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Cannabinoid CB1 Transmission in the Mesolimbic Reward Pathway

Tasha Ahmad, *The University of Western Ontario*

Supervisor: Dr. Steven R. Laviolette, *The University of Western Ontario*

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Abstract

Cannabinoid CB1 receptor (CB1R) transmission within the meso-corticolimbic system plays an important role in forming associative memories, and processing both positive and negative experiences. Opiates generally produce potent rewarding effects and previous evidence suggests that CB1 transmission may modulate the neural reward circuitry involved in opiate reward processing. The ventral tegmental area (VTA), medial prefrontal cortex (mPFC), basolateral amygdala (BLA), and Nucleus Accumbens (NAc) are all implicated in opiate-reward processing, contain high levels of CB1 receptors, and are all modulated by dopamine (DA). Although, CB1 transmission within these areas has been heavily implicated in associative memory and learning, the potential effects of CB1R modulation on these neural regions in regards to opiate related motivational information are not currently understood. Using a combination of unbiased conditioned place preference (CPP) paradigm and pharmacological manipulation, we examined the role of CB1 transmission within these neural circuitries in relation to opiate reward processing.

We report that activation or inhibition of CB1 transmission within the mPFC and BLA bidirectionally regulates the motivational valence of opiates; whereas CB1 activation switched morphine reward signaling into an aversive stimulus, blockade of CB1 transmission potentiated the rewarding properties of normally sub-reward threshold conditioning doses of morphine. Both of these effects were dependent upon DA transmission. Furthermore, CB1-mediated intra-mPFC opiate motivational signaling is mediated through a μ-opiate receptor-dependent reward pathway, or a κ-opiate receptor-dependent aversion pathway, directly within the ventral tegmental area. In contrast, CB1-mediated intra-BLA opiate motivational signaling is mediated through the NMDA transmission in the shell region of NAc (NASh). Finally, using multi-unit *in vivo* electrophysiological recordings in the NASh, we report that the ability of intra-BLA CB1R modulation to control opiate reward salience and motivational valence is associated with distinct reward or aversion neuronal activity patterns and bi-directional regulation of intra-NASh fast-spiking interneurons vs. medium spiny neurons. Our results provide evidence for a novel CB1 mediated motivational valence switching mechanism within the mPFC, and BLA, controlling dissociable subcortical reward and aversion

pathways. Lastly, we report that CB1 mediated reward is localized to the CB1R's located in the posterior region of the VTA.

Keywords:

Cannabinoid, Reward Learning, Dopamine, Medial Prefrontal Cortex, Associative Memory, Emotional Salience, Behavioural Pharmacology, Condition Place Preference, Opiate, Basolateral Amygdala, *In-vivo* Electrophysiology, Nucleus Accumbens Shell, Ventral Tegmental Area

Co-Authorship Statement

Chapter 2:

Entitled “Cannabinoid transmission in the prelimbic cortex bidirectionally controls opiate reward and aversion signaling through dissociable kappa versus μ-Opiate receptor dependent mechanisms” was written by Tasha Ahmad with inputs from Nicole M. Lauzon, Xavier de Jaeger, and Dr. Steven R. Laviolette. Tasha Ahmad performed all experimental procedures and data analyses. Histological analyses were performed by Tasha Ahmad, Nicole M. Lauzon, and Xavier de Jaeger. Dr. Steven R. Laviolette provided intellectual input.

Chapter 3:

Entitled “Bi-directional cannabinoid signaling in the basolateral amygdala controls rewarding and aversive emotional processing via functional regulation of the nucleus accumbens” was written by Tasha Ahmad with inputs from Ninglei Sun, Danika Lyons, and Dr. Steven R. Laviolette. Experimental procedures and data analyses were performed by Tasha Ahmad and Ninglei Sun. Histological analyses were performed by Tasha Ahmad and Danika Lyons. Dr. Steven R. Laviolette provided guidance and intellectual input.

Chapter 4:

Entitled “Cannabinoid reward and aversion in the posterior ventral tegmental area is differentially mediated through dopamine projections to the basolateral amygdala or nucleus accumbens shell” was written by Tasha Ahmad with intellectual inputs from Dr. Steven R. Laviolette.

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List of Abbreviations

2-AG	2-arachidonoylglycerol
α-flu	α-flupenthixol
ANOVA	analysis of variance
AP5	(2*R*)-amino-5-phosphonovaleric acid
AVTA	Anterior ventral tegmental area
BLA	Basolateral amygdala
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid
CBR	Cannabinoid receptor
CB1R	Cannabinoid CB1 receptor
CB2R	Cannabinoid CB2 receptor
CPP	Conditioned place preference
DA	Dopamine
FSI	Fast spiking interneurons
GABA	Gamma-Aminobutyric acid
GLUT	Glutamine
MSN	Medium spiny neurons
NAc	Nucleus accumbens
NACo	Nucleus accumbens core
NASh	Nucleus accumbens shell
NMDA	N-Methyl-D-aspartic acid
mPFC	Medial prefrontal cortex
PLC	Prelimbic cortex
PTSD	Post-traumatic stress disorder
PVTA	Posterior ventral tegmental area
VTA	Ventral tegmental area

Chapter 1

1 General Introduction: Cannabinoids, Opiates, and the Reward Circuitry

1.1 THE ENDOCANNABINOID SYSTEM

In mammalians, the endocannabinoid system is crucial in maintaining health and chemical equilibrium within the body. Endogenous cannabinoids and the receptors they bind to, are found throughout the entire body. The endocannabinoid system enables communication, modulation and coordination between various cell types and receptors. The two major cannabinoid receptors (CBRs) are CB1 and CB2 cannabinoid receptor (CB1R and CB2R respectively). The CBRs are a G-protein coupled, seven trans-membrane domain receptors that are activated by both endogenous and exogenous cannabinoids (Console-Bram, Marcu, & Abood, 2012). The activation of CB1 or CB2 receptors generally blocks adenyl cyclase, and hence prevents signaling through cyclic AMP. The two most widely researched endocannabinoid are 2-arachidonoylglycerol (2-AG) and anandamide, that have been shown to function as retrograde messengers, where they are synthesized and released by postsynaptic dendritic cell bodies and activate CB1Rs located in the presynaptic axon terminals (Marsicano & Lafenetre, 2009). Activation of CB1Rs then modulates signaling by reducing the amount of neurotransmitter released, and the overall effect is dependent on the neurotransmitter secreted by the specific cell type. CB1Rs are distributed throughout the central nervous system (CNS) and are associated with learning, emotional behaviour, and linked to food intake and obesity (Chaperon, Soubrié, Puech, & Thiébot, 1998; Martin, Ledent, Parmentier, Maldonado, & Valverde, 2002; Osei-Hyiaman, Harvey-White, Batkai, & Kunos, 2006; Boyd, 2006). Conversely, CB2Rs are predominantly abundant in the peripheral nervous system (PNS), most notably in the immune and hematopoietic cells (Pertwee, 1997; Osei-Hyiaman et al., 2006). For the purpose of this study we will be focusing on the CB1Rs, as they are primarily present in the brain.

1.1.1 Role of CB1 Receptor in Emotional Processing

A critical aspect of functional health in one's daily life is to present cognitive competence and form appropriate responses to the vast array of incoming sensory information from their surroundings. In order to successfully form coordinated responses, the human brain must assess the emotional valance of the incoming sensory stimuli, which requires the formation of learned associative memories between the stimulus and the environmental

cues (Laviolette & Grace, 2006). Both animal models and human studies have shown that activation of CB1Rs can lead to pronounced effects in associative learning emotional processing. The cannabinoid system as mediated by the activation of CB1Rs have shown to alter emotional behaviour and participate in both learning and memory processes (Martin et al., 2002). Furthermore, CB1R transmission has been implicated to strongly modulate the emotional valence of both rewarding and aversive experiences (Laviolette & Grace, 2006).

Although the CB1Rs are highly localized to the brain, they are not uniformly distributed in the CNS. They are densest in areas responsible for cognition, learning, and memory such as the medial prefrontal cortex (mPFC), basolateral amygdala (BLA), ventral tegmental area (VTA), and nucleus accumbens (Tsou et al., 1998; Fattore et al., 2007; Katona et al., 2001; Fattore et al., 2007; Marsicano & Lafenetre, 2009). Activation of CB1Rs within both the mPFC and BLA have shown to strongly potentiate the emotional salience of normally non-salient events (Tan et. al., 2010, 2014). Furthermore, fear conditioning studies have shown that inhibiting the CB1R with systemic injections of AM 251 (CB1R inverse agonist) in Long-Evans rats resulted in deficits in contextual learning and memory processing (Arenos, Musty, & Bucci, 2006). Moreover, previous research involving pairing of a neutral stimuli (an olfactory cue) with an emotionally salient stimuli (a mild foot shock) have shown that activating CB1Rs with WIN 55,212-2 (synthetic CB1R agonist) in the mPFC, potentiated emotional learning in animals. In contrast, blockade of CB1Rs with AM 251 in the mPFC led to inhibition of emotional learning, as the animals response to the olfactory cue was significantly downregulated (Laviolette & Grace, 2006). These findings outline the key role that CB1Rs play in associative learning and processing emotional salience in the mesolimbic pathway.

1.2 THE MESOLIMBIC REWARD PATHWAY

The mesolimbic reward pathway is the dopaminergic (DAergic) circuitry in the brain. It is a bundle of DAergic fibers that originate from the VTA and innervate higher level limbic structures including mPFC, BLA, and NAc (Gardner, 2005; Grace, Floresco, Goto, & Lodge, 2007; Ikemoto, 2007). Dopamine (DA) receptors are highly expressed in the VTA and play a crucial role in reward-related leaning, and processing emotional and

motivational information (Wise, 2004; Volkow, Wang, & Baler, 2011). Previous research has shown that DA is an integral component of stimulus-reward learning and processing the salience of sensory cues (Berridge & Robinson, 1998; Flagel et al., 2011a).

A vast array of pharmacological data over the years has indicated the existence of a functional interaction between CB1 and DA receptors. Modulation of CB1Rs within the mesolimbic circuitry has shown to play a significant role is DAergic transmission (Hermann, Marsicano, & Lutz, 2002). Activation of CB1Rs in the mPFC by exogenous cannabinoids have been shown to significantly increase extracellular DA levels, and decrease GABA (M Pistis et al., 2002). Similarly, single cell electrophysiological recordings in rats, demonstrated a profound increase in DA firing levels in the VTA, following the administration of a CB1R agonist (French, Dillon, & Wu, 1997; French, 1997). Hence, it is evident that the endocannabinoid system plays a central role in modulating DA levels in the CNS.

The components of the meso-corticolimbic pathway (as discussed below) consist of the VTA-PFC-BLA-NAc circuitry, and they are highly interconnected to one another (see **Fig.1.1**).

1.2.1 Ventral Tegmental Area

The ventral tegmental area (VTA) is part of the midbrain and it is the main site of DA neurons. It's a central component of the reward circuitry, and crucial for associative learning. The VTA sends DAergic efferent to the mPFC, BLA, and NAc, forming the DA cycle. Besides DA, the VTA contains other important receptors such as opiate receptors, CB1Rs, and GABA neurons. Rewarding stimuli such as drugs of abuse often target this area, altering DA levels and hence distorting emotional regulation. Studies involving neuropsychiatric disorders such as ADHD (low levels of DA) and schizophrenia (high levels of DA) often show altered DA activity levels (Kalivas, 1993). Due to the significance of DA transmission throughout the CNS, and the widespread dopaminergic projections of the VTA, the integrity of this neural structure is crucial to proper brain function.

1.2.2 Medial Prefrontal Cortex

The medial prefrontal cortex (mPFC) lies anterior to the frontal lobe and is involved in cognition, executive function, and coding of emotional learning. It is rich with CB1Rs and highly implicated in cue-induced associative learning and memory. Pharmacological manipulation of the CB1Rs in the mPFC with synthetic CB1R agonist resulted in the extinction of fear memories, whereas blockade of CB1Rs in the mPFC potentiated cue-induced fear memory (Lin, Mao, Su, & Gean, 2009). Furthermore, activation of CB1Rs have shown to increase DAergic transmission in the mPFC, while inhibiting CB1Rs has shown the opposite effect (Diana, Melis, & Gessa, 1998). In addition, disturbances in the mPFC CB1 activities have been shown to be implicated in addiction models and associated with neuropsychiatric disorders such as schizophrenia.

1.2.3 Basolateral Amygdala

The basolateral amygdala (BLA) is rich in CB1Rs, processes emotionally salient stimuli, and is involved in encoding and retrieval of reward related memories and consolidation of memory for a variety of tasks such as contextual fear conditioning, taste aversion, and inhibitory avoidance (Campolongo et al., 2009; LaLumiere & Nawar, 2005; McGaugh, 2000). Memory consolidation refers to the stabilization of an item in long-term memory, and it is a necessity in carrying out normal daily activities.

The BLA is a crucial structure in encoding emotional memories and modulating neuronal plasticity related to associative learning. Patch clamp recordings in rats have demonstrated that activation of CB1Rs in the BLA with synthetic CB1 agonist, modulates GABAergic synaptic transmission (Katona et al., 2001). Furthermore, CB1 agonist has shown to lower the overall excitability of efferent neurons in the BLA (Marco Pistis et al., 2004). Lesion studies in the BLA, indicate an impairment in memory acquisition during spatial memory tasks in rats (Maren, 1999; Roozendaal, Portillo-Marquez, & McGaugh, 1996). Taken together, it is evident that the BLA plays an important role in learning and memory.

1.2.4 Nucleus Accumbens

The nucleus accumbens (NAc) plays a vital role in the reward circuitry, contains high levels of both CB1 and DA receptors and it is a key component in consolidation of learning memory. For example, studies have shown that early consolidation of instrumental learning, such as lever pressing in rats require protein synthesis in the NAc. Inhibition of these processes in the NAc, resulted in a disruption of memory consolidation in the animals (Hernandez, Sadeghian, & Kelley, 2002; Salamone, Correa, Farrar, & Mingote, 2007). Furthermore, cue-induced reward during classical conditioning in rats showed a significant increase in DA levels in the NAc during the early phases of memory consolidation (Day, Roitman, Wightman, & Carelli, 2007). In addition CB1 has shown to modulate DA levels in in the NAc. While activation of CB1Rs by a synthetic agonist (WIN 55,212-2) in rats have shown to boost DA release in the NAc, inhibition of CB1Rs in the NAc have shown to suppress ethanol self administration in male adult rats (Malinen & Hyytia, 2008; Sperlagh, Windisch, Ando, & Sylvester Vizi, 2009).

The NAc consists of two distinct areas: shell (NASh) and core (NACo) that serve different functions. These regions will be explored and discussed in Chapter 3.

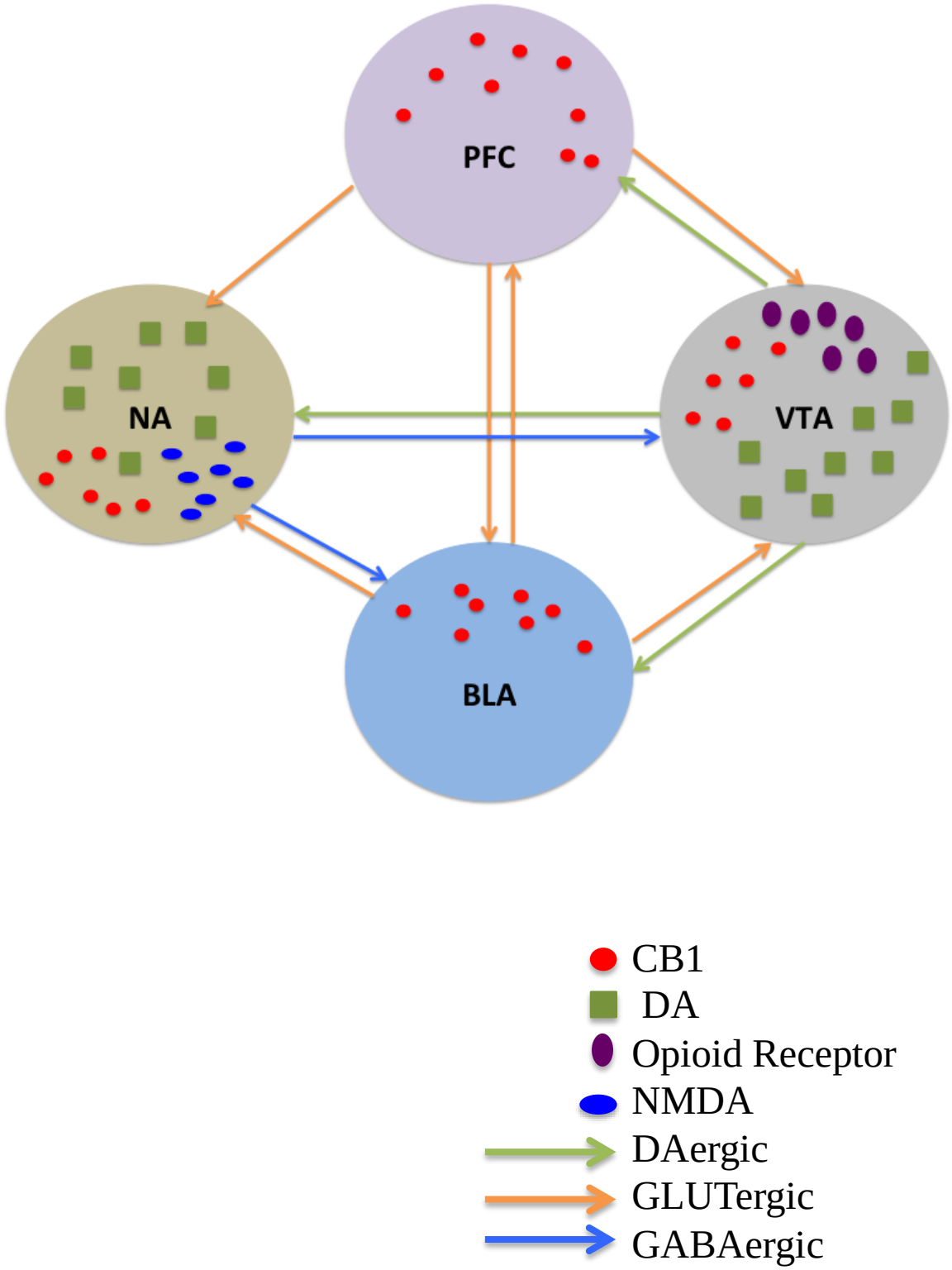

Figure 1.1 Proposed simplified schematic of the mesolimbic circuitry

1.3 CANBIS AND OPIATES

Cannabis, also known as marijuana, is the most commonly used illicit drug in Canada with nearly half of the population (45%) reportedly having used marijuana at least once in their lifetime. Although the acute symptoms of light marijuana use often result in short term attention and executive function deficits, the chronic symptoms in heavy marijuana users can lead to neuropsychological effects and particularly deficits in working memory. THC, the active component in marijuana exerts its effect through the brains CB1 cannabinoid system.

In accordance to the Canadian Pain Society, chronic pain affects 1 in every 5 people in Canada. The most common prescription for chronic pain is opiates, which have various side effects such as nausea, sedation, dependence and addiction. In recent years, there is a growing body of evidence suggesting the use of medical marijuana in conjunction with opiates in the treatment of chronic pain. With both substances having specific side effects, it is very important to explore the interaction of cannabis and opiates.

Opiate addiction studies have shown a disruption in the brains leaning and memory system/mesolimbic pathway (Hyman, Malenka, & Nestler, 2006). Activation of CB1Rs with THC, and μ-opioid receptors with morphine, have both shown to increase the neuronal activity of midbrain DA neurons. Furthermore, μ-opioid receptor signaling is attenuated by CB1 agonist (Rios, Gomes, & Devi, 2006). Studies involving male Wistar rats, indicated that administration of synthetic CB1 antagonist elicited withdrawal symptoms in morphine dependent animals (M Navarro et al., 1998), blocked heroin self-administration and place conditioning in rats and morphine self-administration in mice (Chaperon et al., 1998; Fattore et al., 2007; M Navarro et al., 2001). Furthermore, micro-infusions of CB1 agonist in the NAc and PFC, have shown to attenuate heroin seeking behaviour (Alvarez-Jaimes, Polis, & Parsons, 2008). Hence, it is evident that a functional interaction between DA, CB1 and opioid receptors exist, and exploring this interaction will aid to better understanding these substances, and their role in learning and memory.

1.4 RATIONALE

Although there is a growing body of research indicating the functional interaction between cannabinoid, dopamine and opiate receptors, the specific modulatory mechanisms and pathways of CB1 transmission within the mesolimbic pathway are not well understood. Given CB1 transmission plays a crucial role in reward related learning and memory and is highly implicated in both addiction models and neuropsychiatric disorders, this study will aid in deciphering the specific neural pathways involved.

1.5 HYPOTHESIS

Activation or inhibition of CB1R transmission in the medial prefrontal cortex and basolateral amygdala will modulate DA signaling in the ventral tegmental area and nucleus accumbens pathway, thereby controlling the processing of opiate related motivational information, and associative learning memory.

1.5.1 Objectives

1. Investigate the specific role of CB1 receptor transmission in the mPFC in relation to opiate reward memory, by examining the mPFC→VTA neuronal pathway using an unbiased conditioned place preference paradigm.

2. Investigate the potential role of CB1 receptor transmission in the BLA during the encoding and recall phases of opiate reward conditioning, by employing both a conditioned place preference paradigm and real time *in vivo* electrophysiological recordings.

3. To characterize the location of CB1 and DA receptors involved during cannabinoid related reward in the anterior versus posterior VTA, using intra-VTA microinfusions of synthetic cannabinoid agonist and antagonist.

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Chapter 2

2 Cannabinoid transmission in the prelimbic cortex bi-directionally controls opiate reward and aversion signaling through dissociable kappa versus μ-Opiate receptor dependent mechanisms[1]

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2.1 INTRODUCTION

In the mammalian brain, cannabinoid CB1 receptor and mesolimbic dopamine (DA) transmission functionally interact with opiate-receptor substrates during the processing of motivationally salient learning and memory (Tanda et al., 1997; Rodríguez De Fonseca et al., 2001). The interconnected ventral tegmental area (VTA) and medial prefrontal cortex (mPFC) contain high levels of CB1 receptors. CB1 receptor transmission within these regions can potently modulate rewarding and aversive motivational behaviors and memory formation (Laviolette and Grace, 2006; Zangen et al., 2006; Tan et al., 2010). For example, modulation of CB1 transmission within the prelimbic cortical (PLC) division of the mPFC, increases the emotional salience of fear-related stimuli (Laviolette and Grace, 2006; Tan et al., 2010). In addition, considerable evidence demonstrates functional interactions between CB1 transmission and subcortical DAergic signaling. For example, Δ-9-tetrahydrocannabinol (THC) increases glutamate levels within the mPFC and activates downstream DAergic neuronal activity in the mesolimbic pathway (Diana et al., 1998; Pistis et al., 2001, 2002). Furthermore, *in vivo* extracellular recording studies within the VTA have demonstrated that CB1 receptor activation increases the spontaneous activity of subcortical DA neuronal populations (French et al., 1997).

Although the euphorigenic effects of opiate-class drugs are well established, similar to many other drugs of abuse, opiates also possess aversive stimulus properties (Bechara and van der Kooy, 1987). The VTA serves as a critical neural region for the processing of opiate-related motivational information (Bozarth and Wise, 1981; Laviolette et al., 2004). Within the VTA, opiate-related motivational processing is mediated via heterogeneous opiate-receptor populations. Thus, whereas opiates primarily produce rewarding effects via functional interactions with μ-opiate receptor (MOR) substrates (Gysling and Wang, 1983; Johnson and North, 1992), activation of κ-opiate receptor (KOR) subtypes is linked to the aversive stimulus effects of opioids (Bechara and van der Kooy, 1987; Shippenberg and Elmer, 1998; Davis et al., 2009). Anatomically, MOR-sensitive substrates in the VTA predominantly project to the basolateral nucleus of the amygdala (BLA) whereas KOR-sensitive neuronal substrates predominantly project to the nucleus accumbens (NAc; Ford et al., 2006), suggesting a functional segregation within opiate-dependent

motivational signaling originating from the VTA. Furthermore, efferents from the mPFC to VTA neuronal populations modulate subcortical DA transmission within the mesolimbic pathway, including via direct inputs to DAergic neurons within the VTA (Carr and Sesack, 2000a). Nevertheless, how CB1 transmission within the mPFC may modulate opiate-related motivational information through interactions with subcortical DA substrates is not currently known. Using an unbiased conditioned place preference (CPP) procedure, we examined how pharmacological modulation of CB1 transmission specifically within the PLC division of the mPFC may influence opiate-related reward learning and memory processing. We report that intra-PLC modulation of CB1 receptor transmission bidirectionally controls the motivational valence of opiate-related behavioral conditioning. Whereas CB1 receptor activation switched the motivational valence of morphine from rewarding to strongly aversive, pharmacological blockade of intra-PLC CB1 receptor transmission strongly increased the reward salience of normally sub-reward threshold conditioning doses of morphine. Furthermore, we demonstrate that intra-PLC CB1 transmission bidirectionally controls opiate motivational valence through dissociable MOR versus KOR-dependent substrates, directly within the VTA.

2.2 MATERIALS AND METHODS

2.2.1 Animals and Surgery

All experimental procedures were performed in accordance with Institutional, Federal, and Provincial Animal Care guidelines. Adult male Sprague Dawley rats (350–400 g; Charles River Canada) were anesthetized with an intraperitoneal injection of a ketamine (80 mg/ml)-xylazine (6 mg/kg) mixture, and placed in a stereotaxic device. For intra-PLC microinfusions, two stainless steel guide cannulae (22 gauge) were implanted into the PLC division of the mPFC using the following coordinates (15° angle): from bregma, anteroposterior (AP) +2.9 mm, lateral (LAT) ±1.9 mm, ventral (V) −3.0 mm from the dural surface (Laviolette and Grace, 2006). For experiments involving intra-PLC and intra-VTA microinfusions, rats received two additional cannulae implanted in the VTA using the following coordinates (10° angle): from bregma, AP −5.0 mm, LAT ±2.3 mm, and V −8.0 mm from the dural surface. Dental acrylic and jeweler's screws were used to secure the cannulae to the skull surface.

2.2.2 Drug Treatments

The highly selective CB1 agonist (WIN 55,212-2; Tocris Bioscience) or antagonist (AM251; Tocris Bioscience) were first dissolved in dimethyl sulfoxide then diluted in physiological saline (pH, 7.4). Bilateral microinfusions were performed over a period of 1 min via plastic tubing connected to a 1 μl Hamilton microsyringe. All microinfusions consisted of a total volume of 0.5 μl. Injectors were left in place for an additional 1 min to ensure adequate diffusion of the drug from the injector tip. The μ-opioid receptor antagonist cyprodime hydrochloride (Tocris Bioscience) and κ-opioid receptor antagonist *nor*-binaltorphimine dihydrochloride (Tocris Bioscience) were dissolved in physiological saline, pH 7.4, and micro-infused bilaterally intra-VTA (50 and 500 ng/0.5 μl). Morphine sulfate (Macfarland-Smith) and the broad-spectrum DA receptor antagonist α-flupenthixol hydrochloride (α-flu; Tocris Bioscience) were dissolved in physiological saline. For all experiments involving a pharmacological pretreatment, rats received pretreatments before both saline and morphine conditioning trials. This built-in experimental control mechanism controls for any confounds from potential motivational effects of pretreatment drugs. For CPP conditioning, two doses of morphine were used: a supra-reward threshold dose (5.0 mg/kg, i.p.) that produces robust CPP, and a sub-reward threshold dose (0.05 mg/kg, i.p.) that normally fails to produce a significant CPP (Bishop et al., 2011; Lintas et al., 2012). Systemic morphine was administered immediately after intra-cranial micro-infusions. For DA antagonist treatment, animals received 0.8 mg/kg i.p. α-flu 2.5 hours before conditioning. This dose and time course of α-flu produces no motivational effects in and of itself.

2.2.3 Place Conditioning Procedure

An unbiased, fully counterbalanced CPP procedure was used, as described previously (Bishop et al., 2011; Lintas et al., 2012). Briefly, saline or morphine (systemic or intra-VTA) was paired with one of two environments which differed in terms of color, texture, and smell. Following recovery from surgery, rats were randomly assigned to an experimental group. All rats were exposed to a preconditioning phase where they were placed into a motivationally neutral gray box for 20 min. The following day, the 8 d conditioning phase was commenced. One conditioning environment was white with a

wire-mesh floor covered in woodchips. The alternate environment was black with a smooth Plexiglas floor wiped down with 2% acetic acid immediately before the animal was placed into it. Experimental treatments were counterbalanced such that each animal was randomly assigned to receive morphine in either the white or the black environment and vice versa when receiving saline. As previously reported, rats displayed no baseline preference for either of these environments. During conditioning, rats receive an equal number of morphine-environment versus saline-environment pairings. Therefore, over the 8 d procedure rats receive four 30 min morphine-environment pairings and four 30 min saline-environment pairings. During testing, rats are placed on a narrow gray zone separating the two test environments and times spent in each environment are digitally recorded and scored separately for each animal over a 10 min test session. All rats are tested in a drug free state.

2.2.4 Histology

After completion of experiments, rats were anesthetized with an overdose of euthanyl (sodium pentobarbitol; 240 mg/kg, i.p.) and perfused with isotonic saline followed by 10% formalin. Brains were extracted, sliced at 40 μm, and stained with Cresyl Violet to allow for histological analysis of injection sites. Injector placements were confirmed using light microscopy, and rats with misplaced guide cannulae were excluded from analysis.

2.2.5 Data Analysis

Data were analyzed with either a two-way ANOVA or Student's *t* tests where appropriate. *Post hoc* analyses were performed with Newman–Keuls and Fisher's least significant difference test.

2.3 RESULTS

2.3.1 Intra-mPFC and VTA histological analysis

Histological analysis indicated microinfusion injector cannula placements to be bilaterally localized within the anatomical boundaries of the mPFC and VTA region, as determined by the Atlas of Paxinos and Watson (1986). In Figure 2.1A, we present a

microphotograph showing a representative injector placement within the PLC division of the mPFC. In Figure 2.1B, we present a schematic illustration showing representative intra-mPFC bilateral cannulae placements along the rostral-caudal axis of the mPFC. Rats found to have cannulae placements outside the anatomical boundaries of the mPFC or VTA were excluded from analysis. A total of three rats with misplaced VTA cannulae were excluded from the experimental analyses.

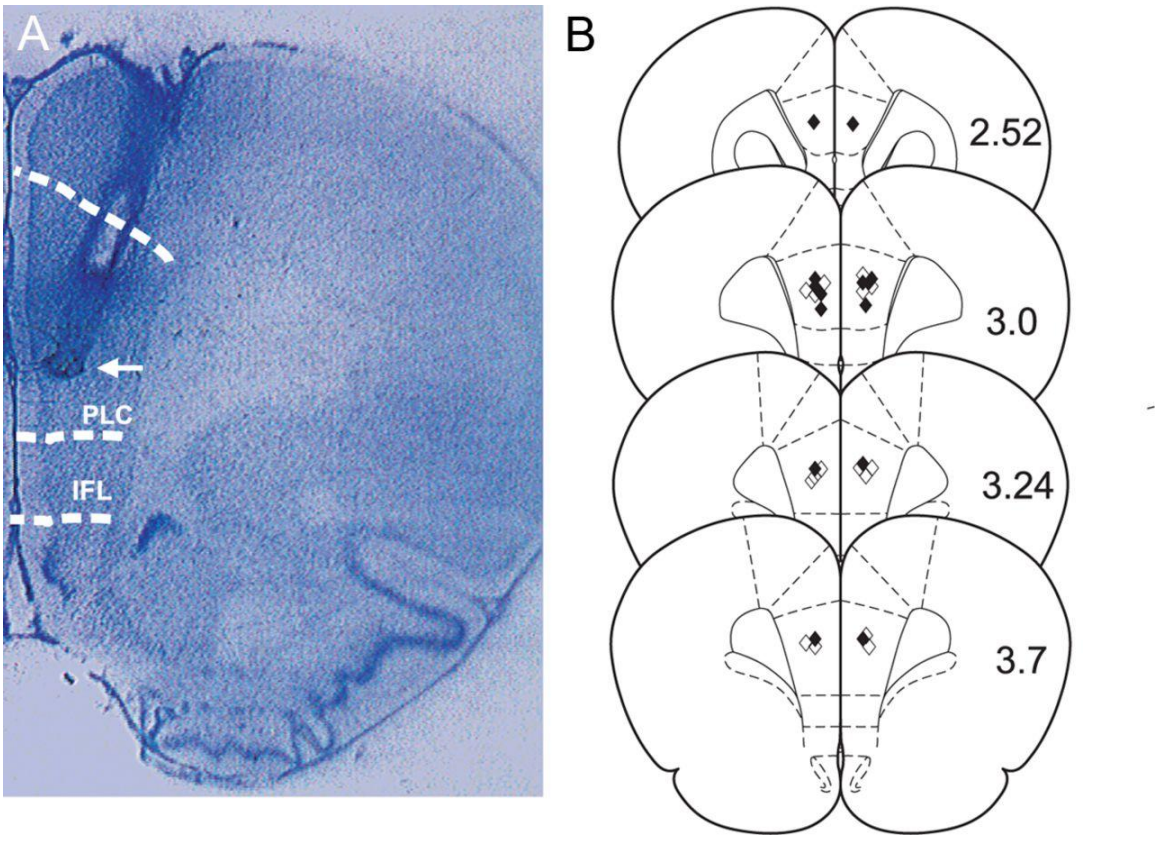


Figure 2.1 Histological analyses of intra-mPFC microinjection sites.

(A) Microphotograph of a representative injector placement within the PLC division of the mPFC. **(B)** Schematic representation of select intra-PLC injector locations; ♦ = 500 ng WIN 55, 212-2 versus 5 mg/kg morphine group, ◊ = 500 ng AM 251 versus 0.05 mg/kg morphine group.

2.3.2 Intra-PLC CB1 receptor activation switches the motivational effects of morphine from rewarding to aversive

We performed bilateral intra-PLC microinfusions of the CB1 agonist WIN 55,212-2 (50 or 500ng/0.5µl), before either sub or supra-reward threshold morphine CPP conditioning. These doses of intra-cranial WIN 55,212-2 are pharmacologically specific and can be blocked by co-administration of selective CB1 antagonists (Laviolette and Grace, 2006; Tan et al., 2011). First, challenging the motivational effects of a sub-threshold conditioning dose of morphine (0.05 mg/kg, i.p.), two-way ANOVA revealed a significant interaction between group and treatment ($F_{(2,43)}$ = 38.6; p < 0.001) on times spent in either saline or morphine paired environments during CPP testing. *Post hoc* analysis revealed that whereas rats receiving a higher dose of WIN 55,212-2 (500ng/0.5µl) demonstrated a significant aversion to morphine-paired environments (n = 8, p < 0.01), this effect was absent in rats receiving a lower dose of WIN 55,212-2 (50ng/0.5µl; n = 7), or vehicle (n = 7) with rats spending equal times in both environments (p > 0.05; Fig. 2.2A). Based upon this dose-dependent effect, we chose the highest behaviorally effective dose of 500ng/0.5µl of WIN 55,212-2 for subsequent behavioral experiments. In our next series of experiments, we microinfused WIN 55,212-2 (500ng) and challenged a suprathreshold conditioning dose of morphine (5.0 mg/kg, i.p.). Two-way ANOVA revealed a significant interaction between group and treatment ($F_{(1,31)}$ = 673.7; p < 0.001). *Post hoc* analysis revealed a highly significant aversion to morphine-paired environments at testing in rats receiving intra-PLC WIN 55,212-2 (n = 8, p < 0.01; Fig. 2.2B). Furthermore, comparing times spent in morphine-paired environments across groups, revealed that rats receiving intra-PLC WIN 55, 212-2 spent significantly less time in morphine-paired environments relative to vehicle controls (n = 7; p < 0.01). Thus, whereas intra-PLC CB1 receptor activation produced no motivational effects in and of itself, activation of CB1 transmission potently and dose-dependently switched the motivational valence of both sub and supra-reward threshold doses of morphine into robust aversive behavioral responses. To control for any potential behavioral effects of intra-PLC WIN 55,212-2, we ran a subsequent control group (n = 8) in which rats received either the previously established effective dose of WIN 55,212-2 (500 ng/0.5 µl) in one environment, or vehicle microinfusions in the control environment.

Statistical analysis revealed that intra-PLC WIN 55,212-2 produced no motivational effects, with rats demonstrating neither preference nor aversion for WIN 55,212-2-paired environments ($t_{(7)} = 2.1$, $p > 0.05$; Fig. 2.2C).

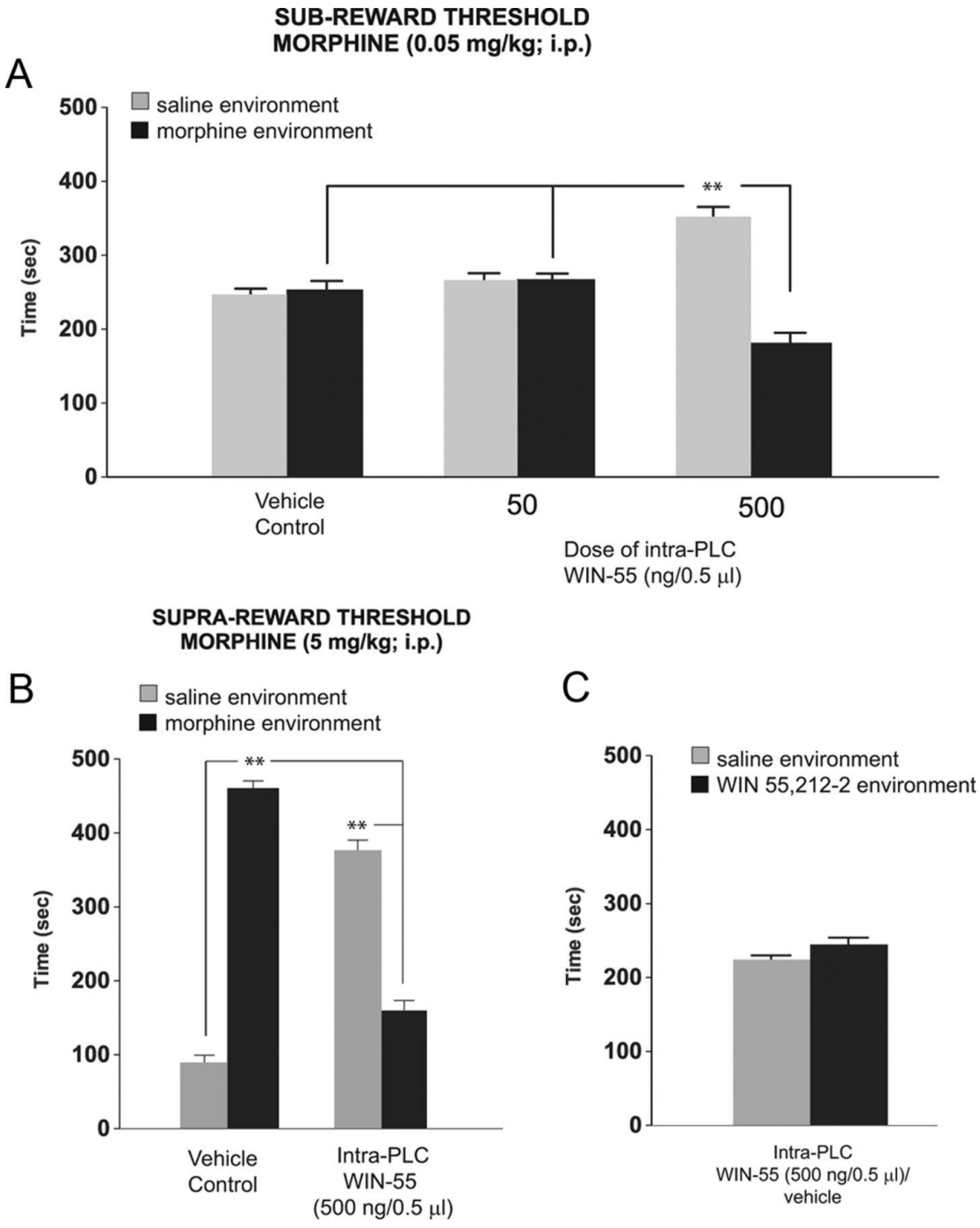

SUB-REWARD THRESHOLD
MORPHINE (0.05 mg/kg; i.p.)
A
saline environment
morphine environment
Time (sec)
500
400
300
200
100
0
**
Vehicle Control
50
500
Dose of intra-PLC WIN-55 (ng/0.5 μl)
SUPRA-REWARD THRESHOLD
MORPHINE (5 mg/kg; i.p.)
B
saline environment
morphine environment
Time (sec)
**
**
Vehicle Control
Intra-PLC WIN-55 (500 ng/0.5 μl)
C
saline environment
WIN 55,212-2 environment
Time (sec)
Intra-PLC WIN-55 (500 ng/0.5 μl)/ vehicle

Figure 2.2 Effects of intra-PLC CB1 receptor activation on morphine CPP conditioning.

(A) Bilateral intra-PLC micro-infusions of the CB1 receptor agonist WIN 55,212-2 (50 and 500ng/0.5µl), dose-dependently produced a morphine aversion against a sub-reward threshold conditioning dose of morphine (0.05 mg/kg, i.p.). Both vehicle controls and rats receiving a lower dose of WIN 55,212-2 (50ng/0.5µl; n = 7) display no significant preference for either environment. Conversely, animals receiving the higher dose of WIN 55,212-2 (500ng/0.5µl; n = 8) display a significant aversion to morphine-paired environments. **(B)** Bilateral intra-PLC microinfusions of WIN 55,212-2 (500ng/0.5µl; n = 8) or vehicle (n = 7) versus a supra-reward threshold dose of morphine (5.0 mg/kg, i.p.) similarly switches the rewarding properties of morphine into aversion, with rats demonstrating robust CPA for morphine-paired environments. **(C)** In control rats receiving intra-PLC WIN 55,212-2 (500ng/0.5µl; n = 8) versus vehicle, no preference for either environment is observed. *$p < 0.05$; **$p < 0.01$, for this and all subsequent figures.

2.3.3 Intra-PLC CB1 receptor blockade potentiates the rewarding properties of morphine

We next examined the potential effects of intra-PLC CB1 receptor blockade on the motivational behavioral effects of either sub or supra-reward threshold conditioning doses of morphine with the selective CB1 antagonist, AM251 (50 –500ng/0.5μl). First, challenging the behavioral effects of a sub-reward threshold conditioning dose of morphine (0.05 mg/kg, i.p.), we administered either 50 or 500 ng/0.5μl directly into the PLC. Two-way ANOVA demonstrated a significant interaction between group and treatment ($F_{(2,43)} = 76.2$; $p < 0.001$) on times spent in either saline or morphine paired environments. *Post hoc* analysis revealed that animals receiving intra-PLC micro-infusions of AM251 (500ng/0.5μl, $n = 8$, $p < 0.001$) spent significantly more time in morphine-paired environments (Fig. 2.3*A*). However, for the vehicle control group ($n = 8$) and rats receiving a lower dose of AM 251 (50ng/0.5 μl, $n = 7$), no CPP was observed. Furthermore, comparing times spent in morphine-paired environments across groups revealed that rats receiving intra-PLC AM251 (500 ng/0.5 μl) spent significantly greater times in morphine-paired environments, relative to controls ($p < 0.01$). Based upon this initial dose–response analysis, we chose the highest behaviorally effective dose of 500ng/0.5μl of AM251 for subsequent behavioral experiments. We next examined the potential effects of intra-PLC AM251 (500ng/0.5μl) against a supra-reward threshold conditioning dose of morphine (5 mg/kg, i.p.). Two- way ANOVA demonstrated a significant interaction $F_{(1,31)} = 15.8$; $p = 0.0005$) be- tween group and treatment, with rats receiving either intra-PLC vehicle or AM251 demonstrating significant CPP for morphine-paired environments ($n = 8$, $p < 0.01$; $n = 8$, $p < 0.01$ respectively; Fig. 2.3*B*). To control for any potential behavioral effects of intra-PLC AM251, we ran a subsequent control group ($n = 8$) in which rats received either the previously established effective dose of AM251 (500ng/0.5μl) in one environment, or vehicle microinfusions in the control environment. Statistical analysis revealed that intra-PLC AM251 produced no motivational effects, with rats demonstrating neither preference nor aversion for AM251-paired environments ($t_{(7)} = 0.89$, $p > 0.05$; Fig. 2.3*C*). Thus, whereas intra-PLC CB1 receptor blockade produced no motivational effects in and of itself, blockade of CB1 transmission potently and dose-dependently potentiated the rewarding properties of a

normally sub-reward threshold conditioning dose of morphine, while having no effect on the rewarding properties of a supra-reward threshold dose of morphine.

In Figure 2.4, we present a summary of the behavioral conditioning effects of intra-PLC CB1 receptor blockade (AM 251) or activation (WIN 55,212-2) on the motivational properties of morphine, showing average difference scores (times in drug minus saline-paired environments), comparing sub-reward threshold morphine effects (Fig. 2.4*A*) or supra-reward threshold morphine effects (Fig. 2.4*B*).

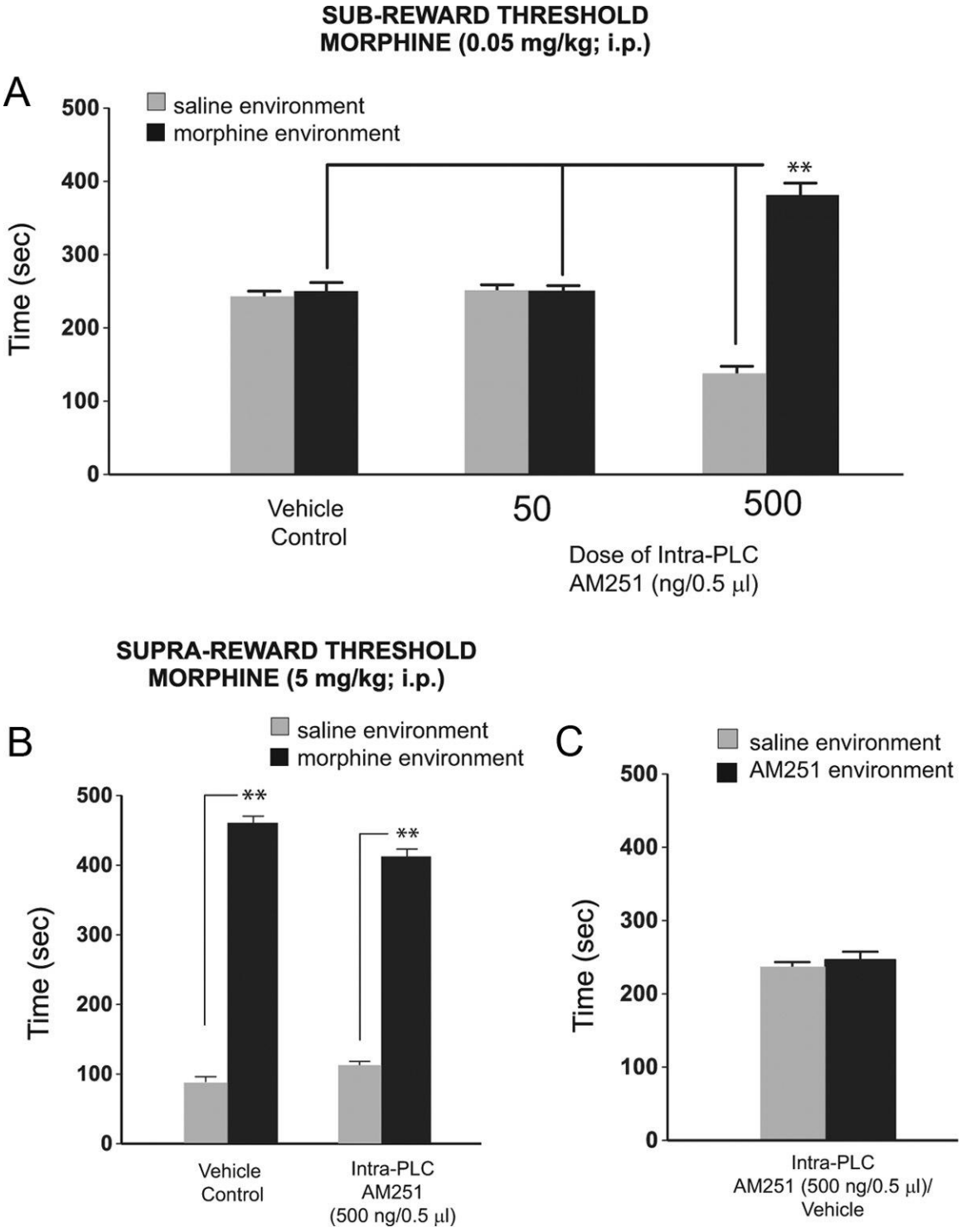

SUB-REWARD THRESHOLD
MORPHINE (0.05 mg/kg; i.p.)
A
saline environment
morphine environment
Time (sec)
500
400
300
200
100
0
**
Vehicle Control
50
500
Dose of Intra-PLC AM251 (ng/0.5 μl)
SUPRA-REWARD THRESHOLD
MORPHINE (5 mg/kg; i.p.)
B
saline environment
morphine environment
Time (sec)
500
400
300
200
100
0
**
**
Vehicle Control
Intra-PLC AM251 (500 ng/0.5 μl)
C
saline environment
AM251 environment
Time (sec)
500
400
300
200
100
0
Intra-PLC AM251 (500 ng/0.5 μl)/ Vehicle

Figure 2.3 Effects of intra-PLC CB1 receptor blockade on morphine CPP conditioning.

(A) Bilateral intra-PLC microinfusions of the CB1 receptor antagonist, AM251 (50ng/0.5µl; *n* = 8, or 500ng/0.5µl; *n* = 7) dose-dependently potentiated the rewarding effects of morphine relative to vehicle controls that displayed no significant preference for either environment. **(B)** Conversely, bilateral intra-PLC microinfusions of AM251 (500ng/0.5µl) versus a supra-reward threshold dose of morphine (5.0 mg/kg, i.p) has no effect on morphine reward conditioning, with both drug (*n* = 8) and vehicle control (*n* = 8) groups demonstrating robust morphine environment CPP. **(C)** In control rats receiving intra-PLC AM251 (500ng/0.5µl; *n* = 8) versus vehicle, no preference for either environment is observed.

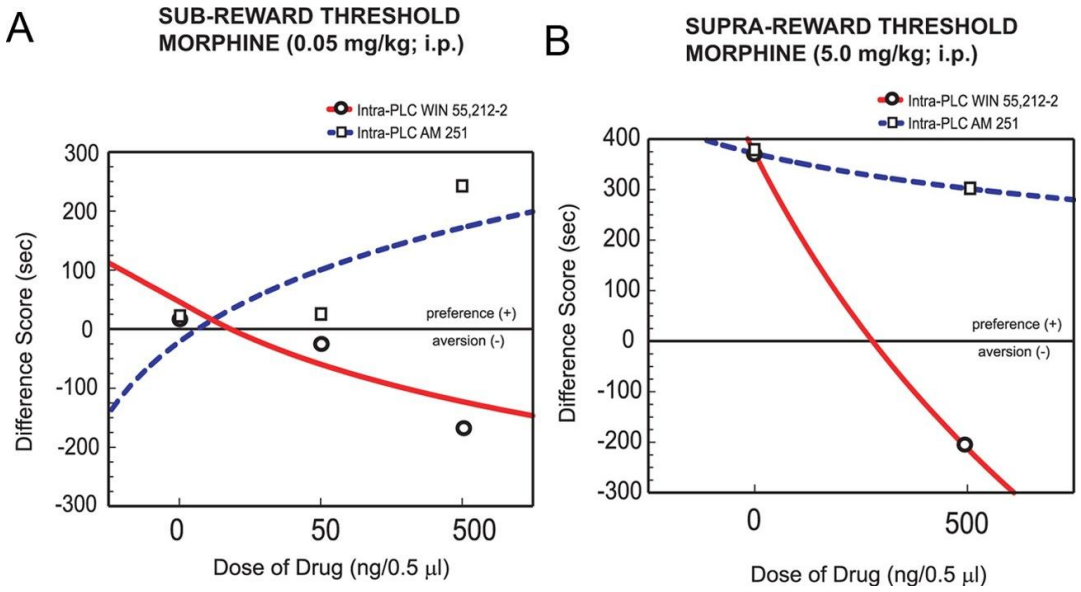


Figure 2.4 Behavioral effects of intra-PLC CB1 receptor activation or blockade on morphine place conditioning.

(A) Summary of the bidirectional behavioral effects of intra-PLC AM-251 (50–500ng/0.5µl) or WIN 55,212-2 (50–500ng/0.5µl) on sub-threshold morphine (0.05 mg/kg, i.p.) reward or aversion effects, presented as difference scores (time in drug minus saline-paired environments). **(B)** Summary of the effects of intra-PLC AM 251 (500ng/0.5µl) or WIN 55 212-2 (500ng/0. µl) on supra-reward threshold (5.0 mg/kg, i.p.) morphine.

2.3.4 CB1 receptor modulation of opiate reward learning is dopamine dependent

Given our findings that intra-PLC CB1 receptor transmission modulates opiate reward and aversion signals (Figs. 2.2, 2.3), we next examined the potential functional interactions between intra-PLC CB1 receptor modulation and DAergic transmission. Accordingly, we challenged both the morphine reward-potentiating and aversion-inducing effects of intra-PLC CB1 receptor modulation by pretreating rats with the broad-spectrum DA receptor antagonist (α-flupenthixol), using a systemic dose (0.8 mg/kg, i.p.) which has been shown previously to block the rewarding properties of opiates in the opiate-dependent/withdrawn state (Laviolette et al., 2004). First, we challenged the ability of intra- PLC WIN 55, 212-2 (500 ng/0.5 ml) to induce morphine place aversions, with α-flu pretreatment (see Materials and Methods) versus supra-reward threshold conditioning dose of morphine (5 mg/kg, i.p.). Two-way ANOVA revealed a significant interaction between treatment and group ($F_{(1,29)}$ = 81.33; $p < 0.001$). *Post hoc* analysis revealed that rats treated with intra-PLC vehicle demonstrated a robust morphine CPP (n = 8; $p < 0.01$), whereas, consistent with our previous results (Fig. 2.2), rats treated with intra-PLC WIN 55,212-2 (n = 7) demonstrated a strong morphine environment aversion ($p < 0.01$; Fig. 2.5*A*). However, in rats pretreated with α-flu (n = 7), the ability of intra-PLC CB1 receptor activation to induce a morphine place aversion was completely blocked, with rats showing no preference or aversion for either environment at testing ($p < 0.05$; Fig. 2.5*A*).

Next, we challenged the ability of intra-PLC AM251 (500 ng/ 0.5 ml) to potentiate the rewarding effects of a sub-reward conditioning dose of morphine (0.05 mg/kg, i.p.), with α-flu pretreatment. Two-way ANOVA revealed a significant interaction between treatment and group ($F_{(1,27)}$ = 257.25; $p < 0.001$). *Post hoc* analysis revealed that rats treated with intra-PLC vehicle demonstrated no preference for environments paired with sub-reward threshold morphine (n = 8; $p > .05$). In contrast, consistent with our previous results (Fig. 2.2), rats treated with intra-PLC AM251 (n = 7) demonstrated a strong morphine place preference ($p < 0.01$; Fig. 2.5*B*). However, in rats pretreated with α-flu (n = 8), the ability of intra-PLC CB1 receptor blockade to potentiate the rewarding

properties of morphine were completely blocked, with rats showing neither preference nor aversion for either environment at testing ($p < 0.05$; Fig. 2.5*B*).

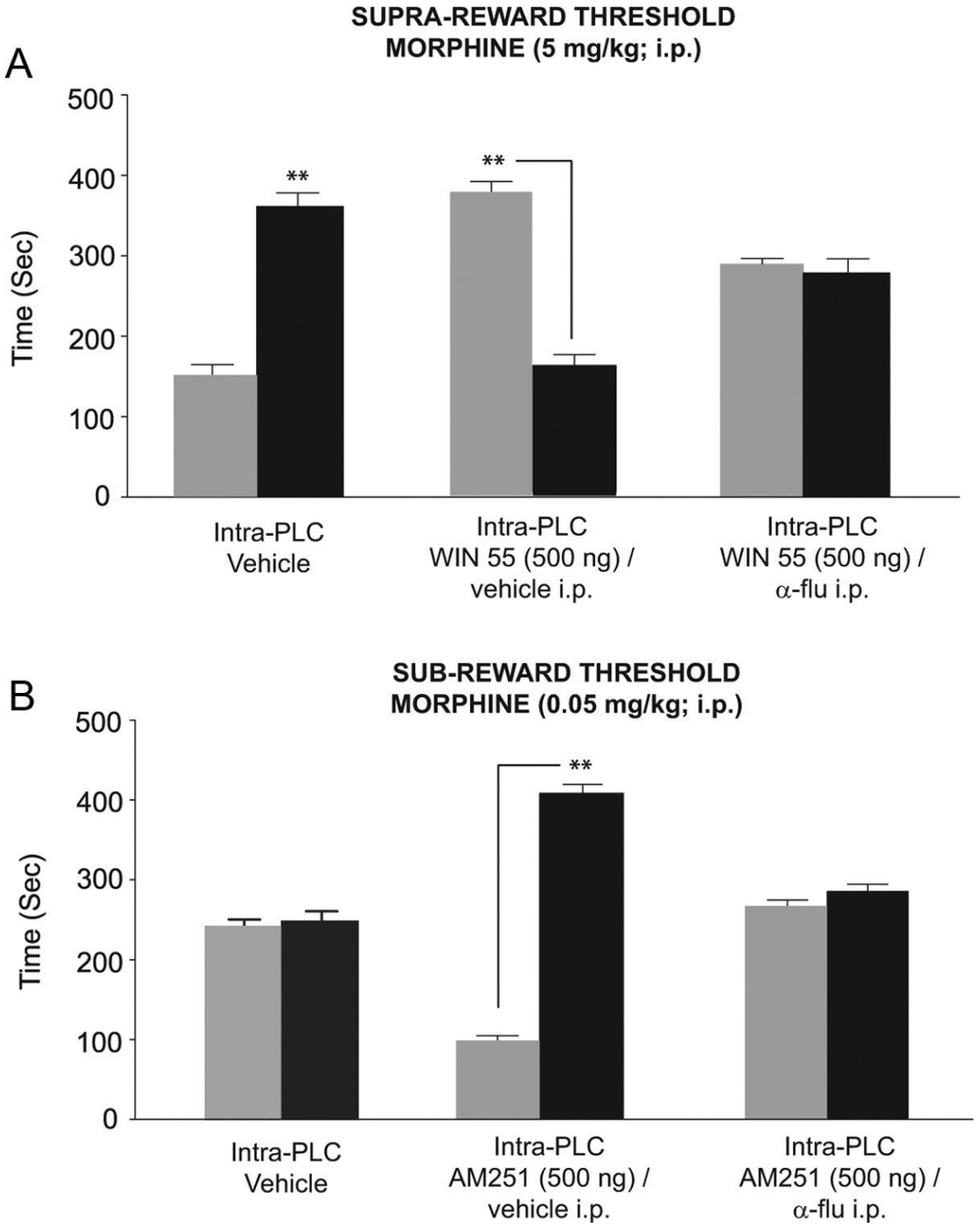

A
SUPRA-REWARD THRESHOLD
MORPHINE (5 mg/kg; i.p.)
Time (Sec)
500
400
300
200
100
0
**
**
Intra-PLC
Vehicle
Intra-PLC
WIN 55 (500 ng) /
vehicle i.p.
Intra-PLC
WIN 55 (500 ng) /
α-flu i.p.
B
SUB-REWARD THRESHOLD
MORPHINE (0.05 mg/kg; i.p.)
Time (Sec)
500
400
300
200
100
0
**
Intra-PLC
Vehicle
Intra-PLC
AM251 (500 ng) /
vehicle i.p.
Intra-PLC
AM251 (500 ng) /
α-flu i.p.

Figure 2.5 Effects of DA receptor blockade on intra-PLC mediated modulation of opiate motivational properties.

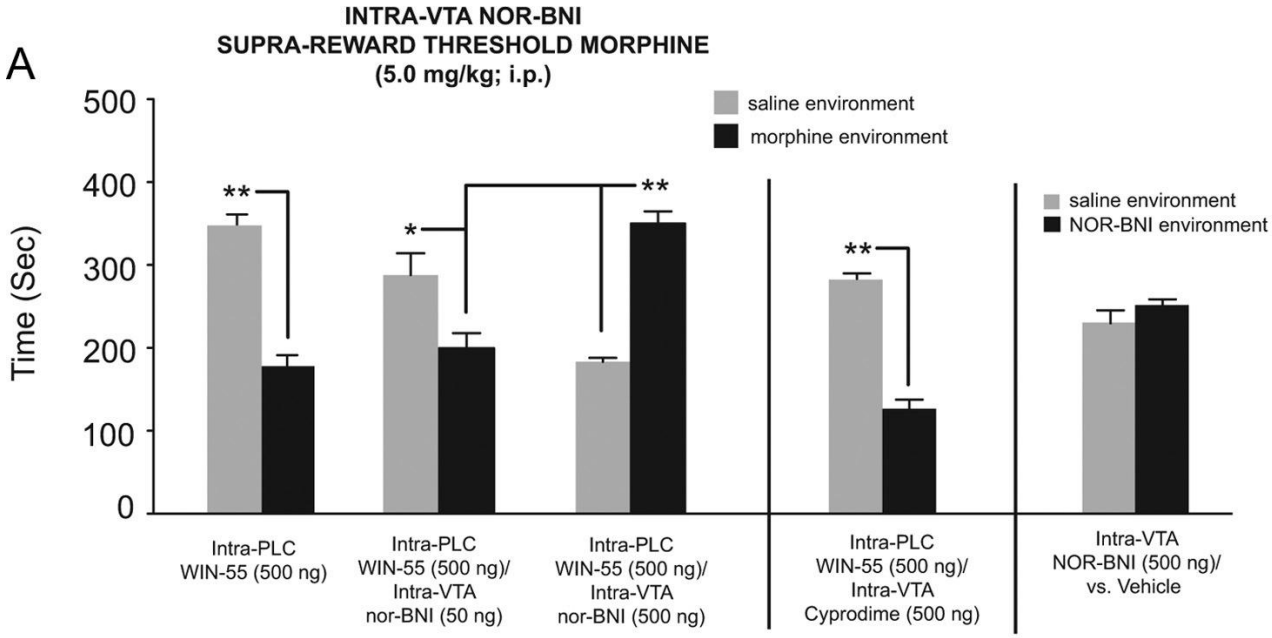
(A) Rats treated with intra-PLC vehicle demonstrate robust CPP for environments paired with supra-reward threshold morphine (n = 8). Relative to vehicle pretreated controls (n = 8), pretreatment with the broad-spectrum DA receptor antagonist α-flu (0.8 mg/kg, i.p.) blocked the ability of intra-PLC WIN 55,212-2 (500ng/0.5μl; n = 7) to induce a behavioral morphine aversion to a supra-reward threshold conditioning dose of morphine (5.0 mg/kg, i.p.). **(B)** Rats treated with intra-PLC vehicle demonstrated no preference for environments paired with sub-reward threshold morphine (n = 8). In contrast, rats treated with intra-PLC AM251 (n = 7) demonstrated a strong morphine CPP ($p < 0.01$; ***B***). However, in rats pretreated with α-flu (n = 8), the ability of intra-PLC CB1 receptor blockade to potentiate the rewarding properties of morphine is blocked.

2.3.5 Intra-PLC CB1-mediated morphine aversions are mediated through κ-opiate receptor transmission in the VTA

Given the well established role of κ-opiate receptor transmission within the VTA in the mediation of opiate-related aversion (Margolis et al., 2003), we next tested whether the ability of intra-PLC CB1 activation to induce morphine-related place aversion was dependent upon a KOR substrate directly within the VTA. Thus, we challenged the aversion-inducing effects of intra-PLC WIN 55,212-2 (500ng/0.5µl) with the highly selective KOR antagonist *nor*-binaltorphimine dehydrochloride (*nor*-BNI) by microinfusing *nor*-BNI into the VTA (50 –500ng/0.5µl) before intra-PLC CB1 receptor activation (see Materials and Methods), using a supra-reward threshold conditioning dose of morphine (5 mg/kg, i.p.). Two-way ANOVA revealed a significant interaction ($F_{(1,27)} = 41$; $p < 0.001$) between group and treatment. *Post hoc* analysis revealed that for rats receiving a lower dose of intra- VTA *nor*-BNI (50ng/0.5µl; $n = 8$), a morphine environment aversion was present ($p < 0.05$), similar, although slightly attenuated, relative to controls receiving only intra-PLC WIN 55,212-2 ($n = 8$; $p < 0.01$; Fig. 2.6*A*). In contrast, for rats receiving a higher dose of intra-VTA *nor*-BNI, morphine environment aversions were completely blocked, and rats displayed a robust morphine CPP ($n = 6$, $p < 0.01$). Thus, blockade of KOR signaling in the VTA dose-dependently reversed the ability of intra-PLC WIN 55, 212-2 to induce morphine environment aversions, and revealed the rewarding behavioral effects of a supra-reward threshold conditioning dose of morphine (Fig. 2.6*A*). To determine the specificity of the intra-VTA KOR mediated effect, we ran an additional control group receiving intra-PLC WIN 55,212-2 with intra-VTA cyprodime hydrochloride, a highly competitive MOR antagonist ($n = 6$). This group displayed a robust morphine-environment aversion; consistent with previous results (Fig. 2.2) demonstrating that intra-VTA MOR transmission is not involved in the aversion-inducing effects of intra-PLC CB1 receptor activation ($t_{(6)} = 14.9$; $p < 0.01$; Fig. 2.6*A*, right).

To control for any potential motivational effects of intra-VTA *nor*-BNI alone, an additional control group ($n = 8$) received bilateral intra-VTA cannulations and received the highest behaviorally effective dose of intra-VTA *nor*-BNI (500ng/0.5µl) in one

conditioning environment, and intra-VTA vehicle in the alternate environment. Analysis of CPP behavior revealed that intra-VTA *nor*-BNI produced no motivational effects in and of itself as rats did not demonstrate any significant difference in times spent in either conditioning environment (Fig. 2.6*A*, right; $t_{(7)} = 1.1814$, $p > 0.05$).


A
INTRA-VTA NOR-BNI
SUPRA-REWARD THRESHOLD MORPHINE
(5.0 mg/kg; i.p.)
saline environment
morphine environment
saline environment
NOR-BNI environment
Time (Sec)
500
400
300
200
100
0
**
*
**
**
Intra-PLC
WIN-55 (500 ng)
Intra-PLC
WIN-55 (500 ng)/
Intra-VTA
nor-BNI (50 ng)
Intra-PLC
WIN-55 (500 ng)/
Intra-VTA
nor-BNI (500 ng)
Intra-PLC
WIN-55 (500 ng)/
Intra-VTA
Cyprodime (500 ng)
Intra-VTA
NOR-BNI (500 ng)/
vs. Vehicle

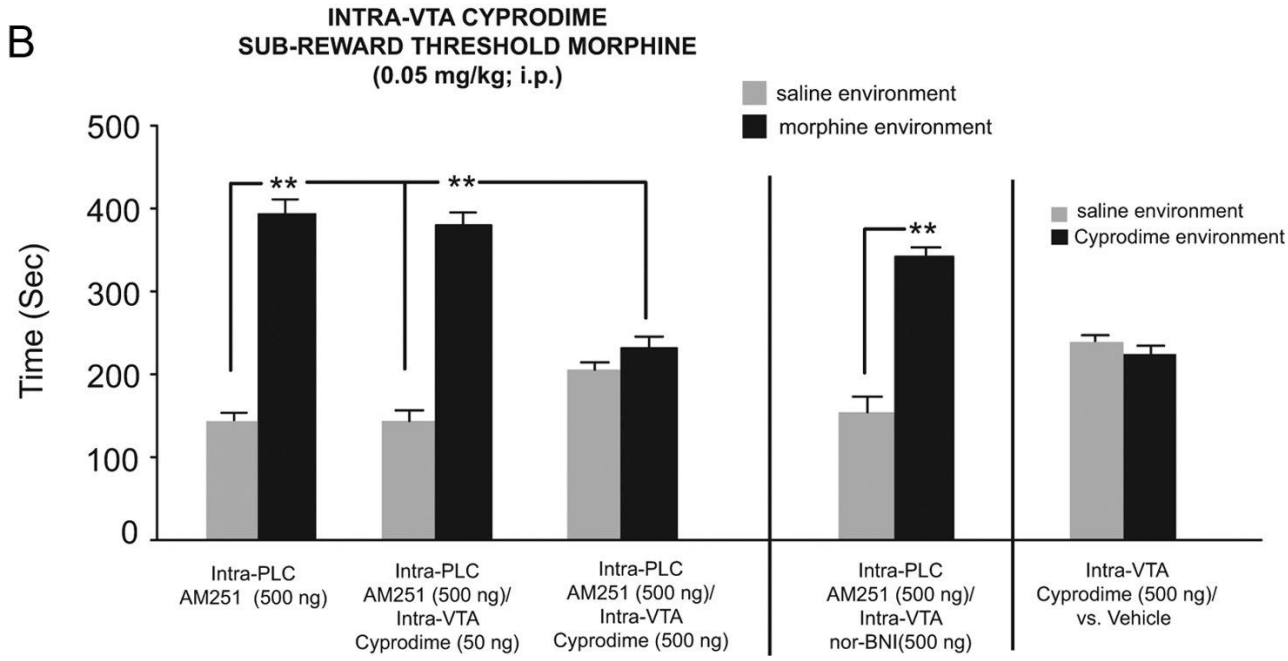

B
INTRA-VTA CYPRODIME
SUB-REWARD THRESHOLD MORPHINE
(0.05 mg/kg; i.p.)
saline environment
morphine environment
saline environment
Cyprodime environment
Time (Sec)
500
400
300
200
100
0
**
**
**
Intra-PLC
AM251 (500 ng)
Intra-PLC
AM251 (500 ng)/
Intra-VTA
Cyprodime (50 ng)
Intra-PLC
AM251 (500 ng)/
Intra-VTA
Cyprodime (500 ng)
Intra-PLC
AM251 (500 ng)/
Intra-VTA
nor-BNI(500 ng)
Intra-VTA
Cyprodime (500 ng)/
vs. Vehicle

Figure 2.6 Effects of intra-VTA κ or μ-opiate receptor blockade on CB1 receptor mediated modulation of opiate reward and aversion behaviors.

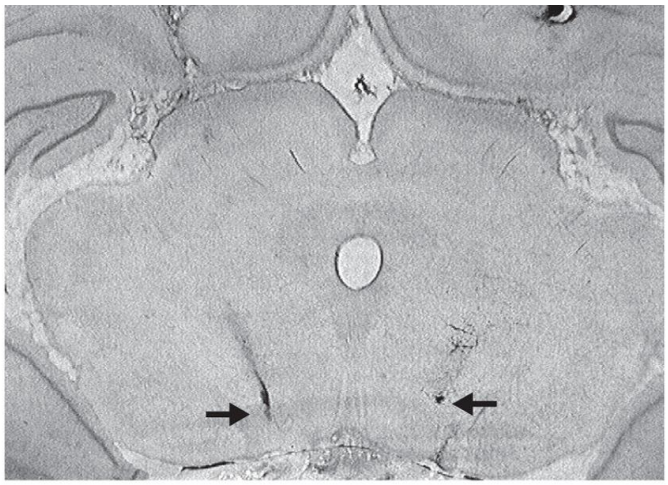
(A) Relative to rats receiving intra-PLC WIN 55,212-2 (500ng/0.5 ml) versus supra-reward threshold morphine (*n* = 8), intra-PLC administration of WIN 55,212-2 (500ng/0.5μl) following intra-VTA administration of the KOR antagonist *nor*-BNI [50 (*n* = 8) or 500 (*n* = 6) ng/0.5μl] dose-dependently blocks the ability of intra-PLC CB1 activation to switch morphine reward signaling into aversion. However, intra-VTA administration of a MOR antagonist, cyprodime (500ng/0.5μl; *n* = 6) fails to reverse the effects of intra-PLC CB1 activation. Intra-VTA administration of the highest effective dose of *nor*-BNI (500 ng/0.5 μl) does not produce any behavioral motivational effects in and of itself (*n* = 8; right). **(B)** Intra-PLC administration of AM251 (500ng/0.5μl) following intra-VTA administration of the MOR antagonist cyprodime [50 (*n* = 7) or 500 (*n* = 7) ng/0.5μl] dose-dependently blocks the ability of intra-PLC CB1 receptor blockade to potentiate sub-reward threshold morphine effects, relative to intra-PLC AM251 alone (*n* = 8). In contrast, intra-VTA administration of the KOR antagonist, *nor*-BNI (500ng/0.5μl; *n* = 6) fails to reverse the effects of intra-PLC CB1 blockade. Intra-VTA administration of the highest effective dose of cyprodime (500ng/0.5μl) does not produce any behavioral motivational effects in and of itself (*n* = 8; right).

2.3.6 Intra-PLC CB1-mediated morphine reward potentiation is mediated through μ-opiate receptor transmission in the VTA

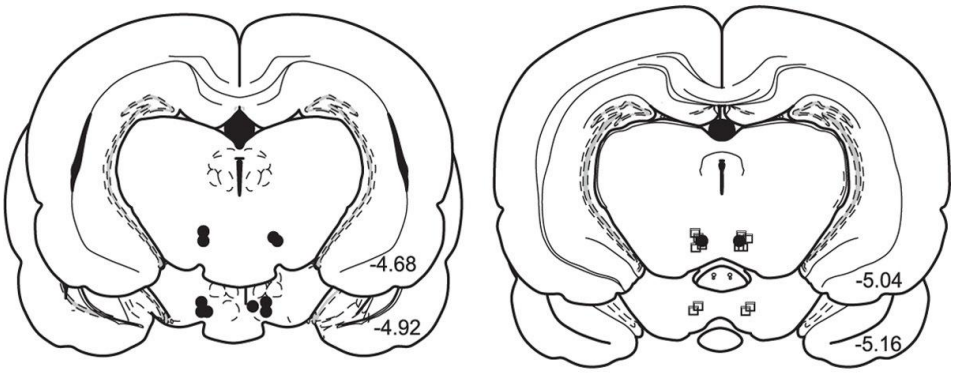
We next tested whether the ability of intra-PLC CB1 blockade to potentiate the rewarding effects of morphine were dependent upon a MOR substrate directly within the VTA. Thus, we challenged the reward-potentiating effects of intra-PLC AM251 (500ng/0.5μl) with the highly selective MOR antagonist cyprodime hydrochloride, by directly microinfusing cyprodime into the VTA (50–500ng/0.5μl) before intra-PLC CB1 receptor blockade (see Materials and Methods), using a sub-reward conditioning dose of morphine (0.05mg/kg; i.p.). Two-way ANOVA revealed a significant interaction ($F_{(1,27)} = 72.7$; $p < 0.001$) between group and treatment. *Post hoc* analysis revealed that for rats receiving a lower dose of cyprodime (50ng/0.5μl), intra-PLC AM251 was able to potentiate the rewarding properties of sub-reward thresh- old morphine, with these rats demonstrating a significant morphine CPP ($n = 7$, $p < 0.05$; Fig. 2.6*B*). However, in rats receiving a higher dose of intra-VTA cyprodime (500ng/0.5μl), the morphine reward potentiating effects of intra-PLC AM251 were completely blocked, with rats showing no preference for morphine-paired environments ($n = 7$; $p > 0.05$; Fig. 2.6*B*). Thus, intra-VTA cyprodime dose-dependently reverses the behavioral effects of intra-PLC CB1 receptor blockade on morphine reward processing. To determine the specificity of the intra-VTA MOR-mediated effect, we ran an additional control group ($n = 6$) receiving intra-PLC AM251 (500 ng/0.5μl) with our previously established effective dose of intra-VTA *nor*-BNI (500ng/0.5μl). These rats dis- played a potentiated morphine reward behavioral response ($t_{(5)} = 8.74$; $p < 0.01$), consistent with previous results (Fig. 2.3), demonstrating that intra-VTA KOR transmission is not involved in the reward potentiating effects of intra-PLC CB1 receptor blockade. To control for any potential motivational effects of intra-VTA cyprodime alone, an additional control group ($n = 8$) received bilateral intra-VTA cannulations and received the highest behaviorally effective dose of intra-VTA cyprodime (500ng/0.5μl) in one conditioning environment, and intra-VTA vehicle in the alternate environment. Analysis of CPP behavior revealed that intra-VTA *nor*-BNI produced no motivational effects in and of itself as rats did not demonstrate any significant difference in times spent in either conditioning environment (Fig. 2.6*B*, right; $t_{(7)} = 0.7281$, $p > 0.05$). Histological analysis of intra-VTA microinfusion locations

revealed injection sites to be within the anatomical boundaries of the VTA as defined by Paxinos and Watson (2006). In Figure 2.7*A*, we present a micro-photograph showing a typical bilateral intra-VTA cannulae placement. In Figure 2.7*B*, we present a schematic summary of intra-VTA microinjection locations. Post experimental histological analysis revealed that effective intra-VTA doses of *nor*-BNI (500ng/0.5µl) were predominantly localized to the anterior VTA (Fig. 2.7*C*). Post experimental histological analysis revealed that effective intra-VTA doses of cyprodime (500ng/0.5µl) were predominantly localized to the posterior VTA (Fig. 2.7*C*).

A



B


-4.68
-4.92
-5.04
-5.16

C

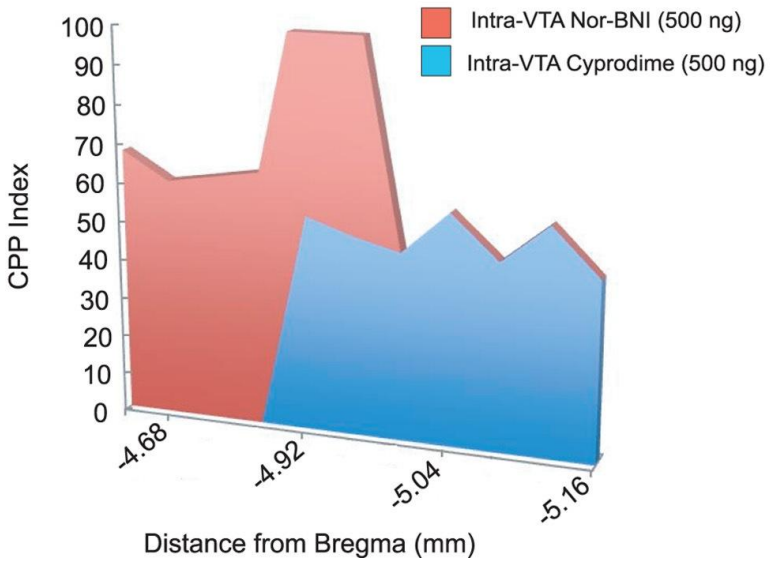

Intra-VTA Nor-BNI (500 ng)
Intra-VTA Cyprodime (500 ng)
CPP Index
100
90
80
70
60
50
40
30
20
10
0
-4.68
-4.92
-5.04
-5.16
Distance from Bregma (mm)

Figure 2.7 Histological analyses of intra-VTA microinfusion locations.
(A) Microphotograph showing representative bilateral intra-VTA infusion locations. **(B)** Schematic summary of intra-VTA microinjector locations; • = intra-VTA *nor*-BNI (500ng/0.5µl); □ = intra-VTA cyprodime (500ng/0.5µl). **(C)** Schematic summary showing relative intra-VTA cannulae placement locations relative to behavioral CPP index score (total time in saline environment + total time in morphine environment/total time in morphine environment; de Jaeger et al., 2013) for rats receiving the behaviorally effective doses of either intra-VTA cyprodime or *nor*-BNI.

2.4 DISCUSSION

We report that CB1 transmission within the PLC division of the mPFC, bi-directionally modulates the motivational behavioral effects of systemic opiates. Although activation of CB1 transmission switched a normally rewarding behavioral effect of morphine into aversion, blockade of CB1 transmission potentiated the rewarding properties of normally sub- reward threshold conditioning doses of morphine. This CB1-mediated switching mechanism was functionally dissociable, DA dependent, and mediated through either a MOR-dependent reward substrate, or a KOR-dependent aversion signaling substrate, directly within the VTA.

2.4.1 Bidirectional control of opiate reward and aversion signals through CB1 receptor transmission in prelimbic cortex

Considerable evidence indicates functional interactions between opiate, cannabinoid, and DAergic transmission during the processing of motivationally salient information (Cheer et al., 2004; Sperlagh et al., 2009; Akirav and Fattore, 2011). Specifically, within the mPFC-VTA circuitry, CB1 receptor transmission modulates downstream DAergic activity within the VTA. In terms of functional interactions between the mPFC and VTA, the mPFC sends descending projections to neuronal subpopulations within the VTA, including both DAergic and non-DAergic neurons (Carr and Sesack, 2000a,b; Sesack and Carr, 2002). Interestingly, CB1 agonists have been reported to increase the spontaneous activity of mPFC neuronal populations and attenuate the inhibitory effects of VTA DAergic stimulation on mPFC neuronal activity, suggesting that systemic cannabinoid activation may remove tonic inhibitory influences of VTA DAergic inputs to the mPFC on cortical neuronal subpopulations (Pistis et al., 2001).

In the context of fear-related learning and memory, considerable evidence implicates a role for intra-mPFC CB1 transmission. Thus, activation of CB1 transmission in the mPFC potentiates normally sub-threshold fear-memory formation behaviorally and associative neuronal conditioning (Laviolette and Grace, 2006). Cannabinoid transmission within the BLA also modulates fear memory processing via modulatory inputs to PLC neurons (Tan et al., 2010). In terms of reward-related learning and memory, CB1 transmission has been shown to modulate the rewarding properties of

opiates, via interactions with mesolimbic circuits. For example, CB1 receptor agonists such as WIN 55,212-2 have been shown to directly excite DA neuron activity within the VTA (French et al., 1997) and potentiate the reward salience of opioids (Caille´ and Parsons, 2006). Nevertheless, most previous studies have used systemic administration of cannabinoid agents and little is known related to how direct CB1 signaling within specific cortical regions, such as the PLC, may modulate reward-related signals.

Given previous evidence showing that CB1 agonists can increase subcortical DAegic transmission (French et al., 1997; Pistis et al., 2001, 2002; Cheer et al., 2004), our initial hypothesis was that CB1 activation would potentiate the reward salience of opiates by directly activating a DA-dependent reward pathway in the VTA. Instead, we observed the opposite effect, with CB1 receptor blockade potentiating opiate-reward salience and CB1 activation instead inducing aversive opiate-related behavioral effects. Nevertheless, given the complexity of CB1-mediated control of neuronal network signaling within cortical regions, several functional explanations are possible. For example, in addition to the well established role of CB1 receptor transmission in inhibiting feed forward, GABAergic inhibitory inputs to principal output neurons (Katona et al., 2001; Freund et al., 2003), CB1 receptors also control presynaptic GLUTergic release in various neural regions, including the hippocampus, BLA, and sensory cortical areas (Domenici et al., 2006). One important source of GLUTergic inputs to the mPFC arises from the BLA. Indeed, CB1 transmission modulates LTP along the BLA-mPFC pathway (Tan et al., 2010, 2011) and is necessary for the effects of intra-PLC CB1 transmission on potentiation of fear-related memory and GLUTergic modulation of opiate reward memory formation (Laviolette and Grace, 2006; Bishop et al., 2011). Thus, one possibility is that intra-PLC CB1 activation may inhibit excitatory inputs to PLC output neuron populations, in turn leading to dysregulated or attenuated PLC signaling to downstream DAergic substrates in the VTA.

In vitro studies have demonstrated bidirectional effects of either CB1 activation or blockade on GLUTergic synaptic strength within the mPFC. Thus, CB1 activation suppresses EPSCs, whereas CB1 blockade increases GLUTergic EPSCs recorded in slice (Auclair et al., 2000). Furthermore, DAergic transmission has been shown to modulate synaptic plasticity within the mPFC (Chiu et al., 2010).

Given the bidirectional behavioral effects observed in the present study, an alternative possibility is that CB1 blockade may in- crease GLUTergic synaptic strength leading to increased PLC output neuron activity to downstream DAergic substrates in the VTA, with CB1 receptor activation producing the opposite functional effect. Although future studies are required to investigate these possibilities, this model could account for AM251-induced activation of down- stream DA signaling and subsequent potentiation of opiate reward salience (by increasing GLUTergic output from the PLC) and by extension, WIN 55,212-2- induced inhibition of GLUTergic output from the PLC and a hypothetical inhibition of downstream DAergic transmission. However, such a mechanism cannot account for the observed switch to a robust, DA-dependent behavioral aversion signal. Furthermore, the rewarding effects of opiates can be mediated in- dependently of DA in the previously opiate-naive state (Laviolette et al., 2004).

2.4.2 Cannabinoid modulation of opiate reward and aversion signals is mediated through separate μ- versus κ-opiate receptor substrates

Opiates, such as morphine, possess rewarding and aversive stimulus properties. Considerable evidence demonstrates dissociable mechanisms within the VTA for the mediation of MOR versus KOR-mediated reinforcing versus aversive behavioral effects. Thus, whereas MOR activation is linked to DA neuron activation via indirect inhibition of inhibitory GABAergic VTA neuronal populations (Johnson and North, 1992), KOR-dependent aversion signals have been linked to direct inhibitory effects on VTA DAergic substrates (Margolis et al., 2003, 2006). Morphine, although showing preferential affinity for the μ-type opiate receptor, also shows affinity for δ and κ receptor subtypes (Yamada et al., 2006), suggesting that multiple opiate receptor pathways may be capable of mediating morphine's behavioral and motivational properties. The aversive effects of systemic morphine have previously been shown to depend on peripheral κ-receptor substrates (Bechara and van der Kooy, 1987; Bechara et al., 1987). The present results demonstrate that central blockade of κ receptors directly within the VTA are capable also of blocking the aversive effects of systemic morphine. Although beyond the scope of the current studies, an interesting question would be whether the ability of intra-PLC cannabinoid activation to induce morphine-aversion effects may be similarly blocked by systemic blockade of κ receptors, extrinsic to centrally localized κ-receptor substrates

within the VTA.

In the present study, we observed that both the reward or aversion-related effects of CB1 receptor modulation were DA-dependent. Previous evidence has suggested that KOR-sensitive DA neurons within the VTA preferentially send recurrent projections back to the mPFC. Although the current study used a systemically administered, broad-spectrum DA receptor antagonist, one possibility is that the aversive effects of intra-PLC CB1 activation, mediated through a KOR-sensitive substrate in the VTA, involves a recurrent pathway from the PLC to select DA neurons in the VTA, which then project back to PLC neuronal substrates. Activation of VTA KORs associated with DAergic neuronal sub-populations selectively reduces DA release in the mPFC, but not in the NAc, further suggesting that aversive opiate-related behavioral effects may be mediated through recurrent mPFC-VTA circuitry (Margolis et al., 2006).

Alternatively, the mPFC sends strong GLUTergic projections to the VTA, which are known to synapse upon both DAergic and non-DAergic (presumably GABAergic) neuronal subpopulations and again, these inputs appear to selectively target recurrent DAergic projections back up to the mPFC (Sesack and Carr, 2002). Given the present findings wherein the CB1-mediated switch from opiate reward to aversion was dependent upon both DAergic and KOR-dependent transmission, intra-PLC CB1 activation may selectively activate efferents to the VTA, which act directly upon KOR substrates associated with VTA DAergic neuronal populations, leading to dysregulation and/or attenuation of DA signaling back up to the mPFC. In contrast, the DA- dependent, reward potentiating effects of intra-PLC CB1 receptor blockade were mediated through MOR, but independent of KOR signaling within the VTA. This would suggest that inhibition of CB1 transmission within the PLC may indirectly activate DA-dependent reward salience signaling in the VTA via MOR substrates, likely associated with non-DA, GABAergic neuronal subpopulations in the VTA, as suggested by previous reports (Gysling and Wang, 1983; Johnson and North, 1992). Interestingly, MOR-associated DAergic neurons within the VTA have been shown to preferentially project to the BLA (Ford et al., 2006). Although future studies are required to examine these issues, one possibility is that opiate reward signals are amplified through a PLC-VTA-BLA circuit, via MOR-mediated

activation of VTA3BLA DA projections. Indeed, we have reported previously that direct activation of DA D1 receptor transmission within the BLA can strongly potentiate normally sub-reward thresh- old morphine conditioning signals (Lintas et al., 2012), suggesting a putative mechanism whereby increased DA input to the BLA may increase opiate-related reward salience, as observed in the present studies. In summary, the present findings add new insights into the role of prefrontal cortical cannabinoid transmission on the modulation of motivationally salient, reward-related memory processing. Furthermore, the present findings suggest that disturbances in CB1 transmission within mPFC circuits may underlie subcortical DAergic dysregulation linked to neuropsychiatric disorders, such as addiction and schizophrenia.

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Chapter 3

3 Bi-directional cannabinoid signaling in the basolateral amygdala controls rewarding and aversive emotional processing via functional regulation of nucleus accumbens [1]

[1] This chapter has been adapted from the submitted journal article: Ahmad, T., Sun, N., Lyons, D., and Laviolette, S.R. (2016). Bi-directional cannabinoid signaling in the basolateral amygdala controls rewarding and aversive emotional processing via functional regulation of the nucleus accumbens. *Addiction Biology (Accepted March 2016, In print)*

3.1 INTRODUCTION

Drugs that modulate the brains cannabinoid system such as marijuana, can powerfully modulate both positive and negative affective states (Hart et al., 2002; Metrik et al., 2011). The cannabinoid CB1 receptor (CB1R) is localized to neural regions responsible for emotional processing, including the basolateral amygdala (BLA) and mesolimbic pathway (Katona et al., 2001). Projections from the BLA to the nucleus accumbens (NAc), not only modulate opiate-reward salience and memory formation (Lintas et al., 2012), but also the activity of NAc neuronal subpopulations via glutamatergic (GLUTergic) projections and dopamine (DA)-dependent receptor transmission (Floresco et al., 2001). Modulation of intra-BLA CB1R transmission strongly modulates rewarding or aversive associative memory formation and associated plasticity mechanisms (Azad et al., 2004; Ramikie & Patel, 2012; Tan et al., 2011). In addition, CB1R transmission modulates the BLA→NAc pathway. For example, administration of CB1R agonists suppresses excitatory BLA control of NAc neuronal activity and inhibits spontaneous activity levels of BLA projection neurons (Pistis et al., 2002, 2004), demonstrating an important role for CB1R modulation of the BLA→NAc circuit.

The NAc serves as a limbic-motor interface where motivationally salient information is converted into goal directed behaviors (Mogenson, Swanson, & Wu, 1983). Approximately 95% of the neuronal population within the NAc is comprised of GABAergic, medium spiny neurons (MSNs) with the remainder consisting of GABAergic or cholinergic fast-spiking interneurons (FSIs). Considerable evidence demonstrates that encoding of aversive vs. rewarding information is linked to differential FSI vs. MSN neuronal activity patterns (Carlezon and Thomas, 2009; Sun and Laviolette, 2015). Nevertheless, the potential effects of CB1R modulation on intra-NAc neuronal encoding of opiate-related motivational information are not currently understood.

In the present study we examined the role of intra-BLA CB1R transmission in the processing of opiate-related motivational behaviors. We report that intra-BLA CB1R transmission bi-directionally controls opiate-related motivational valence; while blockade of BLA CB1R dramatically potentiates the reward salience of normally sub-threshold opiate conditioning doses, activation of CB1R switches the motivational valence of

opiates from rewarding to aversive through DA-dependent mechanisms and were functionally mediated through BLA→NAc GLUTergic projections to the shell region of the NAc (NASh). Furthermore, intra-BLA CB1R transmission bi-directionally controls MSN vs. FSI mediated neuronal correlates of opiate-mediated reward or aversion behaviors both during opiate-related reward conditioning and recall of rewarding or aversive associative memories.

3.2 MATERIALS AND METHODS

3.2.1 Animals and Surgery

All experimental procedures were approved by the Canadian Council on Animal Care. Adult male Sprague Dawley rats (350-400 gm; Charles River, Canada) were anesthetized with a ketamine (80mg/ml)-xylazine (6mg/kg) mixture (i.p), and placed in a stereotaxic device. For microinfusions into the BLA, NASh or NACore, two guide cannulae (22 gauge; PlasticsOne) were implanted bilaterally using the following stereotaxic coordinates (Paxinos and Watson, 2005): for the BLA (0° angle), from bregma: antero-posterior (AP)-2.6mm, lateral (L)±5.0mm, from the dural surface, ventral (V)-7.2mm; for NAcore (8° angle), from bregma AP+1.8mm, L ±2.7mm, from dural surface, V -7.4mm; for NA shell (12° angle), from bregma AP +1.8mm, L ±2.6mm, from dural surface, V -7.4mm. For *in vivo* neuronal recordings, an eight channel micro-wire array (Tucker-Davis) was implanted unilaterally in the NA shell with the following coordinates (Paxinos, 2005): 0° angle, (AP)+2.2mm, lateral (L)±1.2mm, from the dural surface, ventral (V)-7.0mm.

3.2.2 Drug Treatments

The CB1R agonist (WIN 55,212-2, Tocris) and antagonist (AM 251, Tocris) were dissolved in dimethyl sulfoxide (DMSO) and diluted in physiological saline (pH adjusted to 7.4). The dose-ranges of AM 251 and WIN 55 (50-500ng/0.5μl) were based upon previously published reports demonstrating that intra-cranial infusions within this range are pharmacologically specific and behaviorally effective (Laviolette and Grace, 2006). The NMDA receptor antagonist (DL-AP5, Tocris) and DA receptor antagonist, α-flupenthixol (α-flu; Tocris) were dissolved in physiological saline. Bilateral micro-

infusions (0.5 μl volume) were performed over 1 min via a 1 μl Hamilton syringe. Injectors were left in place for an additional 1 min to ensure adequate diffusion from the injector. Morphine sulphate (Macfarland-Smith) was dissolved in physiological saline. Two conditioning doses of morphine were used: a supra-reward threshold dose of 5mg/kg which produces robust morphine CPP, or a sub-reward threshold conditioning dose of 0.05 mg/kg, which alone produces no significant behavioral CPP effects (Bishop, Lauzon, Bechard, Gholizadeh, & Laviolette, 2011; Lintas et al., 2011). For experiments using α-flu, we used a previously characterized dose (0.8 mg/kg; i.p.) administered 2.5 hours prior to conditioning. This dose and administration time produces no motivational effects in and of itself, as previously reported (Laviolette and van der Kooy, 2003).

3.2.3 Place Conditioning

Conditioning experiments used an unbiased conditioned place preference (CPP) paradigm as previously described (De Jaeger et al., 2013; Lauzon, Bechard, Ahmad, & Laviolette, 2013). One environment was white with a wire mesh floor covered with woodchips, and the other environment was black, with a smooth Plexiglass floor wiped down with 2% acetic acid. Rats were randomly assigned to vehicle or morphine-paired environments and received 4 saline/environment and 4 morphine/environment pairings over the 8-day conditioning phase. Rats received intra-BLA drug microinfusions followed by an i.p injection of saline or morphine, before being placed in conditioning environments. For intra-NAc experiments, rats received bilateral microinfusions of AP5 (1 μg/0.5 μl) in either the NACo or NASh immediately prior to conditioning. Following conditioning, rats received a CPP recall test in a drug free state. During CPP testing, the rat is placed on a grey zone, separating the two conditioning environments, and allowed to move freely between the two environments for a period of 10 min. Times spent in each box are digitally recorded (digital timers) for subsequent analysis.

3.2.4 *In-vivo* Electrophysiology Recordings

The *in vivo* micro-wire array recordings followed the procedure previously described (Sun & Laviolette, 2012, 2014). Briefly, 8-channel micro-wire arrays (model MW8, Tucker-Davis) were implanted unilaterally into the NASh, using the above described

stereotaxic procedures. The dimension of the array was 200 μm in length by 500μm in width, consisting of two rows of four micro-wires, separated by 50μm. The array was connected to an RA-16PA pre-amplifier and then sent to a Pentusa Base Station (model RX5, Tucker-Davis). Neuronal spike signals were sampled at 25 kHz/channel with filter settings of 100Hz (high-pass) and 5kHz (low-pass), and were then sent to a window discriminator/amplifier and an audio monitor. The neuronal activity from the NASh was recorded (Open Ex, Tucker- Davis), and stored in a data tank for offline analysis, where spikes were sorted using K-means analysis (OpenSort, TDT). To ensure rats had no pre-existing differences in neuronal responses to conditioning environments, baseline recordings were performed for a period of 10 min for each rat, counterbalanced for the two environments, over 2 days prior to conditioning. Next, during the acquisition phase prior to each conditioning session, baseline recordings (10 min) were performed in the home cage. Baseline recordings were then averaged out for subsequent comparison to recordings performed during conditioning trials. Off-line analyses of NASh neuronal activity parameters involved manually sorting isolated waveforms within each channel to either fast spiking interneurons (FSI), or presumptive medium spiny neurons (MSN). The classification parameters of FSI vs. MSN units were determined through offline spike waveform and firing frequency sorting as described previously (Sun and Laviolette, 2015) and based upon previously reported electrophysiological criteria for identification of FSI vs MSN NAc neuronal units recording *in vivo* (Berke, Okatan, Skurski, & Eichenbaum, 2004; Berke, 2008; Morra, Glick, & Cheer, 2010). Putative MSN vs. FSI neuronal sub-populations were sorted offline using K-means cluster analysis which separates isolated units based upon completion of waveform sorting, single units were further characterized as FSI or MSN units using inter-spike interval (ISI) histograms constructed for each isolated neuronal unit. Sample rastergrams and ISI histograms were prepared using NeuroExplorer. Neurons were manually sorted based on waveform shape and Interval histograms (see Fig.3.3). A subpopulation of neurons met previously established criteria for FSIs (Berke et al., 2004). Neurons with slow waveform shapes (peak widths 120 us; valley widths 265 us) and low firing rates (<5 Hz) were presumed to be medium spiny neurons (MSNs) (Morra et al., 2010).

3.2.5 Histology

At the completion of experiments, rats were anesthetized with an overdose of Euthanyl (Sodium Pentobarbitol, 240 mg/kg; i.p.) and perfused with isotonic saline followed by 10% formalin. Brains were extracted, sliced at 40 μm, and stained with Cresyl Violet to allow for histological analysis of injection sites. Rats with misplaced guide cannulae were excluded from analyses.

3.2.6 Data Analysis

Data were analyzed with either One or Two-way analysis of variance (ANOVA) or student's t-tests where appropriate. Post hoc analyses were performed with Newman-Keuls or Fisher's least significant difference tests, where appropriate.

3.3 RESULTS

3.3.1 Intra-BLA CB1R blockade amplifies the motivational salience of sub-reward threshold morphine

We first examined the effects of intra-BLA CB1R blockade on morphine-related reward processing by challenging the effects of a sub-reward threshold conditioning dose of morphine (0.05 mg/kg; i.p.) with intra-BLA microinfusions of the CB1R antagonist AM 251 (50-500ng/0.5μl; **Fig. 3.1*A***). Two-way ANOVA revealed a significant group x treatment interaction ($F_{(2,41)}$ = 31.5; *p<0.0001*) on times spent in morphine vs. vehicle-paired environments. *Post hoc* analyses revealed that whereas rats receiving a dose of 500ng/0.5μl AM251 spent significantly more time in morphine-paired environments (n=7, *p<0.01*), vehicle controls (n=7) or rats receiving a lower dose of AM 251 (50ng/0.5 □l; n=7) showed no environmental preferences (*p's>0.05*).

Functional BLA→NAc projections have been reported to modulate mesolimbic DA release within the NAc and to control NAc neuronal activation via DA receptor transmission (Floresco et al., 2001; Jones et al., 2010). Accordingly, to determine if our observed effects might depend upon a DAergic transmission mechanism, an additional group of rats (n=7) were pre-treated with the broad-spectrum DA receptor antagonist (□-flu, 0.8 mg/kg, i.p., *see methods*) prior to intra-BLA CB1R blockade and sub-threshold

morphine conditioning. Analysis of CPP scores revealed that DA antagonist pre-treatment blocked the ability of intra-BLA AM 251 to potentiate morphine reward salience (t_6 = 1.03, *p>0.05*; **Fig. 3.1*A***). Next, to control for possible motivational effects of AM 251 in and of itself, a separate control group received intra-BLA vehicle in one environment vs. the effective dose of AM 251 (500ng) in the alternate environment (n=8). Rats showed neither aversion nor preference for AM 251 paired environments (t_7 =1.62, *p>0.05*), indicating that AM 251 produced no motivational effects in and of itself (**Fig. 3.1*A*** far right). Based upon this initial AM 251 dose-response curve, a dose of 500ng/0.5µl was used for all subsequent behavioral experiments.

Next, challenging the effects of the supra-threshold dose of morphine, two-way ANOVA revealed a significant main effect of treatment on times spent in vehicle vs. morphine-paired environments ($F_{(1,29)}$ = 56.2; *p<.0001*). *Post hoc* analyses revealed that both vehicle controls (n=7) and rats receiving intra-BLA AM 251 (n=8) displayed significant morphine CPP (*p's<.01*; **Fig. 3.1*B***). *Post hoc* analyses revealed no significant differences in times spent in environments paired with supra-reward threshold morphine (*p's >.05*). Thus, whereas intra-BLA CB1R blockade potentiated the reward salience of sub-threshold conditioning doses of morphine through a DA-dependent mechanism, this same treatment had no effect on supra-threshold morphine reward conditioning.

3.3.2 Intra-BLA CB1R activation switches opiate motivational valence from rewarding to aversive

We next examined the effects of bilateral intra-BLA CB1R activation with WIN 55 (50-500 ng/0.5µl) on morphine CPP behaviors examining both sub (0.05 mg/kg; i.p.) or supra-reward threshold (5.0 mg/kg; i.p.) doses of morphine (*see methods*). First, examining the effects of intra-BLA WIN 55 on sub-threshold morphine CPP (**Fig. 3.1*C***), two-way ANOVA showed a significant interaction between group and treatment ($F_{(2,41)}$ = 20.97; *p< .0001*). *Post hoc* analysis revealed that animals receiving the higher dose of WIN 55 (500 ng) showed a significant aversion to the morphine-paired environment (CPA) (n=7, *p<.01*), whereas rats receiving vehicle (n=7) or a lower dose of WIN 55 (50 ng; n=7) showed neither preference nor aversion for either environment (*p's>.05*; **Fig. 3.1*C***).

To determine if the aversion-inducing effects of intra-BLA CB1R activation on morphine conditioning depended upon a DAergic transmission mechanism, an additional group of rats (n=8) were pre-treated with α-flu (0.8 mg/kg; i.p.) prior to intra-BLA CB1R activation and sub-threshold morphine conditioning. Analysis of CPP scores revealed that α-flu pre-treatment blocked the intra-BLA CB1R activation induced aversion, with rats demonstrating neither CPP nor CPA for either environment (t_7 = 0.92, *p>0.05*; **Fig. 3.1*C***). To control for possible motivational effects of intra-BLA WIN-55 (500ng) in and of itself, a separate control group received intra-BLA vehicle in one environment vs. WIN 55 in the alternate environment (n=8). Rats showed neither CPP nor CPA for WIN 55 paired environments (t_7 =1.05, *p=.33*), indicating that intra-BLA CB1R activation produced no motivational effects in and of itself (**Fig. 3.1*C*** far right). Based on this initial WIN 55 dose-response curve, for all subsequent experiments, we administered the highest behaviorally effective dose of WIN 55 (500ng/0.5µl) to challenge the effects of morphine.

Next, we challenged the effects of intra-BLA CB1R activation on a supra-reward dose of morphine (5.0 mg/kg; i.p.). Two-way ANOVA revealed a significant main effect of treatment on times spent in morphine vs. vehicle-paired environments ($F_{(1,31)}$= 458.91; *p<.0001*). *Post hoc* analysis revealed a significant morphine CPA in rats receiving intra-BLA WIN 55 (n=8, *p<.01*) whereas intra-BLA vehicle controls demonstrated a robust morphine-environment CPP (n= 8, *p<.01*; **Fig. 3.1*D***). In Figures **3.1*E,F***, we summarize CPP results showing mean 'difference scores' comparing the behavioural effects of CB1R activation/blockade on sub (**3.1*E***) vs. supra-reward threshold (**3.1*F***) conditioning effects on morphine preference or aversion behaviors measured in the CPP test. Thus, while intra-BLA CB1R blockade strongly potentiated the reward salience of normally sub-reward conditioning doses of morphine, intra-BLA CB1R activation, produced the opposite effect, switching a normally rewarding behavioural effect of morphine into a strong place aversion.

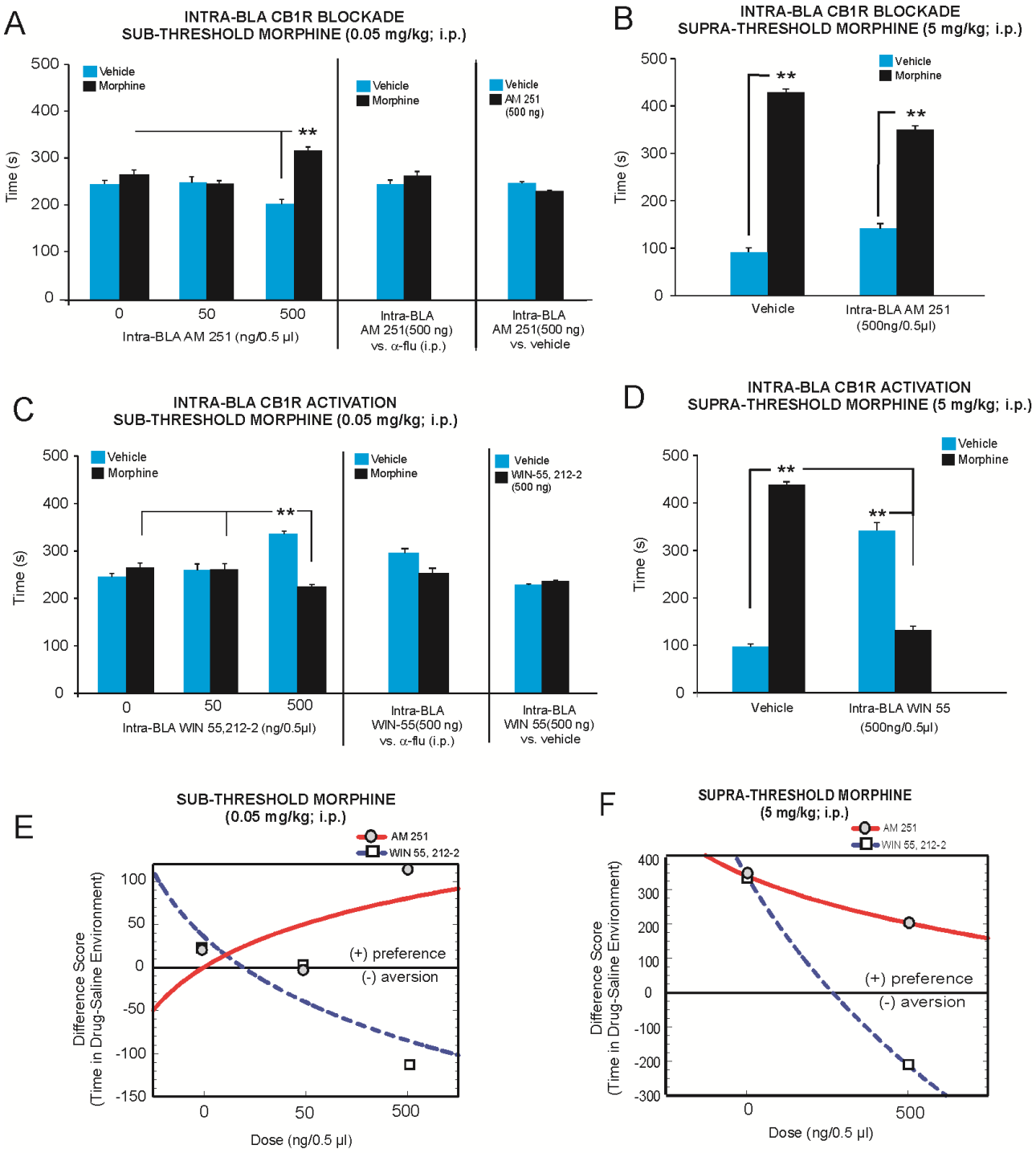

A
INTRA-BLA CB1R BLOCKADE
SUB-THRESHOLD MORPHINE (0.05 mg/kg; i.p.)
Time (s)
Vehicle
Morphine
AM 251 (500 ng)
Intra-BLA AM 251 (ng/0.5 µl)
Intra-BLA AM 251(500 ng) vs. α-flu (i.p.)
Intra-BLA AM 251(500 ng) vs. vehicle
B
INTRA-BLA CB1R BLOCKADE
SUPRA-THRESHOLD MORPHINE (5 mg/kg; i.p.)
Vehicle
Intra-BLA AM 251 (500ng/0.5µl)
C
INTRA-BLA CB1R ACTIVATION
SUB-THRESHOLD MORPHINE (0.05 mg/kg; i.p.)
WIN-55, 212-2 (500 ng)
Intra-BLA WIN 55,212-2 (ng/0.5µl)
Intra-BLA WIN-55(500 ng) vs. α-flu (i.p.)
Intra-BLA WIN 55(500 ng) vs. vehicle
D
INTRA-BLA CB1R ACTIVATION
SUPRA-THRESHOLD MORPHINE (5 mg/kg; i.p.)
Intra-BLA WIN 55 (500ng/0.5µl)
E
SUB-THRESHOLD MORPHINE
(0.05 mg/kg; i.p.)
AM 251
WIN 55, 212-2
Difference Score (Time in Drug-Saline Environment)
(+) preference
(-) aversion
Dose (ng/0.5 µl)
F
SUPRA-THRESHOLD MORPHINE
(5 mg/kg; i.p.)

Figure 3.1 Behavioural effects of intra-BLA CB1R transmission on morphine-related CPP

(A) Dose response analysis of intra-BLA AM 251 (0, 50 and 500ng/0.5µl) versus sub-reward threshold morphine (0.05 mg/kg) conditioning. While rats receiving vehicle (n=7) or 50ng/0.5µl group (n=7), showed no preference for sub-threshold morphine, rats receiving a higher dose of AM 251 (500ng/0.5µl, n=7), showed robust morphine CPP. Pretreatment with α-flu (0.8 mg/kg, n=7), blocked morphine reward potentiation. Intra-BLA AM 251 alone (500ng/0.5µl, n=8) vs. vehicle produced neither preference nor aversions (far right). **(B)** Intra-BLA AM 251 (500ng, n=7) or vehicle controls (n=8) vs. a supra-threshold conditioning dose of morphine (5 mg/kg) both showed significant morphine CPP. **(C)** Dose-response analysis of intra-BLA WIN 55,212-2 (0, 50 and 500ng/0.5µl) vs. sub-threshold morphine (0.05 mg/kg i.p.). Rats receiving intra-BLA vehicle (n = 7) or a lower dose of WIN 55 (50ng/0.5µl; n = 7) showed no CPP for morphine environments, rats receiving a higher dose of WIN 55,212-2 (500ng/0.5µl, n = 7), showed significant CPA. Pretreatment of rats with α-flu (0.8 mg/kg, n=7), blocked morphine CPA. Control rats receiving WIN 55,212-2 (500ng/0.5µl, n = 8) vs. vehicle showed no environmental preferences (far right). **(D)** Bilateral intra-BLA WIN 55,212-2 (500ng/0.5µl) switches a supra-reward threshold conditioning dose (5.0 mg/kg i.p.) of morphine into CPA. While vehicle controls (n=8) showed morphine CPP, rats receiving the WIN 55 (n=8), demonstrated morphine CPA. **(E)** Summary of bidirectional effects of AM 251 and WIN 55,212-2 (0, 50 and 500ng/0.5µl) on subthreshold dose of morphine presented as difference scores (time spent in drug minus saline environments). **(F)** Summary of bidirectional effects of AM 251 or WIN 55,212-2 vs. supra-reward threshold conditioning doses of morphine. * $p < 0.05$ and ** $p < 0.01$, for this and all subsequent figures.

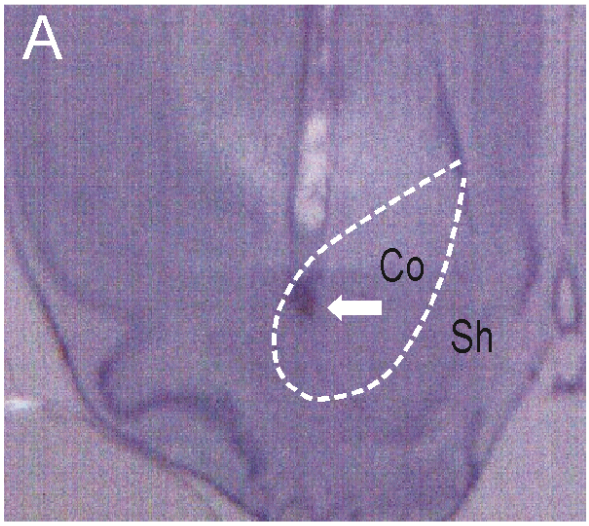
3.3.3 NMDA receptor blockade in the NA shell selectively blocks the effects of intra-BLA Cb1R modulation of opiate reward processing

We next examined the potential role of intra-NAc NMDA receptor transmission on the behavioral effects of intra-BLA CB1R activation or blockade. We first targeted the core region of the NAc (NACo) with bilateral microinfusions of the selective NMDA receptor antagonist, AP5 (see methods), using a dose (1μg/0.5μl) which we have previously reported produces no effects in and of itself on opiate reward processing (Lintas et al., 2012). **Fig. 3.2*A,B*** represents a sample micrograph and schematic summary of intra-NACo cannula placement. The first experimental group received bilateral intra-NACo microinfusions of AP5 (**Fig. 3.2*C,D***) followed by intra-BLA WIN 55 (500ng/0.5μl) or vehicle, vs. the supra-reward threshold dose of morphine (5.0 mg/kg i.p.). Analysis of CPP scores revealed a significant effect of treatment on times spent in morphine vs. saline-paired environments ($F_{(1,29)}$= 194.33, *p<0.0001*). *Post hoc* analyses revealed that both vehicle control and rats receiving intra-NACo AP5 showed significant aversions to normally rewarding conditioning doses of morphine (n's=7, *p's < 0.01*; **Fig. 3.2*C***).

The next group received intra-NACo microinfusion of AP5, and an intra-BLA microinfusion of AM 251 (500ng/0.5μl) against the sub-threshold dose of morphine (0.05 mg/kg i.p.). Analysis of CPP scores revealed a significant effect of treatment on times spent in morphine vs. saline-paired environments ($F_{(1,27)}$= 172.83, *p<0.0001*; **Fig. 3.2*D***). *Post hoc* analyses revealed that both groups showed significant potentiation of normally sub-reward threshold morphine conditioning CPP (n's=7, *p's<0.01*), demonstrating that NMDA transmission in the NACo is not required for either the reward potentiating or aversion inducing effects of intra-BLA CB1R blockade, or activation, respectively.

Next, to examine the potential role of the BLA→NASh pathway in CB1R-induced modulation of opiate motivation, rats received bilateral AP5 (1μg/0.5μl) intra-NASh (**Fig. 3.2*G,H***) prior to intra-BLA WIN 55 (500ng/0.5 μl), vs. the supra-reward threshold dose of morphine. **Fig. 3.2*E,F*** represents intra-NASh injector placement and a schematic summary showing bilateral NASh cannula placements. Analysis of CPP scores revealed a significant interaction between group and treatment ($F_{(1,29)}$ = 73.38, *p<0.0001*; **Fig. 3.2*G***). *Post hoc* analyses revealed that whereas control rats displayed a robust morphine-

environment aversion (n=7; *p<0.01*), rats receiving intra-NASh NMDA receptor blockade displayed neither preference nor aversion for either environment (n=7, *p>0.05*). Next, we challenged the effects of intra-BLA CB1R blockade on morphine reward potentiation vs. intra-NASh NMDA receptor blockade (**Fig. 3.2*H***). Rats received intra-NASh AP5 (1μg/0.5μl), prior to intra-BLA AM 251 (500ng/0.5μl) vs. the sub-reward threshold dose of morphine (n=7). Analysis of CPP scores revealed a significant interaction between group and treatment ($F_{(1,27)}$ = 54.37; *p<.0001*). *Post hoc* analyses revealed that whereas control rats receiving AM 251 showed a significant potentiation in normally sub-reward threshold morphine CPP (n=7 *p<.01*), rats receiving intra-NASh AP5 showed no preference for morphine-paired environments (n=7, *p>.05*). While we have demonstrated previously that intra-NASh AP5 (1μg/0.5μl) fails to block morphine reward in and of itself (Lintas et al., 2012), to rule out any possible motivational effects of intra-NASh AP5 alone, a control group (n=7) received intra-NASh AP5 alone vs. vehicle. Analysis of CPP scores revealed that AP5 alone, failed to produce any motivational effects during the CPP test (**Fig 3.2*H***, t_6=.98, *p>.05*). Together, these results demonstrate that BLA CB1R-mediated modulation of opiate reward processing depends upon an excitatory BLA→NAc pathway, specifically targeting the NASh, and mediated via NMDA receptor transmission.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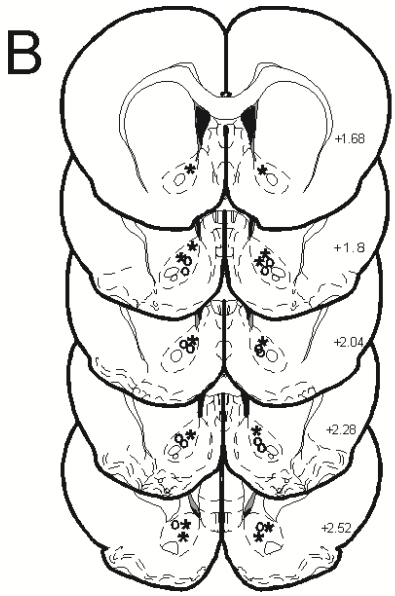
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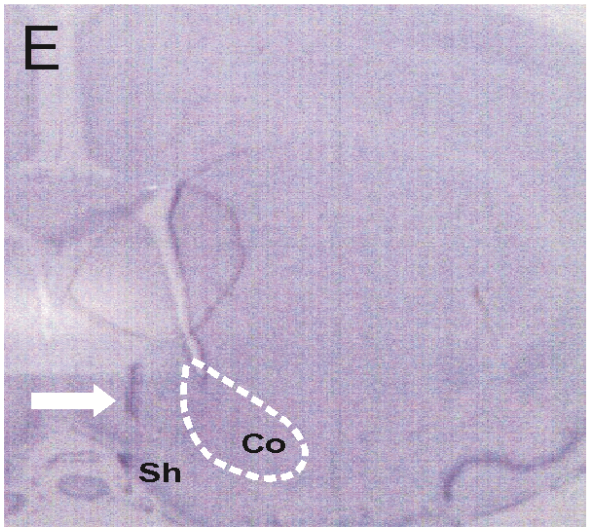
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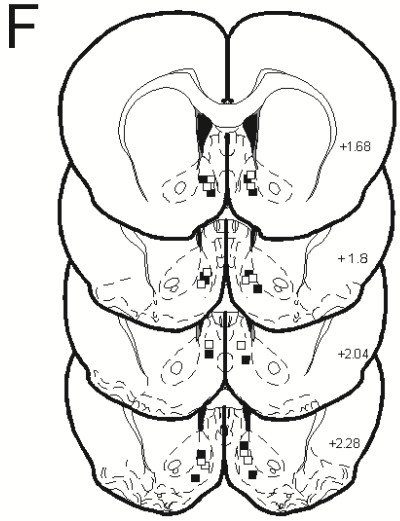
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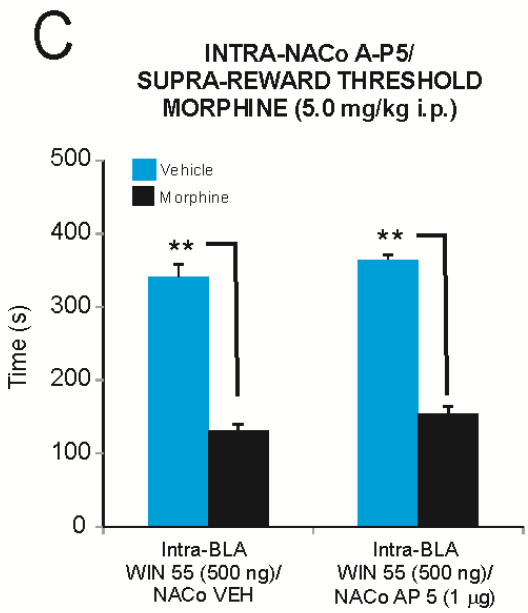
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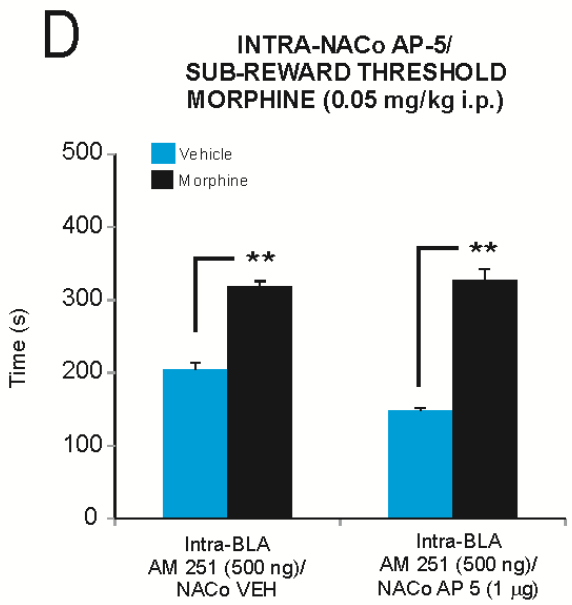
INTRA-NACo A-P5/
SUPRA-REWARD THRESHOLD
MORPHINE (5.0 mg/kg i.p.)
Vehicle
Morphine
Time (s)
500
400
300
200
100
0
**
**
Intra-BLA
WIN 55 (500 ng)/
NACo VEH
Intra-BLA
WIN 55 (500 ng)/
NACo AP 5 (1 μg)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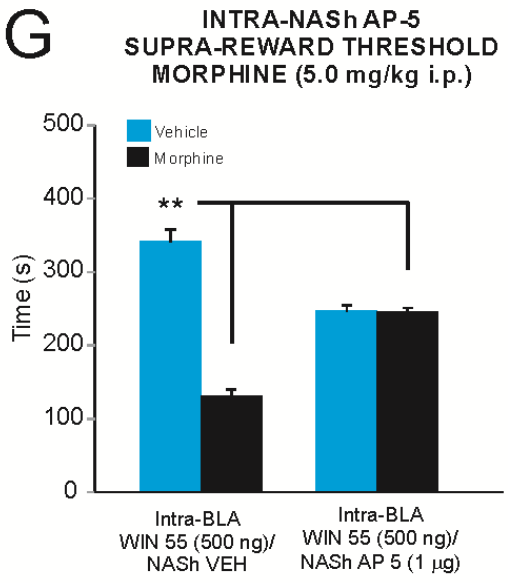
INTRA-NACo AP-5/
SUB-REWARD THRESHOLD
MORPHINE (0.05 mg/kg i.p.)
Vehicle
Morphine
Time (s)
500
400
300
200
100
0
**
**
Intra-BLA
AM 251 (500 ng)/
NACo VEH
Intra-BLA
AM 251 (500 ng)/
NACo AP 5 (1 μg)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INTRA-NASh AP-5
SUPRA-REWARD THRESHOLD
MORPHINE (5.0 mg/kg i.p.)
Vehicle
Morphine
Time (s)
500
400
300
200
100
0
**
Intra-BLA
WIN 55 (500 ng)/
NASh VEH
Intra-BLA
WIN 55 (500 ng)/
NASh AP 5 (1 μg)

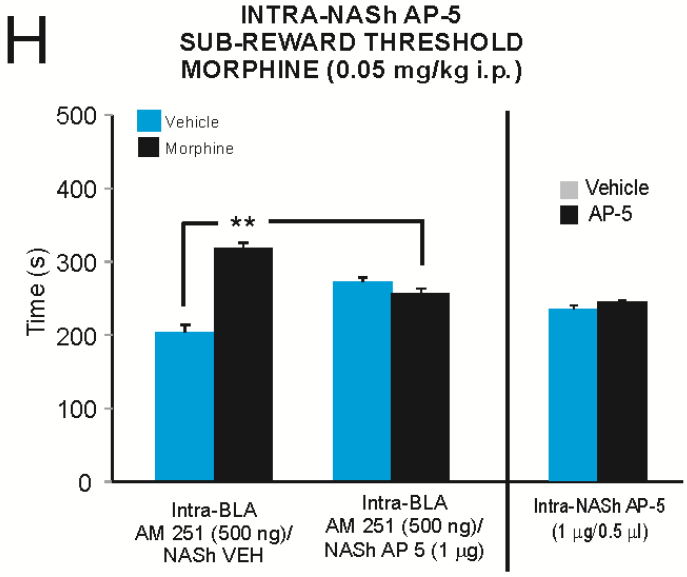
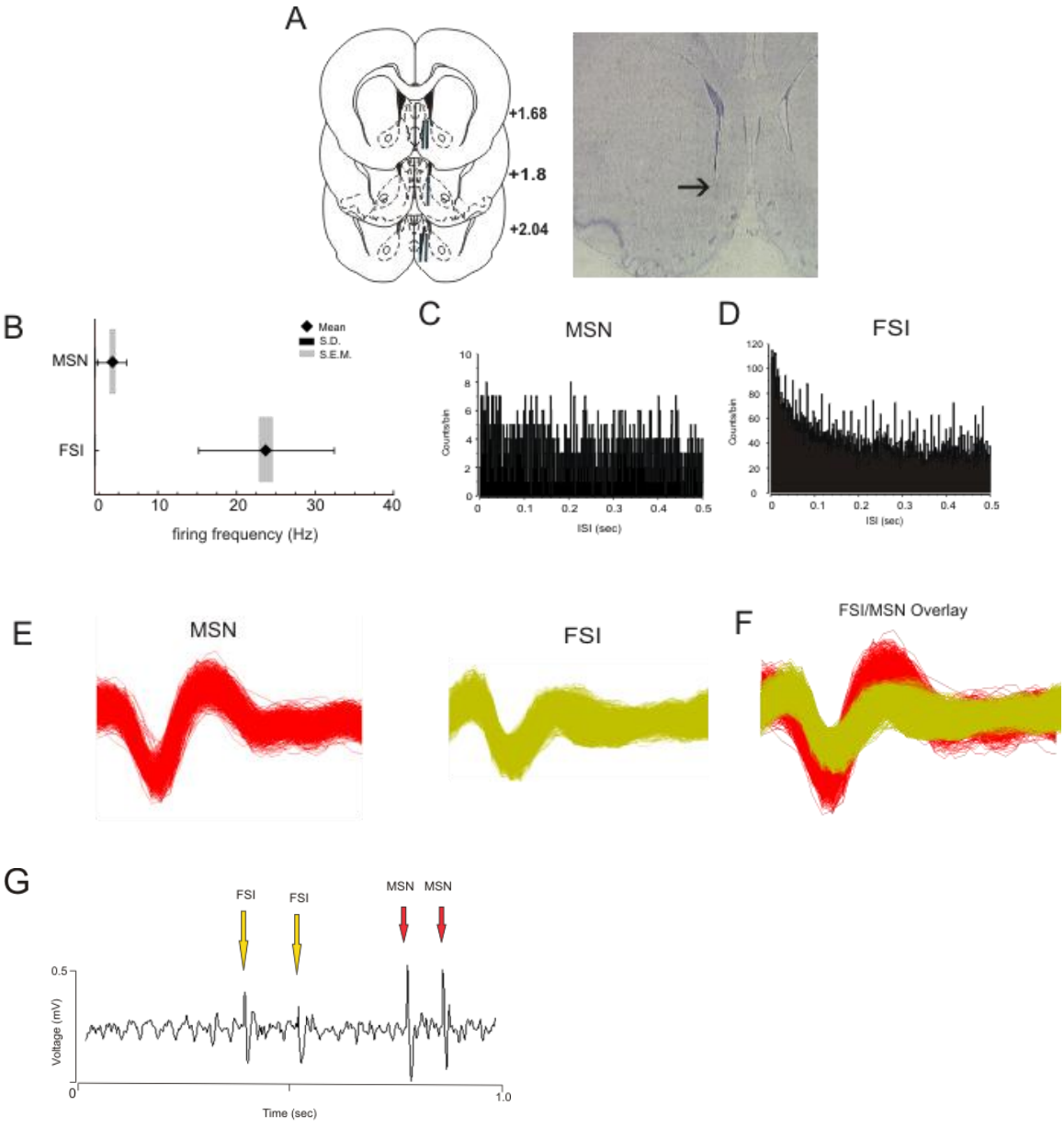

H
INTRA-NASh AP-5
SUB-REWARD THRESHOLD
MORPHINE (0.05 mg/kg i.p.)
Vehicle
Morphine
Time (s)
500
400
300
200
100
0
**
Intra-BLA
AM 251 (500 ng)/
NASh VEH
Intra-BLA
AM 251 (500 ng)/
NASh AP 5 (1 μg)
Vehicle
AP-5
Intra-NASh AP-5
(1 μg/0.5 μl)

Figure 3.2 Effects of intra-NAc NMDA receptor blockade on intra-BLA CB1R-mediated modulation of morphine reward and aversion behaviors

(A) Microphotograph showing a typical intra-NACo injector placement. **(B)** Schematic summary of intra-NACo cannula placements: **O** = NACo placements for 1μg AP5 vs. 500ng WIN 55 vs. supra-threshold morphine. ***** = NACo placements for 1μg AP5 vs. 500ng AM 251 vs. sub-threshold morphine. **(C)** Intra-NACo NMDA receptor blockade fails to block the ability of intra-BLA WIN 55,212-2 (500ng/0.5μl, n = 8) to switch supra-reward threshold morphine CPP into conditioned aversions with both control (n=8) and WIN 55 groups (n=7) showing a significant CPA to morphine-paired environments **(D)** Intra-NACo NMDA receptor blockade (n = 7) fails to block the reward potentiating effects of intra-BLA AM 251 (n=7) during sub-reward threshold morphine conditioning, with both groups showing robust morphine CPP. **(E)** A microphotograph showing a typical intra-NASh injector placement. **(F)** A schematic summary of bilateral intra-NASh cannula placement: □= NASh cannula placements for intra-NASh AP5 vs. 500ng WIN 55,212-2 vs. supra-threshold morphine. ■ = Bilateral NASh cannula placements for intra-NASh AP5 vs. 500ng AM 251 vs. sub-threshold morphine. **(G)** Blockade of intra-NASh NMDA transmission blocks the ability of intra-BLA CB1R activation to switch morphine reward CPP into CPA. While control rats (n=8) showed normal CB1R-mediated morphine CPA, this effect was blocked in rats receiving intra-NASh NMDA receptor blockade (n=7). **(H)** Blockade of intra-NASh NMDA transmission completely blocks the ability of AM 251 (500ng/0.5μl, n = 7) to potentiate sub-reward threshold morphine relative to controls, who show normal reward potentiation effects (n=7). Furthermore, intra-NASh AP 5 (1μg/0.5μl) had no effect on it's own (far right).

3.3.4 Intra-BLA CB1R blockade differentially modulates NASh MSN vs. FSI neuronal activity patterns depending on morphine reward salience

Given our findings demonstrating a functional role for excitatory NMDA transmission within the NASh for the processing of intra-BLA CB1R-dependent effects on opiate reward behaviours (**Fig. 3.2**), we next analyzed the NASh with *in vivo* neuronal recordings to determine the potential effects of intra-BLA CB1R activation or blockade on NASh MSN vs. FSI neuronal activity dynamics (*see methods*, **Fig. 3.3**). A schematic and micrograph showing representative intra-NASh micro-wire placement is presented in **Fig. 3.3*A***. Furthermore, the mean baseline firing frequency for MSN/FSI **(Fig. 3.3*B*)**, and corresponding inter-spike interval histograms (**Fig. 3.3*C,D*)** for MSN vs. FSI neurons are depicted. Moreover sample MSN/FSI waveforms **(Fig 3.3 *E*)**, an overlay **(Fig. 3.3 *F*)**, and sample traces during recording are shown in **Fig. 3.3 *G,H*** respectively.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+1.68
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B
Mean
S.D.
S.E.M.
MSN
FSI
0
10
20
30
40
firing frequency (Hz)
C
MSN
Counts/bin
ISI (sec)
D
FSI
Counts/bin
ISI (sec)
E
MSN
FSI
F
FSI/MSN Overlay
G
FSI
FSI
MSN
MSN
0.5
Voltage (mV)
0
1.0
Time (sec)

Figure 3.3 Histological analyses and characterization of NASh FSI vs. MSN neuronal sub-populations

(A) Schematic of typical unilateral micro-wire NA shell placements for 500ng WIN 55 vs. supra-threshold dose of morphine (blue lines), and microphotograph showing a typical intra-NASh microwire location (black arrow). **(B)** Mean FSI/MSN baseline firing frequency **(C)** A sample MSN inter-spike interval histogram. **(D)** A representative FSI inter-spike interval histogram. **(E)** Sample waveforms for MSN and FSI. **(F)** FSI/MSN waveform overlay. **(G)** A trace recording depicting FSI and MSN

We first examined the effects of sub-reward threshold morphine (0.05 mg/kg) CPP conditioning and memory recall in rats receiving intra-BLA vehicle microinfusions. Post-experimental offline analyses of sorted NASh neuronal units in the intra-BLA vehicle group vs. sub-reward threshold morphine (0.05 mg/kg) revealed totals of n=45 MSN and n=49 FSI units recorded, while offline analyses of NASh neuronal populations from rats receiving intra-BLA AM 251 (500 ng/0.5 μl) vs. sub-reward threshold morphine (0.05 mg/kg) resulted in the analysis of n=20 FSI units and n=39 MSN units over the course of conditioning and CPP recall test phases. Analysis of MSN neuronal population activity over morphine trials for both intra-BLA vehicle and AM 251 animals, showed a significant interaction between group and treatment ($F_{(8,53)}$=10.31, *p<.0001;* **Fig. 3.4*A***). *Post hoc* analysis of vehicle MSN neuronal population activity comparing % change in frequency during morphine CPP conditioning trials relative to baseline, revealed no significant effects of conditioning on MSN firing frequencies across trials (*p's>.05*). In contrast, *post hoc* analysis of CB1R blockade group revealed a significant decrease in MSN activity levels from baseline during all morphine conditioning trials (*p's<.01*). Furthermore, comparing vehicle vs. AM 251 groups revealed rats receiving intra-BLA AM 251 showed significantly attenuated MSN activity levels relative to vehicle controls at all conditioning trials (p's<.01).

We next compared NASh FSI neuronal activity levels comparing intra-BLA vehicle vs. AM 251 groups during conditioning. One-way ANOVA comparing FSI morphine trial recordings across vehicle or AM 251 groups revealed a significant interaction between group and treatment ($F_{(8,71)}$=61.96, *p<.0001*; **Fig. 3.4*B***). *Post hoc* analyses of AM 251 group revealed a significant increase in FSI firing rates during all morphine trials (*p's<.01*), whereas the vehicle group showed no significant changes (*p's>.05*). Furthermore, comparing vehicle vs. AM 251 groups revealed rats receiving intra-BLA AM 251 showed significantly increased FSI activity levels relative to vehicle controls during all trials (Trial 1, 3, 4=p's<,01; Trial 2, p<.05).

The behavioural CPP recall test showed that animals receiving intra-BLA vehicle (n=6) demonstrated no significant differences in times spent in saline vs. morphine environments (t_5=.15, *p>.05*; **Fig. 3.4*C***). In contrast, behavioural testing of animals

receiving intra-BLA AM 251 (n=7), revealed a significant CPP for morphine-paired environments (t_6= 11.25, *p<.0001*; **Fig. 3.4*C***). Moreover, corresponding neuronal population activity recording during the CPP recall test revealed no significant change in either FSI or MSN recordings from baseline for the vehicle control group. However, a divergent FSI vs. MSN population activity levels relative to normalized baselines during exposure to morphine-paired environments was observed for the intra-BLA AM 251 group. Analysis revealed a significant effect of group and treatment ($F_{(4,49)}$= 74.60, *p<.0001*; **Fig. 3.4*D***). *Post hoc* analysis revealed a significant increase in FSI and a decrease in MSN for animals receiving intra-BLA AM 251 (*p<.01*), and no significant change from baseline for the intra-BLA vehicle group (*p>.05*). A sample neuronal rastergram showing the acute inhibitory effect of intra-BLA AM 251 (500ng/0.5 μl) on spontaneous NASh MSN firing activity is presented in **Fig. 3.4*E***. In contrast, the typical acute excitatory effect observed following intra-BLA AM 251 on the spontaneous activity of a representative NASh FSI neuron is presented in **Fig. 3.4*F***.

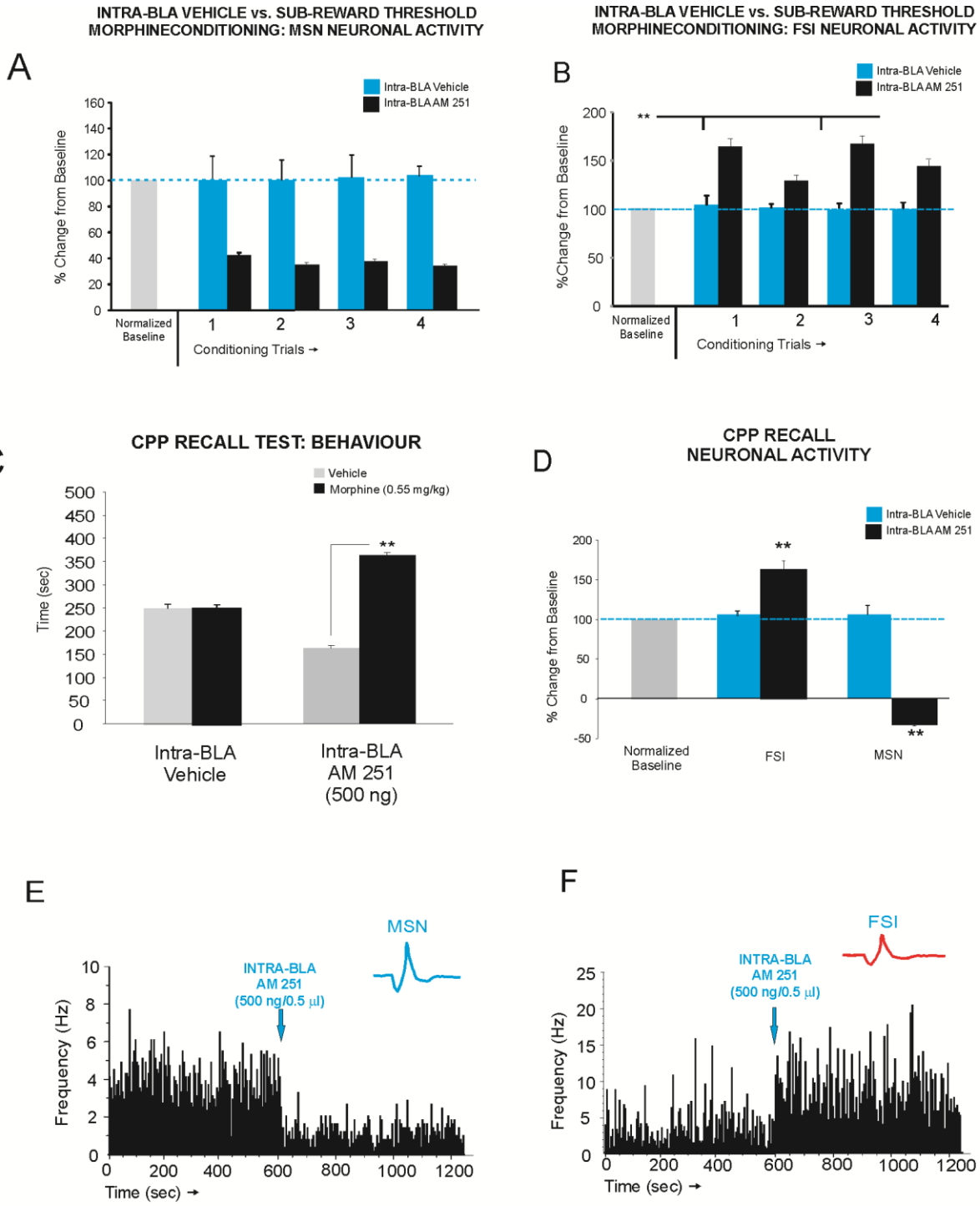

A
INTRA-BLA VEHICLE vs. SUB-REWARD THRESHOLD MORPHINECONDITIONING: MSN NEURONAL ACTIVITY
Intra-BLA Vehicle
Intra-BLA AM 251
% Change from Baseline
Normalized Baseline
Conditioning Trials
B
INTRA-BLA VEHICLE vs. SUB-REWARD THRESHOLD MORPHINECONDITIONING: FSI NEURONAL ACTIVITY
Intra-BLA Vehicle
Intra-BLA AM 251
%Change from Baseline
Normalized Baseline
Conditioning Trials
C
CPP RECALL TEST: BEHAVIOUR
Vehicle
Morphine (0.55 mg/kg)
Time (sec)
Intra-BLA Vehicle
Intra-BLA AM 251 (500 ng)
D
CPP RECALL NEURONAL ACTIVITY
Intra-BLA Vehicle
Intra-BLA AM 251
% Change from Baseline
Normalized Baseline
FSI
MSN
E
MSN
INTRA-BLA AM 251 (500 ng/0.5 µl)
Frequency (Hz)
Time (sec)
F
FSI
INTRA-BLA AM 251 (500 ng/0.5 µl)
Frequency (Hz)
Time (sec)

Figure 3.4 Neuronal FSI vs. MSN population activity patterns during sub-reward threshold morphine (0.05 mg/kg) conditioning and recall testing: intra-BLA AM251 (500ng/0.5µl) vs. vehicle controls.

(A) Summary of MSN recordings during morphine trials revealed no significant changes from baseline for the intra-BLA vehicle group. However, a significant decrease in MSN population activity levels relative to pre-conditioning baseline was observed on all morphine conditioning trials for rats receiving intra-BLA AM 251(500ng/0.5µl). **(B)** Summary of FSI population activity recorded during sub-reward threshold morphine conditioning trials revealed no significant changes from baseline during conditioning trials for intra-BLA vehicle group. In contrast, rats receiving intra-BLA AM 251 showed a significant increase in FSI activity from baseline across all morphine conditioning trials. **(C)** Vehicle control rats showed no preference for vehicle vs. morphine-paired environments during CPP recall testing. However, intra-BLA CB1R blockade resulted in the potentiation of morphine reward CPP. **(D)** Neuronal population activity analyses during CPP recall testing indicted no changes in either FSI or MSN activity levels from pre-test normalized baselines for the vehicle control group. Conversely, neuronal FSI vs. MSN population activity recorded during CPP recall testing shows divergent FSI vs. MSN activity, an increase in FSI and a decrease in MSN activity for the intra-BLA AM 251 group. **(E,F)** Representative MSN **(E)** and FSI **(F)** rastergrams showing representative 10 min neuronal activity level pre and post intra-BLA AM 251 (500ng/0.5µl) microinfusions.

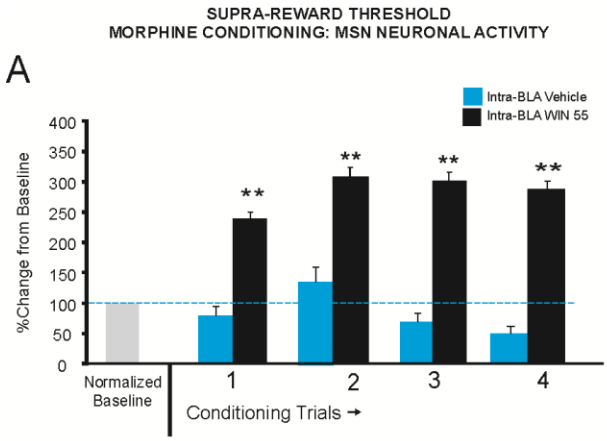
3.3.5 Intra-BLA CB1R activation differentially modulates NASh MSN vs. FSI neuronal activity patterns during morphine reward or aversion learning

We next examined the effects of supra-reward threshold morphine (5.0 mg/kg; i.p.) CPP on NASh MSN vs. FSI neuronal populations in rats receiving intra-BLA vehicle (n=7) and intra-BLA WIN 55 (n=6). Post-experimental offline examination of isolated NASh neuronal populations for vehicle animals resulted in the analysis of n=61 MSN and n=64 FSI and for animals receiving intra-BLA WIN 55 resulted in the analysis of n=31 FSI and n=24 MSN units analyzed over the course of conditioning and CPP test phases. Statistical analysis of MSN neuronal activity during morphine conditioning trials revealed a significant interaction between group and treatment ($F_{(8,152)}$= 87.34, *p<.0001*; **Fig. 3.5*A***). *Post hoc* analyses revealed significant increases in MSN population activity levels from baseline for the intra-BLA WIN 55 group during all morphine conditioning trials (*p's<.01*), and a significant decrease in MSN activity for the 3rd and 4th morphine conditioning trial of vehicle controls (*p's<.05*). Furthermore, comparing vehicle vs. WIN 55 groups revealed that rats receiving intra-BLA WIN 55 showed significantly increased MSN neuronal activity levels relative to vehicle controls during all conditioning trials (p's<.01).

Next, analyses of FSI activity during conditioning trials across both vehicle and experimental group revealed a significant effect between group and treatment ($F_{(8,206)}$= 37.79, *p<.0001;* **Fig. 3.5*B***). *Post hoc* analyses revealed significant decreases in FSI population activity levels during all conditioning trials for the intra-BLA WIN 55 group (*p's<.05*), and a significant increase across all morphine trials for the vehicle control group (*p<.05* for 1st trial, *p's<.01* for the remaining trials). Furthermore, comparing vehicle vs. WIN 55 groups revealed rats receiving intra-BLA WIN 55 showed significantly decreased FSI neuronal activity levels relative to vehicle controls during all conditioning trials (*p's<.01*).

Intra-BLA vehicle control behavioral testing revealed a significant CPP for morphine-paired environments (t_6= 11.25, *p<.0001*; **Fig. 3.5*C***). Consistent with previous findings (**Fig. 3.1*D***), intra-BLA CB1R activation switched the normally rewarding effects of

morphine into a strong conditioned place aversion, with rats (n=6) demonstrating a robust morphine environment aversion during CPP testing (t_5=12.74, *p<0.001*; **Fig. 3.5*C***). Furthermore, corresponding neuronal population activity recording for both groups during the CPP recall test revealed divergent FSI vs. MSN population activity levels relative to normalized baselines during exposure to morphine-paired environments. Analysis revealed a significant effect of group and treatment on relative firing rates ($F_{(4,109)}$= 57.13, *p<.0001*; **Fig. 3.5*D***) with *post hoc* analysis revealing a significant increase in FSI neuronal activity vs. a significant decrease in MSN neuronal population activity levels for the vehicle control group, relative to normalized pre-test baseline levels, and the opposite was observed for intra-BLA WIN 55 group (*p's<.01*, **Fig. 3.5*D***). Moreover, a sample neuronal rastergram showing the acute excitatory effect of intra-BLA WIN 55 (500ng/0.5 μl) on spontaneous NASh MSN firing activity is presented in **Fig. 3.5*E***. In contrast, the typical acute inhibitory effect following intra-BLA WIN 55 on the spontaneous firing activity of NASh FSI neurons is demonstrated in the sample neuronal rastergram presented in **Fig. 3.5*F***. Thus, the effects of intra=BLA CB1R activation on switching morphine reward CPP into CPA, were associated with divergent NASh neuronal effects on MSN vs. FSI population activity: supra-threshold morphine reward CPP acquisition and recall was associated with decreased MSN neuronal activity and increased FSI activity, whereas the CB1R-mediated switch from reward to aversion processing was associated with the opposite pattern of NASh neuronal population activity.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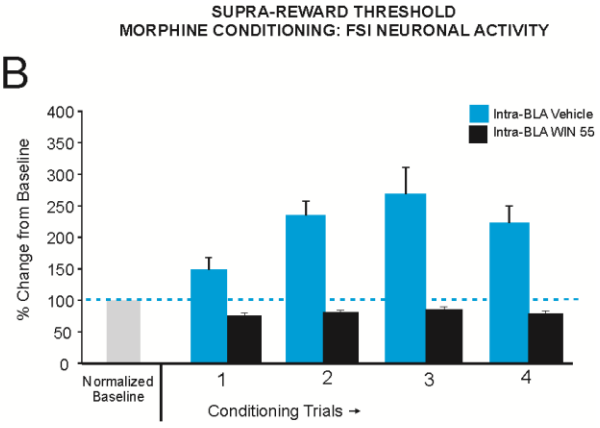
Intra-BLA WIN 55
%Change from Baseline
Normalized Baseline
Conditioning Trials


SUPRA-REWARD THRESHOLD
MORPHINE CONDITIONING: FSI NEURONAL ACTIVITY
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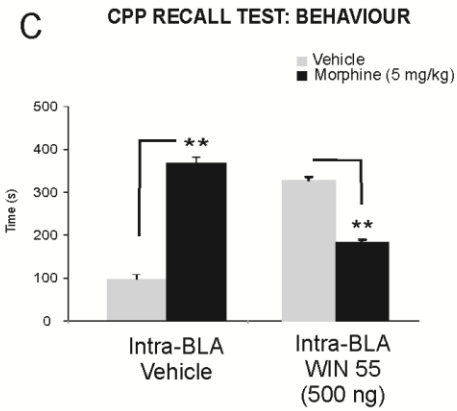
Intra-BLA Vehicle
Intra-BLA WIN 55
% Change from Baseline
Normalized Baseline
Conditioning Trials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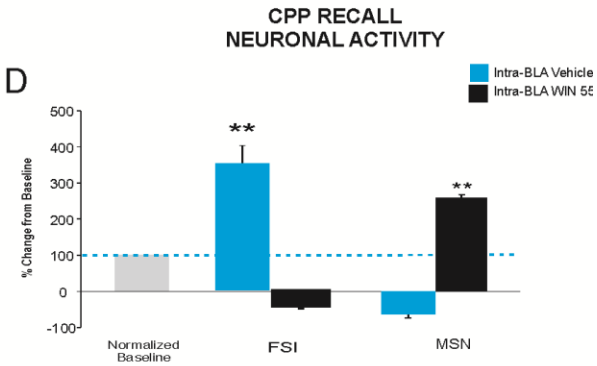
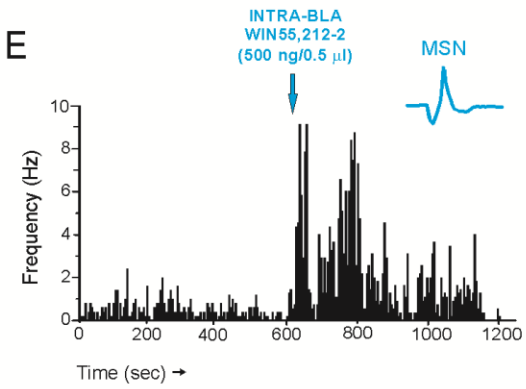
CPP RECALL TEST: BEHAVIOUR
Vehicle
Morphine (5 mg/kg)
Time (s)
Intra-BLA Vehicle
Intra-BLA WIN 55 (500 ng)


CPP RECALL
NEURONAL ACTIVITY
D
Intra-BLA Vehicle
% Change from Baseline
Normalized Baseline
FSI
MSN


E
INTRA-BLA
WIN55,212-2
(500 ng/0.5 µl)
MSN
Frequency (Hz)
Time (sec)

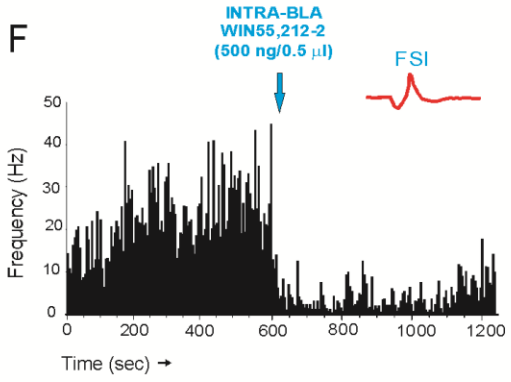

F
INTRA-BLA
WIN55,212-2
(500 ng/0.5 µl)
FSI
Frequency (Hz)
Time (sec)

Figure 3.5 Neuronal FSI vs. MSN population activity patterns during supra-reward threshold morphine (5.0 mg/kg) conditioning and recall testing: intra-BLA WIN 55,212-2 (500ng/0.5µl) vs. vehicle controls.

(A) Intra-BLA CB1R activation resulted in a significant increase in MSN activity in comparison to baseline levels across all four morphine conditioning trials. Conversely, for the intra-BLA vehicle control group, a significant decrease in MSN population activity levels relative to pre-conditioning baseline was observed on conditioning trials 1, 3 and 4. **(B)** A significant decrease in FSI neuronal activity from baseline was observed in rats receiving intra-BLA WIN 55,212-2 (500ng/0.5µl) during morphine reward conditioning trials. In contrast, FSI neuronal population activity is significantly increased relative to normalized pre-conditioning baseline levels during all morphine trials. **(C)** Vehicle control rats show a robust CPP for environments paired with supra-threshold morphine, while BLA CB1R activation produces a significant morphine CPA. **(D)** Neuronal FSI vs. MSN population activity recorded during CPP recall testing shows divergent FSI vs. MSN activity, similar to that observed during conditioning trials. Intra-BLA vehicle controls show increased FSI neuronal activity and a decrease in MSN neuronal activity from baseline. In contrast, intra-BLA CB1 activation causes decreased FSI and increased MSN neuronal activity from baseline. **(E,F)** Sample MSN **(E)** and FSI **(F)** rastergrams depicting representative single neuron responses during 10 min neuronal activity recordings pre and post intra-BLA CB1R activation with WIN 55 (500ng/0.5µl).

3.4 DISCUSSION

The present findings add to a growing body of evidence demonstrating that bi-directional modulation of CB1R transmission can modulate both rewarding and aversion-related emotional processing (Ahmad et al., 2013; Laviolette and Grace, 2006; Tan et al., 2014). Within the mammalian amygdala, CB1Rs are primarily localized to the BLA, but absent in adjacent nuclei such as the central nucleus (Katona et al., 2001). Endocannabinoid signaling within the BLA is involved critically in affective processing (Hill and Patel, 2013). Functionally, BLA CB1R transmission has been shown to modulate feedforward inhibitory presynaptic GABA release and can therefore tightly regulate the excitability of BLA output neurons (Katona et al., 2001). Consistent with the present findings, studies using systemically applied CB1R agonists or antagonists have demonstrated that CB1R transmission can control functional outputs to the NASh. For example, Pistis et al. (2004) reported that BLA→NASh projection neurons were strongly inhibited by WIN 55 administration. Furthermore, BLA-evoked excitatory responses in recorded NASh neurons were similarly inhibited by systemic CB1R agonists, suggesting that activation of CB1R signaling can dampen BLA-dependent excitation of NASh neurons. While these studies examined single unit neuronal responses with systemic drug administration, the present findings are the first study to combine direct intra-BLA CB1R pharmacological activation or blockade, with simultaneous multi-unit neuronal population recordings in the NASh and post-experimental dissociation of FSI vs. MSN neuronal activity patterns.

We found that the ability of BLA CB1R-blockade to potentiate the reward salience of normally sub-reward threshold morphine conditioned effects was associated with concomitant inhibition of MSN and activation of FSI neurons during conditioning and recall phases of morphine-related learning. In contrast, BLA CB1R activation, which switched morphine CPP into CPA behaviors, was associated with the opposite pattern of NASh neuronal population activity. Importantly, BLA CB1R-mediated modulation of NASh FSI vs. MSN neuronal population activity was not simply an effect of acute intra-BLA CB1R blockade or activation, as the same reward vs. aversion-related patterns of MSN/FSI population activity were present during the drug-free memory recall phase, in the absence of any intra-BLA CB1R modulation.

Our results are consistent with evidence demonstrating that reward processing within the NAc is linked to inhibition of MSN neurons, while aversion-related processing is linked to their activation. For example, several studies using single-unit electrophysiological recordings in the NAc have reported that the reinforcing effects of drug self-administration are correlated with transient inhibitory effects on NAc neurons, including studies using heroin (Chang et al., 1997), cocaine (Peoples and West, 1996; Peoples et al., 2007) and naturally rewarding stimuli such as food or sucrose (Carelli et al., 2000; Nicola et al., 1999) as conditioning cues. A consistent finding across these studies is that the most commonly observed pattern of MSN reward-related firing is transient inhibition (for review see Carlezon and Thomas, 2009). Furthermore, systemically or intra-VTA applied opiates such as morphine or heroin have been reported to induce inhibitory effects in a plurality of sampled NAc neurons (Hakan and Henriksen, 1987; 1989; Lee et al., 1999), however these studies did not differentiate between putative FSI vs. MSN neuronal subtypes, which appear to play distinct roles in the associative effects of either rewarding or aversion-related emotional processing (Lansink et al., 2010; Sun and Laviolette, 2015).

Given that intra-BLA and intra-PFC CB1 activation has been shown to strongly potentiate normally non-salient, fear-related associative memories (Draycott et al., 2013), one possibility is that BLA CB1R signaling may induce a general state of negative emotional bias by activating NASh MSN neuronal populations. Indeed, activation of MSN neuronal populations has been linked previously to aversive emotional processing. For example, studies examining the processing of taste aversion learning demonstrated that a majority of NAc neurons displayed excitatory vs. inhibitory response patterns (Roitman et al., 2005; Wheeler et al., 2008). Furthermore, modulating the motivational valence of appetitive (saccharin) taste cues from rewarding to aversive (by inducing cocaine-related conditioned taste aversions), switched the predominant neuronal response pattern from inhibitory to excitatory, suggesting that dynamic switches between reward vs. aversive-related processing may be related to shifts between excitatory vs. inhibitory NAc neuronal response patterns (Wheeler et al., 2008). Furthermore, manipulation of specific molecular signaling pathways within the NAc which in turn increase the general excitability of NAc neurons, have been shown to switch the motivational valence of

conditioning stimuli from rewarding, to aversive (Carlezon and Thomas, 2009). For example, overexpression of CREB within the NAc, which potentiates intrinsic NAc neuronal excitability (Dong et al., 2006), was able to switch the rewarding conditioning effects of cocaine, into aversions, while simultaneously inducing depressive-like behaviors (Pliakas et al., 2001).

While few studies have examined the effects of reward or aversion-related processing on NASh FSI unit activity, it has been reported that dopamine receptor activation can induce FSI unit hyperpolarization (Centonze et al., 2003) and similarly, amphetamine administration strongly potentiates NAc FSI unit activity recorded *in vivo* (Wiltschko et al., 2010). Given our observations that intra-BLA CB1R blockade strongly potentiated the reward salience of normally sub-threshold morphine conditioning doses, through a DA-dependent mechanism, this may suggest that BLA-mediated modulation of NAc neuronal activity may involve modulation of DA release within the NASh, via convergent inputs onto NMDA receptor substrates, consistent with previous reports showing that the BLA modulates NAc neuronal activity rates via convergent NMDA and DA receptor mechanisms (Floresco et al, 2001).

In summary, the present study demonstrates that cannabinoid modulation of amygdala inputs to the NAc, is capable of powerfully disrupting the processing of affective information through both GLUTergic and DAergic mechanisms within the NASh. In summary, the present findings reveal a novel mechanism by which cannabinoid dysregulation within the amygdala may lead to disturbances in affective regulation by altering both the salience and valence of associative cues related to affective processing via NAc neuronal populations.

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Chapter 4

4 Cannabinoid reward and aversion in the posterior ventral tegmental area is differentially mediated through dopamine projections to the basolateral amygdala or nucleus accumbens shell

4.1 INTRODUCTION

Dopamine (DA) neurons are the major components of the brain's reward circuitry and mediate the salience of stimulus reward learning (Berridge & Robinson, 1998; Flagel et al., 2011b). The ventral tegmental area (VTA) contains a high density of DA neurons and sends DA efferent to higher level cortical structures that include the prefrontal cortex (PFC), basolateral amygdala (BLA), and nucleus Accumbens (NAc) which form an interconnected network that is crucial for reward related learning (Grace et al., 2007; Ikemoto & Panksepp, 1999). Drugs of abuse such as nicotine, cannabis, opiates, and cocaine have all been known to exert their effects through the dopamine reward pathway, often altering mesolimbic DA levels (Everitt & Wolf, 2002; Kelley & Berridge, 2002; Koob, Sanna, & Bloom, 1998; R. a. Wise, 1996). Cannabinoid CB1 receptors in particular have shown to modulate DA activity. Systemic administration of THC has shown to excite DA neurons and increase DA firing rate in both the VTA and substantia nigra (French et al., 1997).

More recent studies have shown that DA neurons localized specifically in the posterior region of the VTA (PVTA) is responsible for reward related learning. Nicotine and opiates have been found to be significantly more rewarding when administered in the PVTA rather than the anterior region of VTA (AVTA) (Ikemoto & Wise, 2002). Zangen, Solinas, Ikemoto, Goldberg, & Wise (2006), have shown that THC infusions into the PVTA produced a rewarding effect, while THC infusions in the AVTA produced no effects. Although it is known that cannabinoid activation does produce a behavioural effect, the specific pathways involved in the cannabinoid related reward are unknown.

Since the PVTA sends DAergic projections to both the BLA and specifically the shell region of the NAc (NASh) (Bassareo & Di Chiara, 1997), we wanted to further investigate the possible mechanisms by which cannabinoid reward effects are processed through VTA DAergic outputs. In the present study, we investigated the role of CB1 receptor activation and blockade in the PVTA in relation to cannabinoid related reward and aversion signals, using an unbiased condition place preference (CPP) paradigm in conjunction with behavioural pharmacology. We report that intra-PVTA CB1 activation produced a cannabinoid reward CPP, while CB1 blockade of intra-PVTA produced a

cannabinoid related aversion during the recall phase. Furthermore, the DA antagonist α-flu microinfused intra-BLA, blocked the cannabinoid reward CPP, but not the aversion. In contrast, intra-NASh microinfusion of α-flu blocked the cannabinoid related aversion but not the reward. Thus, cannabinoid related reward and aversion signals are mediated through DAergic PVTA→BLA and PVTA→NASh functional pathways.

4.2 MATERIALS AND METHODS

4.2.1 Animals and Surgery

All procedures performed in the study were in accordance with the Canadian Council on Animal Care and approved by Western University's Animal Care Council. Adult male Sprague Dawley rats (350-400 gm; Charles River Canada) were anesthetized with an intraperitoneal injection (i.p.) of a ketamine (80mg/ml)-xylazine (6mg/kg) mixture, and placed in a stereotaxic device. For double cannulation in the PVTA, two stainless steel guide cannulae (22 gauge) were implanted into the PVTA using the following coordinates (10° angle): from bregma, anteroposterior (AP) -5.5mm, lateral (LAT) ±2.3mm, Ventral (V) -8.0mm from the dural surface. The following coordinates were used for AVTA cannulation (10° angle): AP -4.5mm, LAT ±2.3mm, V -8.0mm from the dural surface. For quadruple cannulation in the BLA-PVTA, two additional cannulae were implanted in the BLA using the following coordinates (0° angle): from bregma, AP -2.6 mm, LAT ±5.0 mm, and V -7.2 mm from the dural surface. For quadruple cannulation in the NASh-PVTA, the two additional cannulae in the NASh were implanted with the following coordinates (12° angle): from bregma, AP +1.8 mm, LAT ±2.6 mm, and V -7.4 mm from the dural surface. Dental acrylic and jeweler's screws were used to secure the cannulae in place.

4.2.2 Drug Treatment

The CB1 agonist WIN 55,212-2 (Tocris Bioscience) and antagonist AM 251 (Tocris Bioscience) were dissolved in dimethyl sulfoxide (DMSO), and diluted in physiological saline (pH adjusted to 7.4) when necessary to obtain the appropriate doses (50ng and 500ng). Bilateral PVTA or AVTA micro-infusions (μg/0.5μl) were performed over a period of 1 minute via plastic tubing connected to a 1μl Hamilton micro-syringe.

Injectors were left in place for an additional 1 minute to ensure adequate diffusion of the drug from the injector tip. The dopamine (DA) receptor antagonist α-Flupenthixol dehydrochloride (α-flu, Tocris Bioscience) was also dissolved in physiological saline (pH 7.4). For DA antagonist treatment, animals received bilateral BLA or NASh microinfusions of α-flu (1μg/0.5μl) prior to receiving intra-PVTA microinfusions of CB1 agonist or antagonist.

4.2.3 Condition Place Preference

All rats were conditioned using the unbiased classical Pavlovian conditioning method condition place preference (CPP), which differentiates between two distinct environments as described previously(Bishop et al., 2011; Steven R Laviolette & van der Kooy, 2004). The two environments used for conditioning varied in smell, texture and colour. One environment was a black box, with a smooth Plexiglass floor wiped down with 2% acetic acid prior to each conditioning session. The other environment was a white box, with a wire mesh floor covered with aspen woodchips. Rats have shown no baseline preference for either of these two boxes (S R Laviolette & van der Kooy, 2003). To adapt the animals to the confinement of these boxes, they were placed in a neutral grey box of the same dimensions for a period of 20 minutes, 24 hours prior to start of the conditioning. During the acquisition phase, animals are conditioned for a period of 8 days counterbalanced between drug and saline paired environments. During the conditioning sessions (in the PVTA or AVTA experiments), animals received bilateral intra-PVTA or intra-AVTA microinfusions of either CB1 agonist, antagonist or saline. Rats are then placed in a drug-paired environment or saline-paired environment alternated over 8 days. During the recall phase, animals are tested drug free, 3-4 days after conditioning. The box used for testing is a combination of the two environments separated by a grey neutral zone in the middle. The animal is placed in the grey zone and allowed to move freely for a period of 10 min between the two environments. The time spent in each environment is then recorded and analyzed.

4.2.4 Histology

At the completion of each experiment, animals were anesthetized with a uthanyl solution and perfused with isotonic saline followed by 10% formalin. Brains were extracted, sliced at 60 μm, and stained with cresyl violet to allow for histological analysis of injection sites. Using light microscopy, location of injector placements is analyzed and any animal with misplaced guide cannulae was excluded from the study.

4.2.5 Data Analysis

Data were analyzed with either a student's *t*-test or *two*-way analysis of variance (ANOVA) where appropriate. *Post hoc* analyses were performed with Newman-Keuls and Fisher's least significant difference test.

4.3 RESULTS

4.3.1 Histological analysis

Histological analysis indicated injector cannulae placements to be bilaterally localized within the anatomical boundaries of the PVTA, AVTA, BLA and NASh region, as determined by the Atlas of Paxinos and Watson (2007). **Fig. 4.1*A***, shows a sample micrograph representing intra-PVTA micro-injector placement and **Fig.4.1*B*** shows a schematic of bilateral intra-PVTA microinjection placements for the CB1 agonist and antagonist groups. The micrograph of the micro-injector placement of the anterior region of the VTA is illustrated in **Fig. 4.2*A***, and the corresponding schematic representing bilateral intra-AVTA microinjection placement if shown in **Fig. 4.2*B***. In **Fig. 4.3*A*** and ***B***, we present a microphotograph showing a representative injector placement within the BLA and a schematic illustration showing a representative intra-BLA bilateral cannulae placements for the α-flu experiments. **Fig. 4.4*A***, shows a micrograph representing intra-NASh micro-injector placement indicated by black arrows and in **Fig. 4.4*B***, we present a schematic of bilateral intra-NASh microinjection placement.

4.3.2 Microinfusion of CB1 agonist in the posterior region of the VTA produced a cannabinoid reward CPP

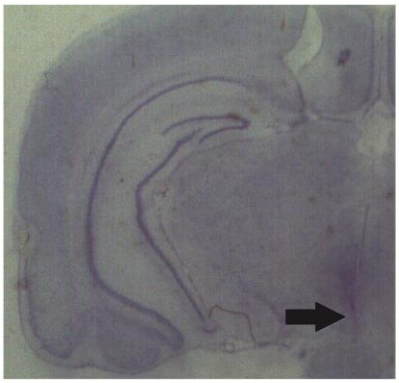
The experiments performed in this study, all used the unbiased condition place preference (CPP) paradigm as outlined earlier in the materials and method section. To determine the potential role of CB1 receptor activation in the posterior region of the ventral tegmental area (PVTA), we performed bilateral intra-PVTA microinfusions of either saline or CB1 agonist WIN 55,212-2 (WIN55, 50 or 500ng/0.5µl). Two-way ANOVA revealed a significant interaction between group and treatment ($F_{(1,27)} = 92.14$; $p < .0001$) on time spent in either the saline or drug paired environment during the recall phase. *Post hoc* analysis revealed that the higher dose of WIN55 (500ng/0.5µl), produced a significant cannabinoid related reward, with animals showing a strong preference for the WIN55 paired environment over the saline paired environment (n=7, $p< .01$, **Fig. 4.1*C***). However, the animals receiving bilateral intra-PVTA WIN55 at the lower dose (50ng/0.5µl) showed no preference for either the saline or drug paired environment (n=7, $p> .05$, **Fig. 4.1*C***). Hence, since the effective dose at which a cannabinoid reward is observed was the higher dose, all subsequent experiments involving WIN55, the dose of 500ng/0.5µl was used.

4.3.3 Intra-PVTA CB1 blockade produced a cannabinoid related aversion

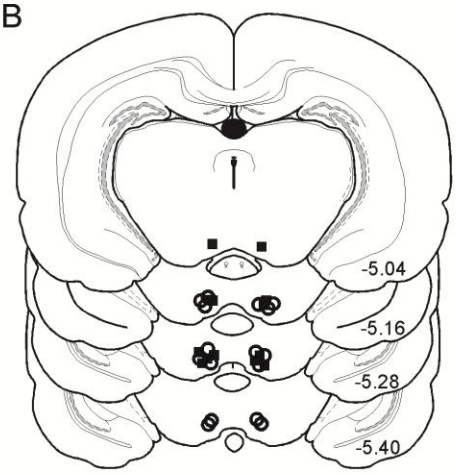
The selective CB1 receptor antagonist AM 251 was used to block cannabinoid transmission in the PVTA. To examine the effects of AM 251, two doses of 50 or 500ng/0.5µl were bilaterally microinfused intra-PVTA in two groups of animals against saline. Statistical analysis showed a significant interaction between group and treatment ($F_{(1,31)} = 176.18$; $p < .0001$) for time spent in either of the two environments during the testing phase. Post hoc analysis revealed that animals receiving the higher dose of AM 251 (500ng/0.5µl), spent a significantly longer time in the saline paired environment over the AM 251 paired environment, in turn demonstrating an AM 251 aversion (n=8, $p< .01$, **Fig. 4.1*D***). Rats receiving the lower dose of AM 251, spent approximately equal amounts of time in both the saline or AM 251 paired environment and hence showed no preference for either environments as summarized in **Fig. 4.1*D*** (n=8, $p> .05$). Thus, for

all subsequent experiments involving AM 251, the effective dose of 500ng/0.5µl was used.

A



B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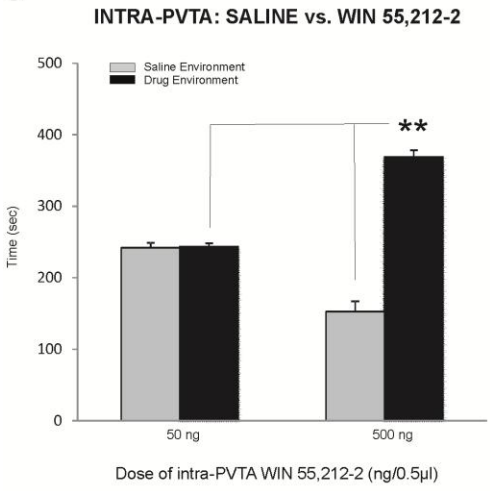
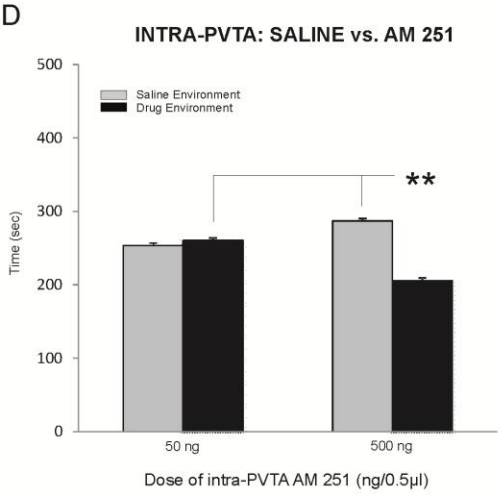
C


INTRA-PVTA: SALINE vs. WIN 55,212-2
Saline Environment
Drug Environment
Time (sec)
500
400
300
200
100
0
**
50 ng
500 ng
Dose of intra-PVTA WIN 55,212-2 (ng/0.5µl)

D


INTRA-PVTA: SALINE vs. AM 251
Saline Environment
Drug Environment
Time (sec)
500
400
300
200
100
0
**
50 ng
500 ng
Dose of intra-PVTA AM 251 (ng/0.5µl)

E

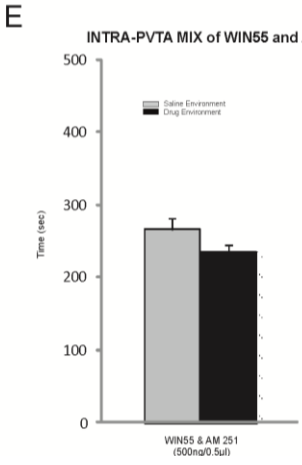

INTRA-PVTA MIX of WIN55 and AM 251
Saline Environment
Drug Environment
Time (sec)
500
400
300
200
100
0
WIN55 & AM 251
(500ng/0.5ul)

Figure 4.1 Bi-directional effects of intra-PVTA CB1 agonist and antagonist and PVTA histological analysis

(A) Micrograph of a typical intra-PVTA injector placement. **(B)** Schematic summary of intra-PVTA cannula placement: ■ = PVTA placements for 500ng/0.5µl WIN 55 vs. Saline; ○ = PVTA placements for 500ng/0.5µl AM 251 vs. Saline. **(C)** Intra-PVTA microinfusion of 50-500ng/0.5µl WIN 55,212-2 vs. saline; the group with the lower dose of CB1 agonist (50ng/0.5µl, n=7), showed no preference for either the saline or WIN 55 paired environment, while the group receiving the higher dose of WIN 55 (500ng/0.5µl, n=7) demonstrated a strong preference for the WIN 55 paired environment over the saline paired environment. **(D)** CB1 antagonist microinfusions intra-PVTA (50-500 ng/0.5µl); rats receiving 50ng/0.5µl AM 251 (n=8) showed no preference for either of the two environment. In contrast, animals receiving 500ng/0.5µl AM 251 intra-PVTA (n=8), displayed a significant preference for the saline paired environment over the AM 251 paired environment. **(E)** Intra-PVTA microinfusion of a mix of WIN 55 and AM 251 (500ng/0.5µl, n=7) showed no cannabinoid related reward or aversion. Hence when combined CB1 agonist and antagonist cancel out the earlier observed effects. * denotes $p<.05$ and ** denotes $p<.01$ for this and all subsequent experiment.

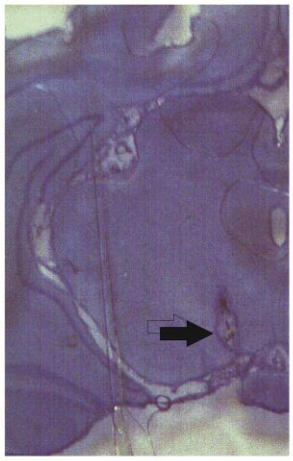
4.3.4 Co-administration of Intra-PVTA CB1 agonist and antagonist microinfusion produced no cannabinoid related reward or aversion

Given the opposing effects observed in the PVTA with separate administration of CB1 agonist and antagonist, we wanted to examine the effects of WIN55 and AM 251 combined. We microinfused intra-PVTA, a combination of WIN55 and AM 251 (500ng/0.5µl) simultaneously against saline, during the conditioning sessions. Our results from the testing phase indicate that rats showed no preference for either the saline or the combined drug paired environment (n=7; t_6=1.48, *p= .19*, **Fig. 4.1*E***). Therefore, it appears that WIN55 and AM 251 combined, cancel out the individual cannabinoid reward and aversive effects observed earlier.

4.3.5 Microinfusions of CB1 agonist and antagonist in the anterior region of the ventral tegmental area produced no behavioural effects

Given our earlier findings that CB1 receptor activation and blockade produced a cannabinoid related reward and aversion (**Fig.1*C*** and ***D***), we next examined the potential effects of CB1 agonist and antagonist in the anterior region of the ventral tegmental area (AVTA). We performed bilateral intra-AVTA microinfusions of WIN55 (500ng/0.5µl) versus saline. Our results indicate that unlike the cannabinoid related reward CPP observed with intra-PVTA microinfusions of WIN55 (500ng/0.5µl), animals receiving the CB1 agonist in the AVTA showed no significant preference for either the saline or drug paired environment (n=8, t_7=2.30, *p= .06*; **Fig.2*C***). Similarly, blockade of CB1 receptors in the AVTA with AM 251 (500ng/0.5µl), produced no cannabinoid related reward or aversion, with animals showing no preference for either the saline or the AM 251 paired environment (n=7, t_6=0.22, *p= .83*; **Fig.2*C***). Thus, the cannabinoid related reward and aversion with WIN55 and AM 251 is only expressed in the posterior region of the VTA and not the anterior region of the VTA.

A



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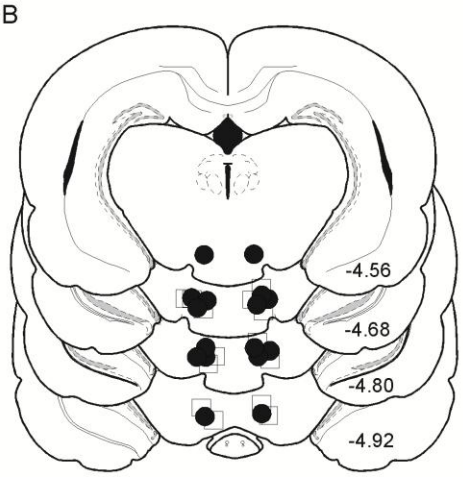
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C

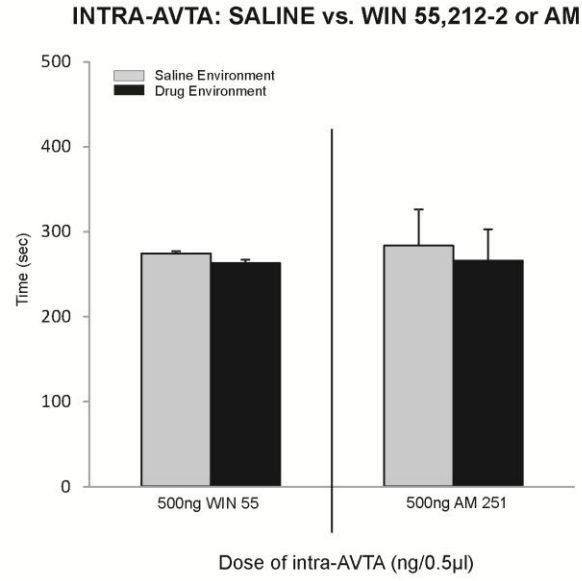

INTRA-AVTA: SALINE vs. WIN 55,212-2 or AM 251
Saline Environment
Drug Environment
Time (sec)
500
400
300
200
100
0
500ng WIN 55
500ng AM 251
Dose of intra-AVTA (ng/0.5µl)

Figure 4.2 Histological analysis and effects of CB1 agonist and antagonist in the AVTA

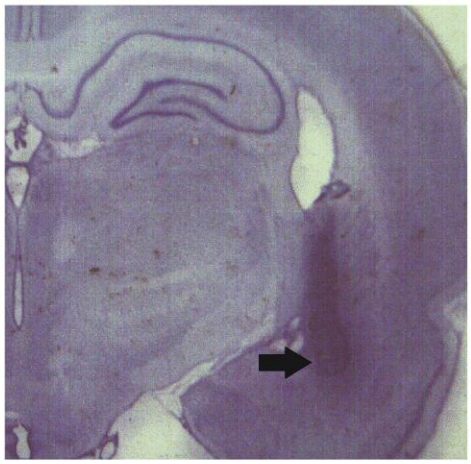
(A) Sample micrograph of an intra-AVTA injector placement. **(B)** Intra-VTA cannula placement schematic summary: ● = intra-AVTA placements for WIN 55 (500ng/0.5µl) vs. saline; □ = intra-AVTA placements for AM 251 (500ng/0.5µl) vs. saline. **(C)** The group of animals receiving intra-AVTA CB1 agonist (500ng/0.5µl, n=8) showed no cannabinoid related CPP. Similarly, rats receiving intra-AVTA CB1 antagonist (500ng/0.5µl, n=7) also showed no preference or aversion to either the saline or drug paired environment.

4.3.6 Cannabinoid related reward learning depends upon dopaminergic transmission within the PVTA-BLA pathway

Previous research has indicated that the VTA sends major Dopaminergic (DAergic) projections to both the basolateral amygdala (BLA) and the Nucleus accumbens (NAc) (Ford, Mark, & Williams, 2006) . To determine if the cannabinoid related reward CPP (WIN55) and the cannabinoid related aversion (AM 251) observed earlier as summarized in **Fig. 4.2** ***B*** and ***C***, are dependent on the PVTA→BLA DAergic pathway, we performed quadruple cannulations (see material and methods). Our first group of animals received bilateral microinfusions of the DA antagonist α-flu (1μg/0.5μl) intra-BLA, followed by a bilateral intra-PVTA microinfusion of either saline or WIN55 (500ng/0.5μl). Two way ANOVA comparing our α-flu/WIN55 group to our earlier group of WIN55 alone (**Fig. 4.1*C***), showed a significant interaction between group and treatment on the time spent in the saline or drug paired environment ($F_{(1,27)}$ = 80.22; $p < .0001$). *Post hoc* analysis indicated that the group of animals that received intra-BLA α-flu (1μg/0.5μl) prior to intra-PVTA WIN55 (500ng/0.5μl) microinfusions displayed no significant cannabinoid related reward CPP (n=7, $p > .05$; **Fig. 4.3*C***), while the group of animals receiving only intra-PVTA WIN55 (500ng/0.5μl), showed a significant preference for the WIN55 paired environment (n=7, $p < .01$; **Fig. 4.1*C***). Thus blocking the DAergic projection to the BLA, successfully blocked the cannabinoid reward CPP.

Next, to examine the possible effects of α-flu on cannabinoid related aversion, we microinfused α-flu (1μg/0.5μl) directly into the BLA, followed by intra-PVTA microinfusions of either AM 251 (500ng/0.5μl) or saline. Statistical analysis indicated that animals in this group still displayed the cannabinoid related aversion observed earlier (**Fig. 4.1*D***), with rats showing a significant preference for the saline paired environment (n=8, t_7=7.89, $p < .0001$; **Fig. 4.3*C***, far right). Hence, blocking DAergic projections in the BLA had no effect on cannabinoid related aversion.

A



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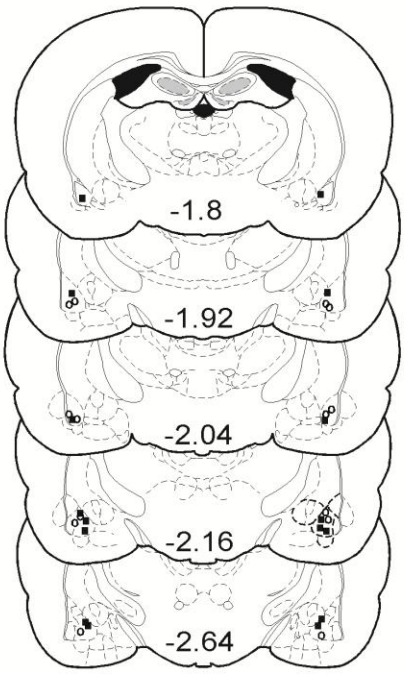
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C

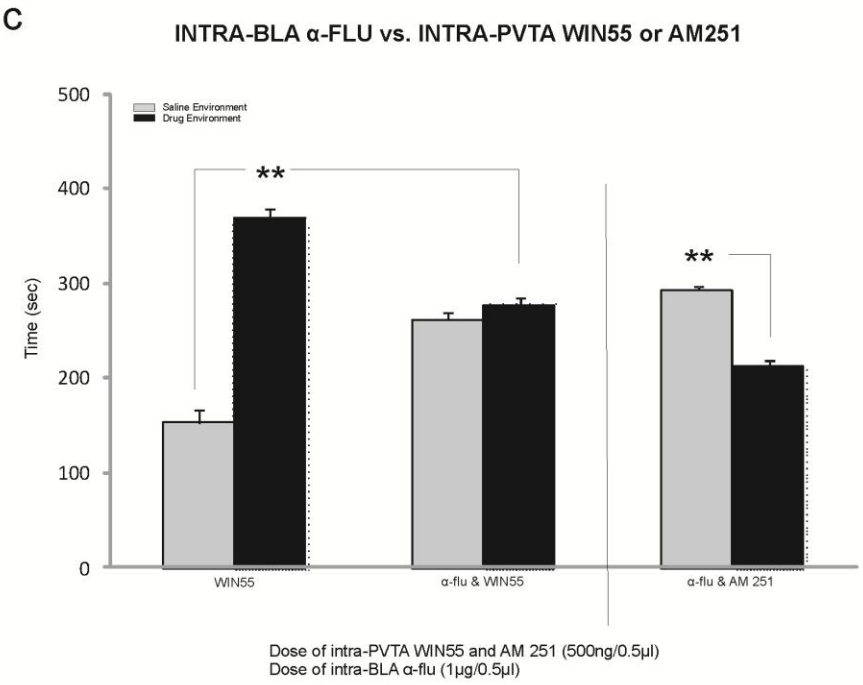

INTRA-BLA α-FLU vs. INTRA-PVTA WIN55 or AM251
Saline Environment
Drug Environment
Time (sec)
500
400
300
200
100
0
**
**
WIN55
α-flu & WIN55
α-flu & AM 251
Dose of intra-PVTA WIN55 and AM 251 (500ng/0.5µl)
Dose of intra-BLA α-flu (1µg/0.5µl)

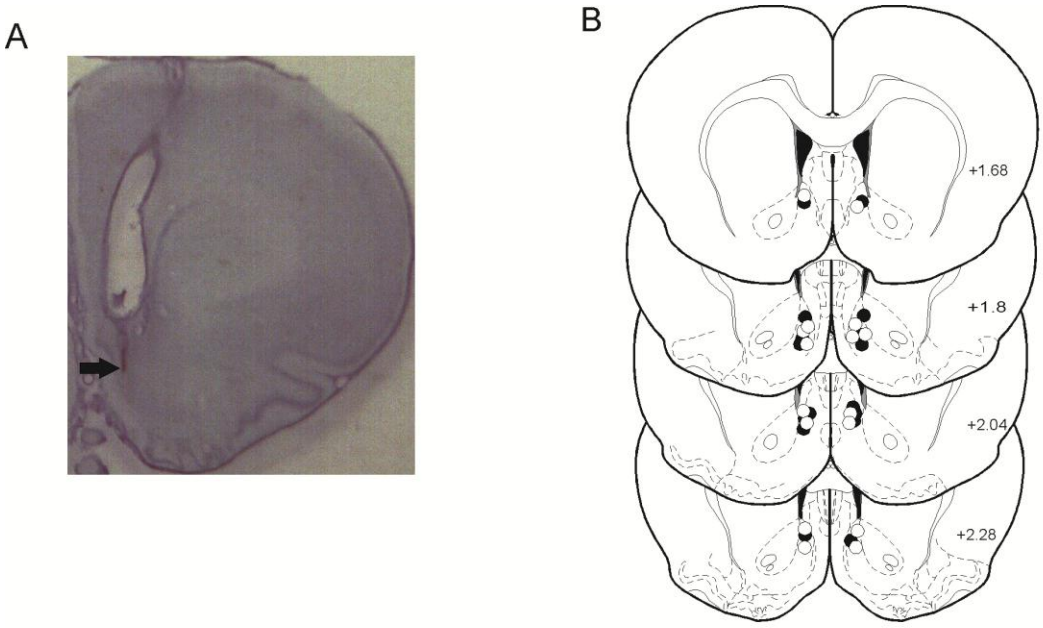
Figure 4.3 Blockade of DA projections to the BLA and histological analysis of the BLA

(A) Micrograph representing a typical intra-BLA injector placement. **(B)** Schematic summary illustrating intra-BLA cannula placement: ○ = BLA placements for α-flu (1µg/0.5µl) vs. intra-PVTA CB1 agonist (500ng/0.5µl); ■ = BLA placements for 1µg/0.5µl α-flu vs. 500ng/0.5µl AM 251. **(C)** Blocking the DA projection from the PVTA to the BLA, successfully blocked the WIN 55 related reward CPP observed with intra-PVTA microinfusion of CB1 agonist (500ng/0.5µl, n=7). However, blocking DA intra-BLA, had no effects on intra-PVTA microinfusion of AM 251 (500ng/0.5µl, n=8), as animals were still displaying a significant preference for the saline paired environment.

4.3.7 Cannabinoid related aversion is mediated through the PVTA-NASh DAergic pathway

The VTA has been implicated to regulate DA release in the NAc and the shell region of the NAc (NASh) has been shown to be involved in aversive motivation. To investigate the potential role of the DAergic projections to the NASh, we administered α-flu (1µg/0.5µl) bilaterally directly into the NAsh, followed by intra-PVTA microinfusions of either AM 251(500ng/0.5µl) or saline. Two-way ANOVA indicates a significant interaction between group and treatment ($F_{(1,31)}$ = 36.01; $p < .0001$) on times spent in saline and drug paired environment during the recall phase. *Post hoc* analysis revealed that animals receiving the α-flu prior to the CB1 antagonist showed no preference for either of the two environments (n=8, $p > .05$; **Fig. 4.4*D***), while rats that only received the CB1 antagonist showed a significant preference for the saline paired environment over the AM 251 paired environment, displaying a cannabinoid related aversion (n=8, $p < .01$; **Fig. 4.4*D***, far left). Therefore, α-flu successfully blocked the aversion observed earlier (**Fig. 4.1*D***) with AM251.

Subsequently, to examine the effects of blocking DA in the NASh in conjunction with CB1 agonist administration; we microinfused α-flu (1µg/0.5µl) intra-NASh, followed by intra-PVTA microinfusions of WIN55 (500ng/0.5µl). Statistical analysis showed that rats in this group showed a significant preference for the WIN55 paired environment (n=8, t_7=9.54, $p < .0001$; **Fig. 4.4*D***, far right), much like the group that did not receive DA antagonist (**Fig. 4.1*C***). Hence, α-flu in the NASh, had no effect on cannabinoid reward CPP.


A
B
+1.68
+1.8
+2.04
+2.28

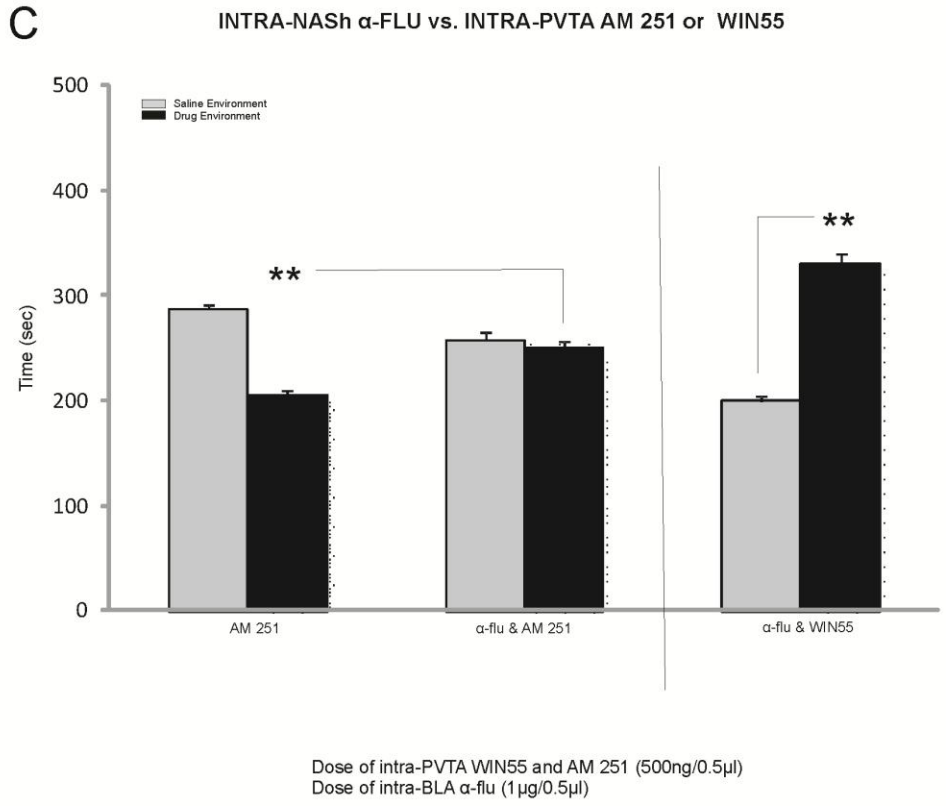

C
INTRA-NASh α-FLU vs. INTRA-PVTA AM 251 or WIN55
Saline Environment
Drug Environment
Time (sec)
500
400
300
200
100
0
**
**
AM 251
α-flu & AM 251
α-flu & WIN55
Dose of intra-PVTA WIN55 and AM 251 (500ng/0.5µl)
Dose of intra-BLA α-flu (1µg/0.5µl)

Figure 4.4 Histological analysis of the NASh and the effects of blocking DA projections to the NASh on CB1 agonist and antagonist intra-PVTA microinfusion.

A. Sample micrograph of intra-NASh injector placement. **B.** Intra-NASh cannula placement: ⭘ = NASh placements for the group of animals receiving intra-NASh α-flu (1µg/0.5µl) vs. AM 251 (500ng/0.5µl); ● = NASh placements for intra-NASh α-flu (1µg/0.5µl) vs. WIN 55 (500ng/0.5µl). **C.** Blocking the DA projections in the intra-PVTA-NASh pathway, successfully blocked the cannabinoid related aversion with intra-PVTA AM 251 (500ng/0.5µl, n=8), as animals showed no aversion towards either environment. Conversely, blocking DA projections intra-PVTA-NASh pathway had no effect on cannabinoid related reward with intra-PVTA WIN55 microinfusion (500ng/0.5µl, n=8), as rats showed a strong preference for the drug environment over the saline environment, and hence a cannabinoid reward CPP.

4.4 DISCUSSION

A large body of literature has suggested a functional interaction between cannabinoids and DA. The cannabinoid system plays a major role in mediating neuronal excitability, inhibition and synaptic transmission, and most of CNS cannabinoid activity is regulated by the CB1 receptor (CB1R). Animal research has indicated that activation of the CB1R with an exogenous cannabinoid agonist, have been shown to inhibit presynaptic GLUTergic transmission in the VTA (Melis et al., 2004). Furthermore, both systemic and intra-cranial administrations of the highly potent CB1R agonist WIN 55 has been shown to regulate DA neurotransmission in several midbrain sub-regions (Ahmad, Lauzon, de Jaeger, & Laviolette, 2013; Fanarioti et al., 2014). Similarly, electrophysiological studies using HU210 (potent cannabinoid agonist), WIN 55, and THC (active ingredient in marijuana) have shown a robust increase in DA firing rate within the VTA (Cheer, Kendall, Mason, & Marsden, 2003; Gessa, Melis, Muntoni, & Diana, 1998; Wu & French, 2000). The VTA is the main site of DA neurons, however the DA neurons involved in the reward learning pathway are mostly localized within the posterior region of the VTA (PVTA); studies have shown that opiate related reward (Zangen, Ikemoto, Zadina, & Wise, 2002) and cannabinoid related reward (Zangen et al., 2006) are expressed when microinjected into the PVTA, and not the AVTA. Taken together, these findings support our results that WIN55 microinfused intra-PVTA, dose dependently produced a cannabinoid reward CPP **(Fig. 4.1*C*)**. In contrast, WIN55 administered intra-AVTA produced no rewarding effects (**Fig. 4.2*C***).

In terms of CB1 blockade, antagonism of CB1R located on both inhibitory and excitatory axon terminal target the midbrain DA system; blocking CB1R has shown to reduce cue-induced reinstatement of drug seeking behaviour (Lupica & Riegel, 2005) and block extinction of conditioned taste aversion (Kobilo, Hazvi, & Dudai, 2007). The potent CB1R antagonist AM 251 has been shown to significantly suppress food intake (Chambers, Koopmans, Pittman, & Sharkey, 2006), reduce food-seeking behaviour (Chambers, Sharkey, & Koopmans, 2004), and inhibit methamphetamine self-administration in rats (Vinklerová, Nováková, & Sulcová, 2002). However, the direct effects of CB1 antagonist within the VTA are not well documented. Our findings

illustrated that intra-PVTA AM 251 microinfusion, dose dependently produced a cannabinoid related aversion (**Fig. 4.1*D***), while having no rewarding or aversive effects when administered intra-AVTA (**Fig. 4.2*C***).

To further analyze the observed effects with WIN55 and AM 251, we examined the DA projections from the PVTA to the BLA and NASh. The BLA plays a crucial role in memory and learning processes, and hence a major component in the reward pathway and behavioural output. Intracellular recordings have shown that stimulation of PFC, inhibits behavioural output by activating GABAergic interneurons within the BLA (Grace & Rosenkranz, 2002). Furthermore, the BLA mediates DA efflux to the PFC, originating from the VTA (Phillips, Ahn, & Howland, 2003), drugs of abuse such as opiates share the common VTA-BLA-NAc pathway (Feltenstein & See, 2008), and DA projections from the VTA have shown to influence rewarding and emotional behaviour by modulating BLA-evoked changes in the PFC (Floresco & Tse, 2007). We found that by blocking DA projections within the BLA, we blocked the cannabinoid related reward observed with WIN55 (**Fig. 4.3*C***). Conversely, inhibiting DA neurons in the BLA, had no effects with the cannabinoid related aversion induced by intra-PVTA microinfusion of AM 251(**Fig. 4.3*C***, far right). Thus DA efferent in the PVTA→BLA pathway mediates the rewarding properties of CB1R activation, but has no effects on the aversive properties of CB1 antagonism. Activation of CB1R, inhibits GABA release (Katona et al., 2001), and in turn prolongs DA level activation in the PVTA→BLA mesolimbic pathway, resulting in a behavioural output of cannabinoid related reward CPP.

Since the PVTA sends DA projections to multiple brain areas that serve various functions, the DA neurons also respond differently based on areas they innervate. To account for the cannabinoid related aversion, we examined the PVTA→NASh pathway. Lammel, Ion, Roeper, & Malenka (2011) have shown that DA cells projecting to the NASh responds to both rewarding and aversive stimulus. Furthermore, a review presented by Murray & Bevins (2010) showed that cannabinoid can present their effects in both positive and negative signals; whereas low doses of THC elicited a cannabinoid reward CPP, a higher dose of THC produced a conditioned place aversion (Elsmore & Fletcher, 1971). Our findings show that blocking DA neurons within the NASh, blocked

the cannabinoid related CPA induced by intra-PVTA AM 251 microinfusions (**Fig. 4.4*C***), while having no effect on cannabinoid related reward CPP. These results suggest that aversive stimuli are processed through the PVTA→NASh pathway. Previous pharmacological research indicates that CB1 activation promotes an increase in the CREB cycle in the NASh, promoting reward related behaviour such as CPP (Barrot et al., 2011). However, deactivation of CB1R, would lead to a decrease in the CREB cycle, modulating DA neurons, suppressing reward, and resulting in an aversion response. This parallels our findings of the CB1 antagonist AM 251 intra-PVTA microinfusion resulting in a cannabinoid related aversion.

4.5 CONCLUSION

In summary, we report that intra-PVTA microinfusions of WIN 55,212-2 dose-dependently produced a cannabinoid reward CPP, while intra-PVTA microinfusions of the CB1 antagonist AM 251 produced a cannabinoid related CPA. Neither of these effects was observed when repeated in the AVTA, supporting the notion that reward related learning is primarily conducted through the PVTA region. The intra-PVTA cannabinoid reward CPP and CPA were blocked using the broad band DA antagonist α-flu within the PVTA→BLA and the PVTA→NASh pathway respectively. The present study provides dose dependent evidence for CB1R activation and blockade within the PVTA, and it's dependence on DA neurons to execute its modulatory effects. Our findings present a functional relationship between CB1R and DA within the mesolimbic system. Furthermore, our results indicate that dose dependent pharmacological manipulations can result in both rewarding and aversive signals, depending on the area of projection.

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Chapter 5

5 General Discussion

5.1 SUMMARY OF FINDINGS

5.1.1 Cannabinoid Transmission in the mPFC Controls Opiate Signaling

Chapter 2 explores the role of CB1 transmission within the mPFC in relation to opiate reward using an unbiased classical conditioning paradigm. Cannabinoid, dopamine (DA), and opiate receptor pathways play an integrative role in emotional learning, associative memory, and sensory perception. Modulation of cannabinoid CB1 receptor transmission within the mPFC regulates the emotional valence of both rewarding and aversive experiences. Furthermore, CB1 receptor substrates functionally interact with opiate-related motivational processing circuits, particularly in the context of reward-related learning and memory. Considerable evidence demonstrates functional interactions between CB1 and DA signaling pathways during the processing of motivationally salient information. However, the role of mPFC CB1 receptor transmission in the modulation of behavioral opiate-reward processing is not currently known. Given the functional interaction between cannabinoids and opiates in establishing place preference conditioning and attenuating morphine self administration (Chaperon, Soubrié, Puech, & Thiébot, 1998; Navarro et al., 2001), we hypothesized that activation of CB1 receptors within the mPFC would potentiate the rewarding effects of a sub-threshold dose of morphine, that under normal circumstances does not produce any effects.

Our findings however, indicated an opposite effect to our initial expectations. We found that activation of CB1 receptors intra-mPFC using a synthetic CB1 agonist made both a sub and supra reward threshold dose of morphine highly aversive. In contrast, inhibiting CB1 receptors by micro-infusing a synthetic CB1 antagonist intra-mPFC potentiated the rewarding effects of a subthreshold dose of morphine, while having no effects on the suprathreshold dose of morphine. Blocking DAergic projections from the VTA to the mPFC, blocked our observed reward and aversion signals, indicating that they are indeed DA dependent. We further explored the μ-opioid receptor, which has been shown to be excitatory, and the κ-opioid receptor which has been shown to be inhibitory (Ford, Mark, & Williams, 2006). Blockade of μ-opioid receptor intra-VTA, blocked the earlier potentiation of the subthreshold dose of morphine observed with intra-mPFC CB1 antagonist. Conversely, blocking the κ-opioid pathway by micro-infusing a κ-opioid

receptor antagonist intra-VTA resulted in the ability of the CB1 agonist to potentiate the sub reward threshold dose of morphine, and the earlier observed morphine aversion was no longer present.

Hence we report that CB1 modulated intra-mPFC opiate motivational signaling is mediated through a dissociable μ-opiate receptor dependent reward pathway, or a κ-opiate receptor dependent aversion pathway, directly within the VTA. Our results provide evidence for a novel CB1-mediated motivational valence switching mechanism within the mPFC, controlling dissociable subcortical reward and aversion pathways (Ahmad, Lauzon, de Jaeger, & Laviolette, 2013).

5.1.2 Bi-directional Cannabinoid Signaling in the BLA Controls Rewarding and Aversive Emotional Processing

Functional connections between the BLA and NAc are involved critically in opiate-reward processing. In the BLA, inhibitory GABAergic substrates are inhibited by cannabinoid CB1R activation and can modulate BLA projections to various limbic regions, including the NAc. High frequency activity in BLA efferents can modulate neuronal activity in the NAc via activating both DA and NMDA receptors (Floresco, Blaha, Yang, & Phillips, 2001). However the potential role of CB1R transmission in the regulation of opiate-related memory formation via the BLA→NAc circuit is not understood. Using an unbiased conditioned place preference paradigm (CPP) in rats, we examined the effects of intra-BLA CB1R modulation by either direct pharmacological activation (using CB1 agonist WIN 55,212-2) or blockade of CB1R transmission (using CB1 antagonist AM 251). We report that intra-BLA CB1R activation switches normally rewarding effects of morphine into strongly aversive effects. In contrast, CB1R blockade strongly potentiates normally sub-reward threshold effects of morphine. Next, using targeted microinfusions of an NMDA receptor antagonist to either the core (NACo) or shell (NASh) sub-divisions of the NAc, we found that selective blockade of NMDA transmission in the NA shell, but not core, prevented both intra-BLA CB1 blockade-mediated opiate reward potentiation and CB1 activation-mediated aversion effects.

Finally, using multi-unit, *in vivo* electrophysiological recordings in the NASh, we report that the ability of intra-BLA CB1R modulation to control opiate reward salience and motivational valence is associated with distinct reward or aversion neuronal activity patterns and bi-directional regulation of intra-NASh fast-spiking interneurons (FSI) vs. medium spiny neurons (MSN). These findings identify a unique mechanism whereby bi-directional BLA CB1R transmission can regulate opiate-related motivational processing and control affective states through functional modulation of mesolimbic neuronal activity.

5.1.3 Cannabinoid Related Reward and Aversion Signals in the Posterior VTA is Mediated through DAergic Projections to the BLA and NASh

The ventral tegmental area (VTA) has functional DAergic projections to the basolateral amygdala (BLA), and nucleus accumbens (NAc). It is a critical neural region responsible for mediating both rewarding and aversive related behavioural processing and cannabinoids are known to modulate the activity of the dopamine (DA) neuronal populations within the VTA. Previous research has shown that cannabinoid activation via THC administration in the posterior region of the VTA (PVTA), produced rewarding behavioural effects, while the same activation in the anterior region of VTA (AVTA), produced no effects (Zangen, Solinas, Ikemoto, Goldberg, & Wise, 2006). Hence, a functional dissociation between posterior and anterior VTA does exist. Using an unbiased conditioned place preference (CPP) procedure combined with behavioural pharmacology, we administered either a CB1 agonist (WIN-55,212-2) or antagonist (AM 251) into the PVTA or AVTA of Sprague-Dawley rats. CB1R activation in the PVTA with WIN 55,212-2 (50-500ng) produced a dose-dependent cannabinoid reward CPP, while blockade of CB1R with AM 251 (50-500ng) produced a dose-dependent aversion. Interestingly, when WIN 55,212-2 and AM 251 were micro-infused in the AVTA, no cannabinoid reward or aversion effects were observed. To examine the PVTA→BLA and PVTA→NAc pathways, we used the broad spectrum DA receptor antagonist α-flupenthixol to block DA transmission in either the NAc or BLA. Intra-BLA micro-infusions of α-flu (1μg), blocked the earlier observed cannabinoid reward CPP, but not the cannabinoid antagonist-related aversion. Conversely, intra-NASh micro-infusions of

α-flu (1μg), blocked the aversion observed with intra-PVTA CB1 antagonist administration, but not the rewarding effects of intra-PVTA WIN-55. Thus, our findings demonstrate a functional dissociation between PVTA DA outputs to either the NASh or BLA. Furthermore, while the rewarding effects of intra-PVTA CB1 activation depend upon a PVTA→BLA pathway, the aversive effects of CB1 receptor blockade depend upon PVTA DA outputs to the NASh.

5.1.4 Limitations

The current available literature on receptors of interest such as CB1 and DA are often investigated in isolation. The brain is a complex structure, with a multitude of activated neuronal pathways and release of various neurotransmitters simultaneously. Thus, a significant limitation to our study is that we are unable to account for the coexistence and release of other neurotransmitters. For example: the mPFC, NAc and VTA are all rich in serotonin receptors that may have overlapping signaling pathways with DA. Hence, although we have accounted for cannabinoid transmission modulating DA levels, we did not account for the possible co-release of other neurotransmitters such as serotonin. *In vivo* electrophysiological studies have shown that activation of serotonin receptors increases DA activity levels (Prisco, Pagannone, & Esposito, 1994), and CB1R is co-expressed in high density with both DA and serotonin receptors (Hermann, Marsicano, & Lutz, 2002). It is plausible to consider that CB1, DA, and serotonin receptors may concurrently interact with one another in the VTA, NAc, or mPFC, modulating their downstream effects through cyclic AMP and other signaling cascades, suggesting an alternative explanation to emotional processing mechanisms.

The bi-directional effects of CB1 transmission observed in our study are all DA-dependent, since using a broadband DA antagonist often blocked both the reward and aversion signals. Thus, another limitation to the study is that we did not differentiate between D1 and D2 receptor subtypes. In opiate naïve animals, activation of D1 receptor subtype has shown to potentiate the rewarding effects of opiates. However, when opiate dependence was achieved, potentiating the rewarding effects of opiates was switched to a D2 receptor subtype (Lintas et al., 2012). Therefore, it is important to differentiate

between the D1/D2 subtypes, in order to better understand the underlying mechanisms of the DAergic reward pathway.

5.2 Conclusion

Cannabinoids are one of the most abundant receptors in humans. Disruptions in CB1 levels are implicated with many neuropsychiatric disorders, addiction studies, and deficits in learning and memory. The goal of this thesis was to characterize CB1 transmission in the mesolimbic reward circuitry in relation to the motivational effects of opiates. We explored the mPFC, BLA, VTA, and NAc circuitry. Our findings indicate novel bi-directional CB1 mediated mechanism in the mPFC, and BLA with functional interconnections to the VTA and NASh that control opiate signaling. These results will contribute to the growing body of research concentrated on the biphasic characteristic of cannabinoids and further help elucidate their role in reward related learning.

5.3 FUTURE DIRECTIONS

Although our research has been successful in characterizing CB1 transmission in the mesolimbic pathway, many critical questions remain. It is important to note that our current results are dose dependent, and two doses of 50 and 500ng of CB1 agonist and antagonist were used. We should incorporate a mid range dose of 100ng for both CB1 agonist WIN 55,212-2 and antagonist AM 251 to determine a more comprehensive dose curve.

Furthermore, future studies are required to more precisely characterize the mechanism by which intra-BLA CB1R transmission may regulate DA release patterns within the NAc. For instance, intra-NAc D1 vs. D2 receptor subtypes have been reported to differentially regulate activity states of MSN vs. FSI neuronal subpopulations and drug-reward related behaviours (Smith et al., 2013; Calipari et al., 2016). Activation of intra-NAc D1-containing MSN neurons has been demonstrated to promote reward-related behaviours whereas activating D2-containing MSN’s have been shown to oppose these effects and/or induce aversive effects. While beyond the scope of the present study, future studies using selective blockade of D1 vs. D2 MSN neuronal subpopulations may yield additional

insights into how BLA CB1R-dependent signaling may control accumbens processing of reward vs. aversion-related motivational signals.

Next, although our posterior VTA reward findings parallel those of Zangen et. al. (2006), further studies are required to determine the neuronal pathways taken by the CB1 antagonist mediated aversive signals and CB1 agonist mediated reward. Since cannabinoid and opiate receptors mediate overlapping pharmacological responses, it would be beneficial to examine the μ and κ-opioid receptor pathway in terms of cannabinoid reward and aversion signals. It is quite possible that the CB1 and opioid receptors can interact directly with one another, modulating each other's function when co-expressed in the same cell. To test this theory, we would activate the CB1R and block the μ-opioid receptor pathway by intra-PVTA simultaneous micro-infusions of WIN 55,212-2 and the μ-opioid receptor antagonist cyprodime. If our cannabinoid related reward is via the μ-opioid reward pathway, we would expect a block in the observed reward CPP. Conversely to explore the possibility of the CB1 related aversion in relation to κ-opioid inhibitory pathway, we would micro-infuse simultaneously intra-PVTA, the CB1 antagonist AM 251 and the κ-opioid receptor antagonist *nor*-binaltorphimine. Similarly, if the CB1 related aversion observed is mediated by the κ-opioid pathway, we would expect to see no CPP or CPA.

Lastly, to further explore the role of CB1 transmission in the mPFC in mediating DA levels in the VTA, we can employ single cell *in vivo* electrophysiological recordings in the VTA to examine firing levels of DA at various doses of CB1 microinfusions for both rewarding and aversive signals.

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Tasha Ahmad

EDUCATION

Ph.D in Neuroscience – Neurobiology and Electrophysiology **2016**
Western University – London, ON

B.Sc. Contemporary Science – Psychology and Physics **2010**
Ryerson University – Toronto, ON

B.Sc. in Integrative Biology **2008**
University of Toronto – Toronto, ON

HONOURS and AWARDS

Canadian Consortium for the Investigation of Cannabinoids **2015**
Trainee Conference Support Award ($1,000)

International Cannabinoid Research Society **2014,2015**
Travel Award ($500 each year)

NSERC CGSD Fellowship **2013**
($70,000)

Ontario Graduate Scholarship **2012**
($15,000)

Western research Graduate Scholarship **2010-2014**
($7,000/year)

Ryerson Dean's list **2009-2010**

RELATED WORK EXPERIENCE

Laboratory Coordinator – Department of Anatomy and Cell Biology **2011-2013**
University of Western Ontario – London, ON

Teaching Assistant – Department of Psychology **2010-2011**
University of Western Ontario – London, ON

Teaching Assistant – Department of Biology and Chemistry **2009-2010**

Ryerson University – Toronto, ON

Research Assistant – Department of Medical Physics **2008-2009**

Ryerson University – Toronto, ON

CONFERENCE PRESENTATIONS

Society for Neuroscience – Chicago, Illinois **October 2015**

- Cannabinoid reward and aversion in the PVTA is differentially mediated through dopamine projections to the BLA and NA shell

International Cannabinoid Research Society – Wolfville, Nova Scotia **June 2015**

- In-vivo electrophysiological recordings during CB1 agonist and antagonist microinfusions coupled with morphine training.

International Cannabinoid Research Society (ICRS) – Baveno, Italy **June 2014**

- CB1 transmission in the BLA and recording from the NA shell

South Ontario Neuroscience Association (SONA) – London, Ontario **May 2014**

- CB1 transmission in the BLA and recording from the NA shell

London Health Research Day – London, Ontario **March 2014**

- Memory formation in nucleus accumbens shell vs. core

Society for Neuroscience – San Diego, California **November 2013**

- Cannabinoid CB1 transmission in the basolateral amygdala controls the motivational properties of opiates via functional excitatory inputs to the nucleus accumbens shell

Anatomy and Cell Biology Research Day – London, Ontario **March 2013**

- Cannabinoid CB1 transmission in the basolateral amygdala controls the motivational properties of opiates via functional excitatory inputs to the nucleus accumbens shell

Society for Neuroscience – New Orleans, Louisiana **October 2012**

- Cannabinoid transmission in the prelimbic cortex bi-directionally controls opiate reward and aversion signaling through dissociable kappa versus μ-opiate receptor dependent mechanisms

London Health Research Day – London, Ontario **March 2012**

- Rewarding effects of opiates in relation to the CB1 receptor communication in the basolateral amygdala and prefrontal cortex

Society for Neuroscience – Washington, District of Columbia **November 2011**

- CB1 transmission in the mPFC modulates the motivational effects of opiates

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