepared with saline at a concentration of 20 mg/ml and 1 mg/ml, respectively, and administered subcutaneously (sc) at a volume of 1 ml/kg. All cannabinoid related compounds were dissolved in a vehicle mixture of ethanol, Tween80 and physiological saline in a 1:1:18 ratio. The drugs were first dissolved in ethanol then Tween 80 was added to the solution and the ethanol was evaporated off with a nitrogen stream after which the saline was added. The final VEH consisted of 1:9 (Tween: saline). URB597 (0.3 mg/ml), PF 3845 (10 mg/ml; Ramesh et al. 2011), AM251 (1 and 2.5 mg/ml), and AM4113 (1 and 2.5 mg/ml) were administered intraperitoneally (ip) at a volume of 1 ml/kg. The doses of URB597 (0.3 mg/kg) and PF3845 (10 mg/kg) were chosen on the basis of their ability to provide maximal inhibition of FAAH and concomitant elevation of AEA in rats when administered 2 hrs prior (Ahn, Johnson, Mileni, Beidler, Long, Mckinney, et al., 2009; Fegley et al., 2005; Kathuria et al., 2003).

AM6527 (2.5 mg/ml) was administered by oral gavage at a volume of 2 ml/kg (5 mg/kg). The dose of AM6527 was selected on the basis of a report by Sink et al. (2010) that when delivered orally AM6527 was approximately half as potent as when delivered ip.

Apparatus

The conditioning boxes were rectangular (60 x 25 x 25 cm$^3$) and made of black Plexiglas with a wire-mesh lid (as previously described in Parker, Burton, Sorge, Yakiwchuk, & Mechoulam, 2004). During conditioning, single black metal floors made of a grid or hole pattern were used as contextual cues. During pretest, test and reinstatement trials, split black metal floors equally divided into a half grid/half hole pattern were used. A camera mounted (1.5 m) directly over top of the boxes and fire-wired to a computer recorded their movement. Ethovision software was
used to define box perimeters and assign a neutral floor zone for pretest, test and reinstatement trials. All movement was tracked using Ethovision software.

Procedure

Prior to all experiments, rats received a 15 min pretest using the split grid/hole floor to detect any floor biases. Ethovision software tracked their movement and measured the time spent on each floor for the duration of the pretest. Rats were then assigned to a pretreatment drug group and drug floor (grid, hole; being the floor which would be paired with withdrawal) matched on the basis of initial pretest preferences. Rats with a bias of more than 250 sec for either floor were removed.

*Experiment 1: Effect of URB597 (1A) and PF3845 (1B) on the establishment of an acute naloxone-precipitated MWD induced CPA*

A three day conditioning cycle was used to obtain the naloxone precipitated MWD induced CPA. The rats received the appropriate pretreatment injection two hr prior to both the saline conditioning trial (Day 1) and the naloxone–precipitated morphine withdrawal conditioning trial (Day 3) to ensure any difference in the amount of time spent on the drug floor at test was not due to any rewarding or aversive effects of the pretreatment drug itself. On the first day, the floor opposite the assigned drug floor was paired with a sc saline injection. Rats were administered VEH, 0.3 mg/kg URB597, or 10 mg/kg PF3845 by ip injection 2 hr prior to 1 ml/kg sc saline. Ten min later each rat was placed on the saline-paired floor for 45 min in the conditioning box and Ethovision recorded their movements. On the second day, 24 hr post-saline injection, all rats received a high dose of morphine (20 mg/kg sc) and were placed in an empty shoebox cage. The rats were monitored for signs of respiratory distress and returned to their home cage once fully
ambulatory. On the third day, 24 hr post-morphine, the floor assigned as the MWD floor was paired with a sc naloxone injection. As on the saline trial, rats received VEH, URB597, or PF3845 by ip injection 2 hr prior to 1mg/kg sc naloxone. Ten min later all rats were placed on the MWD-paired floor for 45 min and Ethovision tracked their movement. The final groups were: Experiment 1A: VEH (n=12), URB597 (n=12); Experiment 1B: VEH (n=12), PF3845 (n=12). Five days later, all rats were given daily 15 min test trial with the split grid/hole floor for 3 days. On each occasion, rats received a sc injection of saline in the conditioning room 10 min prior to test. Ethovision software tracked their movement and measured the time spent on each floor for the duration of the test.

Experiment 2: Effect of AM251 and AM4113 on the establishment of an acute naloxone-precipitated MWD induced CPA

As in Experiment 1, a three day conditioning cycle was used to obtain the naloxone-precipitated morphine withdrawal CPA. On Days 1 and 3, the rats received the appropriate pretreatment drug 30 min prior to the saline or naloxone injection. The pretreatment conditions were: VEH (n=10), 1 mg/kg AM251 (n=12), 2.5 mg/kg AM251 (n=8), 1 mg/kg AM4113 (n=12) or 2.5 mg/kg AM4113 (n=9). As in Experiment 1, Ethovision software tracked the total distance moved during each conditioning trial. Beginning 5 days after conditioning, rats received daily 15 min test trials with the split grid/hole floor for 3 days as described in Experiment 1.

Experiment 3: Effect of AM251 and AM4113 on reinstatement of a CPA by a naloxone-precipitated MWD prime

As in Experiment 1, a three day conditioning cycle was used to obtain the naloxone-precipitated morphine withdrawal CPA; however, no pretreatment drugs were administered during
conditioning. Beginning 5 days after conditioning, rats received daily 15 min test trials with the split grid/hole floor until the place aversion extinguished (6 days). On each occasion, rats received a sc injection of saline in the conditioning room 10 min prior to test. Since the experiment aimed to evaluate the potential of the CB₁ antagonist to interfere with reinstatement of a previously established CPA, rats with an aversion of less than 120 sec for the MWD-paired floor on the first test trial were removed from the experiment. Test trials continued until the CPA extinguished (defined by lack of significant (p>0.05) paired t-tests) for two consecutive days.

A week following the last extinction trial, the rats were tested for reinstatement of the CPA. On reinstatement Day 1, they received a saline prime test. On Day 2, they were injected sc with 20 mg/kg morphine in their home cage. On Day 3, they received the naloxone precipitated MWD prime test (1 mg/kg naloxone sc). On both Day 1 and Day 3, the rats were injected ip with VEH (n=17), 2.5 mg/kg AM251 (n=15), or 2.5 mg/kg AM4113 (n=16) 30 min prior to saline or naloxone which was given 10 min prior to the 15 min test. Ethovision software tracked their total distance moved and the time spent on each floor for the duration of the test.

Experiment 4: Effect of AM6527 on establishment of a CPA

As in Experiment 1, a three day conditioning cycle was used to obtain the naloxone-precipitated morphine withdrawal CPA. Rats received the appropriate pretreatment drug (VEH, 5 mg/kg AM6527) 1 hr prior to both the saline conditioning trial (Day 1) and the naloxone-precipitated morphine withdrawal conditioning trial (Day 3). Ethovision software tracked the total distance moved during each trial. The groups were: VEH (n=11) and AM6527 (n=12). Beginning 5 days after conditioning, rats received daily 15 min test trials with the split grid/hole floor for 3 days as described in Experiment 1.
Data analysis

For each experiment aimed at assessing the effect of the pretreatment drug on the establishment of naloxone precipitated MWD induced CPA, the number of seconds spent by each rat on the saline paired floor and on the MWD paired floor was entered into a mixed factor Analysis of Variance (ANOVA) with the within group factor of floor (saline/MWD) and the between groups factor of Drug (VEH and appropriate compound [s]) and Test Trial (1-3). Subsequent main effects analyses were conducted as appropriate. Statistical significance was set at p<0.05.

Results

Experiment 1: Effect of URB597 (Experiment 1A) or PF3845 (Experiment 1B) on the establishment of a naloxone-precipitated MWD induced CPA

FAAH inhibition did not modify the strength of a naloxone-precipitated MWD induced place aversion. As is evident in Figure 1A and 1B, neither URB597 nor PF3845 prevented the CPA. A 2 x 2 x 3 mixed factors ANOVA for Experiment 1A with URB597 and for Experiment 1B with PF3845 revealed only a significant main effect of floor (Experiment 1A: $F(1, 22) = 30.4; p < 0.001$; Experiment 1B: $F(1, 22) = 7.1; p = 0.014$), but no significant floor by drug interactions. Overall, the rats displayed a CPA following a single pairing with naloxone-precipitated MWD, but FAAH inhibition did not attenuate that aversion.

Experiment 2: Effect of AM251 and AM4113 on the establishment of a naloxone-precipitated MWD induced CPA

AM251 (at 2.5 mg/kg, but not 1 mg/kg) and AM4113 (at both 1 and 2.5 mg/kg) interfered with the establishment of the naloxone-precipitated MWD-induced CPA. Figure 2
presents the mean (+ sem) number of seconds spent on the saline-paired and the MWD-paired floor during each test trial for each pretreatment group. The \(5 \times 2 \times 3\) mixed factor ANOVA with the between groups factors of pretreatment drug (VEH, 1 mg/kg AM251, 2.5 mg/kg AM251, 1 mg/kg AM4113, 2.5 mg/kg AM251) and the within groups factors of floor and trials revealed only significant effects of floor, \(F(1, 46)=38.5; p<0.001\), and a drug by floor interaction \(F(4, 46)=2.9; p=0.032\). Because of the lack of an effect of trials (or interaction with any other factor), the pooled number of seconds on the MWD paired floor and on the saline paired floor for each drug condition was analyzed as a paired-t test. Overall, rats pretreated with VEH \((p<0.001)\) and 1 mg/kg AM251 \((p<0.01)\) spent significantly less time on the MWD paired floor than the saline paired floor; however, the rats pretreated with 2.5 mg/kg AM251, 1 mg/kg AM4113 or 2.5 mg/kg AM4113 did not show a CPA. Evaluation of the total distance moved on the saline conditioning trial revealed no significant differences in motor activity between the pretreatment drugs.

Experiment 3: Effect of AM251 and AM4113 on reinstatement of the CPA by a naloxone-precipitated MWD prime

The naloxone-precipitated MWD-induced CPA was reinstated by the prime following extinction; however, neither AM251 nor AM4113 interfered with or potentiated the reinstatement of the CPA. Figure 3 presents the floor aversion on the test trials for the naloxone-precipitated MWD-induced CPA. The rats displayed a significant CPA as assessed by paired t-tests on Test Days 1 \((p<0.001)\), 2 \((p=0.013)\), and 4 \((p=0.027)\), but not on Days 3, 5 or 6.

Figure 4 presents floor preferences on the naloxone-precipitated MWD reinstatement trial that occurred 9 days following the last extinction trial. On both the saline trial (not depicted) and MWD trial, the rats were pretreated with VEH, AM251 or AM4113 30 min before the test. On
the saline trial, a 2 x 3 mixed factor ANOVA with the within groups factor of floor (saline-paired, MWD-paired) and the between groups factor of pretreatment drug (VEH, AM251, AM4113), revealed no significant differences. On the MWD reinstatement trial, the 2 x 3 mixed factor ANOVA revealed only a significant main effect of floor, $F(1, 45)=6.1; p=0.02$, but no interaction. Rats spent significantly less time on the MWD-paired floor than the saline-paired floor, however, this aversion was not modified by the pretreatment drug. Evaluation of the total distance moved on the saline reinstatement trial revealed no significant differences in motor activity between the pretreatment drugs.

*Experiment 4: Effect of AM6527 on establishment of a naloxone-precipitated MWD-induced CPA*

AM6527 interfered with the establishment of the naloxone-precipitated MWD-induced CPA. The floor preferences on the test trials for establishment of the naloxone-precipitated MWD-induced CPA are found in Figure 5. The 2 x 2 x 3 mixed factor ANOVA with the between groups factors of pretreatment drug (VEH, AM6527) and the within groups factor of floor (saline-paired, MWD-paired) and trial, revealed a significant main effect of floor, $F(1, 21) = 9.6; p = 0.005$, and a significant drug by floor interaction, $F(1,21)=4.7; p = 0.04$. To analyze the interaction, a paired t-test pooled across trials revealed that rats pretreated with VEH ($p<0.01$), but not AM6527, spent significantly less time on the MWD-paired floor than the saline paired floor. Evaluation of the total distance moved on the saline conditioning trial and the MWD conditioning trial by an independent samples t-test revealed a significant difference in motor activity between the pretreatment drugs on both the saline conditioning trial, $t(21)=2.9; p<0.01$ (Mean [+sem] cm : VEH = 9961[+638]; AM6527: 6749 [+162]) and on the MWD conditioning trial, $t(21) = 2.1; p =0.049$ (Mean [+sem] cm : VEH = 5346[+385]; AM6527: 4447 [+157]).
Oral administration of 5 mg/kg of AM6527, not only interfered with the establishment of the CPA, but also suppressed locomotor activity relative to VEH during conditioning.

Discussion

The present findings are the first to show that antagonism of the CB₁ receptor is capable of interfering with the acquisition of the motivationally aversive state of acute morphine dependence as quantified by the place conditioning paradigm. Specifically, rats having received AM251 (at 2.5, but not 1 mg/kg), AM4113 (at both 1 and 2.5 m/kg) or oral AM6527 (at 5 mg/kg) prior to conditioning did not show a one-trial naloxone-precipitated MWD-induced CPA. Only orally administered AM6527 also suppressed locomotor activity during conditioning. These findings are in agreement with prior studies demonstrating the ability of antagonism of the endocannabinoid system to attenuate opioid self-administration (Caillé & Parsons, 2003; De Vries et al., 2003; Navarro et al., 2001; Solinas et al., 2003) and conditioned place preference (Chaperon et al., 1998; Mas-Nieto et al., 2001; Navarro et al., 2001; Singh et al., 2004).

Interestingly, however, although antagonism of the endocannabinoid system with the CB₁ antagonist SR141716 has been shown to block reinstatement of opioid drug seeking (De Vries et al., 2003; Fattore et al., 2003), the current findings suggest that this phenomenon may be exclusive to the rewarding properties of opioids. Indeed, following establishment and extinction of the CPA, none of the antagonists tested interfered with (or potentiated) reinstatement of the aversion. The apparent dissociations between reinstatement of CPP and CPA, and the establishment and reinstatement of the CPA found in the present study, suggests that each of these processes may be engaging distinct brain regions or a combination of distinct brain regions.
Although the manifestation of withdrawal is associated with changes in the cAMP pathway (Nestler & Aghajanian, 1997), it is unlikely that attenuation of the establishment of the CPA was mediated by an inhibition of intrinsic cellular activity and increased expression of cAMP since the inverse agonist, AM251, and the neutral antagonists, AM4113 and AM6527, were all effective in attenuating establishment of the CPA. As previously noted, neutral antagonists have been found to lack such effects on intrinsic cellular activity (Chambers et al., 2007). This suggests that the present findings may be attributed solely to the blockade of endocannabinoid binding, although the specific neurons and brain circuits involved in mediating these effects remain to be elucidated.

Somewhat surprisingly, although consistent with the present findings implicating the efficacy of CB₁ receptor antagonism in preventing establishment of the morphine withdrawal CPA, the FAAH inhibitors, URB597 and PF3845, did not interfere with establishment of the CPA. This finding is inconsistent with those prior studies demonstrating the ability of FAAH inhibitors to block naloxone-precipitated somatic withdrawal symptoms in morphine-dependent mice (Ramesh et al., 2011). Several factors could contribute to these discrepant findings including the type of species used (mice vs. rats), precipitation from chronic vs. acute dependence, and the brain regions involved in the manifestation of physical vs. motivational morphine withdrawal. Indeed, a dissociation between the brain regions involved in mediating physical and motivational opiate dependence has been described with regions such as the periaqueductal gray (Wei, Loh, & Way, 1972, 1973), dorsal thalamus (Bozarth & Wise, 1984), and locus coeruleus (Maldonado, Stinus, Gold, & Koob, 1992) responsible for mediating physical aspects of withdrawal, and the nucleus accumbens and amygdala (Stinus et al., 1990) identified as important in mediating the motivational or emotional aspects of withdrawal.
Recently, Trang et al. (2006) also implicated the depletion of calcitonin-gene related peptide in the spinal cord to the manifestation of opioid physical dependence, for which endocannabinoid tone was shown to help mediate. Therefore, it is possible that the brain regions involved in physical withdrawal are more sensitive to modulation of endocannabinoid tone than those implicated in motivational withdrawal.

To date, two brain regions have been identified as having a role in the manifestation of motivational opioid withdrawal, the nucleus accumbens (NAcc) and the extended amygdala (Gracy et al., 2001; Koob, 2009b; Stinus et al., 1990; Valverde, Tzavara, Hanoune, Roques, & Maldonado, 1996). It is well known that increased dopamine within the NAcc plays an important role in mediating the rewarding effects of a variety of drugs of abuse, including opiates. Specifically, opiates such as morphine, bind to presynaptic mu-opioid receptors on GABAergic neurons in the ventral tegmental area (VTA) where they inhibit GABA release onto dopaminergic neurons that project to the NAcc, resulting in an increased release of dopamine (Ford, Mark, & Williams, 2006; Johnson & North, 1992; Madhavan, Bonci, & Whistler, 2010; Shoji, Delfs, & Williams, 1999). Similarly, just as elevation of dopamine in the NAcc is responsible for the rewarding effects of morphine, disruption of the dopaminergic system may be responsible for mediating the aversive effects of morphine, termed a within-system neuroadaptation (Koob, 2009b). Within the mesolimbic dopamine system, cannabinoid receptors have been found to be located predominantly on presynaptic GABAergic and glutamatergic neurons where their activation inhibits neurotransmitter release (Maldonado et al., 2006). Through these mechanisms, endocannabinoids have been found to indirectly modulate dopamine transmission by inhibiting GABA release onto dopaminergic neurons in the VTA in a manner similar to morphine. Additionally, endocannabinoids also act on glutamatergic neurons within
the NAcc itself, inhibiting the release of glutamate projecting to GABAergic neurons regulating dopaminergic neuronal activity in the VTA (Maldonado et al., 2006). As a result, contrary to our findings, it would be expected that antagonism of the endocannabinoid system within the NAcc would result in a decrease of dopamine release and the manifestation of an aversive affect. Therefore, it is unlikely that the attenuation of a morphine withdrawal-induced CPA by the CB₁ antagonist is mediated by its action within the NAcc. Indeed, Hou et al. (2009) have reported that lesions to the NAcc were unable to impair CPA in acute morphine dependent rats. However, before endocannabinoid blockade within the NAcc can be ruled out, it should be noted that there is evidence to suggest that CB₁ and mu-opioid receptors within the NAcc core may allosterically interact through G-protein coupled heterodimeric receptor complexes to block the effects of concurrent antagonist treatments (Schoffelmeer et al., 2006). Indeed, consistent with the present findings, Schoffelmeer et al. (2006) reported that SR141716 blocked the antagonistic effect of naloxone at mu-opioid receptors and, similarly, naloxone prevented the antagonistic action of SR141716 at CB₁ receptors regulating inhibition of GABA and glutamate release from superfused NAcc core slices. However, further studies are required in order to establish unequivocal evidence.

A second brain region attributed to mediating the aversive effects of opioid withdrawal is the extended amygdala. This entity comprising the central nucleus of the amygdala, the bed nucleus of the stria terminalis, and a transition zone in the nucleus accumbens shell, has been implicated in mediating the negative effects on reward termed ‘antireward’ which is based on the hypothesis that brain stress systems are recruited to limit reward function and maintain a state of hedonic homeostasis, an example of a between-system neuroadaptation (Koob, 2009b; Koob & Le Moal, 1997, 2001). The central nucleus of the amygdala (CeA), in particular, has been
identified as having a central role in the establishment of naloxone-precipitated morphine withdrawal-induced CPA, as lesions to this brain region result in impaired acquisition of the CPA (Watanabe et al., 2002; Xu et al., 2012). Furthermore, increases in c-fos expression in the CeA have been shown to parallel the development of CPA in acute and chronic morphine dependent rodents (Frenois et al., 2002, 2005; Ishida et al., 2008; Jin et al., 2004, 2005). Antagonism of the endocannabinoid system has also been found to attenuate the anxiogenic effects produced by systemic naloxone when administered directly to the CeA (Zarrindast et al., 2008), providing evidence not only for a role of the CeA in mediating the aversive effect of opioids but also for a role of blockade of the endocannabinoid system to ameliorate its occurrence. Finally, in agreement with the inability of CB₁ antagonists to prevent reinstatement of a CPA, the involvement of the CeA in withdrawal induced CPA has been limited to its acquisition, suggesting that reinstatement of the CPA may be mediated by a different brain region.

In conclusion, the present findings provide additional evidence for the ability of the cannabinoid system to modulate opiate addiction processes. Specifically, blockade of the CB₁ receptor, but not an increase in anandamide through inhibition of FAAH, is able to prevent the motivationally aversive effects of acute MWD. Furthermore, the ability of AM4113 and AM6527 to effectively attenuate establishment of the naloxone-precipitated MWD-induced CPA suggests that neutral antagonism of the CB₁ receptor is sufficient in mediating the effects, providing potential for neutral CB₁ antagonists in the treatment of opiate dependence.
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Figure 1. Mean (± sem) time spent in seconds on the saline-paired floor and the MWD-paired floor for Experiment 1A (VEH, 0.3 mg/kg URB597) and Experiment 1B (VEH, 10 mg/kg PF3845) during each 15 min test trial.
Figure 2. Mean (± sem) time spent in seconds on the saline paired floor and the MWD-paired floor for each pretreatment drug (VEH, 1 mg/kg AM251, 2.5 mg/kg AM251, 1 mg/kg AM4113, 2.5 mg/kg AM4113) during each 15 min test trial in Experiment 2. Asterisks indicate a significant difference between the saline and morphine withdrawal paired floors. ***p < 0.001, **p < 0.01
Figure 3. Mean (± sem) time spent in seconds on the saline paired-floor and the MWD- paired floor during each 15 min test trial in Experiment 3. Asterisks indicate a significant difference between the saline- and MWD-paired floors. *p < 0.05, ***p < .001
Figure 4. Mean (± sem) time spent in seconds on the saline-paired floor and the MWD-paired floor for each pretreatment drug (VEH, 2.5 mg/kg AM251, 2.5 mg/kg AM4113) during the 15 min MWD-reinstatement trial in Experiment 3.
Figure 5. Mean (± sem) time spent in seconds on the MWD-paired floor for each pretreatment drug (VEH, 5 mg/kg AM6527 po), during each 15 min test trial in Experiment 4. Asterisks indicate a significant difference between the saline and MWD-paired floors. **p < 0.01
Double dissociation of monoacylglycerol lipase inhibition and CB$_1$ antagonism in the central amygdala, basolateral amygdala and the interoceptive insular cortex on the affective properties of acute naloxone-precipitated morphine withdrawal in rats

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Abstract

Both CB₁ receptor antagonism and agonism, in particular by 2-arachidonoylglycerol (2-AG), have been shown to reduce somatic symptoms of morphine withdrawal. Here we evaluated the effects of both systemic pretreatment with the monoacylglycerol lipase (MAGL) inhibitor MJN110 (which selectively elevates 2-AG) and central administration of both MJN110 and the CB₁ antagonist (AM251) on the affective properties of morphine withdrawal. Acute morphine withdrawal induced place aversion occurs when naloxone is administered 24 hr following a single exposure to a high dose of morphine. Systemic pretreatment with the MAGL inhibitor, MJN110, prevented the aversive effects of acute morphine withdrawal by a CB₁ receptor dependent mechanism. Furthermore, in a double dissociation, AM251 infusions into the central amygdala (CeA), but MJN110 infusions into the basolateral amygdala (BLA), interfered with the naloxone-precipitated morphine withdrawal induced place aversion. As well, MJN110, but not AM251, infusions into the interoceptive insular cortex (IC, a region known to be activated in acute morphine withdrawal) also prevented the establishment of the place aversion by a CB₁ mechanism of action. These findings reveal the respective sites of action of systemically administered MJN110 and AM251 in regulating the aversive effects of morphine withdrawal.
Introduction

Withdrawal from opiates has been shown to be a driving force in the maintenance of opiate addiction (eg, Koob, 2009a,b). In animal models, morphine withdrawal (MWD) can be produced by terminating chronic exposure to morphine or by administering an opiate antagonist to morphine pretreated animals. Indeed, morphine withdrawal symptoms can be observed in both humans (Heishman et al, 1990; June et al, 1995) and other animals (Eisenberg, 1982; Martin & Eades, 1964) when naloxone is administered several hours following a single exposure to a high dose of morphine. The withdrawal is apparent not only by behavioral symptoms of abstinence, but also by the ability of such withdrawal to serve as an aversive motivational stimulus. Parker et al. (2002) demonstrated that the aversive properties of naloxone precipitated MWD were evident up to 48 hr after a single injection of morphine, but not saline, in a conditioned place aversion (CPA) paradigm.

There is growing evidence that activation of the endocannabinoid system (eCB) may ameliorate symptoms of opiate addiction. The eCB system consists of two receptors (CB₁ and CB₂), the eCBs, anandamide (AEA; Devane et al, 1992) and 2-arachidonoylglycerol (2-AG; Sugiura et al, 1995) and the enzymes that regulate their synthesis and degradation (Ahn et al, 2008). CB₁ antagonism interferes with a naloxone precipitated MWD CPA (Wills et al, 2014) and with somatic symptoms of MWD in rats (Mas-Nieto et al. 2001; Rubino et al. 2000). However, somewhat paradoxically THC (Bhargava 1976a, 1976b), AEA (Vela et al, 1995) and 2-AG (Yamaguchi et al, 2001) have also been reported to reduce the intensity of MWD somatic symptoms in mice. When systemically administered, AEA and 2-AG are rapidly degraded by fatty acid amide hydrolase (FAAH; Cravatt et al, 1996) and monoacylglycerol lipase (MAGL; Dinh et al, 2002) respectively. Blocking these catabolic enzymes produces a prolonged elevation
of the respective eCB. Ramesh et al. (2011) reported that both FAAH (PF-3845) and MAGL (JZL184) inhibitors were effective in attenuating precipitated MWD somatic symptoms in mice; although the MAGL inhibitor was more effective than was the FAAH inhibitor. While FAAH inhibition does not significantly modify the establishment of a naloxone precipitated MWD CPA (Wills et al, 2014), the potential of MAGL inhibition to interfere with the affective component of acute naloxone precipitated MWD has not been evaluated in rats. Recently, a potent and selective MAGL inhibitor, MJN110 (Niphakis et al, 2013), has been developed that selectively elevates 2-AG by 10-fold, but not AEA. In rat brains, doses of 5 mg/kg (ip) and greater, produced maximal inhibition of MAGL for up to 12 hr following administration. MJN110 also inhibits the alternative 2-AG hydrolase, ABHD6, but with ~300-fold lower potency than MAGL. MJN110 has been shown to inhibit both acute and anticipatory nausea in rat gaping models (Parker et al, 2014) and to alleviate mechanical allodynia in a rat model of diabetic neuropathy (Niphakis et al, 2013).

The experiments reported here evaluated the potential of systemic administration of MJN110 and central administration of both MJN110 and the CB₁ antagonist, AM251, to interfere with the affective properties of MWD in rats. The aim was to determine the site of action of agonism and antagonism of the eCB system in regulating a naloxone precipitated MWD CPA. Considerable evidence implicates the amygdala in the neurocircuitry of the negative reinforcement associated with MWD (e.g., Koob, 2009a,b). Therefore, we evaluated the potential of intracranial administration of MJN110 and AM251 into both the basolateral amygdala (BLA) and the central nucleus of the amygdala (CeA) to interfere with the aversive affective effects of MWD. As well, both the BLA and the CeA receive input from the interoceptive insular cortex (IC; Mcdonald, 1998; McDonald, Shammah-Lagnado, Shi, & Davis, 1999), which also has been implicated in
both addiction and nausea processes (Contreras et al., 2007). Since CB₁ agonism in the BLA (Ganon-Elazar & Akirav, 2009) but CB₁ antagonism in the CeA (Zarrindast et al., 2008) produces stress-relieving and anxiolytic effects in rats in aversive environments, we predicted that MJN110 in the BLA, but AM251 into the CeA, would reduce the aversive properties of MWD. Inactivation of the interoceptive IC has recently been demonstrated to prevent the acquisition of a naloxone precipitated MWD CPA in rats (Li et al., 2013). Therefore, we also predicted that elevation of the regulatory neurotransmitter 2-AG by intracranial administration of MJN110 into the interoceptive IC (Allen, Saper, Hurley, & Cechetto, 1991; Cechetto & Saper, 1987; Contreras et al., 2007) would also interfere with the establishment of a naloxone precipitated MWD CPA.

**Materials and methods**

**Subjects**

Subjects were male Sprague-Dawley rats weighing between 350-450g. Animals were housed individually as described in Wills et al. (2014). All animal procedures were approved by the Animal Care Committee of the University of Guelph and adhere to the guidelines of the Canadian Council of Animal Care.

**Drugs**

Morphine, naloxone and vehicle (VEH) were prepared as previously described in Wills et al. (2014). For the systemic experiments (Experiments 1 & 2), the MAGL inhibitor, MJN110, and CB₁ antagonist, AM251, were prepared in VEH at a final concentration of 10 mg/ml and 1 mg/ml, respectively. The concentration of MJN110 was selected on the basis of its ability to maximally inhibit MAGL activity in rats *in vivo* (Niphakis et al., 2013), while the concentration of AM251 has been previously found to have no effect on the establishment of a naloxone-
precipitated conditioned place aversion (Wills et al, 2014). For the intracranial experiments (Experiment 3-4), MJN110 and AM251 were prepared in the same VEH at a concentration of 5 μg/μl and 0.25 μg/μl, respectively; for Experiment 5, MJN110 and AM251 were prepared in the same VEH at a concentration of 2 μg/μl and 0.1 μg/μl, respectively.

**Surgery**

For Experiments 3-5, rats were anesthetized with isoflurane gas and prepared for intracranial surgery as described in detail in Limebeer et al. (2012). Once rats were stabilized in the stereotaxic frame in the flat skull position (Paxinos & Watson, 1998), small bilateral holes were drilled into the exposed skull and stainless steel guide cannulas (22G, 6 or 8mm below pedestal) were lowered into the CeA (Experiment 3), BLA (Experiment 4) or the interoceptive IC (Experiment 5) using the following coordinates relative to Bregma, CeA: -2.2 mm anteroposterior (AP), +4.3mm mediolateral (ML), and –6.0 mm dorsoventral (DV) from the skull surface; BLA: -2.3mm AP, +5.0mm ML, -6.5mm DV from the skull; interoceptive IC (10º divergent angle): -0.5mm AP; + 5.0mm ML; –4.5mm DV from the skull. The guide cannulas were stabilized to the skull using six screws and dental cement. Once the dental cement hardened, stainless steel dummies were inserted into the guide cannulas to prevent obstruction.

**Histology**

Guide cannula placements in Experiments 3-5 were determined through the histological examination of brain tissue as described in detail in Limebeer et al. (2012). Prior to perfusion, rats were microinfused with Chicago blue dye to verify diffusion of the drug was localized to the CeA, BLA and interoceptive IC; analyses revealed an average spread of 0.35mm in the CeA and BLA, and 0.75mm in the interoceptive IC in each of the AP, ML, DV coordinate planes.
Following, rats were deeply anaesthetized with a lethal dose of Euthansol (85 mg/kg ip) and transcardially perfused with PBS buffer (0.1 M) followed by 4% formalin. Brains were removed and stored in a 20% sucrose and 4% formalin solution overnight at room temperature, after which they were preserved at a temperature of 4°C until sectioned. Brains were frozen and sliced into 60 μm sections using a CM1850 Leica cryostat and relevant slices were mounted onto gelatin-subbed glass microscope slides. Slide tissue sections were then stained with thionin, cover-slipped, and examined using a Leica MZ6 Stereomicroscope. Rats with improper cannula placement were excluded from the study. All n’s reported in the manuscript reflect the post-histology numbers per group.

Apparatus

The conditioning apparatus was a plain black rectangular box as previously described in Wills et al. (2014) with chambers differentiated solely by the floor texture. Removable floors were used to transition the boxes from conditioning cycles to pretest/test trials. During conditioning, single black metal floors made of a grid or hole pattern were used as contextual cues. During pretest and test trials, split black metal floors equally divided into a half grid/half hole pattern were used. Ethovision software was used to define box perimeters and assign a neutral floor zone between the two floors for pretest and test trials.

Procedure

All procedures are as described in detail in Wills et al. (2014). Prior to all experiments, rats received a 15 min pretest. Rats were assigned to a pretreatment drug group and drug floor matched on the basis of initial pretest preferences. Rats with a bias of more than 200 sec for
either floor were removed from the experiment. In all experiments, Ethovision software tracked activity and measured the time spent on each floor.

**Experiment 1: Potential of systemic MJN110 to produce a CPP or a CPA**

The rats (n=8) were injected with 10 mg/kg MJN110 on one day and VEH on another day (24 hr apart; counterbalanced order) two hr prior to placement in the conditioning box with the grid or the hole floor (counterbalanced) for 45 min. Twenty-four hr after the final conditioning day of the single conditioning trial, the rats were given daily 15 min test trials with the split grid/hole floor for 3 days. On each test, rats received an ip injection of VEH 2 hr prior to being placed in the box.

**Experiment 2: Effect of systemic MJN110 and the interaction of systemic MJN110 and AM251 on the establishment of a MWD CPA**

A three-day conditioning cycle was used to obtain the MWD CPA. On the first day, the floor opposite the assigned drug floor was paired with a sc saline injection (saline floor). The rats were administered an ip injection of VEH (n=12), 10 mg/kg MJN110 (n=11) or 10 mg/kg MJN110 + 1 mg/kg AM251 (n=12), 2 hr (MJN110 or VEH) or 30 min (AM251) prior to the saline injection. The inclusion of a group treated with AM251 alone was omitted based on our previous findings reporting no significant effect of 1 mg/kg AM251 on the establishment of a MWD CPA (Wills et al, 2014). Ten minutes after the saline injection, the rats were placed in the conditioning chamber for 45 min. On the second day, 24 hr post-saline injection, all rats received a high dose of morphine (20 mg/kg, sc) in an empty shoebox cage. The rats were monitored for signs of respiratory depression and stimulated when required to prevent decease (<5% mortality rate). Finally, on the third day, 24 hr post-morphine, the assigned drug floor was paired with an injection of naloxone (MWD floor). As on the saline trial, the rats received VEH, MJN110, or
MJN110 + AM251, 2 hr (MJN110 or VEH) or 30 min (AM251) prior to the naloxone injection (1 mg/kg), which occurred in the conditioning room 10 min prior to being placed in the conditioning box for 45 min. The final groups were: VEH ($n = 12$), MJN110 ($n = 11$), and MJN110 + AM251 ($n = 12$). Five days later, rats were given daily 15 min tests as described above, with the exception that rats received a sc injection of saline 10 min prior to being placed in the box.

**Experiment 3: Effect of MJN110 and AM251 on the establishment of a MWD CPA when delivered to the CeA**

As in Experiment 2, a three-day conditioning cycle was used to obtain the MWD CPA. One hour prior to both the saline conditioning trial (Day 1) and the MWD conditioning trial (Day 3), rats received bilateral microinfusions of VEH ($n = 8$), 2μg MJN110 ($n = 10$), or 0.1μg AM251 ($n = 6$) into the CeA at a rate of 0.2 μl/min for 2 min. Injector tips were inserted to extend 2 mm beyond the length of the guide cannula. Following the two-min infusion period, the injectors were left in place for an additional min to ensure full diffusion of the drug from the injector. Rats were injected with saline (Day 1) or naloxone (Day 3) 10 min prior to being placed in the conditioning boxes for 45 min. Beginning 5 days after conditioning, rats received daily 15 min tests as described above.

**Experiment 4: Effect of MJN110, AM251 and MJN110 – AM251 on the establishment of a MWD CPA when delivered to the BLA**

The procedures were identical to those of Experiment 3 except that VEH ($n = 8$), 2μg MJN110 ($n = 10$), 0.1μg AM251 ($n = 7$), and 2μg MJN110 – 0.1μg AM251 ($n = 8$) were bilaterally infused into the BLA.
**Experiment 5: Effect of MJN110, AM251, and MJN110-AM251 on the establishment of a MWD CPA when delivered to the interoceptive IC**

The procedures were identical to those of Experiment 3 except that VEH (n=10), 2 μg MJN110 (n=8), 0.1μg AM251 and 2μg MJN110 – 0.1μg AM251 were bilaterally infused into the interoceptive IC at a rate of 0.5 μl/min for 2 min.

**Data Analysis**

In each experiment, the time (sec) spent on each floor during each of the three test trials was entered into a three factor mixed design, with the between group factor of pretreatment drug and the within group factors of floor (saline-paired or MWD-paired) and test trial. As well, the activity measures during conditioning trials were entered into a mixed factors analysis of variance (ANOVA) with the between groups factor of pretreatment drug and the within-groups factor of conditioning trial (saline or MWD) for each experiment. Significance was set at \( p < 0.05 \).

**Results**

*Experiment 1: MJN110 does not produce a CPP or CPA*

Single trial place conditioning with MJN110 did not produce a significant preference or aversion for the drug paired floor, \( F(1, 6) = 0.22 \), ns. Pooled across test trials, rats spent an equal amount of time on the VEH paired floor (M = 411.32 sec, ± sem) and the MJN110 paired floor (M = 373.96 sec, ± sem), and the order of conditioning (VEH first vs. MJN110 first) did not significantly alter this effect. An evaluation of activity during both conditioning trials revealed no motoric effects of MJN110 compared to VEH.
**Experiment 2: Systemic MJN110 interferes with the establishment of a MWD CPA which is reversed by AM251**

As seen in Figure 1, MJN110 significantly interfered with the establishment of the naloxone precipitated morphine MWD CPA, an effect that was reversed by the CB₁ antagonist AM251. The ANOVA revealed a significant floor by drug interaction, \( F(2, 32) = 7.40; p = 0.002 \). Rats pretreated with VEH \((p < 0.001)\), or MJN110 + AM251 \((p = 0.017)\), but not MJN110 alone spent significantly less time on the MWD-paired floor than the saline-paired floor. Evaluation of activity during the conditioning trial revealed significant effects of trial, \( F(1, 32) = 273.3; p < 0.001 \), drug, \( F(2,32) = 11.4; p < 0.001 \), and a significant trial by drug interaction, \( F(2,32) = 8.0; p < 0.01 \). Overall, rats were significantly less active during MWD conditioning than saline conditioning. Additionally, pretreatment with MJN110+AM251 significantly reduced activity compared to VEH and MJN110 on the both the saline trial \((p < 0.001)\) and on the MWD trial \((p’s < .0.05)\). Group MJN110 did not differ from VEH on any trial.

**Experiment 3: AM251 delivered to the CeA interferes with the establishment of a MWD CPA**

As seen in Figure 2, central administration of AM251 into the CeA interfered with the establishment of a naloxone precipitated MWD CPA. The ANOVA revealed a significant floor x drug interaction, \( F(2, 21) = 4.6; p =0.02 \). VEH and MJN110 treated rats \((p’s < 0.01)\), but not the AM251 treated rats, spent significantly less time on the MWD floor than the saline floor, pooled across tests. Analysis of the mean distance traveled during conditioning revealed only a significant effect of trial, \( F(1, 21) = 116.8, p< 0.001 \); rats were significantly less active during
the MWD trial than the saline trial. There was no effect of MJN110 or AM251 on activity on either the saline or MWD conditioning trial.

**Experiment 4: MJN110 delivered to the BLA interferes with the establishment of a MWD CPA**

As seen in Figure 3, central administration of MJN110 into the BLA interfered with the establishment of a naloxone precipitated MWD CPA and this effect was reversed by co-administration with AM251. The ANOVA revealed a significant floor by drug interaction, $F(3, 29) = 3.1, p = 0.04$. Rats administered VEH, VEH-AM251 and MJN110-AM251 but not MJN110, bilaterally to the BLA one hr prior to conditioning spent significantly less time on the MWD paired floor than the saline floor ($p$’s < 0.05). Evaluation of conditioning activity, revealed a significant effect of trial, $F(1, 29) = 56.4, p < 0.001$. Overall, rats were significantly less active on the MWD conditioning trial compared to the saline conditioning trial and drug pretreatment did not alter this on either trial.

**Experiment 5: MJN110 delivered to the interoceptive IC interferes with the establishment of a MWD CPA**

As seen in Figure 4, MJN110 administered to the interoceptive IC interfered with the naloxone precipitated MWD CPA and this effect was reversed by co-administration with AM251. The ANOVA revealed a significant floor x drug interaction, $F(3, 29) = 3.6, p =0.03$. Pooled across trials, VEH, VEH-AM251 and MJN110-AM251 displayed a significant aversion for the MWD paired floor ($p$’s < 0.05), but group MJN110 did not show an aversion. Evaluation of activity during conditioning revealed a significant effect of trial, $F(1, 29) = 156.1, p < 0.001$; drug, $F(3, 29) = 4.1, p = 0.02$; and a significant trial by drug interaction, $F(3, 29) = 4.5, p=0.01$. Overall, rats were significantly less active on the MWD trial than the saline trial. Furthermore, rats treated
with AM251 or MJN110-AM251, were significantly less active on the saline trial, but not the MWD trial, than rats that were treated with VEH or MJN110.

**Discussion**

Systemic administration of the MAGL inhibitor, MJN110, prevented the establishment of a naloxone precipitated MWD CPA, an effect that was reversed by the CB₁ antagonist AM251. The ability of MJN110 to interfere with the CPA is not due to rewarding properties of the inhibitor *per se*, since MJN110 produced neither a place preference nor aversion. As well, intracranial administration of MJN110 (2 μg, bilaterally) into the BLA and into the interoceptive IC, central areas implicated in the negative effects of dependence (Contreras et al, 2007; Koob, 2009b; Li et al, 2013; Li et al, 2009) also prevented the naloxone precipitated MWD CPA. Although rats were less active on the MWD conditioning trial than on the saline conditioning trial in each experiment, MJN110 did not modify their activity relative to VEH controls. Consequently, the elevation of 2-AG by MAGL inhibition (Niphakis et al, 2013) appears to counteract the aversive properties of MWD. The ability of MAGL inhibition to alleviate aversive effects of MWD is in agreement with previous studies investigating the ability of MAGL inhibition to reduce somatic symptoms of MWD in morphine-dependent mice, whether spontaneous or precipitated by naloxone (Ramesh et al, 2011, 2013).

Contrary to the BLA and interoceptive IC, intra-CeA administration of the CB₁ receptor antagonist AM251, and not the MAGL inhibitor MJN110, interfered with the naloxone precipitated MWD CPA. While rats were significantly less active during MWD conditioning, intra-CeA AM251 did not significantly modify activity during conditioning relative to VEH rats. The ability of AM251 to attenuate the aversive properties of MWD are in agreement with
systemic studies conducted by Wills et al (2014), and identifies the CeA as a neural substrate mediating the effect of AM251 on MWD. Indeed, the role of the CeA in contributing to the aversive state of MWD has been demonstrated in lesion (Watanabe et al, 2002; Xu et al, 2012), and c-fos studies (Frenois et al, 2002; Ishida et al, 2008; Jin et al, 2004, 2005). Since intra-CeA AM251 has been shown to antagonize the anxiogenic effects produced by systemic naloxone in the elevated-plus maze (Zarrindast et al, 2008), it is likely that it also reduced the anxiety associated with MWD in the present study.

Within the BLA, MJN110 reduced the aversive properties of MWD through a CB₁-dependent mechanism; likely by elevating 2-AG. These findings are in agreement with a previous study reporting the ability of CB₁ agonism within the BLA to prevent stress-enhanced aversive learning (Ganon-Elazar & Akirav, 2009). Considering the modulatory role of the endocannabinoid system in regulating the hypothalamic-pituitary-adrenal (HPA) axis in this region (Hill & Tasker, 2012), it is possible that BLA 2-AG reduces the aversive properties of MWD by acting to constrain HPA axis activity.

The double dissociation between the ability of CB₁ receptor agonism (elevated 2-AG through MAGL inhibition) in the BLA and CB₁ receptor antagonism in the CeA to interfere with a MWD CPA is consistent with the opposite pattern of c-fos expression in these regions during naloxone precipitated MWD in rats; specifically, c-fos expression was significantly enhanced in the CeA, whereas it was slightly decreased in the BLA (Frenois et al., 2002). Given that cannabinoids are produced pre-synaptically and act retrogradely to inhibit neurotransmitter release (GABA or glutamate), it is feasible that the CB₁ receptor antagonist reduces MWD by disinhibition of GABA release in the CeA, while 2-AG reduces MWD by inhibition of GABA release in the BLA - ultimately restoring neuronal activation to baseline levels in both regions.
Indeed, CB₁ receptors have been found to modulate GABA transmission in both regions (Azad et al., 2003; Katona et al., 2001; Roberto et al., 2010), and the complex interplay between cannabinoid mediated inhibition and excitation of the amygdala in the regulation of anxiety states is largely disrupted in MWD (see Ruehle, Rey, Remmers, & Lutz, 2012; for a review). Alternatively, given the more essential role of the CeA in mediating aversive withdrawal and the connectivity between the CeA and BLA (Jin et al., 2005; Wenzel et al., 2011), it is also possible that intra-BLA MJN110 may interfere with MWD by indirectly inactivating the CeA as would be the direct effect of AM251 in the CeA. Indeed, inhibition of GABA release in the BLA would elevate excitation of pyramidal neurons projecting to the intercalated cells, which would lead to greater GABA release within the CeA (as described in detail in Katona et al, 2001).

Unlike the amygdala, the insular cortex has only recently been implicated in addiction processes. Naqvi et al. (2007) were the first to propose that the insula may be critical to nicotine addiction in human stroke patients. The insula had been implicated in representation of interoceptive states (Craig, 2002). In pre-clinical animal models, Contreras et al. (2007) reported that inactivation of the interoceptive IC blocked the expression of both LiCl induced malaise in rats and of amphetamine induced conditioned place preference, suggesting that the interoceptive IC is involved in modulation of a “state of well being”. Inactivation of the interoceptive IC has recently been demonstrated to prevent the acquisition of a naloxone precipitated MWD CPA in rats (Li et al, 2013). Since naloxone precipitated MWD also produces malaise in rats as indicated by conditioned gaping (McDonald et al, 1997), it is conceivable that elevation of 2-AG in this region reduced the aversive properties of MWD thereby interfering with the CPA. Indeed, 2-AG and MJN110 (but not anandamide or FAAH inhibition) exogenously delivered to the interoceptive IC has been reported to reduce the establishment of LiCl-induced conditioned
gap reactions, a measure of nausea in rats, by a CB\textsubscript{1} dependent mechanism of action (Sticht, Limebeer, Rafla, & Parker, 2015; Sticht et al., 2016).

The current findings revealed the respective sites of action of systemically administered MJN110 (BLA and interoceptive IC) and AM251 (CeA) to interfere with the establishment of a one-trial acute naloxone precipitated MWD CPA. Given the role of the endocannabinoid system and these brain regions in emotional processing and interoceptive deviations from normal, it is suggested that these effects are mediated by the ability of these compounds to counteract the aversive properties of MWD. Ultimately, these findings reveal a complex relationship for the endocannabinoid system in regulating opiate withdrawal in distinct brain regions that warrants further investigation.
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Figure 1. Mean (± sem) time spent in seconds on the saline-paired floor and the MWD-paired floor for each pretreatment drug group (VEH, 10 mg/kg MJN110, 10 mg/kg MJN110+ 1 mg/kg AM251 during conditioning) during each 15 min test trial. Asterisks indicate a significant difference between the saline and morphine withdrawal paired floors. *p < 0.05, ***p < 0.001
Figure 2. A) CeA cannula placements for all rats included in the experiment. B) Mean (± sem) time spent in seconds on the saline-paired floor and the MWD-paired floor for each pretreatment drug group (VEH, 2 μg MJN110, 0.1μg AM251 into the CeA during conditioning) during each 15 min test trial. Asterisks indicate a significant difference between the saline and morphine withdrawal paired floors. **p < 0.01
Figure 3. A) BLA cannula placements for all rats included in the experiment. B) Mean (± sem) time spent in seconds on the saline-paired floor and the MWD-paired floor for each pretreatment drug group (VEH, 2 µg MJN110, 0.1 µg AM251, 2 µg MJN110 – 0.1 µg AM251 into the BLA during conditioning) during each 15 min test trial. Asterisks indicate a significant difference between the saline and morphine withdrawal paired floors. *p < 0.05
Figure 4. A) Interoceptive IC cannula placements for all rats included in the experiment. B) Mean (± sem) time spent in seconds on the saline-paired floor and the MWD-paired floor for each pretreatment drug group (VEH, 2 µg MJN110, 0.1µg AM251, 2µg MJN110 – 0.1µg AM251 into the interoceptive IC during conditioning) during each 15 min test trial. Asterisks indicate a significant difference between the saline and morphine withdrawal paired floors.

*p < 0.05, **p < 0.01
CB$_1$ receptor antagonism in the bed nucleus of the stria terminalis interferes with affective opioid withdrawal in rats

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Abstract

The bed nucleus of the stria terminalis (BNST) is a region of the extended amygdala that is implicated in addiction, anxiety and stress related behaviours. This region has been identified in mediating the aversive state of naloxone-precipitated morphine withdrawal (MWD) and cannabinoid type 1 (CB₁) receptors have been found to modulate neurotransmission within this region. Previous findings suggest that the CB₁ antagonist/inverse agonist, AM251, administered systemically or by infusion into the central nucleus of the amygdala (CeA) prevented the aversive affective properties of MWD as measured by conditioned place aversion learning. As well, when administered systemically or by infusion into the basolateral nucleus of the amygdala (BLA) or the interoceptive insular cortex, the monoacylglycerol lipase (MAGL) inhibitor, MJN110 (which elevates 2-arachidonlylglycerol), also prevented a naloxone-precipitated MWD induced place aversion. Given the connectivity of these regions and the BNST, the present study sought to determine whether cannabinoid modulation of the BNST would also prevent the affective properties of naloxone precipitated MWD-induced place aversion learning. Prior to conditioning trials, rats received intra-BNST infusions of AM251, in Experiment 1, or MJN110 in Experiment 2. AM251, but not MJN110, prevented the establishment of the MWD-induced place aversion. The current findings emphasize an important role for the BNST in opioid withdrawal and suggest that the ameliorative effects of systemically administered CB₁ antagonists are mediated, in part, by their actions within this region.

Keywords: opioid withdrawal, conditioned place aversion, cannabinoid type 1 receptor antagonist, monoacylglycerol lipase inhibition, extended amygdala, rat
Introduction

Opioid addiction is maintained by the prevention of the aversive withdrawal state which is characterized by feelings of dysphoria, anxiety and restlessness (Koob & Le Moal, 2006). Although both symptoms of physical and affective withdrawal are present, addicts will often relapse to drug use long after symptoms of physical withdrawal have dissipated (Koob & Le Moal, 1997), which emphasizes an important role for the affective component of withdrawal in mediating continued drug use. Opioid withdrawal in rats (Mcdonald et al., 1997; Meyer & Sparber, 1977), as in humans (Heishman et al., 1990; June et al., 1995), can be precipitated by the opioid antagonist naloxone, following the administration of even a single high dose of morphine. This withdrawal, when paired with a contextual environment 24-48 hours post-morphine, can produce a robust conditioned place aversion (CPA); a model of acute dependence and affective opioid withdrawal in rats (Azar et al., 2003; Parker et al., 2002; Parker & Joshi, 1998). Compounds currently used in the treatment of opioid withdrawal (eg. buprenorphine) are effective at preventing the establishment of this place aversion (Stinus et al., 2005), and consequently, this model can be used to identify alternative non-opioidergic pharmacological treatments and the neural correlates attributed to mediating the affective withdrawal state.

A potential pharmacological target which has shown promise in treating opioid withdrawal is the endocannabinoid system (Wills & Parker, 2016). This system is one of the main neuromodulators of the central nervous system, where it acts as a retrograde inhibitor of neurotransmitter release (López-Moreno et al., 2008). It is composed of the cannabinoid (CB) type 1 and CB2 receptors, bound by the endogenously produced ligands anandamide and 2-arachidonoylglycerol (2-AG), which are degraded by the catabolic enzymes fatty acid amide hydrolase and monoacylglycerol lipase (MAGL), respectively (Ahn, McKinney, & Cravatt,
In rats, acute pharmacological inhibition of MAGL with MJN110 (Niphakis et al., 2013) has been found to be effective in reducing affective morphine withdrawal (MWD) as evidenced by its ability to prevent the establishment of a naloxone-precipitated CPA (Wills et al., 2016). This effect was prevented with pretreatment of the CB₁ antagonist AM251, at a dose which did not produce an effect on its own, suggesting that it is acting to elevate endogenous 2-AG to agonize the CB₁ receptor. Furthermore, although somewhat paradoxical, the establishment of a naloxone-precipitated morphine withdrawal CPA was prevented in CB₁ receptor knock-out mice (Ledent et al., 1999) and with acute pretreatment of CB₁ receptor antagonist/neutral antagonists (AM251, AM4113, AM6527) in rats (Wills et al., 2014), suggesting that both agonism and antagonism of the CB₁ receptor is able to ameliorate symptoms of affective opioid withdrawal. To explain this discrepancy, we evaluated the site of action of these compounds (Wills et al., 2016).

The negative affective state of opioid withdrawal is thought to be mediated not only by alterations within brain reward systems but also by the recruitment of brain stress regions, such as the extended amygdala (Koob, 2008). The extended amygdala, comprised of the bed nucleus of the stria terminalis (BNST), the central amygdala (CeA) and a transition zone in the posterior part of the nucleus accumbens (Heimer & Alheid, 1991), represents an interface between emotional cortical and limbic structures (such as the insula and basolateral amygdala), and hypothalamic and extrapyramidal motor systems (Alheid, De Olmos, & Beltramino, 1995; McDonald, Shammah-Lagnado, Shi, & Davis, 1999). Indeed, expression of the immediate early gene, c-fos, is enhanced in the BNST and CeA during the development of a naloxone precipitated CPA (Frenois, Cador, Caille, Stinus, & Le Moine, 2002; Frenois, Stinus, Di Blasi, Cador, & Le Moine, 2005; Gracy, Dankiewicz, & Koob, 2001; Ishida et al., 2008; Jin et al.,
2004, 2005), while lesions to the CeA (Watanabe et al., 2002; Xu et al., 2012), and bilateral intra-BNST delivery of noradrenergic β1 & 2 antagonists or lesions to the noradrenergic bundle innervating the BNST (Aston-Jones et al., 1999) impair its acquisition. Considering the prominent role of the extended amygdala in the establishment of a naloxone-precipitated morphine withdrawal CPA and the localization of CB\textsubscript{1} receptors within this region (Patel, Cravatt, & Hillard, 2005; Puente et al., 2010; Tsou, Brown, Sanudo-Pena, Mackie, & Walker, 1998), we evaluated the effects of AM251 and MJN110 delivered to the CeA and the basolateral amygdala (BLA) on the establishment of the naloxone-precipitated morphine withdrawal CPA (Wills et al., 2016). When delivered bilaterally to the BLA or the interoceptive insular cortex, MJN110 (2 µg), but not AM251 (0.1 µg) interfered with the CPA; however, when delivered bilaterally to the CeA, AM251 (0.1 µg), but not MJN110 (2 µg) interfered with the CPA. Since the CeA, BLA and the interoceptive insular cortex all project to the BNST (Gungor, Yamamoto, & Paré, 2015), and CB\textsubscript{1} receptor activation strongly inhibits both excitatory and inhibitory synaptic transmission within this region (Puente et al., 2010), we hypothesized that endocannabinoid modulation within the BNST would interfere with affective withdrawal. Consequently, the current experiment aimed to evaluate the potential of intra-BNST administration of the same effective dose of AM251 (0.1 µg) or the MAGL inhibitor, MJN110 (2 µg), to interfere with a naloxone-precipitated morphine withdrawal induced CPA.

**Method**

**Subjects**

Subjects were male Sprague–Dawley rats (Charles River Lab, St Constant, Quebec) weighing between 350 and 450 g. Animals were individually housed in opaque plastic cages with food (Envigo 2014 Rodent Diet, 14% protein) and water ad-libitum. Rats were maintained
on a reverse light-dark cycle (lights off at 7 am.) and the colony room was kept at an ambient temperature of 21°C. All animal procedures were approved by the Animal Care Committee of the University of Guelph and adhere to the guidelines of the Canadian Council of Animal Care.

Drugs

Morphine and naloxone were prepared with saline at a concentration of 20 and 1 mg/ml, respectively, and administered subcutaneously (sc) at a volume of 1 mg/ml. The MAGL inhibitor, MJN110 (2 µg; Sticht et al, 2016; Wills et al., 2016) and CB₁ antagonist, AM251 (0.1 µg; Wills et al., 2016) were prepared in a vehicle (VEH) consisting of 1:1:18 mixture of ethanol, Tween 80 and Saline (with the ethanol evaporated off) making the final mixture 1:9 Tween:Saline. Both compounds were microinfused over a 2 min period into the BNST at a rate of 0.25 µl/min, for a final volume of 0.5 µl. AM251 (Experiment 1) and MJN110 (Experiment 2) were infused before both the saline and the naloxone-precipitated MWD trial. Given that these pretreatments are experienced in both chambers, any confounds from the potential motivational effects of the pretreatment drugs in and of themselves are controlled.

Surgery

Rats were anesthetized with isoflurane gas and prepared for intracranial surgery as previously described (Limebeer et al., 2010). Once rats were stabilized in the stereotaxic frame in the flat skull position, small bilateral holes were drilled into the exposed skull and stainless steel guide cannulas (22 G, 6mm below pedestal) were lowered into the BNST at a 20° convergent angle using the following coordinates relative to Bregma (Paxinos & Watson, 1998): - 0.3mm anteroposterior, + 4.0mm mediolateral, and -4.7mm dorsoventral from the skull surface. The guide cannulas were stabilized to the skull using six screws and dental cement. Once dental
cement hardened, stainless steel dummies were inserted into the guide cannulas to prevent obstruction.

**Histology**

Guide cannula placements were determined through the histological examination of brain tissue. Before perfusion, rats were microinfused with Chicago blue dye to verify diffusion of the drug was localized to the BNST; analyses revealed an average spread of 0.35mm in each of the anteroposterior, mediolateral, and dorsolateral coordinate planes. The rats were then deeply anesthetized with a lethal dose of Euthansol (85 mg/kg i.p.) and transcardially perfused with PBS buffer (0.1 M) followed by 4% formalin. Brains were removed and stored in a 20% sucrose and 4% formalin solution overnight at room temperature, after which they were preserved at a temperature of 4 °C until sectioned. Brains were frozen and sliced into 60 μm sections using a CM1850 Leica cryostat and relevant slices were mounted onto gelatin-subbed glass microscope slides. Slide tissue sections were then stained with thionin, cover-slipped, and examined using a Leica MZ6 Stereomicroscope. Rats with improper cannula placement in either or both hemispheres were excluded from the study; 13 rats were removed from Experiment 1 and 14 rats were removed from Experiment 2. All n’s reported in the manuscript reflect the post-histology numbers per group.

**Apparatus**

An unbiased conditioning apparatus was used. The apparatus was a black rectangular Plexiglas box (60 x 25 x 25 cm) with a wire mesh lid and chambers differentiated solely by the floor texture. Removable floors were used to transition the boxes from conditioning cycles to pretest/test trials. During conditioning, single black metal floors made of a grid or hole pattern
were used as tactile cues. During pretest and test trials, split black metal floors equally divided into a half grid/half hole pattern were used. A camera mounted directly above the boxes and firewired to a computer recorded rat movement and Ethovision software was used to define box perimeters and assign a third neutral floor zone 5 cm on either side of the center where the floors join between the two floors for pretest and test trials. Therefore, the final measurement of preference provided time spent in three zones.

Procedure

Place conditioning was conducted using an unbiased place conditioning procedure with assignment to floors (grid vs hole) counterbalanced among the pretreatment groups. Two days prior to the first conditioning trial, the rats received a 15 min pretest on the split grid/hole floor to determine their baseline preference which did not significantly differ between the two floors (Experiment 1: mean grid=397 sec, mean hole=394 sec, t (25) =0.12, ns; Experiment 2: mean grid=412 sec, mean hole=388 sec, t (26) =0.99). Rats with a bias of more than 250 s for either floor (2 rats in each experiment) were removed from the experiments.

Experiment 1: Effect of AM251 on the establishment of a MWD CPA when delivered to the BNST

A three-day conditioning cycle was used to obtain the MWD CPA. On the first day, the rats were injected with saline (1 ml/kg s.c.) 10 min prior to being placed in the conditioning box with the saline-paired floor for 45 min. Twenty four hours later, rats received a high dose (20 mg/kg s.c.) of morphine and remained in their home cage, while being monitored constantly. On the third day, twenty four hours post morphine, the rats were injected with naloxone (1 mg/kg s.c.) 10 min prior to being placed in the conditioning box with the MWD floor for 45 min.
Fifteen minutes (consistent with Wills et al., 2016) prior to both saline and naloxone conditioning trials, rats were bilaterally microinfused with 0.1 µg AM251 ($n = 13$) or VEH ($n = 13$) into the BNST at a rate of 0.25 µl/min for 2 min. Injector tips were inserted to extend 2mm beyond the length of the guide cannula. Following the 2 min infusion period, the injectors were left in place for an additional minute to ensure full diffusion of the drug from the injector.

The place preference test trials began four days later. The rats were given daily 15 min test trials with the split grid/hole floor for 3 days. On each test, rats received a s.c injection of saline 10 min before being placed in the box.

Experiment 2: Effect of MJN110 on the establishment of a MWD CPA when delivered to the BNST

The procedures were identical to those of Experiment 1 except that 2 µg MJN110 ($n = 14$) or VEH ($n = 13$) were bilaterally microinfused into the BNST 60 min (consistent with Wills et al., 2016) prior to both saline and naloxone precipitated MWD conditioning and this group was compared with the VEH group ($n=13$) collected in Experiment 1.

Data Analysis

In each experiment, the time spent in seconds on each floor during each of the three test trials was entered into a mixed three factor analysis of variance (ANOVA), with the between group factor of pretreatment drug and the within group factors of floor (saline-paired or MWD-paired) and test trial. As well, activity measures during conditioning trials were entered into a mixed factor ANOVA with the between groups factor of pretreatment drug and the within-groups factor of conditioning trial (saline or MWD) for each experiment. Significance was set at $p<0.05$. 
Results

Experiment 1: AM251 delivered to the BNST interferes with the establishment of a MWD CPA

Intra-BNST microinfusion of AM251 significantly interfered with the establishment of a MWD CPA. Figure 1 presents the mean number of seconds that rats spent on the MWD paired floor or the saline paired floor on each trial when administered intra-BNST infusions of VEH or AM251 (1A), as well as cannula placements for each rat included in the data analysis (1B). The mixed factor ANOVA revealed only a significant floor by pretreatment drug interaction, $F(1, 24) = 6.4, p = 0.02$; therefore, the file was split by drug for analysis of each drug individually. This analysis revealed that pooled across trials, rats pretreated with VEH ($p = 0.02$), but not AM251, spent significantly less time on the MWD paired floor than the saline paired floor.

An analysis of the conditioning activity data (not depicted) revealed a significant main effect of trial, $F(1, 24) = 102.0, p<0.01$; indicating total distance travelled was significantly reduced during the MWD conditioning trial than during the saline conditioning trial, but pretreatment drug group did not significantly modify activity.

Experiment 2: MJN110 delivered to the BNST does not interfere with the establishment of a MWD CPA

Intra-BNST administration of MJN110 (at a dose that did interfere with MWD CPA when delivered to the BLA; Wills et al., 2016) did not interfere with the establishment of the MWD CPA. Figure 2 presents the mean number of seconds that rats spent on the MWD paired floor or the saline paired floor on each trial when administered intra-BNST infusions of VEH or MJN110 (1A), as well as cannula placements for each rat included in the data analysis (1B). The mixed factor ANOVA revealed only a significant main effect of floor, $F(1, 25) = 7.2, p = 0.01$;
but no floor by drug interaction, $F(1, 25) = 0.8$, or floor by drug by trial interaction, $F(2, 50) = 0.2$. Therefore, the files were not split by drug for further analysis. Pooled across conditioning trials and drug pretreatment groups, the rats spent significantly less time on the MWD paired floor than the saline paired floor.

An analysis of the conditioning activity data (not depicted) revealed only a significant main effect of trial, $F(1, 25) = 83.6, p<0.01$, indicating total distance travelled was significantly reduced during the MWD conditioning trial than during saline conditioning trial, but the pretreatment group did not modify this effect.

**Discussion**

Systemic (Wills et al., 2014) and intra-CeA (Wills et al., 2016) administration of the CB₁ antagonist/ inverse agonist, AM251, interferes with a MWD CPA. Since the CeA and the BNST are interconnected components of the extended amygdala, we evaluated the potential of the same dose of AM251 (0.1 ug) to interfere with a MWD CPA as well. Intra-BNST infusions of AM251 prevented the MWD CPA in Experiment 1. On the other hand, intra-BNST infusions of the MAGL inhibitor, MJN110 (Niphakis et al., 2013), which elevates 2-AG did not prevent MWD-induced aversion at the same dose that did prevent the MWD-induced floor aversion when delivered to the BLA or to the interoceptive insular cortex (Wills et al., 2016), which are both interconnected with the BNST (Dong et al., 2001; McDonald et al., 1999). Since systemic MJN110 also prevents MWD-induced floor aversions (Wills et al., 2016), elevated 2-AG may reduce the aversive effects of MWD in the BLA and the interoceptive insular cortex, but not in the BNST.
Although a higher dose of MJN110 was not tested in the current experiment, we can be confident this dose of MJN110 elevated 2-AG levels in the BNST. Sticht et al. (2016) delivered the same dose of MJN110 (2 µg, bilaterally) to the interoceptive insular cortex and measured 2-AG levels released in tissue 60 min later; 2-AG was significantly elevated in this region and this dose prevented nausea based conditioned gaping reactions at the same dose. As well, the same dose of MJN110 (2 µg, bilaterally administered) did reduce the naloxone-precipitated MWD CPA when administered to the BLA or to the interoceptive insular cortex in our prior work (Wills et al., 2016). While a direct comparison of the level of MAGL mRNA expression between these regions has not been conducted, MAGL expression has been found to correlate with CB$_1$ receptor expression (Dinh et al., 2002; Gulyas et al., 2004), which is more abundant in the BLA than in the BNST (Matsuda, Bonner, & Lolait, 1993).

There are few investigations of the role of the endocannabinoid system in the BNST, and consequently, it is difficult to determine how AM251 administration may be acting to interfere with the establishment of the naloxone-precipitated MWD CPA. However, given that AM251 was administered to the BNST on both saline and MWD conditioning trials, any rewarding or aversive properties of the drug itself would have been associated with both floors and would likely not contribute to its ability to interfere with the CPA.

It is intriguing that while CB$_1$ receptor antagonism interfered with the CPA, elevation of 2-AG through inhibition of MAGL activity did not modify its establishment. Since AM251 would be acting non-specifically at CB$_1$ receptors that may be activated by either 2-AG or anandamide (AEA), it is difficult to exclude the possibility that 2-AG may be contributing to the establishment of the naloxone-precipitated CPA. While prior experiments by our lab suggest that inhibition of FAAH activity (which elevates AEA) with PF3845 and URB597 was unable to
modify a naloxone-precipitated MWD CPA when administered systemically (Wills et al., 2014), future investigations will be required to elucidate the role of AEA in the BNST in response to naloxone-precipitated MWD. Additionally, it would be interesting to evaluate the effects of a dual FAAH/MAGL inhibitor (such as JZL195; Long, Nomura, et al., 2009) in the BNST to elucidate the role of endocannabinoid crosstalk in mediating affective opioid withdrawal.

The BNST is a component of the extended amygdala which plays an important role in the regulation of reward, stress and anxiety (McElligott & Winder, 2009; Silberman & Winder, 2013). Given that cannabinoids are able to modulate both excitatory and inhibitory transmission within the BNST (Puente et al., 2010), it is possible that AM251 produced its effects by disinhibiting or elevating GABA or glutamate release; however, the present study cannot address the mechanism. We might speculate that blocking CB₁ receptors on GABAergic neurons may promote transmission of this inhibitory neurotransmitter in this region resulting in a suppression of anxiety-like responding. To support this hypothesis, it has been found that inhibition of GABA release from the CeA to the BNST through the activation of presynaptic dynorphin receptors located on CeA afferents promotes anxiogenesis (Li et al., 2012). Alternatively, although glutamate antagonism in the BNST is anxiolytic (Kim et al., 2013), the potential for AM251 to enhance glutamatergic transmission within certain subregions of the BNST may represent an alternative mechanism of action. Indeed, photostimulation of glutamatergic projection neurons from the BLA decreases anxiety-like behavior in the elevated-plus maze, while photoinhibition of this same projection reduces it (Kim et al., 2013). Future work with conditional knockout models are necessary to determine the actual mechanism underlying the potential of CB₁ antagonism of the BNST to reduce the aversive effects of acute naloxone precipitated MWD.
It is important to note, however, that there are findings based upon other measures than MWD that are in contradiction to those reported here. Gomes-de-Sousa et al (2016) reported that microinjections of AM251 into the BNST enhanced tachycardia caused by restraint stress and that microinjections of the MAGL inhibitor, JZL 184 into the BNST decreased the heart rate response produced by stress (Gomes-de-Souza et al., 2016). Therefore, it will be necessary in future research to investigate the effect of manipulations of the endocannabinoid system in the BNST to modify anxiogenic behaviors in rats using other behavioral measures.

In addition to the ability of cannabinoids to modulate GABA and glutamate activity within the BNST, it is also possible that AM251 is acting to directly or indirectly reduce or interfere with the effects of noradrenaline (NA) or corticotropin-releasing factor (CRF) within this region. Both neurotransmitters are elevated in the BNST during acute drug withdrawal (Aston-Jones et al., 1999; Delfs et al., 2000; Nobis, Kash, Silberman, & Winder, 2011; Olive, Koenig, Nannini, & Hodge, 2002), have been directly or indirectly implicated in mediating the aversive state of opioid withdrawal (Aston-Jones et al., 1999; Park et al., 2013), and have been reported to be influenced by cannabinoid modulation (Kupferschmidt, Klas, et al., 2012; Kupferschmidt, Newman, et al., 2012; Oropeza, Mackie, & Van Bockstaele, 2007; Oropeza, Page, & Van Bockstaele, 2005; Page et al., 2007). Specifically, NA neurotransmission is elevated in the BNST during opioid withdrawal and bilateral intra-BNST delivery of noradrenergic β 1 & 2 antagonists or lesions to the noradrenergic bundle innervating the BNST impair the establishment of an opioid withdrawal-induced CPA (Aston-Jones et al., 1999; Delfs et al., 2000). While it remains to be established whether cannabinoid modulation alters NA neurotransmission within this region, CB1 receptor agonism has been found to enhance extracellular NA in associated regions such as the prefrontal cortex (Oropeza et al., 2007, 2005;
Page et al., 2007; Stamatakis et al., 2014). Finally, although the evidence for the ability of cannabinoid modulation to interfere with the MWD CPA by influencing CRF activity is indirect, some reports suggest an interaction is possible. Indeed, CRF injected directly into the BNST is capable of enhancing anxiety-like behavior as measured by the acoustic startle response (Lee & Davis, 1997), morphine withdrawal-potentiated startle is known to be mediated by enhanced CRF and NA activity (Park et al., 2013), and CRF activity within the BNST has been attributed to mediating anxiety-like behavior (Sink et al., 2012; Walker, Miles, & Davis, 2009) and dysphoria in nicotine withdrawal (Qi et al., 2016). Furthermore, intracerebroventricular administration of AM251 reduced anxiety-like behavior in the elevated plus maze produced by intracerebroventricular CRF or cocaine withdrawal (Kupferschmidt, Newman, et al., 2012); an effect hypothesized to be mediated by an extrahypothalamic CRF system such as the extended amygdala. Future studies should further investigate the role of CRF and NA activity in the BNST in mediating aversive opioid withdrawal and evaluate the role of the endocannabinoid system in modulating these behaviors.

In summary, the current findings suggest that CB₁ receptor antagonism/inverse agonism in the BNST is capable of interfering with the aversive state of opioid withdrawal as evidenced by its ability to interfere with the establishment of a naloxone-precipitated MWD CPA. Future studies will need to address the mechanism through which this occurs.
Acknowledgements

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Figure 1. A) Mean (±SEM) time spent in seconds on the saline-paired floor and the MWD-paired floor for each pretreatment drug group (VEH, 0.1 μg AM251 into the BNST during conditioning) during each 15 min test trial. B) Cannula placements for all rats included in the experiment.
Figure 2. A) Mean (±SEM) time spent in seconds on the saline-paired floor and the MWD-paired floor for each pretreatment drug group (VEH, 2 μg MJN110 into the BNST during conditioning) during each 15 min test trial. B) Cannula placements for all rats included in the experiment.
CHAPTER 5
General Discussion

This dissertation aimed to expand on the growing body of knowledge suggesting a role for cannabinoid modulation in the treatment of opioid addiction through an evaluation of cannabinoid-induced reductions in affective opioid withdrawal. The main findings from the research presented herein reveal that enhancement of endocannabinoid tone, specifically 2-AG through the inhibition of MAGL activity, or blockade of the CB₁ receptor with neutral antagonists/inverse agonists, is capable of interfering with the establishment of a naloxone-precipitated MWD CPA in rats. Furthermore, the ability of MAGL inhibition to interfere with the CPA was mediated, in part, by its actions within the BLA and interoceptive IC, while the effect of the CB₁ receptor antagonist, AM251, was mediated, in part, by its actions within the CeA and BNST. Because it is well accepted that a naloxone-precipitated MWD CPA is a measure of affective withdrawal and has predictive validity assessing the ability of a pharmacological compound to attenuate this aversive state (Stinus et al., 2005), these findings suggest that these compounds may have therapeutic potential in the treatment of opioid withdrawal. However, before any conclusions can be drawn, a discussion of the potential mechanisms that may be mediating these effects is required.

Of first importance is a discussion regarding the somewhat paradoxical effect of MAGL inhibition and CB₁ receptor antagonism to similarly interfere with the MWD CPA. This finding, although surprising, is consistent with previous reports on the ability of CB₁ receptor agonism and antagonism to mutually reduce somatic symptoms of opioid withdrawal. As revealed in later experiments, this effect may be attributable to a
dissociation of the ability of these compounds to reduce affective withdrawal in regions implicated in mediating the aversive withdrawal state. However, while agonism vs. antagonism interfered with the MWD CPA in either region, the reverse did not enhance the CPA. It is important to clarify that while no enhancements of the naloxone-precipitated MWD CPA were observed, the paradigm and procedures employed would be sensitive to detecting an enhancement of the CPA. In establishing the place aversion through the use of one conditioning cycle, the aversion is reliably produced but not at the limits of detection (i.e. does not produce a ceiling effect). This can be exemplified by pointing out that an additional conditioning cycle is able to significantly enhance the place aversion produced by a single conditioning cycle as employed in the current dissertation (Parker et al., 2002; Parker & Joshi, 1998). Ultimately, the findings presented reveal a complicated role for the endocannabinoid system in affective morphine withdrawal.

**Role of AEA vs. 2-AG in affective morphine withdrawal**

Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) represent the two main endogenous ligands of the endocannabinoid system. While both ligands target the CB1 receptor, it is now generally accepted that each ligand regulates behavior through distinct cannabinoid mechanisms (Kinsey, Long, Cravatt, & Lichtman, 2010; Long, Nomura, et al., 2009). In response to morphine treatment, a dissociation in the levels of these ligands in brain regions relevant to addiction (i.e. limbic areas) is noted with decreases in 2-AG and increases in AEA content being reported following acute morphine administration and withdrawal (Viganò et al., 2003, 2004), indicating that morphine administration is having opposing effects on the mechanisms regulating AEA and 2-AG homeostasis (Welch, 2009). Indeed, decreased FAAH activity was additionally reported during withdrawal periods when AEA levels were high
Conversely, the reverse is observed for 2-AG during chronic morphine administration with enhancements of 2-AG and no changes in AEA reported (Viganò et al., 2004).

The findings presented in this dissertation partially agree with a dissociation of the role of these endocannabinoids in mediating affective opioid withdrawal in that systemic administration of a MAGL inhibitor (elevates 2-AG), but not a FAAH inhibitor (elevates AEA) interfered with the establishment of the CPA (Wills et al., 2014, 2016). Given the null effect of the two FAAH inhibitors tested (PF3845 & URB597) and the ability of these compounds to fully inhibit brain FAAH activity and elevate AEA at the dose and timing that these compounds were administered (Ahn, Johnson, Mileni, Beidler, Long, McKinney, et al., 2009; Fegley et al., 2005; Kathuria et al., 2003), the current experiments did not evaluate the ability of centrally administered FAAH inhibitors to interfere with the establishment of a naloxone-precipitated MWD CPA. However, given the ability of AM251 administration into the CeA and BNST to interfere with the CPA, it would be interesting to evaluate the effects of a FAAH inhibitor in these regions to elucidate the role of this endocannabinoid in these regions. Although elevated 2-AG did not enhance the CPA, AM251 would be acting non-specifically at CB₁ receptors activated by AEA and 2-AG, thus it is possible that 2-AG may still be involved in mediating affective withdrawal. Indeed, certain behavioral effects observed to be mediated by activation of the endocannabinoid system are the result of crosstalk between AEA and 2-AG (Long, Nomura, et al., 2009). Consequently, the investigation of a dual FAAH/MAGL inhibitor administered systemically and intra-cranially would be an additional avenue worth exploring.
Extended Amygdala and Associated Regions as the Locus of Interaction

Investigations into the brain regions mediating the effects of the cannabinoid compounds tested were driven by our systemic findings revealing the effectiveness of the CB₁ receptor antagonists/neutral antagonists to interfere with the establishment of the naloxone-precipitated MWD CPA. In finding this somewhat surprising effect, our focus shifted from an investigation into the brain regions well known to contribute to reward and addiction processes, such as the nucleus accumbens (NAcc) and ventral tegmental area (VTA), to those better known to be involved in aversive behavior, such as the extended amygdala. Indeed, the positive reinforcing effects of cannabinoid agonists are thought to be mediated by indirectly enhancing the release of dopamine in the NAcc through their actions within the NAcc and VTA (Maldonado et al., 2006), and opioid withdrawal is associated with diminished dopamine transmission within these brain regions. Consequently, one would expect the administration of CB₁ receptor antagonists to the NAcc and VTA to worsen rather than reduce affective withdrawal. Owing to the more limited understanding of cannabinoid involvement in the extended amygdala in relation to drug addiction and withdrawal, and its emerging role in drug addiction processes and affective opioid withdrawal in particular (Koob & Le Moal, 2001), an investigation into regions such as the CeA ensued. However, in light of the allosteric interactions reported between the µ-opioid receptor and the CB₁ receptor in the NAcc (i.e. the ability of an antagonist to interfere with the actions of a reciprocal antagonist; Schoffelmeer et al., 2006), an investigation into the ability of CB₁ antagonists to interfere with the naloxone-precipitated MWD CPA when administered to this region may be warranted.

An additional caveat to the investigation of the NAcc and the VTA as the locus of interaction was the inability of CB₁ receptor antagonists to interfere with the MWD prime
induced reinstatement of the CPA. The NAcc along with the VTA and the medial prefrontal cortex (mPFC) have been implicated in mediating heroin- and cocaine-prime induced reinstatement (Shaham et al., 2003). Given CB$_1$ receptor antagonists have been found to be effective in reducing reinstatement of opioid-primed drug seeking (De Vries et al., 2003; Fattore et al., 2003), we hypothesized that they may be additionally effective in reducing reinstatement of the CPA. While it remains to be determined whether the mechanisms mediating the reinstatement of a preference or aversion are different, similar brain regions could be involved. Consequently, the inability of the CB$_1$ receptor antagonists to modify reinstatement of the CPA indicated that these compounds may be acting in regions other than the NAcc and VTA.

**Possible Mechanisms of Action**

A conditioned place aversion provides a measure of affective learning. Consequently, in determining how modulation of the endocannabinoid system is able to interfere with the establishment of a naloxone-precipitated MWD CPA, one must consider whether the compound is interfering with the aversive state, or whether the compound is interfering with Pavlovian learning. In each of the experiments reported above, the pretreatment (CB$_1$ antagonist, MAGL or FAAH inhibitor) was administered on both the saline trial and on the naloxone trial, therefore, any affective effects of the pretreatment itself are equally distributed between the two chambers. Yet, one might argue that the pretreatment rather than interfering with the aversive withdrawal state, may be simply preventing the formation of the association itself. This is especially important when considering pharmacological manipulations of the endocannabinoid system which
are known to be involved in altering short and long term synaptic plasticity (Maldonado et al., 2006; Sidhpura & Parsons, 2011).

**MAGL Inhibition.** Although the exogenous administration of systemic CB$_1$ receptor agonists prior to a task is associated with impairments in working and short-term memory, and consequently may impair memory acquisition, a different picture emerges with the selective enhancement of endogenous AEA and 2-AG (Mechoulam & Parker, 2013; Riedel & Davies, 2005). Indeed, studies looking at memory acquisition in the Morris water maze (MWM) and object recognition (OR) task of MAGL knock-out mice have reported improved performance on these tasks indicating enhanced learning (Pan et al., 2011). Similarly, pharmacological inhibition of MAGL activity prior to training produced no memory impairments in the OR task (Busquets-Garcia et al., 2011), and produced only minor impairments in the MWM when tested at high doses (Wise et al., 2012). Together, these findings suggest the ability of the MAGL inhibitor, MJN110, to interfere with the establishment of the naloxone-precipitated MWD CPA when administered systemically are unlikely due to its ability to impair the acquisition of learning.

On the other hand, manipulations which modulate the level of endogenous 2-AG are associated with changes in affective state. Indeed, inhibition of 2-AG synthesis through the genetic deletion of diacylglycerol lipase α (DAGLα) produces an anxiogenic phenotype (Jenniches et al., 2016; Shonesy et al., 2014), while systemic administration of MAGL inhibitors have been found to be anxiolytic and promote positive affect (Busquets-Garcia et al., 2011; Kinsey, O’Neal, Long, Cravatt, & Lichtman, 2011). This is particularly true in instances where environmental aversiveness is high (Sciolino, Zhou, & Hohmann, 2011; Sumislawski, Ramikie, & Patel, 2011), and may be attributable to the fact that 2-AG also plays an important role in the regulation and habituation of the stress response where it acts to constrain HPA axis activation.
(Hill & Tasker, 2012). In fact, a similar dysregulation of 2-AG signaling (2-AG enhancement) is observed during chronic stress (Morena, Patel, Bains, & Hill, 2016) as when rats are administered chronic morphine. Thus, it has been suggested that any memory impairing effects produced by endogenous cannabinoids may actually be related to their ability to reduce the memory enhancing effects of emotionality, rather than their ability to interfere with memory processes per se (Morena & Campolongo, 2014). Consequently, the ability of systemic MAGL inhibition to interfere with the establishment of a naloxone-precipitated MWD CPA is likely due to its ability to interfere with the aversive affective state of morphine withdrawal.

Coincidentally, support for the ability of endocannabinoids to interfere with the enhancing effects of emotionality on memory acquisition comes from a study involving the BLA. Specifically, intra-BLA administration of the CB1 agonist, WIN55,212-2, twenty minutes prior to an elevated platform stressor impaired the memory enhancing effects of the stressor on the learning of a light-dark inhibitory avoidance task in rats; conversely, intra-BLA administration of the CB1 receptor antagonist, AM251, enhanced it (Ganon-Elazar & Akirav, 2009). Furthermore, intra-BLA administration of the CB1 agonist and antagonist produced no significant effects on the acquisition of the memory task alone in the absence of the stressor. The ability of WIN55,212-2 to impair stress-enhanced memory acquisition was accompanied by a reduction of stress-induced corticosterone release, suggesting the effects produced by cannabinoid modulation were not due to specific impairments in memory acquisition but instead resulted from their ability to constrain HPA axis activation. However, it is important to note that the effects of CB1 receptor activation on aversive memory acquisition in the BLA are not
consistently reported. Indeed, microinfusion of WIN55,212-2 into the BLA prior to training has actually been found to enhance the acquisition of subthreshold footshock fear learning in rats (Tan et al., 2011). Ultimately, these findings support the notion that the ability of intra-BLA MAGL inhibition to interfere with the establishment of the MWD CPA is unlikely due to its ability to produce memory impairments per se but is more likely due to the ability of MJN110 to reduce aversive withdrawal. As might be expected in a withdrawn state, naloxone-precipitated MWD is associated with enhanced corticosterone release and glucocorticoid receptor signaling (Navarro-Zaragoza, Hidalgo, Laorden, & Milanés, 2012), so the ability of the endocannabinoid system to reduce stress-induced emotionality is possible.

Within the BLA, cannabinoids have been found to modulate both GABAergic and glutamatergic synaptic transmission (Azad et al., 2003; Domenici et al., 2006; Katona et al., 2001). As a result, MJN110 could be acting to modify both GABA and glutamate release within the BLA to interfere with the establishment of the naloxone-precipitated MWD CPA. When considering GABAergic transmission, 2-AG has been found to mediate stress and glucocorticoid-induced suppression of spontaneous inhibition by reducing neurotransmitter release from GABAergic interneurons synapsing onto glutamatergic principal neurons (Di et al., 2016; Patel, Kingsley, Mackie, Marnett, & Winder, 2009). 2-AG mediated suppression of inhibition was associated with an enhancement of anxiety-like behavior (Di et al., 2016) and, consequently, the reduction in GABA release may underlie the ability of BLA endocannabinoids to enhance emotionality and emotional memory formation. On the other hand, 2-AG mediated suppression of amygdala glutamatergic activity is associated with anxiolytic efficacy and stress-resilience (Bedse et al., 2017; Bluett et al., 2017). Indeed, BLA spontaneous excitatory postsynaptic current (sEPSC) frequency is positively correlated with anxiogenic behavior in the
light/dark box assay in mice and MAGL inhibition with JZL184 is associated with a reduction in sEPSC frequency and enhancement in anxiolytic behavior (Bedse et al., 2017). Furthermore, stress-induced increases in BLA sEPSC frequency is reduced in JLZ184 incubated brain slices and is associated with reduced stress-induced anxiety-like behavior in mice in-vivo (Bluett et al., 2017). While it remains to be experimentally determined, these findings suggest that MJN110 is interfering with affective withdrawal by preferentially modulating glutamate release within this region.

Much less is known regarding the role of the endocannabinoid system in the interoceptive IC. This region has been described to be an important neural substrate in the representation of the internal physiological state and the processing of conscious emotion (Craig, 2002). Not surprisingly then, elevation of 2-AG content in the interoceptive IC with the administration of the MAGL inhibitor, MJN110, interfered with the establishment of lithium-chloride-induced conditioned gaping in rats; a measure of nausea (Sticht et al., 2016). The ability of MJN110 to interfere with nausea was unlikely due to memory impairments since delivery of the compound to the interoceptive IC was unable to interfere with taste avoidance learning. Given the findings of this study using the same compound and dose as was administered in the current dissertation, and the ability of morphine withdrawal to produce nausea as evidenced by its ability to produce conditioned gaping (McDonald et al., 1997), these findings suggest that intra-interoceptive IC MJN110 interferes with the establishment of the naloxone-precipitated MWD CPA by interfering with the malaise produced by morphine withdrawal in this region.
**CB₁ Receptor Antagonists.** As alluded to earlier in this dissertation, the ability of CB₁ receptor antagonists to interfere with the establishment of the naloxone-precipitated MWD CPA is somewhat surprising considering the role of the endocannabinoid system in learning and affective processes. Indeed, when administered systemically, CB₁ receptor antagonists are well known to reverse the memory impairments produced by CB₁ receptor agonists or act as memory enhancers (Riedel & Davies, 2005) and are typically attributed to producing anxiogenic-like behavior if having any effect on emotionality (Moreira & Wotjak, 2010; Viveros, Marco, & File, 2005). Consequently, finding that systemic administration of CB₁ receptor antagonists/inverse agonists were able to interfere with affective withdrawal, we sought to identify their locus of action to help determine how they may be mediating their effects.

In identifying that the effects of the CB₁ receptor antagonist, AM251, were mediated by its actions in the extended amygdala, we are able to posit that the CB₁ antagonists are interfering with the effects of corticotropin-releasing factor (CRF) within these regions. Specifically, intracerebroventricular (i.c.v.) AM251 has been shown to interfere with the anxiety-like behavior produced by i.c.v. CRF and cocaine withdrawal in the elevated-plus maze – an effect that was attributed to actions within an extrahypothalamic brain stress system (Kupferschmidt, Newman, et al., 2012) – and CRF activity within the CeA and BNST is implicated in mediating aversive withdrawal behavior (Heinrichs et al., 1995; Huang et al., 2010; McNally & Akil, 2002; Qi et al., 2016). As a result, it is reasonable to hypothesize that AM251 is acting to interfere with the establishment of the naloxone-precipitated MWD CPA by interfering with the actions of CRF within these regions. Given that CRF₁ receptor overexpression produces an anxious phenotype in rats that is associated with enhanced baseline glutamatergic transmission in the CeA (Natividad et al., 2017), it is possible that CB₁ receptor antagonists could be mediating their effects by
disrupting glutamate transmission in this region. However, the exact mechanism by which AM251 interferes with the actions of CRF remains to be elucidated. Future studies on the potential of AM251 to interfere with the effects of CRF downstream of CRF receptor binding through the modulation of GABA or glutamate transmission are required.

While the ability of CB$_1$ receptor antagonists to interfere with affective opioid withdrawal by mitigating the actions of CRF in the CeA and BNST seems promising, an alternative hypothesis is that μ-opioid and CB$_1$ receptors form heterodimeric receptor complexes in these regions. Indeed, as previously mentioned, μ-opioid and CB$_1$ receptors have been found to form heterodimeric receptor complexes (Hojo et al., 2008; Rios et al., 2006) and within the nucleus accumbens core, where μ-opioid and CB$_1$ receptors are found to co-localize (Navarro et al., 1998; Pickel, Chan, Kash, Rodriguez, & MacKie, 2004; Rodriguez, Mackie, & Pickel, 2001), application of a CB$_1$ receptor antagonist (SR141716A) blocked the ability of a μ-opioid receptor antagonist (naloxone) to modify GABA and glutamate release (Schoffelmeer et al., 2006). In support of this hypothesis, μ-opioid and CB$_1$ receptors have been reported to be co-localized in the CeA, but not in the BLA (Navarro et al., 1998). Thus, if the CB$_1$ receptor antagonists we tested are able to block the effects of naloxone at the μ-opioid receptor, this could interfere with the establishment of the CPA since spontaneous morphine withdrawal alone is unable to produce a CPA following a single conditioning trial (Stinus, Caille, & Koob, 2000). Upon closer examination of the studies which reported the ability of acute CB$_1$ antagonist administration to reduce opioid withdrawal, it becomes apparent that withdrawal was precipitated by naloxone in the presence of the antagonist (Trang et al., 2006; Wills et al.,
2014, 2016). However, while this remains a possibility worth exploring, this type of interaction would be unable to explain the ability of intracerebroventricular AM251 to reverse the anxiogenic effects produced by spontaneous cocaine withdrawal (Kupferschmidt, Newman, et al., 2012), albeit the mechanisms mediating anxiety produced by cocaine withdrawal may be different than opioid withdrawal. One additional possibility is that this receptor dimerization interferes with the ability of CRF to produce aversive affective withdrawal.

Finally, an additional note should be made in light of a study conducted by Seely et al. (2012) which reported that AM251 and rimonabant bind with mid-nanomolar affinity to μ-opioid receptors. With the possibility that these cannabinoid antagonists/inverse agonists may be acting as direct antagonists at the μ-opioid receptor, it was advised that studies employing high doses of AM251 and rimonabant be interpreted with caution. However, considering the 500-fold higher affinity of AM251 for the CB₁ receptor and its anticipated Ki value of ~2 μM for the μ-opioid receptor (Seely et al., 2012), we can be confident that our findings are not attributable to the actions of AM251 at the μ-opioid receptor given the dose and concentration of AM251 that we administered systemically (2.5 mg/kg) and intracranially (0.1 µg/µl) and the inability of AM251 to interfere with a naloxone-precipitated MWD CPA when administered to the BLA or the interoceptive IC at the same or at a higher concentration (0.25 µg/µl), respectively (Wills et al., 2016). Furthermore, it remains to be determined whether the additionally effective neutral CB₁ receptor antagonists tested would bind directly to the μ-opioid receptor.

**Future Experiments**

Although the current experiments suggest that endocannabinoid modulation is able to prevent the establishment of a naloxone-precipitated MWD CPA, a greater understanding of the
mechanisms underlying these effects remains to be elucidated. As discussed previously, the ability of the MAGL inhibitor to interfere with the MWD CPA when microinfused into the BLA and interoceptive IC is hypothesized to be mediated by its ability to inhibit glutamatergic neurotransmission since CB$_1$ receptor and 2-AG mediated inhibition of GABA activity is known to produce anxiety-like behavior in these regions (Di et al., 2016; Rey, Purrio, Viveros, & Lutz, 2012), while 2-AG mediated inhibition of glutamate activity is anxiolytic and promotes stress-resilience (Bedse et al., 2017; Bluett et al., 2017). Consequently, to test this hypothesis, electrophysiological experiments should investigate the degree to which bath application of a MAGL inhibitor is able to modify inhibitory and excitatory post-synaptic potentials in BLA and interoceptive IC slices obtained from rats experiencing naloxone-precipitated MWD.

Similarly, a greater understanding of the mechanisms mediating the effect of the CB$_1$ receptor antagonist is also required. Initially, it would need to be determined whether µ-opioid and CB$_1$ receptors are able to functionally interact in the CeA and BNST to rule out the possibility that these receptors are forming heterodimeric receptor complexes in these regions. One way in which this could be achieved is by quantifying the interaction of AM251 and naloxone on the release of radiolabeled GABA and glutamate in superfused slices of the CeA and BNST (see Schoffelmeer et al., 2006). If AM251 is able to block the effect of naloxone on neurotransmitter release in these regions, then it is possible that the ability of AM251 to reduce the establishment of the naloxone-precipitated MWD CPA is mediated by such interactions.

If µ-opioid and CB$_1$ receptors do not functionally interact, experiments using conditional CB$_1$ receptor knock-out (KO) mutants specifically for GABA and glutamate
neurons (see Rey et al., 2012) should be performed to determine whether the effects of the CB1 receptor antagonists are mediated by their actions on either cell type. Additionally, null and conditional CB1 KO mutants could be used in combination with electrophysiology to determine if and how CB1 receptors may influence evoked and applied CRF mediated GABA and glutamate neurotransmission in CeA and BNST slices.

It would also be interesting to evaluate the ability of the neutral CB1 receptor antagonists, that were administered systemically (AM4113, AM6527), to interfere with the MWD CPA when microinfused into the CeA and BNST to confirm their efficacy is maintained in these regions. Additionally, while systemic FAAH inhibition did not modify the establishment of the naloxone-precipitated MWD CPA, an evaluation of its effects when administered to the CeA and BNST would be of interest.

Finally, although CB1 receptor antagonists did not modify reinstatement of the CPA using naloxone-precipitated MWD drug primes, future experiments should investigate the ability of these compounds to interfere with footshock stress-induced reinstatement given that the CeA and BNST are known to have a central role in mediating this behavior (Erb, Shaham, & Stewart, 2001; Shaham, Erb, & Stewart, 2000; Shaham et al., 2003). However, this considered, it should be noted that CB1 receptor antagonists have been reported to be unable to modify footshock-induced reinstatement of cocaine drug-seeking (De Vries et al., 2001; Kupferschmidt, Klas, et al., 2012).

Clinical Implications

The proper management of opioid withdrawal symptoms would remove its capacity to serve as a negative reinforcer in continued drug use and improve detoxification in addicts. The
findings presented in this dissertation suggest that pharmacological manipulation of the endocannabinoid system could serve as an alternative treatment to classical opioid compounds in the treatment of opioid detoxification. However, although both neutral CB₁ receptor antagonists and a MAGL inhibitor were able to reduce affective opioid withdrawal, the inconsistent findings reported on the ability of CB₁ antagonists/inverse agonists to reduce somatic and affective withdrawal requires a more rigorous investigation into the therapeutic utility of neutral CB₁ receptor antagonists. Furthermore, given that acute CB₁ receptor antagonist treatments have been reported to precipitate both spontaneous affective and somatic withdrawal (Navarro et al., 1998, 2001), chronic antagonism of the endocannabinoid system during the development of dependence using neutral antagonists should be investigated. Clearly, more studies conducted on the ability of neutral CB₁ receptor antagonists to reduce somatic and affective opioid withdrawal are required, including a better understanding of their mechanism of action when administered acutely during opioid withdrawal.

Alternatively, pharmacological inhibition of MAGL activity may seem like a more promising approach to CB₁ receptor antagonism in the treatment of opioid withdrawal. Unfortunately, recent studies reveal that the effects of MAGL inhibition on affective opioid withdrawal are also inconsistent. Specifically, a study by Gamage et al. (2015) investigating the ability of the MAGL inhibitor, JZL184, to reduce the establishment of a naloxone-precipitated MWD CPA in morphine withdrawn mice found no effect. However, given that these discrepant findings of MAGL inhibition were obtained by different laboratories using different procedures, compounds (JZL-184vs.MJN110), and species (mice vs.rats), more research into the potential of MAGL inhibition in reducing affective opioid withdrawal will be required in order to elucidate
its role. Indeed, the MAGL inhibitor employed in the Gamage study has been found to be ineffective at elevating MAGL in rats (Long, Li, et al., 2009).

Additionally, unlike FAAH inhibitors, MAGL inhibitors have been found to produce cannabimimetic side effects (eg. hypomotility, hyperreflexia; Long, Li, et al., 2009) and can lead to the development of dependence and tolerance with repeated administration (Schlosburg et al., 2010). Consequently, this would limit the therapeutic utility of MAGL inhibitors to acute treatment. In light of this, Gamage et al. (2015) and Ramesh et al. (2013) tested the combinations of low doses of FAAH and MAGL inhibitors, or dual FAAH/MAGL inhibitors, for their effectiveness in reducing withdrawal maximally without additional side effects. Indeed, this combination of catabolic enzyme inhibitors proved to be highly effective in reducing somatic withdrawal (including jumping, paw flutters, head shakes, diarrhea, weight loss) and was absent of adverse effects, but proved to have little effectiveness in reducing affective withdrawal when tested in the place aversion paradigm (Gamage et al., 2015). However, given that this same lab reported that MAGL inhibitors were unable to modify affective withdrawal further studies should be conducted on the utility of dual FAAH/MAGL compounds. Ultimately, when considering pharmacological interventions that may aid in the treatment of opioid withdrawal detoxification, dual FAAH/MAGL inhibition (at low doses) may be most promising.

Conclusions

Owing to the body of research indicating that endocannabinoid modulation may have potential in the treatment of somatic opioid withdrawal, this dissertation aimed to evaluate the role of the endocannabinoid system in affective opioid withdrawal. The findings presented herein revealed that systemic elevation of endogenous 2-AG through the inhibition of MAGL activity, as well as antagonism/neutral antagonism of the CB$_1$ receptor, is able to interfere with the
establishment of a naloxone-precipitated MWD CPA; a model of affective opioid withdrawal. These effects were mediated, in part, by the actions of the CB₁ antagonist in the CeA and BNST, and the MAGL inhibitor in the BLA and the interoceptive IC. Although the precise mechanism through which these effects occur remains to be elucidated, a review of the literature suggests that these manipulations are interfering with the CPA by reducing the negative affective state. Ultimately, this suggests that MAGL inhibition and neutral CB₁ receptor antagonism may have potential in the treatment of opioid withdrawal. However, given that the effects of the CB₁ receptor antagonists may be mediated by their ability to interfere with the actions of naloxone at the µ-opioid receptor and these compounds have been found to precipitate somatic opioid withdrawal, future research should focus on inhibition of MAGL activity as a viable alternative to synthetic opioids in the treatment of opioid withdrawal.
CHAPTER 6

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