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Psychotropic Effects of Cannabis Components on the Mesolimbic Dopaminergic System

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Abstract

The two most abundant phytochemical compounds in cannabis are cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC). THC is the primary psychoactive component of cannabis and is a partial CB_1 receptor (CB_1R) agonist. THC is believed to be responsible for the motivational and dependence-producing effects of cannabis and causes psychotomimetic and affective processing disturbances. Conversely, CBD, is non-psychoactive, acts as a $5\text{-}HT_{1A}$ receptor agonist, antagonizes CB_1Rs, and possesses both anti-psychotic and anxiolytic properties. The neural substrate believed to be responsible for many of the effects of cannabis is the dopaminergic, mesolimbic reward pathway which is responsible for the regulation of cognition and emotion. Specifically, the shell region of the nucleus accumbens (NASh) and the ventral tegmental area (VTA) are important brain areas involved in motivation, reward, aversion, and fear-related behavioural processing. Using a combination of behavioural, electrophysiology and molecular techniques, the first chapter evaluates the effects of direct infusions of CBD into the NASh. Intra-NASh CBD blocked the formation of fear memory through a $5\text{-}HT_{1A}$-dependent mechanism by functionally modulating the activity of neuronal activity dynamics directly in the VTA. In the second chapter, we examined the effects of THC in either the anterior NASh (aNASh), known as the "hedonic hotspot", or posterior NASh, known to be involved in aversion. We demonstrate that aNASh THC produced rewarding behavioural effects and modulated reward salience through a μ-opioid-receptor-dependent mechanism, whereas THC in the pNASh produced aversive behavioural effects through a κ-opioid-receptor-dependent mechanism. ICV infusions of THC caused aNASh MSN activity to decrease and increased the power of ɣ-oscillations on the local field potential but caused pNASh increased MSN activity and decreased the power of ɣ-oscillations on the local field potential. Finally, in the third chapter, we provide a characterization of how THC differentially regulates fear-related memory formation and cognitive processing via distinct Akt-dependent vs. GSK3-dependent signaling pathways, in the aNASh vs. pNASh, respectively. Together, these data provide a novel neuronal, molecular, behavioural and anatomical characterization of the effects of CBD and THC directly within the mesolimbic circuitry and reveals critical new insights into the mechanisms by which THC and CBD regulate affective and cognitive behaviours.

Co-Authorship Statement

Chapter 2: Entitled "Cannabidiol Modulates Fear Memory Formation Through Interactions with Serotonergic Transmission in the Mesolimbic System" was written by Christopher Norris with inputs from Dr. Michael Loureiro, Jordan Zunder and Dr. Steven R. Laviolette. Christopher Norris performed all experiments and data analyses except electrophysiology which was performed by Dr. Michael Loureiro and Dr. Cecilia Kramar. Dr. Justine Renard performed some of the cannulation surgeries. Dr. Steven R. Laviolette and Dr. Walter Rushlow provided intellectual input.

Chapter 3: Entitled "Δ^9-tetrahydrocannabinol (THC) Regulates Reward and Aversion Processing via Dissociable Opioid Receptor Substrates and Neuronal and Oscillatory Modulation in Distinct Striatal Sub-Regions" was written by Christopher Norris with input from Dr. Hanna J. Szkularek and Dr. Steven R. Laviolette. Christopher Norris performed all experiments and data analyses. Surgeries were performed by Christopher Norris with help from Dr. Hanna J. Szkudlarek. Histological analyses were performed by Christopher Norris and Brian Pereira. Dr. Steven R. Laviolette and Dr. Walter Rushlow provided intellectual input.

Chapter 4: Entitled "Δ^9-tetrahydrocannabinol (THC) Regulates Memory, Anxiety and Sensorimotor Gating via Dissociable Modulation of the Wnt and mTOR Signaling Pathways in the Nucleus Accumbens Shell" was written by Christopher Norris with input from Roger Hudson and Dr. Steven R. Laviolette. Christopher Norris performed all experiments and data analyses. PPI and PPF programs were made by Roger Hudson. Surgeries were performed by Christopher Norris with help from Roger Hudson and Dr. Hanna J. Szkudlarek. Histological analyses were performed by Christopher Norris, Roger Hudson and Dinat Khan. Dr. Steven R. Laviolette and Dr. Walter Rushlow provided intellectual input.

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List of Abbreviations

2-AG	2-arachidonoylglycerol
5-HT$_{1A}$	Serotonin 1A receptor
Akt	Protein Kinase B
α-flu	α-flupenthixol
aNASh	anterior nucleus accumbens shell
ANOVA	analysis of variance
BLA	basolateral amygdala
cAMP	cyclic adenosine monophosphate
CaMKII	Ca^{2+}/ calmodulin-dependent protein kinase II
CB	cannabinoid
CBD	cannabidiol
CBR	cannabinoid receptor
CB$_1$R	cannabinoid receptor 1
CB$_2$R	cannabinoid receptor 2
CPA	conditioned place aversion
DA	dopamine
EPM	elevated plus maze
ERK	extracellular signal-related kinase
FAAH	fatty acid amide hydrolase
GABA	γ-aminobutyric acid
hiPSC	human-induced pluripotent stem cell
KOR	κ-opioid receptor
LFP	local field potential
MOR	μ-opioid receptor
MSN	medium spiny neuron
mTOR	mammalian target of rapamycin
NAc	nucleus accumbens
NACo	nucleus accumbens core
NASh	nucleus accumbens shell
mPFC	medial prefrontal cortex

p70s6K   ribosomal protein S6 kinase

pNASh   posterior nucleus accumbens shell

PPI   pre-pulse inhibition

PPF   pre-pulse facilitation

PTSD   post-traumatic stress disorder

THC Δ^9- tetrahydrocannabinol

VTA   ventral tegmental area

CHAPTER 1

1   General Introduction

1.1 CANNABIS USE AND PHYTOCHEMICAL PROPERTIES

Cannabis is the most widely used illicit drug in the world, with 182 million users globally in 2016 (United Nations Office on Drugs and Crime, 2018). As several countries prepare for full legalization of recreational use, rates of cannabis consumption are likely to increase. Heralding legalization is a widespread attitude shift that cannabis use is harmless. The effects of cannabis on the body and brain, however, are complex and still poorly understood. Previous research has established that, like most other drugs of abuse, cannabis is addictive (Maldonado, Berrendero, Ozaita, & Robledo, 2011) and can cause users to seek increasing amounts of the drug despite negative consequences. Additionally, heavy cannabis use, particularly during periods of adolescent brain development, has been demonstrated to increase the likelihood of developing schizophrenia (Arseneault, 2002). Conversely, cannabis is frequently used to treat pain (Allan et al., 2018; Ware et al., 2010), and some recent evidence suggests it may also be beneficial for PTSD and schizophrenia (Blessing, Steenkamp, Manzanares, & Marmar, 2015; Bonn-Miller, Vujanovic, & Drescher, 2011; Haney & Evins, 2016; Iseger & Bossong, 2015; Schubart et al., 2014; Zuardi, Crippa, Hallak, Moreira, & Guimaraes, 2006).

One of the biggest challenges to studying the effects of cannabis is that the plant is known to contain over 100 distinct phytochemicals (Radwan et al., 2009), each with their own unique pharmacology. Thus, to fully understand the potential therapeutic or detrimental effects of cannabis on mental health, it is crucial to characterize the neuropharmacological, anatomical and neurophysiological effects of each individual compound. Nevertheless, the two most prevalent and best characterized phytochemicals in cannabis are Δ^9-tetrahydrocannabinol (THC), the primary psychoactive compound and the source of the dependence-producing properties, and cannabidiol (CBD), the most abundant, but non-psychoactive, compound. THC is also believed to be responsible for the pro-psychotic, anxiogenic and aversive properties of cannabis (Childs, Lutz, & de Wit, 2017; Freeman et al., 2015; Renard, et al., 2017; Schramm-Sapyta et al., 2007). CBD, however, is known to possess anti-psychotic (Zuardi et al., 2006) and anxiolytic (Campos et al., 2013; Crippa et al., 2011) properties, and can modulate the processing of associative fear memories (Das et al., 2013; Norris et al., 2016). CBD also modulates or reverses some of the negative side effects of THC administration. For example, co-administration of CBD with THC, inhibits THC-induced paranoia and memory loss (Englund et al., 2013). In addition, our

laboratory has shown previously that THC and CBD can produce diametrically opposing effects on dopamine (DA) neuronal activity states and on the activation states of several molecular signaling pathways linked to neuropsychiatric disorders, such as glycogen synthase kinase-3 (GSK3), Protein Kinase B (Akt), mammalian target or rapamycin (mTOR) and p70-S-6-Kinase (p70S6K), with THC inhibiting and CBD activating these pathways in distinct mesocorticolimbic brain regions, respectively (Renard et al., 2016, 2017). Thus, despite the structural similarity and common botanical origins of these phytocannabinoids, they are capable of exerting opposing effects on many neuronal and molecular substrates linked to neuropsychiatric disorders, such as schizophrenia, anxiety, depression and addiction.

The underlying pharmacological mechanisms of THC and CBD are also distinct. THC exerts partial agonist activity on two receptors in the endocannabinoid (eCB) system, the cannabinoid$_1$ receptor (CB$_1$R) and the cannabinoid$_2$ receptor (CB$_2$R) (Pertwee, 2008). Many of the effects of THC, however, also rely on interactions with other neurotransmitter systems. For example, reward from low doses of THC is not present in μ-opioid knockout rats and aversion from high doses of THC is not present in κ-opioid receptors knockout rats (Ghozland et al., 2002).

CBD, however, has much more diverse effects. It is an agonist of the serotonin receptor 5-HT$_{1A}$ (Campos & Guimarães, 2008; Russo, Burnett, Hall, & Parker, 2005), an agonist of PPARγ (O'Sullivan, 2016), a negative allosteric modulator of CB$_1$ (Tham et al., 2018), and it prevents the degradation of endogenous cannabinoids through the inhibition of the enzyme fatty acid amide hydrolase (FAAH) (Leweke et al., 2012). Increased 5-HT$_{1A}$ transmission is believed to be the key determinant in response to serotonin reuptake inhibitors when treating depression (Blier & Abbott, 2001). Additionally, 5-HT$_{1A}$ receptor agonists possess anxiolytic properties (File & Gonzalez, 1996) and 5-HT$_{1A}$ knockout mice show increased anxiety that cannot be rescued by antidepressant treatment (Toth, 2003). Moreover, antipsychotic drugs that stimulate 5-HT$_{1A}$ receptors induce dopamine release in the mesolimbic reward pathway, which likely plays a role in their effectiveness at treating schizophrenia (Bantick, De Vries, & Grasby, 2005; Bantick, Deakin, & Grasby, 2001; Li, Ichikawa, Dai, & Meltzer, 2004; Rollema, Lu, Schmidt, Sprouse, & Zorn, 2000). Taken together, this evidence suggests that the activity of CBD at 5-HT$_{1A}$ receptors might be the primary mechanism responsible for the putative therapeutic effects of cannabis on

psychiatric illness. Nevertheless, the diverse and wide range of effects of THC and CBD necessitates in depth investigations into their influence in the brain.

1.2   THE ENDOCANNABINOID SYSTEM

The biological system that most cannabinoids interact with is known as the endocannabinoid (eCB) system and, in mammalian species, is crucial to the normal function of the organism, both centrally and peripherally. Indeed, eCB receptors and ligands are present everywhere in the body and serve primarily as neuromodulators of other neurotransmitter systems. CB_2Rs were originally thought to only be expressed in the periphery, while this remains their primary locus, some are present on microglia in the central nervous system (CNS) (Cabral, Raborn, Griffin, Dennis, & Marciano-Cabral, 2008). CB_1Rs, however, were originally thought to only be expressed in the CNS but more recent evidence has demonstrated they are also expressed throughout the body (Herkenham et al., 1990; Pagotto, Marsicano, Cota, Lutz, & Pasquali, 2006). Both CBRs are G-protein coupled receptors and respond to a diverse range of exogenous and endogenous cannabinoids. Activation of these receptors typically blocks adenyl cyclase and decreases levels of cyclic adenosine monophosphate (cAMP). Changes in cAMP is linked to dozens of different molecular processes within the neuron that serve diverse functions.

The two primary eCBs in the brain are anandamide (AEA) and 2-arachidonoylglycerol (2-AG), which both act as retrograde messengers at the synaptic cleft. They are synthesized and released by the postsynaptic neuron and activate CB_1Rs on the presynaptic terminal, modulating the release of other neurotransmitters, regulating neural excitability, and stimulating the glia-neuron interaction (Ohno-Shosaku, Tanimura, Hashimotodani, & Kano, 2012). CB_1Rs are associated with social behaviour (Litvin, Phan, Hill, Pfaff, & McEwen, 2013; Wei, Allsop, Tye, & Piomelli, 2017), reward (Sanchis-Segura, Cline, Marsicano, Lutz, & Spanagel, 2004), learning (Bergado Acosta, Schneider, & Fendt, 2017), emotional behaviour (Rubino et al., 2008; Ruehle, Rey, Remmers, & Lutz, 2012), memory (Marsicano & Lafenêtre, 2009; Morena & Campolongo, 2014) and sensorimotor gating (Ortega-Álvaro et al., 2015). THC has substantial binding affinity to both CBRs, but CB_1Rs appear to be responsible for the neuropsychiatric effects of cannabis consumption.

Anatomically, CB_1Rs are present throughout the brain but are not uniformly distributed. There are high concentrations of the receptor in the structures that compose the mesolimbic reward pathway, which is responsible for appetitive processing, cognition and memory (Tsou, Brown, Sañudo-Peña, Mackie, & Walker, 1998). For example, activation of CB_1Rs in the nucleus accumbens (NAc), an important area within the mesolimbic pathway, impairs contextual fear learning (Pedroza-Llinás, Méndez-Díaz, Ruiz-Contreras, & Prospéro-García, 2013), and, conversely, activating CB_1Rs in the medial prefrontal cortex (mPFC) potentiates the learning of an olfactory associative fear memory (Laviolette & Grace, 2006). Additionally, CB_1R signaling in the mesolimbic pathway has been demonstrated to play a significant role in drug reward. For example, amphetamine-induced dopamine release in the shell region of the NAc (NASh) was shown to be dependent on CB_1R activation (Kleijn et al., 2012) and blockade of CB_1Rs in the NAc was shown to potentiate morphine sensitization behaviours (Haghparast, Azizi, Hassanpour-Ezatti, Khorrami, & Naderi, 2009).

1.3   THE MESOLIMBIC REWARD PATHWAY

The mesolimbic reward pathway is a dopaminergic (DAergic) circuit in the brain responsible for motivation, emotion, cognition and memory (Wise, 2004). It consists of the A10 DAergic neurons which originate in the ventral tegmental area (VTA) and connect to areas in the striatum and forebrain such as, the NAc, the mPFC and, the amygdala (Grace, Floresco, Goto, & Lodge, 2007; Ikemoto, 2007). Previous research has implicated DA signalling in the mesolimbic pathway in reward learning (FitzGerald, Dolan, & Friston, 2015); fear learning and memory (Fadok, Darvas, Dickerson, & Palmiter, 2010; Pignatelli et al., 2017); and social behaviour (Manduca et al., 2016). A simplified diagram of the mesolimbic reward pathway can be seen in **Figure 1.1**.

Extensive evidence has demonstrated substantial functional interactions between the DA and eCB systems. For example, CB_1Rs and DA receptors are frequently co-expressed on neurons in the forebrain, indicating substantial crosstalk between the neurotransmitter systems (Hermann, Marsicano, & Lutz, 2002). Additionally, the eCB system acts as a modulator of afferent inputs to dopamine neurons and regulates DA neurotransmission (Covey, Mateo, Sulzer, Cheer, &

Lovinger, 2017). Furthermore, DA/ eCB interaction is vital for proper decision-making, as eCBs regulate inhibitory and excitatory input into the mesolimbic pathway (Hernandez & Cheer, 2015). The effects of administration of exogenous cannabinoids also suggests substantial interaction between the eCB and DA systems. For example, acute administration of exogenous cannabinoids, such as THC, causes strong DA efflux, while chronic THC administration is associated with the blunting of DA transmission (van de Giessen et al., 2016). Taken together, the evidence strongly indicates that the eCB systems plays a significant role in regulating dopamine signaling in the brain.

1.3.1 The Nucleus Accumbens

The NAc serves as a key integratory area in the mesolimbic pathway. It receives and consolidates information from the VTA, the mPFC and the amygdala. Traditionally, the NAc was seen as the brain's primary "reward centre" but subsequent research demonstrated it also plays a complex role in decision-making and motivational salience processing. Likewise, the NAc plays a significant role in drug reward and addiction-related phenomena (Quintero, 2013; Ren et al., 2013), including mediating the rewarding properties of THC (Zangen, Solinas, Ikemoto, Goldberg, & Wise, 2006). However, NAc activity is also required for processing aversion-related information (Al-Hasani et al., 2015; Qi et al., 2016) and for processing long term memories of associative fear learning (Fadok et al., 2010). In addition, the NAc processes appetitive information from other areas in the mesolimbic pathway and changes the likelihood, efficiency and vigor of goal orientated behaviours. For example, the NAc does not directly control food motivation but does mediate approach behaviour, sustained attention, effort, and task learning (Hernandez, Sadeghian, & Kelley, 2002; Salamone, Correa, Farrar, & Mingote, 2007; Salamone & Correa, 2012). Essentially, the NAc motivates individuals toward stimuli that result in positive or rewarding outcomes and away from stimuli that result in negative or aversive outcomes. Additionally, CB_1Rs have been previously demonstrated to play a significant role in NAc function. For example, CB_1 knockout mice lack morphine induced NAc dopamine release (Mascia et al., 1999) but administration of a synthetic CB_1 agonist increases NAc dopamine release (Sperlágh, Windisch, Andó, & Sylvester Vizi, 2009).

Anatomically, the NAc is divided into two distinct areas: the shell (NASh) and the core (NACo). The NACo encodes motor information related to reward to facilitate the acquisition of the reward and serves as a limbic-motor interface (Mannella, Gurney, & Baldassarre, 2013; Nestler, Hyman, Holtzman, & Malenka, 2015). The NASh, however, is believed to be involved in the affective and cognitive aspects of reward and aversion-related processing. For example, the NASh is responsible for "like" and "dislike" reactions and motivational salience, the form of attention that motivates the individual towards or away from a stimulus (Floresco, 2015; Ikemoto & Panksepp, 1999; Reynolds & Berridge, 2002; Saddoris, Cacciapaglia, Wightman, & Carelli, 2015). The shell also plays a much larger role in drug reward and addiction (Crofton et al., 2017; Gaetano Di Chiara et al., 2004). Importantly, within the shell itself, recent evidence has demonstrated anatomically and functionally distinct sub-regions. For example, the anterior pole of the NASh has been characterized as a "hedonic hotspot" because localized pharmacological stimulation of the structure with eCBs or select opioid agonists, creates potent rewarding effects. The functional division within the structure is believed to be caused by differential concentrations of specific opioid receptor subtypes, specifically, the 'mu' receptor and the 'kappa' receptor subtypes, which are independently responsible for signaling rewarding or aversive motivational signals in the mesolimbic circuitry, respectively (Peciña & Berridge, 2000; Smith & Berridge, 2007).

Despite evidence for these regional differences in terms of affective processing in the anterior vs. posterior shell regions, little is known regarding how specific cannabinoids, such as THC, might differentially impact these striatal sub-regions in terms of reward or aversion processing. These functional differences are the focus of the studies described in chapters 3 and 4 of this thesis. At a cellular level, the NA is mainly composed of the GABAergic inhibitory medium spiny neurons (MSNs). MSNs are primarily divided into two subpopulations: cells primarily expressing D_1 DA receptors and cells primarily expressing D_2 receptors. Traditionally, D_1 MSNs were thought to mediate reward and positive reinforcement and D_2 MSNs were believed to mediate aversion and negative reinforcement (Kravitz & Kreitzer, 2012; Volman et al., 2013). More recent evidence, however, has suggested that this dichotomy does not apply to all reward related behaviours (Kupchik et al., 2015; Soares-Cunha, Coimbra, David-Pereira, et al., 2016). For example, activation of D_2 expressing neurons in the NA increased motivation, suggesting that both

subpopulations are important for motivational salience (Soares-Cunha, Coimbra, David-Pereira, et al., 2016).

1.3.2 The Ventral Tegmental Area

The VTA is a structure in the midbrain that acts as the main site for DAergic neurons and plays a central role in reward and goal-oriented behaviours. The VTA is one of the primary sites acted on by drugs of abuse, including THC (Zangen et al., 2006), and is required for the acquisition and expression of many drug-related behaviours (Oliva & Wanat, 2016). Previous evidence has demonstrated that the VTA is also involved in conditioned fear responses, indicating that the structure is involved in processing negative or aversive associations as well (N. A. Chen et al., 2016; Matulewicz, Orzeł-Gryglewska, Braszka, Zawistowski, & Jurkowlaniec, 2015; Oliveira, Reimer, & Brandão, 2009). Although the actions of the DA neurons in the VTA are the best characterized in terms of motivational processing, the VTA also contains opiate receptors (ORs), CB_1Rs and GABA receptors, localized on both DAergic and GABAergic neuronal populations. Anatomically, the VTA is heavily connected with other areas of the mesocorticolimbic system and sends afferent connections to the mPFC, BLA and NAc. The NAc also sends GABAergic projections back into the VTA, creating a circuit loop that enables both areas to affect the neuronal activity states in the other. The VTA/NAc circuit plays a key role in drug reward (Ishikawa et al., 2013; Owesson-White et al., 2009) and addiction (Britt & Bonci, 2013; Stuber, Britt, & Bonci, 2012). The specific influence of this circuit on associative fear memory processing is discussed further in Chapter 2.
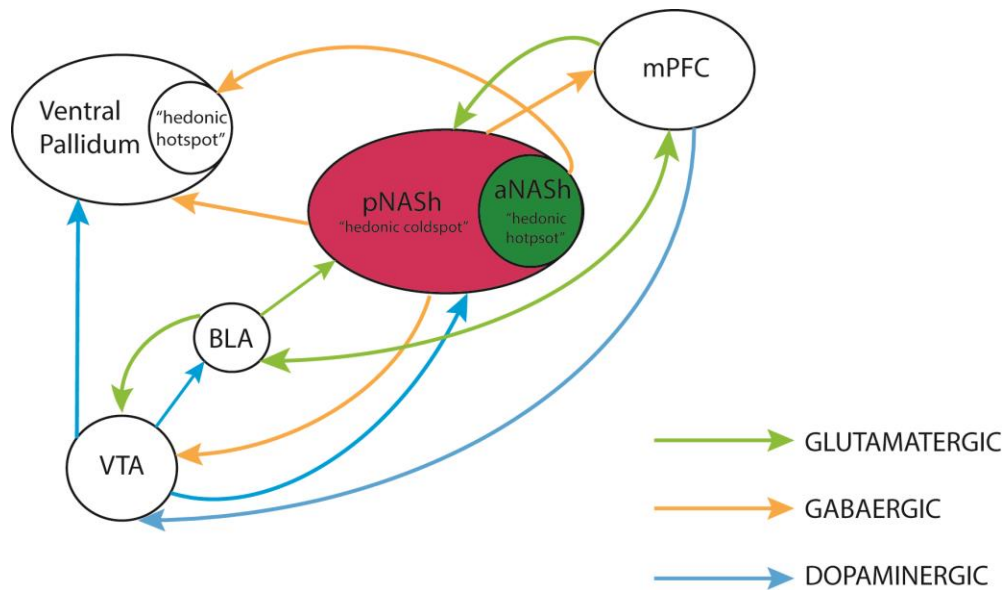
Figure 1.1 A proposed simplified version of the mesolimbic circuit. A diagram demonstrating the highly interconnected nature of the mesolimbic circuit, including the unique connections between the hedonic hotspots in the NASh and the ventral pallidum

1.4 MOLECULAR SIGNALLING PATHWAYS ASSOCIATED WITH THE EFFECTS OF PHYTOCANNABINOIDS

Molecular signalling cascades within neurons are responsible for the basic functions governing the activity of the cell, including production and phosphorylation of specific proteins. These pathways can be affected by exogenous compounds, including cannabinoids, in a variety of ways. Signalling proteins act in a complex and highly interconnected manner referred to as an 'interactome'. The highly interconnected nature of many cellular signalling pathways can make the effects of individual molecules difficult to characterize, but recent studies have elucidated the role of many proteins in neuronal activity, specifically as they relate to neuropsychiatric disorders and their symptoms.

For example, the Wingless/ Integrated (Wnt) signalling pathways are signal transduction mechanisms that pass signals into a cell from a variety of cell surface receptors. Three distinct Wnt pathways have currently been discovered: canonical Wnt, which causes the accumulation of β-catenin in the cytoplasm and eventual translocation into the nucleus to alter transcription; the

Wnt/ Jun N-terminal kinase (JNK) pathway, which plays a role in cell morphology; and the Wnt/calcium pathway, which regulates the levels of calcium inside the cell (Rao & Kühl, 2010). Dysfunction of the Wnt pathways has been linked to numerous psychiatric illnesses such as schizophrenia, bipolar disorder and autism (Mulligan & Cheyette, 2016). Moreover, blocking glycogen synthase kinase 3 (GSK3), an important molecule in Wnt signaling, directly in the NASh, has been shown to alleviate depression and addiction related symptoms (Crofton et al., 2017).

Another important signalling pathway linked to the cognitive symptoms of neuropsychiatric disorders is the mammalian target of rapamycin (mTOR). mTOR signaling regulates cell metabolism, proliferation, and growth. mTOR nucleates two distinct protein complexes: mTOR complex 1 (mTORC1), which acts as a cell energy sensor and controls protein synthesis based on the availability of nutrients (Kim et al., 2002); and the mTOR complex 2 (mTORC2), which phosphorylates protein kinase B (Akt) and is involved in the regulation of the cytoskeleton and cellular metabolism. Dysregulation of mTOR signalling has been implicated in addiction and many neuropsychiatric disorders, including schizophrenia and depression (Bockaert & Marin, 2015; Meffre et al., 2012; Ryskalin, Limanaqi, Frati, Busceti, & Fornai, 2018). Additionally, the rapid antidepressant effect of ketamine has been demonstrated to be mediated by mTOR (Li et al., 2010). Interestingly, both THC and CBD alter the Wnt and mTOR pathways, which may potentially explain their neuropsychotropic properties. For example, previous studies have demonstrated that chronic exposure to THC during adolescent neurodevelopment inhibits GSK3 and mTOR signalling pathways directly in the mPFC (Renard et al., 2017). Furthermore, CBD can block amphetamine-induced behavioural sensitization through activation of mTOR or p706K pathways directly in the nucleus accumbens shell (Renard et al., 2016). Taken together, this evidence implicates the Wnt and mTOR pathways as being important players in affective and cognitive processing within the mesocorticolimbic circuitry.

1.5 SUMMARY AND RATIONALE

As discussed above, the specific phytochemical compounds in cannabis can exert a wide range of effects throughout the brain and strongly alter behaviour, cellular activity, and intra-cellular signalling pathways. The mesolimbic reward pathway is of importance given its role in

cognition, motivation, reward, emotion and memory processing. Although considerable research attention has been dedicated to studying the phytochemical compounds in cannabis, we still understand relatively little about how THC and CBD exert their effects on cognitive and affective processing within the mesocorticolimbic circuitry. Given the well-established potential for both detrimental and therapeutic effects of cannabis-derived phytochemicals, the studies reported in this thesis aim to more clearly elucidate how and where THC and CBD may produce neuropsychiatric effects within the mesocorticolimbic system.

1.5.1 General hypothesis

My overarching hypothesis is that CBD and THC will differentially affect cognition, emotional associative memory and affective processing via dissociable molecular, neuronal, pharmacological, and anatomical mechanisms directly in the mammalian NAc. **My thesis will address this general hypothesis with the following three specific research aims:**

AIM 1. Investigate and characterize the effects of intra-NASh CBD on the formation of associative fear memory through functional interactions with the serotonergic 5-HT_{1A} signalling pathway and associated modulation of VTA neuronal network dynamics. (Chapter 2)

AIM 2. Investigate and characterize the behavioural and neuronal effects of intra-NASh THC on reward and aversion processing within anatomically dissociable regions of the NASh and identify the potential role of separate opioid receptor substrates underlying these dissociable effects. (Chapter 3)

AIM 3. Investigate and differentiate the localized molecular signalling mechanisms underlying the effects of THC in the anterior vs. posterior poles of the NASh as it relates to fear-related associative memory, anxiety and sensorimotor gating phenomena. (Chapter 4)

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CHAPTER 2

2 Cannabidiol Modulates Fear Memory Formation Through Interactions with Serotonergic Transmission in the Mesolimbic System[1]

2.1 INTRODUCTION

There is ongoing debate regarding the potential therapeutic potential of cannabis-derived phytochemicals in the treatment of neuropsychiatric conditions. Central to this debate is emerging evidence demonstrating that separate phytochemical constituents of cannabis may possess differential pharmacological and psychotropic effects. Although delta-9-tetrahydrocannabinol (THC) is considered the primary psychoactive component of marijuana, cannabis contains over 100 distinct compounds (ElSohly & Slade, 2005; Radwan et al., 2009). Of these, cannabidiol (CBD), which is considered non-psychoactive, is the most abundant. In contrast to THC, CBD can act as an antagonist or inverse agonist of the CB1 receptor (CB1R; (Pertwee, 2008; Thomas, Gilliam, Burch, Roche, & Seltzman, 1998), is an agonist at the 5-HT$_{1A}$ receptor (Russo et al., 2005) and decreases cellular reuptake and hydrolysis of the endocannabinoid anandamide, potentiating its central effects (Bisogno et al., 2001). In terms of psychotropic profiles, THC is associated with transient and long-term psychotomimetic effects (Bhattacharyya et al., 2012; Murray, Morrison, Henquet, & Di Forti, 2007) whereas clinical and pre-clinical research has shown that CBD can produce antipsychotic and anxiolytic effects (Campos, Ferreira, & Guimarães, 2012; Casarotto, Gomes, Resstel, & Guimarães, 2010; Crippa et al., 2011; Fogaca, Reis, Campos, & Guimarães, 2014; Gomes et al., 2015; Leweke et al., 2012; Mechoulam, Peters, Murillo-Rodriguez, & Hanuš, 2007; Renard et al., 2016; Schubart et al., 2014; Zuardi et al., 2006, 2012). In addition, CBD may modulate emotional memory processing and decrease symptoms associated with emotional memory disorders such as post-traumatic stress disorder (PTSD; Betthauser, Pilz, & Vollmer, 2015; Blessing et al., 2015).

While the precise neuroanatomical regions responsible for CBD's actions are not known, considerable evidence implicates the nucleus accumbens (NAc) as an important site for CBD's modulatory effects on various cognitive and behavioural phenomena (Bhattacharyya et al., 2009; Guimarães, Zuardi, Del Bel, & Guimarães, 2004; Mijangos-Moreno, Poot-Aké, Arankowsky-Sandoval, & Murillo-Rodríguez, 2014; Pedrazzi, Issy, Gomes, Guimarães, & Del-Bel, 2015; Valvassori et al., 2011). For example, CBD attenuates THC-induced dysregulation of the ventral striatum during verbal recall tasks (Bhattacharyya et al., 2009) and increases c-fos and adenosine

levels in rodent NAc (Guimarães et al., 2004; Mijangos-Moreno et al., 2014). In addition, CBD blocks amphetamine-induced oxidative stress in the NAc (Valvassori et al., 2011) and intra-NAc CBD attenuates the disruptive effects of amphetamine on pre-pulse inhibition (PPI) (Pedrazzi et al., 2015). Nevertheless, the precise functional and pharmacological mechanisms by which CBD may produce these effects in the mesolimbic system are not currently understood.

In the present study, we investigated the potential effects of CBD on fear-related memory formation and how CBD may modulate neuronal activity states within the mesolimbic circuitry. Using a combination of behavioural conditioning and in vivo neuronal electrophysiological recordings, we report that intra-NAc CBD dose-dependently blocks the formation of associative fear memories and blunts the activity levels of DAergic neuronal activity in the ventral tegmental area (VTA). Furthermore, these effects were dependent upon intra-NASh 5-HT$_{1A}$ receptor transmission and functional interactions between the NASh and GABAergic transmission in the VTA.

2.2 MATERIALS AND METHODS

2.2.1 Animals and Surgery

Male Sprague–Dawley rats (300–350 g; Charles River, Senneville, QC, Canada) were anesthetized with ketamine (80 mg/ml)–xylazine (6 mg/kg; intraperitoneally) and placed in a stereotaxic apparatus. An incision was made to expose the skull, and stainless steel guide cannulae (22 G; Plastics1) were implanted into the NASh using the following stereo- taxic coordinates (12° angle, mm from bregma): antero- posterior (AP) +1.8, lateral (LAT) ± 2.6, and ventral (V) − 7.4 from the dural surface and the following coordinates for the VTA (10° angle, mm from bregma): AP − 5.0, LAT ± 2.6, and V − 8.0 from the dural surface. Coordinates were based on the Atlas of Paxinos and Watson (2005). Rats in the NASh-VTA disconnection groups received single uni- lateral NASh cannulation and contralateral VTA cannulation. All experimental procedures were performed in accordance with the regulations of the Canadian Council on animal care (CCAC) and the University of Western Ontario.

2.2.2 Drug Administration

The broad-spectrum DA receptor antagonist, α-flupenthixol (α-flu; Tocris), the $GABA_A$ antagonist, bicuculline methiodide (Tocris), and the $GABA_A$ antagonist, hydroxysaclofen (Tocris), were dissolved in physiological saline (pH adjusted to 7.4). The 5-HT1A receptor antagonist, NAD 299 hydro- chloride (Tocris), CBD (Tocris), and CB1R antagonist, SR141716A (rimonabant, RIM), were first dissolved in dimethyl sulfoxide (DMSO) and then diluted in PBS for a final 1% DMSO in PBS vehicle (VEH) solution. Microinfusions were performed over 1 min through a Hamilton microsyringe. To ensure adequate diffusion, injector cannulae were left in place for an additional 1 min.

2.2.3 Olfactory Fear Conditioning

We used a previously described olfactory fear conditioning paradigm to measure fear memory formation (Draycott et al., 2014; Lauzon, Bishop, & Laviolette, 2009). Two distinct environments were used. Environment A was a 30 in× 30 in Plexiglass box with black spots on a white background and environment B was a 30 in× 30 in Plexiglass box with black and white stripes. The designated shock environment had a metallic grid shock floor, while the designated test environment had a smooth gray Plexiglas floor. The environments were assigned as 'shock' and 'test' in a counterbalanced manner such that all rats with 'shock' environment A were tested in environment B and all rats with 'shock' environment B were tested in environment A. On day 1 (habituation), rats were given sham microinfusions into the NASh and habituated to both conditioning environments for 30 min. Footshocks were delivered at an intensity of 0.8 mA (for 1 s), which we have previously reported to produce highly robust conditioned freezing behaviours (Draycott et al., 2014; Lauzon et al., 2009). On day 2 (conditioning phase), rats were returned to the room and, immediately before being placed in previously assigned 'shock' environment, rats received intra-NASh microinfusions of CBD (1, 10, and 100 ng/ 0.5 µl); the DA antagonist α-flu (100 ng–1.0 µg/0.5 µl), alone or in combination with CBD; the 5-HT1A antagonist NAD 299 hydrochloride (10–100 ng/0.5 µl), alone or in combination with CBD (100 ng). Two odors were delivered during conditioning, almond and peppermint. One odor was presented with a footshock (CS+) and the other was presented in the absence of a footshock (CS -). After 1 min in the 'shock' environment, the CS− odor was presented for 20 s. After 2 min, the CS+ odor was presented for 19 s followed by a 1 s footshock delivered through the shock floor.

This cycle was repeated five times. On day 3 (test phase), rats were placed in the previously assigned test environment. They were given 1 min to explore the environment before odor presentations began, during which time baseline levels of freezing behaviour were recorded. Both CS+ and CS− odors were presented in a fully counter- balanced order for 5 min and amounts of time rat's spent freezing (lack of movement except for respiration) was recorded and analyzed with ANY-MAZE video software for offline analysis.

2.2.4 Footshock Sensitivity Tests

To ensure that intra-NASh infusions of CBD, NAD 299, or α-flu were having no effect on baseline sensitivity to footshock stimulation during fear memory acquisition, separate control groups received either bilateral intra-NASh microinfusions of VEH, CBD (100 ng/0.5 μl), NAD 299 (500 ng/0.5 μl), or α-flu (1000 ng/0.5 μl) alone immediately before a sensitivity testing phase wherein rats received five test shock administrations (0.8 mA, 1 s, in the absence of any cue delivery), once per minute over a total of 5 min. During this time, levels of freezing behaviour, total distance traveled, and the number of jumping events were recorded with ANY-MAZE video software and analyzed offline.

2.2.5 *In Vivo* Electrophysiological Recordings

In vivo single-cell extracellular VTA recordings were performed as described previously (Loureiro, Renard, Zunder, & Laviolette, 2015; Tan, Bishop, Lauzon, Sun, & Laviolette, 2009). Briefly, rats were anesthetized with urethane (1.4 g/kg, intraperitoneally) and placed in a stereotaxic frame with body temperature maintained at 37 °C. A scalp incision was made, and holes were drilled in the skull above the NASh and VTA. For intra-NASh microinfusion of CBD (100 ng/0.5 μl), a 1 μl Hamilton syringe was slowly lowered at the same coordinates used for behavioural studies. For intra- VTA extracellular recording, glass microelectrodes (with an average impedance of 6–8MΩ) filled with a 2% Pontamine Sky Blue solution were lowered using a hydraulic micro- positioner (Kopf 640) at the following flat skull stereotaxic coordinates (in mm from bregma): AP − 5.3, LAT ± 0.7 from midline, ventral (V) − 7.0 to − 8.5 from the dural surface. Extracellular signals were amplified using a MultiClamp 700B amplifier (Molecular Devices) and recorded through a Digidata 1440A acquisition system (Molecular Devices) using the pClamp 10 software. Extracellular recordings were filtered at 1 kHz and

sampled at 25 kHz. VTA DA neurons were identified according to well-established electrophysio- logical features (Jalabert et al., 2011; Ungless, Ungless, Magill, & Bolam, 2004): (1) a relatively long action potential width (>2.5 ms); (2) a slow spontaneous firing rate (2–5 Hz); (3) a biphasic waveform consisting of a notch on the rising phase followed by a delayed after potential; and (4) a single irregular or bursting firing pattern. VTA GABA interneurons were also characterized based on previously reported criteria: (1) a narrow action potential width (<1 ms); (2) relatively fast firing rates (typically between 10 and 20 Hz); and (3) the absence of bursting activity. Neurons that failed to clearly meet the aforementioned criteria for VTA DA or VTA non-DA electrophysiological neuronal properties were excluded from post experimental analyses. Recordings analyses were accomplished using the Clampfit 10 software. Response patterns of isolated VTA neurons following intra-NASh CBD micro-infusions were determined by comparing neuronal frequency rates between the 5 min preinfusion vs postinfusion epochs. Classification of drug-infusion effects used a criterion of a ≥10% increase in firing frequency postinfusion to be classified as an increase; a ≤10% decrease to be classified as a decrease. Neurons showing firing frequency parameters within these cut-off points were classified as 'no change'. For VTA DA neurons, we also analyzed the bursting rate and number of spikes within each burst. The onset of a burst was defined as the occurrence of two consecutive spikes with an interspike interval of <80 ms. For histological analysis of extracellular VTA neuronal recording sites, recording electrode positions were marked with iontophoretic deposit of Pontamine Sky Blue dye (−20 µA, continuous current for 12–15 min). Rats were then perfused transcardially with isotonic saline followed by 10% formalin. Brains were removed and stored in a 25% sucrose-formalin solution before sectioning (40 µm sections) on a freezing cryostat. Following this, sections were stained with neutral red and infusion and/or neuronal recording sites were confirmed with light microscopy.

2.2.6 NASh–VTA Functional Disconnection Studies

Previous research has established the functional connectivity between the NAc and the VTA, whereby GABAergic projections from the NAc are capable of modulating VTA DAergic and non-DAergic neuronal activity states (Kalivas, Churchill, & Klitenick, 1993; Nauta, Smith, Faull, & Domesick, 1978). To determine if the behavioural effects of intra-NASh CBD were due to modulatory influences from the NASh→VTA, we performed a functional disconnection

procedure, similar to one described previously (Rosen et al., 2015). In this procedure, to disconnect the effects of intra-NASh CBD from intra-VTA GABAergic transmission, experimental groups had micro- injector cannulae placed unilaterally in the NASh of one hemisphere, and the VTA of the contralateral hemisphere. Group 1 received intra-VTA VEH followed by intra-NASh VEH. Group 2 received intra-VTA VEH followed by intra- NASh CBD (100 ng/0.5 µl). Group 3 received intra-VTA microinfusion of the GABAA receptor antagonist bicuculline methiodide (50 ng/0.5 µl) with the selective GABAB antagonist hydroxysaclofen (100 ng/0.5 µl), followed by intra-NASh CBD (100 ng/0.5 µl). Group 4 received the intra-VTA bicuculline/hydroxysaclofen mixture followed by intra- NASh VEH. All groups received the intra-VTA treatment 2 min before intra-NASh treatments. The hemispheres for unilateral/contralateral cannulations were counter-balanced within groups to control for laterality. All groups were then subjected to the same olfactory fear conditioning paradigm described above.

2.2.7 Data Analysis

Data were analyzed with one- or two-way ANOVA, followed by *post hoc* paired-samples t-tests or Newman–Keuls tests, where appropriate.

2.3 RESULTS

2.3.1 Histological Analyses

Histological analysis revealed injector placements localized within the anatomical boundaries of the shell subdivision of the NAc or VTA, according to (Paxinos and Watson (2005). **Figure 2.1** presents a microphotograph displaying a typical injector placement within the NASh, relative to the 'core' subdivision. **Figure 2.1** displays a schematic illustration showing representative intra-NASh cannulae placements along the rostral–caudal axis of the NAc. Black circles represent VEH control rats receiving for shows a micro- photograph displaying typical bilateral intra-NASh injector placements.
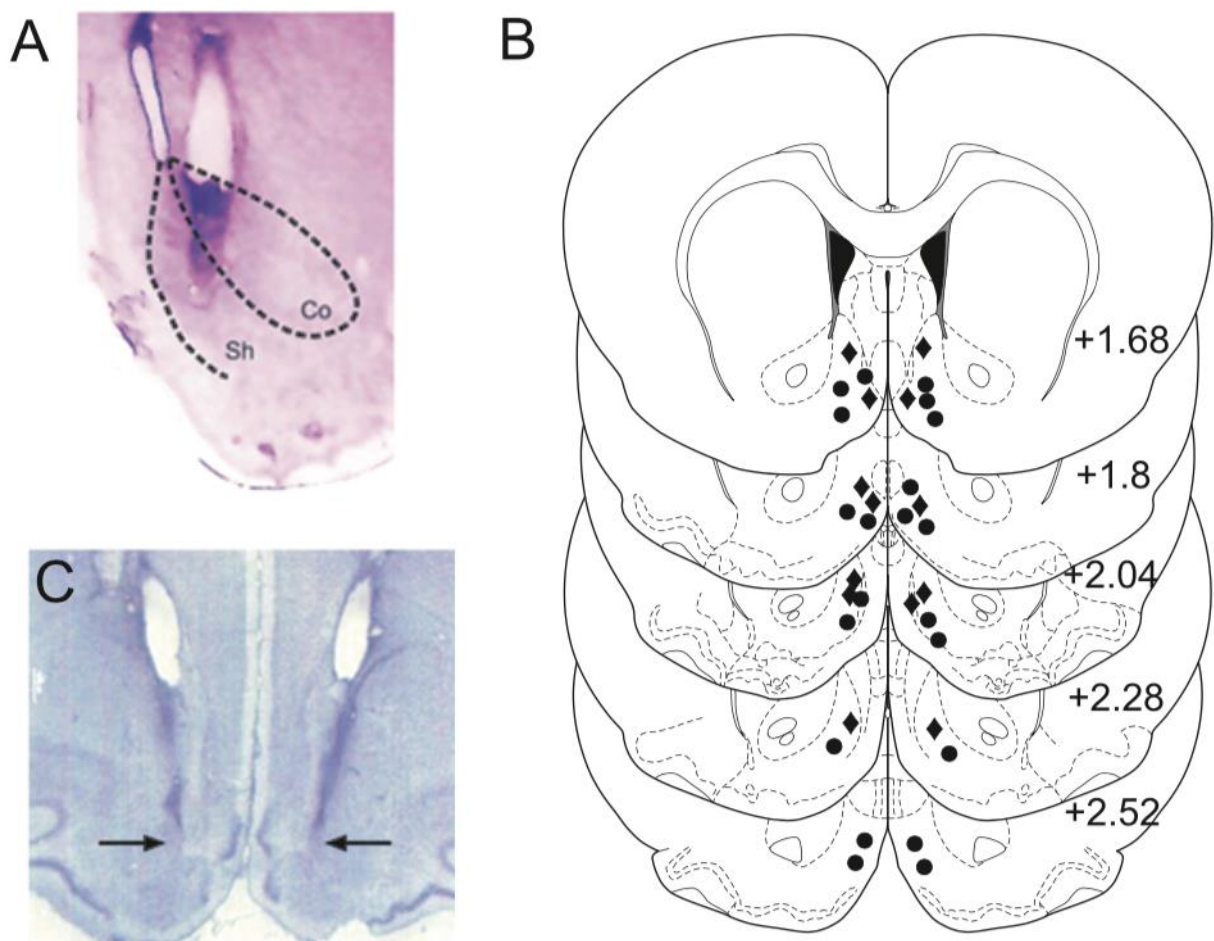
Figure 2.1 Histological analysis of intra-NASh microinjection sites. A, Microphotograph of a representative injector placement within the shell subdivision of the NAc. B, Schematic representation of select intra-NASh injector locations; • = 100ng CBD group, ♦ = 100ng CBD + 500ng NAD299 group. C, Microphotograph of representative intra-NASh bilateral cannulae placements.

2.3.2 Intra-NASh CBD Dose-Dependently Blocks the Formation of Associative Fear Memory

We first examined the potential effects of intra-NASh CBD on the acquisition of associative fear memory (see Materials and Methods). Using a wide dose range of bilateral intra-NASh CBD (1 ng–100 ng/0.5 μl), we challenged the acquisition of fear memory by administering CBD immediately before the fear conditioning session. ANOVA comparing percentages of time spent

freezing during testing revealed a significant main effect of group ($F_{(7,287)} = 5.75$, $p = 0.0001$). *Post hoc* analyses revealed that whereas rats treated with intra-NASh VEH ($n = 6$) and rats treated with a lower dose of CBD (1 ng/0.5 µl, $n = 7$) spent significantly greater amounts of time freezing in the presence of the CS+ (*p*'s <0.05, 0.01, respectively), rats receiving either 10 ng ($n = 8$) or 100 ng ($n = 6$) of intra-NASh CBD showed no associative freezing in response to CS− vs CS+ cue presentations (*p*'s > 0.05; **Figure 2.2a**). In addition, associative freezing in response to CS+ presentations were significantly lower relative to VEH controls for both the 10 ng and 100 ng CBD groups (*p*'s < 0.01; **Figure 2.2a**). Thus, bilateral intra- NASh CBD dose-dependently blocks the formation of conditioned fear memories measured with conditioned freezing behaviours. Accordingly, we selected the highest behaviourally effective dose of 100 ng/0.5 µl as the challenge dose for subsequent behavioural and electrophysiological experiments.

2.3.3 Intra-NASh CBD Modulates Fear Memory Formation Through a 5-HT$_{1A}$-Dependent Mechanism

Given previous evidence demonstrating that CBD produces its pharmacological effects via the 5-HT$_{1A}$ receptor (Russo et al, 2005), we next challenged the effects of intra-NASh CBD (100 ng) with co-administration of the selective 5-HT$_{1A}$ receptor antagonist, NAD 299 (10–100 ng/0.5 µl). ANOVA comparing percentages of time spent freezing during testing revealed a significant main effect of treatment ($F_{(1,37)} = 13.9$; $p < 0.001$). *Post hoc* analyses revealed that rats receiving either the lower (10 ng/0.5 µl) or higher (100 ng/0.5 µl) dose of NAD 299 (10 ng, $n = 7$; 100 ng, $n = 6$) with CBD, demonstrated robust associative freezing behaviours in response to CS+ presentations, relative to VEH controls ($n = 7$, *p*'s < 0.01; **Figure 2.2b**). To control for the potential effects of NAD 299 alone, a separate control group received bilateral intra-NASh NAD 299 (100 ng/0.5 µl; $n = 8$) alone, before training. These rats displayed normal conditioned freezing behaviour, freezing significantly more to CS+ presentations ($p < 0.05$). Next, to examine the possible involvement of DA receptor transmission in the effects of intra-NASh CBD, we challenged the effects of intra-NASh CBD with the broad-spectrum DA receptor antagonist, α-flu (100–1000 ng/0.5 µl). ANOVA revealed a significant main effect of group ($F_{(3,50)} = 5.65$; $p < 0.01$) on times spent freezing to CS+ vs CS− presentations. *Post hoc* analyses revealed that rats receiving co-administration of CBD (100 ng/0.5 µl) with either the lower dose of α-flu (100 ng/ 0.5 µl; $n = 6$) or the higher dose of α-flu (1000 ng/0.5 µl; $n = 8$) showed no

associative freezing during CS+ presentations (p's > 0.05; **Figure 2.2b**). To control for any potential effects of α-flu alone on fear memory formation, a separate control group ($n = 8$) received intra-NASh α-flu alone (1000 ng/0.5 μl). This group demonstrated significant associative freezing behaviours ($p < 0.05$; **Figure 2.2b**). Thus, whereas 5-HT$_{1A}$ receptor blockade reversed the effects of CBD-induced block of fear memory acquisition, DA receptor blockade had no effect. In addition, neither the highest behaviourally effective doses of NAD 299 nor α-flu produced any effects on fear memory acquisition in and of themselves.

Beyond the 5-HT$_{1A}$ receptor system, previous studies have suggested that CBD may produce pharmacological actions on the CB1 receptor system (McPartland, Duncan, Di Marzo, & Pertwee, 2015). To examine if the effects of CBD on fear memory acquisition may be mediated through a CB1 receptor substrate, separate groups of rats received intra-NASh CBD (100 ng/0.5 μl) co-administered with the selective CB1 antagonist, RIM (50–500 ng/0.5 μl; $n = 9$, $n = 10$, respectively). Comparing the effects of RIM/CBD co-administration on freezing behaviours revealed a significant effect of group ($F_{(2,47)} = 10.53$; $p < 0.01$), with post hoc analyses revealing that neither dose of RIM reversed the effects of CBD on fear memory blockade, relative to VEH controls, with both groups displaying no associative freezing in response to CS+ vs CS− presentations (p's > 0.05; **Figure 2.2b**). To ensure that the highest doses of CBD, NAD 299, or α-flu were not producing any unconditioned effects on footshock sensitivity during the fear memory acquisition phase, separate control groups received footshock sensitivity tests (see Materials and Methods) following bilateral intra-NASh microinfusions of VEH ($n = 8$), CBD (100 ng/0.5 μl, $n = 7$), NAD 299 (500 ng/0.5 μl, $n = 8$), or α-flu (1000 ng/ 0.5 μl, $n = 8$). Group comparisons with ANOVA revealed no significant group differences in the amount of freezing behaviour following footshock (0.8 mA) administration ($F_{(3,29)} = 1.10$; $p > 0.05$; **Figure 2.2b**); total distance traveled during the sensitivity testing ($F_{(3,29)} = 0.92$; $p > 0.05$; **Figure 2.2d**) or total average number of jumping events during footshock administrations ($F_{(3,29)} = 0.25$; $p > 0.05$; **Figure 2.2b**). Thus, neither CBD, NAD 299 nor α-flu produced any observable alterations in footshock sensitivity during fear memory acquisition training.
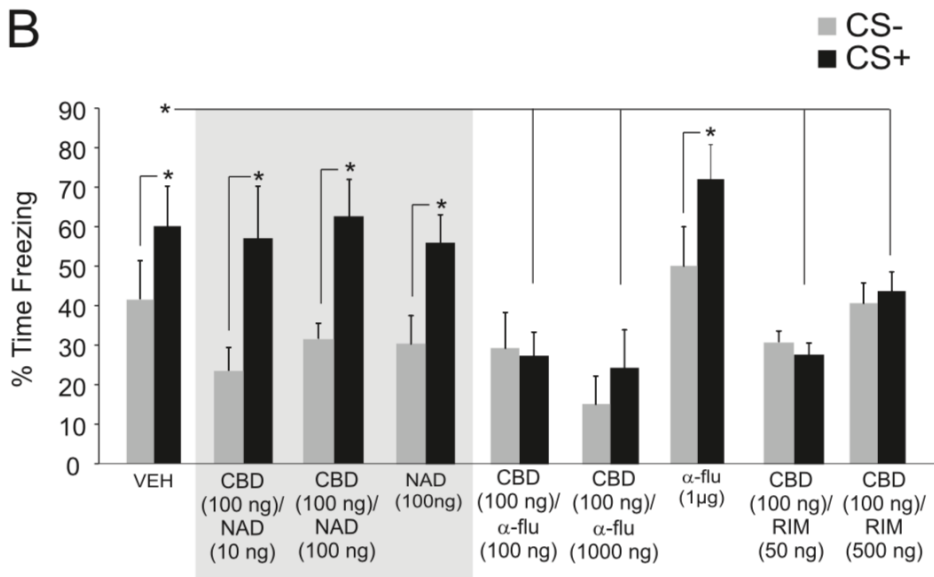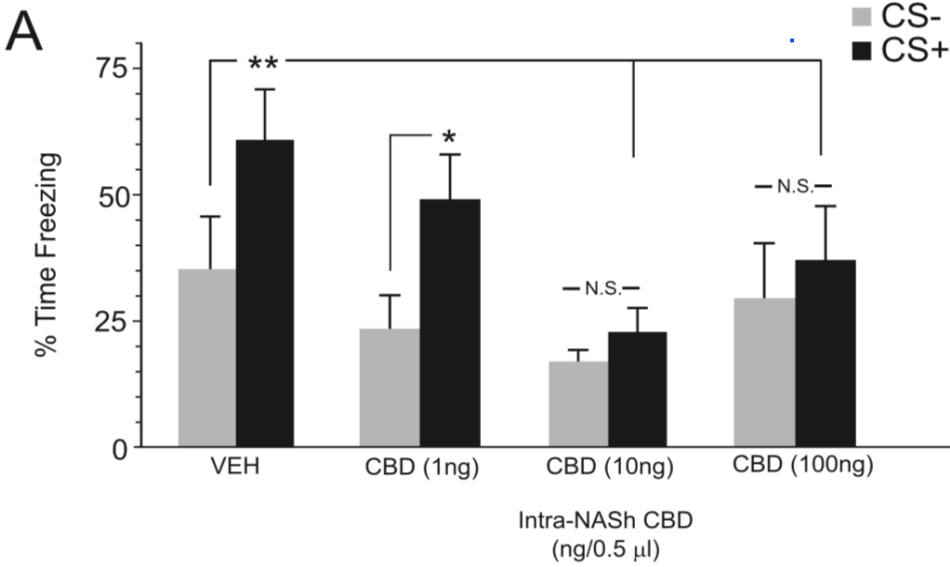
Figure 2.2 Effects of intra-NASh CBD on olfactory fear memory acquisition: modulation by 5-HT$_{1A}$ receptor transmission. A, Relative to vehicle control rats, bilateral administration of intra-NASh CBD (1-100 ng/ 0.5 µl) dose-dependently blocks the acquisition of supra-threshold (0.8 mA) olfactory associative fear memory. **B**, The effects of intra-NASh CBD on associative fear memory formation are dose-dependently reversed by co-administration of a selective 5-HT$_{1A}$ receptor antagonist (NAD 299, 10- 100 ng/ 0.5µl), but not by the broad-spectrum DA receptor antagonist, α-flu (100-1000 ng/0.5 µl) or the CB1 receptor selective

antagonist, rimonabant (50-500 ng/0.5 µl). Neither intra-NASh NAD 299 (500 ng) nor α-flu (1000 ng) administered alone produce any effects on fear memory formation. $* = p < .05$; $** = p < .01$. Bars represent means ± standard error of the mean (S.E.M.) for this and all subsequent figures.

2.3.4 Intra-NASh CBD Modulates DAergic vs Non-DAergic Neuronal Activity States in the VTA

We next performed *in vivo*, single-unit neuronal recordings in the VTA to determine the potential effects of intra-NASh CBD administration on spontaneous neuronal activity patterns in isolated populations of DAergic vs non-DAergic VTA neurons (see Materials and Methods; **Figure 2.3a**). Accordingly, our previously determined behaviourally effective dose of CBD (100 ng) was microinfused into the NASh while simultaneously recording single-cell extracellular VTA neuronal units. A total of $n = 15$ VTA-DA neurons were recorded and analyzed following intra-NASh CBD micro- infusions. A cell was considered to have changed its firing rate if there was a minimum of 10% difference in frequency rate from baseline. Using this previously established and reported criterion (Draycott et al., 2014; Tan et al., 2009), qualitative analysis revealed that 60% of DA neurons showed decreased activity, 7% increased, and 33% were unchanged (**Figure 2.3b**). Subgroup analyses of average frequency recorded during 5 min pre- vs post-intra-NASh microinfusions revealed that CBD significantly decreased the firing rate ($t_{(14)} = 3.13$, $p < 0.01$; **Figure 2.3c**) and bursting rate ($t_{(14)} = 2.76$, $p < 0.05$) without significantly decreasing the number of spikes per burst ($t_{(14)} = 2.06$, p = 0.06) (Figure 2.3d). Thus, intra-NASh CBD (100 ng) causes a predominantly inhibitory effect on VTA DAergic neuronal frequency and bursting rates. In **Figure 2.3e**, we present a rastergram of a single VTA DA neuron showing a typical decreased activity pattern following intra-NASh CBD microinfusion. We next recorded and analyzed presumptive VTA GABA neurons ($n = 15$) during intra-NASh CBD (100 ng) administration. Qualitative analysis of overall population activity revealed that 27% of non-DA neurons decreased activity, 40% increased, and 33% demonstrated no significant change in frequency (**Figure 2.3f**). Statistical analyses of neuronal sub- populations recorded 5 min pre- vs post-NASh microinfusions showed that while CBD did not significantly modify the collective (group) firing frequency of VTA GABA interneurons ($t_{(14)} = 0.04$; p= 0.97) separate sub-

population analyses revealed that neurons showing increased activity were significantly elevated in firing frequency relative to baseline ($t_{(5)} = 3.8$; $p < 0.01$; **Figure 2.3g**), whereas neurons showing either decreased ($t_{(3)} = 2.9$; $p > 0.05$) or no change in activity ($t_{(4)} = 0.55$; $p > 0.05$) were not significantly changed from preinfusion baseline levels (data not shown). Collectively, these data demonstrate that intra-NASh CBD induces heterogeneous effects on presumptive VTA GABAergic neurons. However, a plurality of these neurons (40%) significantly increased their spontaneous firing rates. In **Figure 2.3h**, we present a rastergram showing a typical non-DAergic neuronal response pattern following intra-NASh CBD microinfusion.
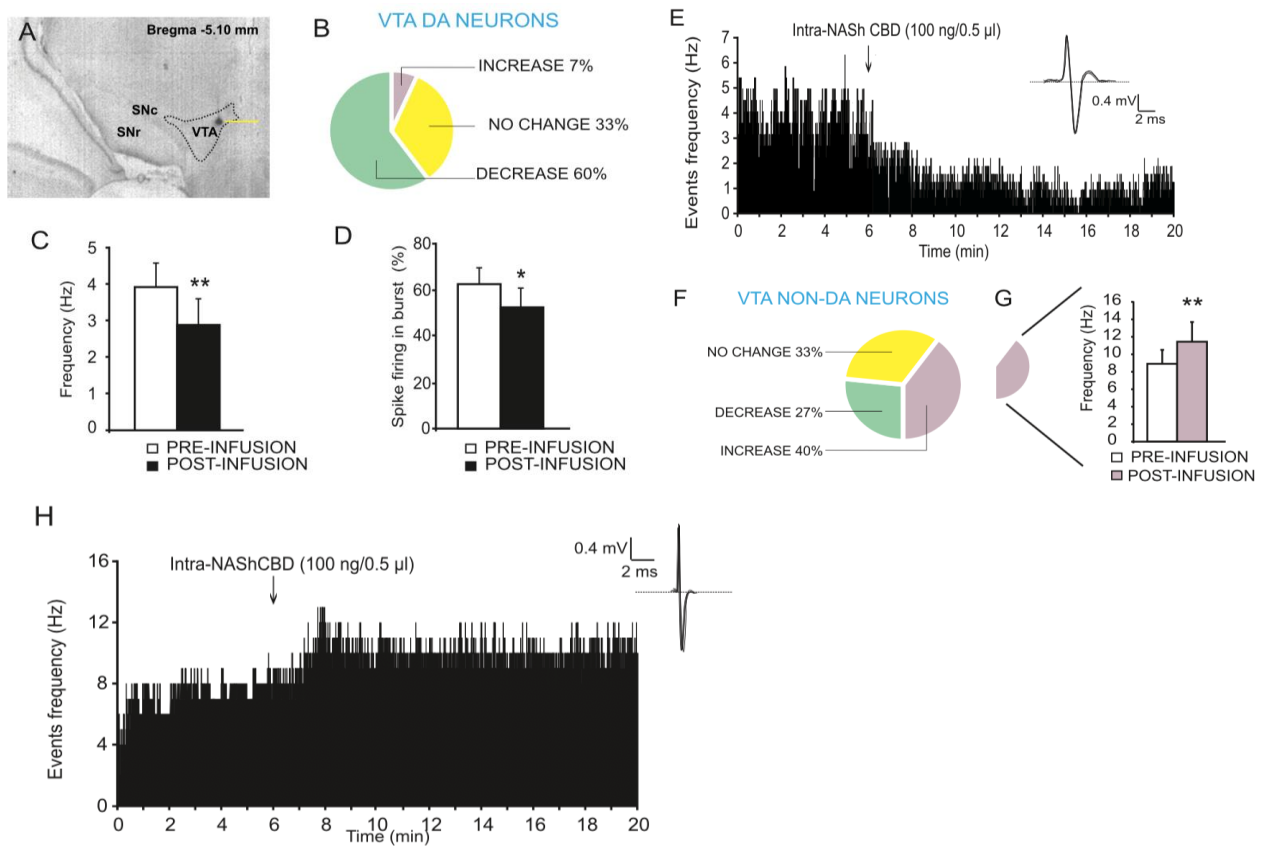
Figure 2.3 Effects of intra-NASh CBD on DAergic and non-DAergic neuronal activity patterns in the ventral tegmental area. A, Representative microphotograph showing typical intra-VTA in vivo DA neuron recording location (indicated by black arrow). **B,** Group summary of all VTA DA neuronal effects showing distribution of neuronal response patterns (no change in frequency, decreased activity, increased activity) across experimental conditions. **C,** Intra-NASh CBD significantly decreased spontaneous VTA DA neuronal firing frequency and **D,** bursting

rates. **E,** Sample rastergram showing typical VTA DAergic neuronal response pattern following intra-NASh CBD (100 ng/0.5 ml) administration. **F,** Summary of non-DA neuronal group response patterns following intra-NASh CBD administration. **G,** A sub-population of non-DAergic VTA neurons show a significant increase in spontaneous firing frequency following intra-NASh CBD (100 ng/0.5 ml). **H,** Sample rastergram showing a typical VTA non-DA neuronal response pattern following intra-NASh CBD administration

2.3.5 CBD Modulates DAergic Neuronal Activity in the VTA Selectively Through NASh 5-HT$_{1A}$ Transmission

We next sought to determine how the effects of co-administration of selective 5-HT$_{1A}$ or DA receptor antagonists on CBD-mediated behaviours might influence VTA DAergic neuronal activity. Accordingly, we performed in vivo, single-unit intra-VTA neuronal recordings to determine the potential effects of intra-NASh CBD, NAD 299/CBD, or α-flu/CBD co-administration on VTA DA neuron firing frequency and bursting levels (see Materials and Methods), using our behaviourally effective doses of CBD (100 ng/0.5 μl), NAD 299 (100 ng/0.5 μl), or α-flu (1 μg/0.5 μl). We sampled a total of $n = 44$ VTA DA neurons (VEH group, $n =10$ cells; CBD (100 ng) group, $n =15$ cells; CBD (100 ng) + NAD 299 (100 ng) group, $n =10$ cells; CBD (100 ng) + α-flu (1000 ng), $n = 9$ cells). A summary of VTA DAergic neuronal activity profiles after intra-NASh microinfusions are represented in **Figure 2.4a**. For rats receiving intra-NASh VEH, 50% of neurons showed no change, 30% increased, and 20% showed decreased activity. For rats receiving intra-NASh CBD, 60% of neurons decreased firing frequency, 33% showed no change, and 7% increased frequency. In rats receiving intra- NASh CBD+NAD 299, neurons decreasing, increasing, or showing no change in activity levels were 60%, 20%, and 20%, respectively. In rats microinfused with CBD + α-flu, 78% of the recorded cells decreased their firing frequency, 22% showed no change, and no cells demonstrated increased frequency. Analyses of pre- vs postinfusion activity rates for VTA DA neurons revealed average changes from baseline of +7% for rats treated with VEH, −27% for rats treated with CBD, +3% with CBD + NAD 299, and −23% for rats microinfused with CBD + α-flu (**Figure 2.4b**). ANOVA comparing groups revealed a significant main effect of treatment on VTA DA neuron firing frequency rates ($F_{(3,43)} = 3.57$, $p < .05$). Post hoc analysis revealed that rats treated with intra-NASh CBD alone or with CBD + α-flu showed significantly decreased firing frequencies relative

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to baseline (p's < .05). However, rats treated with CBD + NAD 299 showed significantly increased firing frequency relative to these groups, and no significant difference from baseline levels (p < 0.05; **Figure 2.4b**). Thus, cotreatment with NAD 299, but not α-flu, reversed CBD-induced reductions in spontaneous DA neuron frequency rates. Analysis of VTA DA neuron bursting rates revealed average changes from baseline of −4%, −35%, +29%, and −40% for rats treated with intra-NASh VEH, CBD, CBD + NAD 299, and CBD + α-flu, respectively (**Figure 2.4c**). ANOVA comparing groups revealed a significant effect of treatment ($F_{(3,43)} = 4.24$, $p < 0.01$) and *post hoc* comparisons showed that bursting rates were significantly different between rats treated with CBD vs CBD + NAD 299 ($p < .05$) and between CBD + α-flu and CBD + NAD 299 groups ($p < .05$). Thus, cotreatment with NAD 299, but not α-flu, reversed CBD-induced reductions in spontaneous DA neuron bursting rates. Next, to examine if intra-NASh α-flu or NAD 299 had any effects on VTA DA neuron frequency or bursting rates in and of themselves, separate control groups received either intra-NASh NAD 299 (500 ng/0.5 μl; $n = 10$ neurons) or α-flu (1000 ng/0.5 μl; $n = 9$ neurons). For intra-NASh NAD 299, qualitative analysis revealed that 10% of neurons showed increased, 30% decreased, and 60% no change in frequency (**Figure 2.4d**). For intra-NASh α-flu, qualitative analysis revealed that 20% of neurons increased, 35% decreased, and 45% showed no changes (**Figure 2.4d**). ANOVA revealed no significant treatment effect on VTA DA neuron firing rates across groups ($F_{(2,28)} = 0.63$; $p > .05$; **Figure 2.4e**). ANOVA analysis of bursting rates similarly revealed no significant treatment effect on DA neuron burst rates across groups ($F_{(2,26)} = 1.33$; $p > 0.05$; **Figure 2.4f**). Thus, neither intra-NASh NAD 299 nor α-flu had any effects on VTA DA neuron activity parameters in and of themselves. Representative neuronal rastergrams showing single VTA DA neuronal response patterns following intra-NASh CBD (100 ng alone; **Figure 2.4g**); CBD (100 ng) + NAD 299 (100 ng; **Figure 2.4h**); and CBD (100 ng) + α-flu (1000 ng; **Figure 2.4i**) demonstrate typically observed neuronal activity patterns following these treatments. Taken together, these results show that intra-NASh CBD decreases VTA DA neuronal frequency and bursting rates. These effects were blocked by confusion of a 5-HT$_{1A}$ antagonist but remained unchanged by the confusion of a DA receptor antagonist.
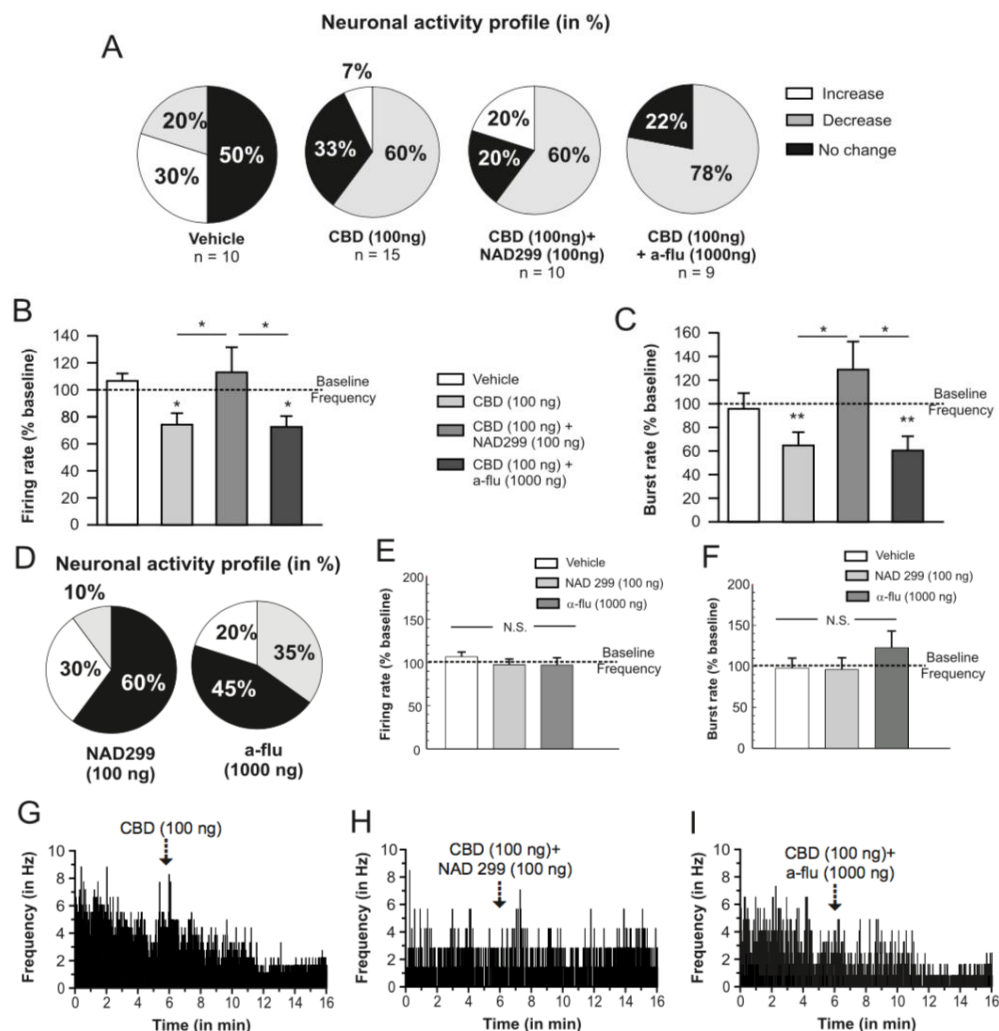
Figure 2.4 Effects of intra-NASh CBD and serotonergic/dopaminergic antagonists on VTA DAergic neuronal activity patterns in the ventral tegmental area. A, Summary of experimental neuronal groups showing relative changes (no change, increase or decrease) firing frequencies following intra-NASh pharmacological treatments. **B**, Intra- NASh CBD alone (100 ng/0.5 µl) caused a significant decrease in spontaneous VTA DA neuronal firing frequency rates. This inhibitory effect was reversed by co-administration of the effective dose of NAD 299 (500 ng/0.5 µl). In contrast, co-administration with the DA receptor antagonist, α-flu (1 µg/0.5 µl) had no effect on CBD's inhibition of VTA DA neuron activity. **C**, Comparing VTA DA neuron bursting rates revealed that intra-NASh CBD (100 ng/0.5 µl) significantly decreases VTA DA neuron bursting rates. Again, this effect was significantly reversed with co-administration of NAD 299 (500 ng/0.5 µl). In contrast, co-administration with the DA receptor antagonist, α-flu (1 µg/0.5 µl) had no effect on CBD's inhibition of VTA DA neuron bursting rates. **D**, Intra-

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NASh NAD 299 (500 ng) or α-flu (1000 ng) produced heterogeneous effects on spontaneous VTA DA neuron activity patterns. However, neither drug alone produced any significant effects on firing frequency (E) or bursting rates (F). **G**, Sample rastergram showing a typical VTA DA neuron inhibitory response pattern following intra-NASh CBD. **H**, Sample rastergram showing a typical VTA DA neuron response following co- administration of CBD with NAD 299 and **I**, sample rastergram showing a typical response pattern following co-administration of CBD with α-flu.

2.3.6 Functional Disconnection of the NASh→VTA Pathway Reverses the Effects of Intra-NASh CBD on Fear Memory Formation Medium

Medium spiny neurons (MSNs) in the NAc send GABAergic terminals to VTA neurons (Kalivas et al., 1993; Nauta et al., 1978). Given our previously described finding (**Figures 2.3f–h**) that intra-NASh CBD caused significant increases in non-DA, presumptive VTA GABAergic neurons, we hypothesized that the behavioural effects of intra-NASh CBD may depend on NAc→VTA GABAergic projections and therefore depend on GABA receptor transmission directly in the VTA. Accordingly, using a functional cerebral disconnection procedure (see Materials and Methods), we examined whether intra-VTA blockade of GABAergic transmission may modulate the ability of intra-NASh CBD to block associative fear memory in rats with unilateral cannulae implantations into the NASh and VTA. Rats received a combination of intra-VTA bicuculline (50 ng) and saclofen (100 ng) to block intra-VTA GABA$_{A/B}$ receptor transmission immediately before a unilateral micro- infusion of intra-NASh CBD (100 ng) in the contralateral hemisphere (**Figure 2.5a**). Histological analysis revealed intra-VTA injector locations within the anatomical boundaries of the VTA or NASh as defined by Paxinos and Watson (2005) (**Figures 2.5b and c**). ANOVA comparing behavioural associative freezing scores across groups revealed a significant main effect of group ($F_{(3,38)}$ =3.261; $p \leq 0.05$; **Figure 2.5d**) on percentages of time spent freezing to either CS+ or CS− presentations. Post hoc analyses revealed that VEH control rats (NASh/ VEH vs VTA/ VEH; $n = 6$) demonstrated robust associative freezing behaviours in response to CS+ presentations (p < 0.01). In contrast, rats

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receiving the intra-NASh CBD ($n = 6$; 100 ng/ 0.5 µl) with intra-VTA VEH showed no associative fear memory ($p > 0.05$), consistent with previous results. However, rats receiving intra-VTA GABA$_{A/B}$ antagonists before intra- NASh CBD (100 ng/ 0.5 µl) ($n = 6$) demonstrated robust associative freezing behaviours in response to CS+ presentations ($p < 0.05$), demonstrating that contralateral blockade of VTA GABA$_{A/B}$ transmission is sufficient to reverse the behavioural effects of intra-NASh CBD on blockade of associative fear memory formation. Finally, to ensure that intra-VTA GABA$_{A/B}$ receptor blockade did not in itself influence fear memory formation, a separate group received intra-VTA GABA$_{A/B}$ antagonist treatment before intra-NASh VEH. Similar to VEH/ VEH-treated controls, these rats displayed significant associative fear responses during CS+ presentations ($n = 6$; $p < 0.05$).
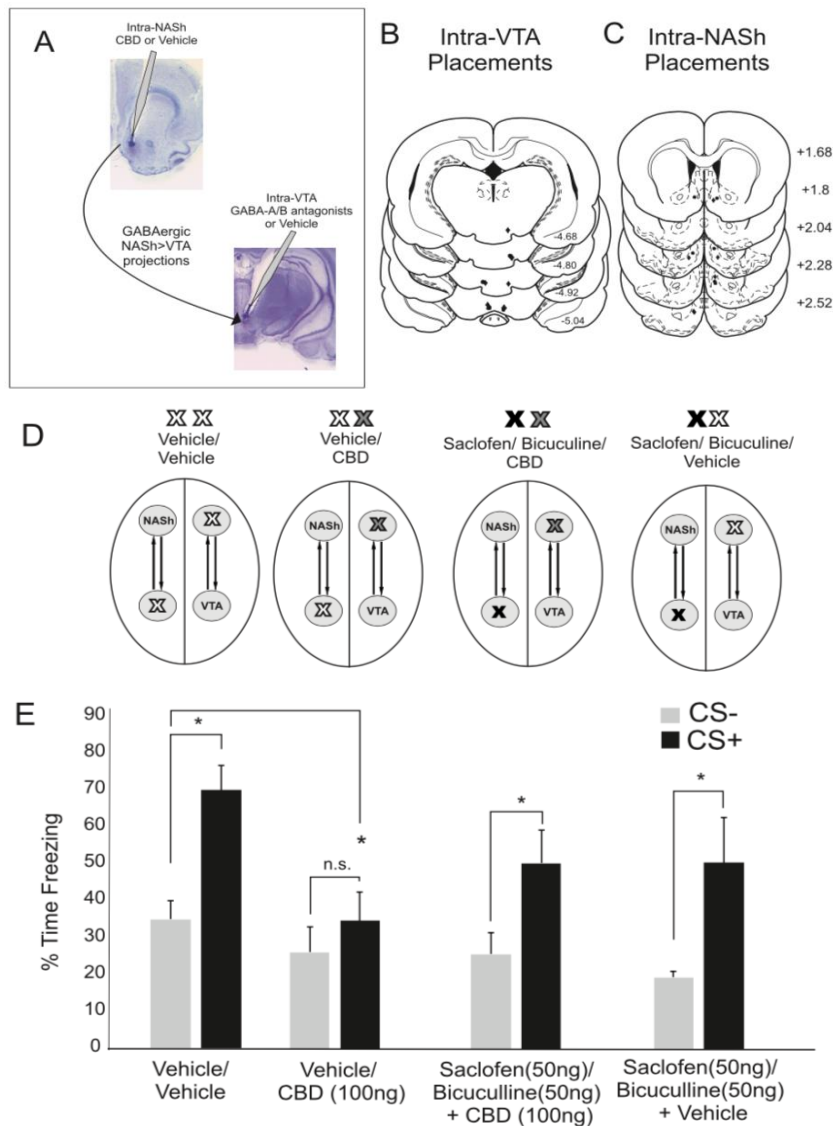
Figure 2.5 Disconnection of NASh/VTA GABA$_{A/B}$ transmission restores associative fear memory formation. A, Summary of experimental design for NASh-VTA disconnection procedure with sample microphotographs showing representative intra- VTA and intra-NAc microinjector placements. **B,C,** Schematic summaries showing unilateral placements for intra-VTA microinfusions of the GABA$_{A/B}$ antagonist mixture and intra-NASh CBD microinfusion locations (see methods) ● = 100ng CBD+ 50ng Saclofen/ 50ng Bicuculine group, ♦ = 100ng CBD + saline group. **D,** Schematic representation of experimental groups with description of contralateral intra-VTA vs. intra-NASh treatments. **E,** Consistent with previous results (Figure.

2.2a), intra-NASh CBD effectively blocks formation of associative fear memory in rats receiving intra-NASh CBD vs. contralateral intra-VTA VEH, relative to VEH/VEH controls. In contrast, rats receiving intra-NASh CBD vs. intra-VTA GABA$_{A/B}$ receptor blockade in the contralateral hemisphere, show no blockade of fear memory formation. In addition, rats receiving intra-NASh CBD vs. contralateral VTA GABA$_{A/B}$ receptor blockade demonstrate normal fear memory formation.

2.4 DISCUSSION

In the present study, we report that intra-NASh CBD blocks the formation of fear-related memory via a 5-HT$_{1A}$ receptor- dependent mechanism. Furthermore, we found that intra-NASh CBD decreased spontaneous VTA DAergic neuronal activity, both in terms of frequency and bursting levels, and conversely, increased the spontaneous activity of non-DAergic, presumptive VTA GABAergic neurons. CBD's modulation of VTA DAergic neuronal activity was dependent on intra-NASh 5-HT$_{1A}$ transmission, but not DAergic or CB$_1$ receptor transmission, as only blockade of 5-HT$_{1A}$ transmission reversed the effects of CBD on VTA DAergic activity states and restored associative fear memory formation. Finally, consistent with our electrophysiological findings, functional disconnection of GABAergic transmission between the contralateral NASh and VTA reversed the effects of intra-NASh CBD on associative fear memory formation. Taken together, these findings demonstrate several new insights into the potential role for CBD in the modulation of emotional memory processing within the mesolimbic pathway, implicating serotonergic transmission as an important mediator of these effects. Our findings are consistent with previous research demonstrating a role for CBD in modulating emotional memory. For example, Stern, Gazarini, Takahashi, Guimarães, and Bertoglio (2012) reported that systemically administered CBD could block reconsolidation of associative fear memories in rats in a temporally specific manner. Das et al. (2013) reported that systemic CBD could enhance the consolidation of fear-related extinction learning in human subjects. Marinho, Vila-Verde, Fogaça, and Guimarães, (2015) recently reported that CBD microinfusions into the prelimbic or infralimbic regions of rat prefrontal cortex could induce either anxiolytic or anxiogenic effects through a 5-HT$_{1A}$-dependent mechanism. The present findings are the first report that CBD can

produce effects on fear-memory acquisition directly in the NAc, via 5-HT$_{1A}$-dependent modulation of VTA neuronal activity.

2.4.1 CBD Modulates Conditioned Freezing Behaviour Through a 5-HT$_{1A}$-Dependent Mechanism in the NAc Considerable

Considerable evidence links the pharmacological and behavioural effects of CBD to the serotonergic system. For example, CBD is a 5-HT$_{1A}$ receptor agonist (Russo et al., 2005) and systemic CBD modulates the reward-facilitating effects of morphine via 5-HT$_{1A}$ receptors in the dorsal raphe nucleus (Katsidoni, Anagnostou, & Panagis, 2013). In addition, CBD has been shown to modulate anxiety-like behaviours through actions on the 5-HT$_{1A}$ receptor (Fogaca et al., 2014; Marinho et al., 2015). Consistent with the current findings, previous evidence has demonstrated that CBD's ability to interact with the mesolimbic system involves 5-HT$_{1A}$ receptor transmission. For example, 5-HT$_{1A}$ activation in the NASh blocks apomorphine-induced behaviours in rodents (Fujita et al., 2008), inhibits amphetamine-induced DA release in the rat NAc (Ichikawa, Kuroki, Kitchen, & Meltzer, 1995), and reduces striatal DA synthesis (Johnson, Tsai, Shahan, & Azzaro, 1993). Given these effects of 5-HT$_{1A}$ activation, blockade of 5-HT$_{1A}$ transmission would be expected to counteract the effects of CBD, removing the inhibitory effects of intra-NASh CBD on VTA DAergic activity. This prediction is consistent with our electro-physiological findings showing that blockade of 5-HT$_{1A}$ transmission restores VTA DAergic tone in the presence of CBD and reverses the behavioural effects of CBD on fear memory formation. In contrast, co-administration of a DA receptor antagonist or selective CB$_1$ receptor antagonist failed to reverse the behavioural effects of CBD. Previous studies have reported that CBD can act as an antagonist or inverse agonist of the CB$_1$ receptor (Pertwee, 2008; Thomas et al., 1998). However, other reports have found that CBD has relatively low binding affinity and/or activity at the CB1 receptor (Bisogno et al., 2001; Thomas et al., 2007). The present findings would suggest that, at least within the NASh, CBD produces its effects through non-CB$_1$ receptor-dependent mechanisms.

2.4.2 CBD Modulates Neuronal Activity Patterns in the VTA Through a 5-HT$_{1A}$-Dependent Mechanism

Interestingly, previous studies using systemic CBD administration have reported both effects of CBD on spontaneous activity patterns in VTA neuronal populations (French, 1997) or increases in mesolimbic DA release (Murillo-Rodríguez, Millán-Aldaco, Palomero-Rivero, Mechoulam, & Drucker-Colín, 2006). These discrepancies are likely due to differences in administration routes, as the present study exclusively used targeted microinfusions into the NASh vs systemic administration. However, it is of interest that systemically applied CBD may be interacting with neural circuits extrinsic to the mesocorticolimbic system, which may in turn produce different effects on DAergic transmission patterns. Within the VTA, we found that behaviourally effective doses of intra-NASh CBD were sufficient to inhibit VTA DAergic activity levels while simultaneously increasing the activity states of presumptive inhibitory VTA GABAergic neurons. This effect is consistent with previous observations demonstrating that blunting or potentiating VTA DAergic neuronal activity can potently modulate the formation of associative fear memories. For example, we have reported that activation of CB1 receptors in the rat prefrontal cortex can simultaneously block the formation of normally supra-threshold conditioned freezing behaviours and inhibit subcortical DAergic activity (Draycott et al., 2014). In contrast, overstimulation of DAergic transmission in the PFC can amplify normally non-salient associative fear conditioning cues (Lauzon et al., 2009).

The present findings suggest that modulation of VTA DAergic activity via CBD/ 5-HT$_{1A}$-dependent mechanisms may similarly regulate emotional salience and the acquisition of conditioned freezing behaviour via functional interactions with select VTA neuronal populations. For example, by blunting VTA DAergic neuronal activity during the acquisition of associative fear memory, CBD may serve to block conditioned freezing behaviours. Future studies are required to more precisely characterize the effects of CBD across these distinct learning phases of emotional memory processing. How might accumbal 5-HT$_{1A}$ transmission modulate upstream DAergic mesolimbic transmission? Anatomical evidence demonstrates that NAc GABAergic MSNs send functional GABAergic projections to the VTA (Kalivas et al., 1993; Nauta et al., 1978). In the present study, we found that intra-NASh CBD increased the activity rates of presumptive, non-DA GABAergic neurons in the VTA. Accordingly, we hypothesized that intra-

NASh CBD may regulate VTA DAergic activity (and corresponding fear-related associative memory formation) via indirect modulation of GABAergic transmission substrates in the VTA. To test this, we performed a disconnection study wherein intra-NASh CBD effects were disconnected from the contralateral hemisphere with combined intra-VTA microinfusions of GABA$_{A/B}$ receptor antagonists. Consistent with this hypothesis, disconnection blockade of VTA GABAergic transmission was capable of reversing the effects of intra-NASh CBD on fear memory formation. While beyond the scope of the present study, one interesting implication from the present results is that 5-HT$_{1A}$ receptor activation within the NASh might similarly modulate downstream VTA neuronal activity states and fear memory formation. Future studies are required to investigate these possibilities.

2.4.3 Implications of CBD's Modulatory Role on Mesolimbic Activity

The present study adds to a growing body of preclinical and clinical evidence demonstrating that CBD can modulate activity states of the mesolimbic DA system, and, in turn, regulate emotional processing. Importantly, the ability of CBD to inhibit mesolimbic DAergic activity and blunt fear- related memory formation is particularly intriguing given the large body of evidence demonstrating that the primary psychoactive phytochemical in cannabis, THC, produces pro-psychotic effects (Kuepper et al., 2011). Furthermore, in direct contrast to the effects of CBD, THC has been shown to induce a state of hyperactive mesolimbic DAergic activity following adolescent neurodevelopmental exposure (Renard et al., 2017). In addition, we have recently reported that intra-NASh CBD blocks hyper-DAergic activity and psycho- motor sensitization induced by amphetamine exposure (Renard et al., 2016). Several clinical and preclinical reports have suggested that CBD can reduce anxiety and may serve to ameliorate symptoms associated with emotional memory disorders such as PTSD. For example, CBD has been reported to facilitate the extinction of previously acquired traumatic or associative fear memories (Bitencourt, Pamplona, & Takahashi, 2008; Das et al., 2013; Stern et al., 2012). While currently no clinical evidence has reported specific long-term therapeutic effects of CBD in PTSD patient populations, PTSD has been associated with dysregulation of 5-HT$_{1A}$ expression levels in brain regions responsible for emotional memory and processing, such as the amygdala (Sullivan et al., 2013). Furthermore, preclinical evidence has shown also that genetic deletion of the 5-HT$_{1A}$ receptor in mice leads to potentiated reactivity to fear-related conditioning cues (Klemenhagen,

Gordon, David, Hen, & Gross, 2006). Future research is required to more precisely characterize the mechanisms by which CBD and 5-HT$_{1A}$ receptor substrates may interact and how these processes may, in turn, regulate emotional memory formation. In summary, the present study reveals several novel insights regarding the functional effects of CBD on emotional memory processing and identifies a 5-HT$_{1A}$ receptor mechanism within the NASh as an underlying mechanism by which CBD may regulate mesolimbic activity and modulate the formation of associative emotional memories. Furthermore, these findings underscore growing evidence demonstrating that differential phytochemical constituents of cannabis (CBD vs THC) can produce divergent and opposing effects on DAergic activity states and emotional memory behaviours.

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3   The Bivalent Rewarding and Aversive properties of Δ^9-
tetrahydrocannabinol are Mediated Through Dissociable
Opioid Receptor Substrates and Neuronal Modulation
Mechanisms in Distinct Striatal Sub-Regions

3.1 INTRODUCTION

Δ^9-tetrahydrocannabinol (THC), the primary psychoactive component of cannabis, strongly modulates affective processing via interactions with the mesolimbic circuitry. THC can produce both rewarding and aversive effects in humans and other animals (Chen, Paredes, Lowinson, & Gardner, 1991; Lepore, Vorel, Lowinson, & Gardner, 1995; Parker & Gillies, 1995) and strongly modulates the mesolimbic dopamine (DA) system. For example, acute THC administration in the rodent NAc strongly activates ventral tegmental area (VTA) DAergic neuronal activity (Fitoussi, Zunder, Han, & Laviolette, 2018) and systemic THC administration directly activates VTA DA neurons (French, 1997). In humans, chronic cannabis use has been shown to induce salience sensitization to cues associated with cannabis administration, via sensitization of the mesolimbic system (Filbey et al., 2016). Nevertheless, the precise neuroanatomical and pharmacological mechanisms by which THC regulates affective processing in the mesolimbic pathway are not currently understood.

The primary pharmacological target of THC, CB_1 receptors (CB1R), are widely distributed throughout the brain, including the mesolimbic circuitry. Substantial evidence implicates the nucleus accumbens (NAc) as an important area for THC's effects on cognitive and affective processing. For example, systemic THC causes opioid receptor (OR)- dependent DA efflux in the NAc (Chen et al., 1990; Chen, Paredes, Lowinson, & Gardner, 1991) and chronic THC exposure alters synaptic plasticity within the NAc (Hoffman, Oz, Caulder, & Lupica, 2003). Importantly, the NAc is involved in both reward and aversion processing, particularly the medial shell sub-region (NASh), which is implicated in motivational salience, the processing of emotional stimuli, reinforcement, and addiction (Baliki et al., 2013; Bozarth, 2015; Calipari et al., 2016; Saddoris et al., 2015).

The accumbens is a highly complex limbic structure involved in both reward and aversion signaling. Previous evidence has identified the anterior pole of the NASh as a region containing high concentrations of μ-opioid receptors (Arvidsson et al., 1995). Thus, stimulation of the anterior NASh with a μ-opioid receptor (μOR) agonist produces reward while stimulation of the posterior NASh with a κ-opioid (κOR) agonist, produces aversion (Castro & Berridge, 2014). In addition, there is significant co-localization of CB1Rs and ORs within the NASh region (Pickel,

Chan, Kash, Rodríguez, & Mackie, 2004). However, how an exogenous cannabinoid like THC might influence striatal affective processing through CB1-OR signaling remains unknown. Given that THC serves as a partial CB1 receptor agonist, this evidence may suggest that the differential affective stimulus effects of THC might depend upon differential activation of anatomically distinct OR subtypes, within the mammalian NAc.

At the neuronal level, medium spiny neuron (MSN) activity states within the NAc are functionally linked to reward and aversion processing, with reward states characterized by decreased MSN activity levels, and aversive states associated with increased activity (Tasha Ahmad, Sun, Lyons, & Laviolette, 2017; Carlezon & Thomas, 2009). Additionally, neuronal population activity within the NAc, specifically γ-oscillations, are associated with the processing of reward-related information (Kalenscher, Lansink, Lankelma, & Pennartz, 2010) and disturbances in γ-oscillations are linked to various neuropsychiatric disturbances, including schizophrenia-related affective dysregulation (Sun et al., 2011).

In the present study, we investigated the effects of direct microinfusions of THC on reward and aversion conditioning, social behaviour and neuronal activity states within the NASh. Using a combination of behavioural assays and *in vivo* neuronal electrophysiology, we report that THC infused into the anterior NASh produces μ-opioid receptor dependent reward effects, potentiates morphine reward salience, decreases medium spiny neuron activity and increases the power of high frequency γ-oscillations. In contrast, THC in the posterior NASh produces κOR dependent aversion effects, impairs social recognition memory, increases medium spiny neuron activity and decreases the power of high frequency γ-oscillations in local field potential recordings. These findings reveal critical new insights into the underlying neuronal and pharmacological mechanisms responsible for the bivalent motivational effects of THC, directly in the NAc.

3.2 MATERIALS AND METHODS

3.2.1 Animals and Surgery

Male Sprague Dawley rats (300 to 350 g; Charles River, Quebec, Canada) were used in compliance with the Canadian Council for Animal Care and institutional guidelines. Rats were

housed under controlled conditions (12-hour light/dark cycle and food/water access ad libitum). Rats were anesthetized with a mixture of ketamine (80 mg/ml) and xylazine (6 mg/ml) and placed in a stereotaxic device. The anatomical criteria for defining the anterior vs. posterior poles of the NASh were based upon previously reported studies which have anatomically defined these sub-regions based upon pharmacologically defined dissociations between positive vs. negative affective behavioural outcomes (Castro *et al*, 2016; Castro and Berridge, 2014; Mahler *et al*, 2007; Reynolds and Berridge, 2002; Smith and Berridge, 2007). In addition, pilot CPP studies conducted in our laboratory revealed that an anterior-posterior demarcation at > 2.5 mm from bregma (anterior) vs. <1.5 mm from bregma (posterior) represented the most reliable boundary for producing THC-related CPP vs. THC-related CPA behaviours. Stainless steel guide cannula (22 gauge; PlasticsOne) were implanted bilaterally into either the aNASh at the following coordinates (Paxinos & Watson, 2005) : anteroposterior (AP): +2.5 mm from bregma, lateral (L): ±2.6 mm, dorsal-ventral (DV): -7.4 mm from the dural surface, or the pNASh at the following coordinates: (AP): +1.5 mm from bregma, (L): ±2.6 mm, (DV): -7.4. Guide cannulae were held in place using jeweler's screws and dental acrylic. After completion of behavioural experiments, rats received an overdose of pentobarbital (240 mg/kg, i.p.) and were perfused with isotonic saline followed by 10% formalin. Brains were extracted and post-fixed 24 hrs before being placed in a 25% formalin-sucrose solution for one week. Brains were sliced (60 μm) using a cryostat and stained with Cresyl violet. Injector tips placements were localized using a light microscope. Rats with cannula placements found outside the anatomical boundaries of the NASh were excluded from data analysis.

3.2.3 Drug Administration

Rats received intra-NASh microinfusions with either THC (Cayman Chemical, 10-100ng/0.5 µl), cyprodime (CYP; Tocris; 500ng or 1µg/ 0.5µl), *nor*-binaltorphimine dihydrochloride (nor-BNI; Tocris; 500ng or 1µg/ 0.5µl), THC co-administered with CYP, or THC co-administered with nor-BNI, immediately prior to conditioning. Intra-NASh drugs were delivered via a 28-gauge microinfusion syringe over a period of 1 minute. Microinjectors were left in place for an additional 1 min to ensure adequate diffusion from the tip. All intracerebral infusions were 0.5 µL total volume per side. Morphine sulfate (Macfarland-Smith) was administered i.p. The intra-cranial dose selections for the above compounds were based upon our previously published or

piloted dose-response curves using these compounds (Ahmad, Lauzon, de Jaeger, & Laviolette, 2013; Norris et al., 2016) which demonstrated maximal behavioural efficacy and the absence of non-specific behavioural side effects. For morphine CPP experiments, rats were injected with either morphine (0.05mg/kg) or saline on alternating conditioning days.

3.2.4 Conditioned Place Preference

All rats were conditioned using an unbiased, fully counterbalanced place conditioning procedure as described previously (Ahmad et al., 2013; Laviolette and van der Kooy, 2003). The two conditioning environments differed in smell, texture and color. One environment was black, with a smooth Plexiglass floor wiped down with 2% acetic acid prior to each conditioning session. The other environment was white, with a wire mesh floor covered with woodchips. Prior to commencement of CPP conditioning, rats are given a preconditioning phase where they are placed into a motivationally neutral gray box for 20 min, 24h prior to start of conditioning. CPP conditioning consists of 4 drug-environment and 4 vehicle-environment pairings once per day, alternating over an 8-day period. Environmental conditioning exposures are fully counterbalanced for both environment assignment and drug/ vehicle presentations. During the CPP test phase, rats are placed in a neutral gray zone separating the drug and vehicle environments and allowed to move freely for a period of 10 min between environments. Times spent in each environment are digitally recorded and analyzed offline. All rats are tested in a drug-free state. For experiments examining the effects of intra-NASh THC and/or opioid receptor challenges, experimental groups received intra-NASh microinfusions of either THC (10-100ng /0.5 µl), cyprodime (500ng-1µg/ 0.5µl), nor-BNI (500ng-1µg/ 0.5µl), THC co-administered with cyprodime, or THC co-administered with nor-BNI vs. VEH microinfusions. A follow-up CPP experiment examine the potential effects of intra-NASh THC on morphine reward salience, using a sub-threshold conditioning dose of morphine (0.05 mg/kg, i.p.) which we have previously reported does not produce significant morphine CPP in and of itself (Ahmad *et al*, 2013; Ahmad and Laviolette, 2017; Loureiro *et al*, 2016a). For these experiments, THC or VEH control groups received either anterior or posterior THC or VEH microinfusions before being injected with either sub-threshold morphine (0.05mg/kg) or saline on alternating conditioning days. Thus, the potential motivational effects of intra-NASh THC (rewarding or aversive) were balanced across both saline and morphine conditioning sessions.

3.2.5 Sucrose Preference Test

Prior to testing, rats were water deprived for 12 hrs. At testing, rats were given two bottles of a 2% sucrose solution to drink for 12 hrs. After exposure to the sucrose solution, rats were micro-infused with either THC (100ng/0.5µl) or vehicle. They were then placed back in their cages and given access to one bottle of regular water or one bottle of 2% sucrose solution. After 1 hr, bottles were removed and the weight of the bottles pre- vs. post-test were compared to determine the amount of each liquid consumed to determine any increased preference for sucrose.

3.2.6 Sociability and Social Memory

Testing was performed in a rectangular, three-chambered box. For the sociability test, rats were placed in the middle chamber for 5 minutes. Following habituation, an unfamiliar male rat was placed in one of the side chambers in a rectangular plexiglass cage. The location of the rat was counterbalanced between subjects. The subject was then infused with either THC (100ng/ 0.5µl) or vehicle in either their aNASh or pNASh. The subject was then allowed to explore the entire apparatus for 8 minutes. Entries were defined as all four paws present in one chamber. Behavioural performance was expressed using sociability scores (i.e., difference between times spent in stranger vs. empty compartments). Next, to evaluate social recognition, each rat was tested in an 8-minute session to evaluate social memory. A second, unfamiliar rat was placed in the previously empty chamber. The test rat had a choice between the previously encountered rat versus the novel, unfamiliar rat. Times spent in each chamber were recorded, and a social recognition score (i.e., difference between times spent in the nonfamiliar vs. familiar rat chamber) was calculated for each rat. Times spent in each chamber were recorded with a video-tracking system (ANY- maze) during all tests. A sociability (time spent with the other rat vs. time spent with the empty cage) and social recognition score (time spend with the novel rat vs. time spent with the novel rat) was calculated for each rat.

3.2.7 *In Vivo* Electrophysiological Recordings

In-vivo extracellular recordings were performed as described previously (Lintas et al., 2012; Loureiro, Kramar, Renard, Rosen, & Laviolette, 2016b; Loureiro et al., 2015). Rats were

anesthetized with urethane (1.4 g/kg, i.p.) and placed in a stereotaxic apparatus with body temperature maintained at 37°C. A scalp incision was made to remove the skin above the skull, and holes were drilled in the skull above the NASh and the cranial ventricle. For intra-cranial ventricle (ICV) microinfusions of THC (1 µg/µL), a 10 µL gastight Hamilton syringe was slowly lowered into the cranial ventricle (15° angle): AP: -0.9 mm from bregma, LAT ±2.7 mm, DV: -3.8 mm from the dural surface. For intra-NASh extracellular recording, glass micro-electrodes (with an average impedance of 6 to 8 MΩ) filled with a 2% Pontamine Sky Blue solution were lowered using a hydraulic micro-positioner (Kopf 640) at the following flat skull stereotaxic coordinates: AP: +1.5 or +2.5 mm from bregma, LAT: ± 0.8 mm, DV: -6.0 to -8.0 mm from the dural surface. Extracellular signals were amplified using a MultiClamp 700B amplifier (Molecular Devices) and recorded through a Digidata 1440A acquisition system (Molecular Devices) using pClamp 10 software. Extracellular recordings were filtered at 1 kHz and sampled at 25 kHz. NASh medium spiny neurons were identified using previously established criteria. Any cells with a spike width of less than 1ms and more than 2ms were excluded from analysis. The electrode was used to simultaneously record local field potentials (LFP). Recording analyses were performed with Clampfit 10 software. Response patterns of isolated NASh neurons and LFPs to microinfusion of THC alone or in combination with either CYP or nor-BNI were determined by comparing neuronal frequency rates and local field potentials (LFP) oscillatory patterns between the 10-minute pre- vs. post-infusion recording epochs. A cell was considered to have changed its firing rate if there was a minimum of 20% difference in frequency rate from baseline. The electrode was used to simultaneously record LFPs. LFP signals were uploaded to NeuroExplorer decimated at $n = 25$. LFP signals then had a Butterworth filter applied and a Gaussian filter of 4 for smoothing. For histological analysis of extracellular NASh neuronal recording sites, recording electrode positions were marked with iontophoretic deposit of Pontamine Sky Blue dye (−20 µA, continuous current for 12–15 minutes). Brains were removed and post-fixed 24 h before being placed in a 25% formalin-sucrose solution for one week before sectioning (60 µm). Following this, sections were stained with neutral red and infusion/ neuronal recording sites were confirmed with light microscopy.

3.3 RESULTS

3.3.1 Histological Analysis

Histological analysis revealed injector placements localized within the anatomical boundaries of the shell subdivision of the NASh. Sample NASh microinfusion locations and schematic illustration of several experimental groups across the anterior-posterior axis of the NASh are shown in **Figure 3.1**.
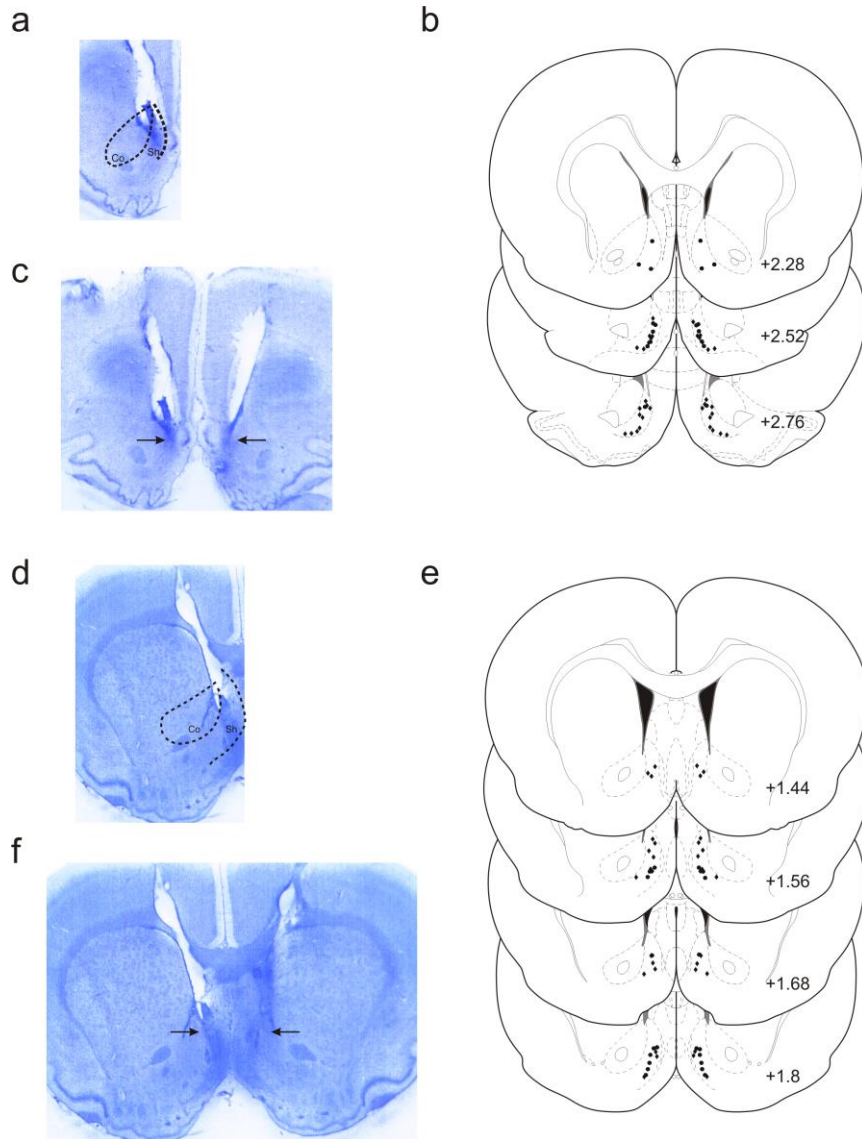
Figure 3.1 Histological analysis of intra-NASh microinjection sites. A, Microphotograph of representative injector placement within the anterior portion of the nucleus accumbens shell. **B,** Schematic representation of select intra-anterior-NASh injector locations; ● = 100 ng THC group, ◆ = 100 ng THC + 1 µg CYP. **C,** Microphotograph of representative intra-anterior-NASh

bilateral cannulae placements. **D,** Microphotograph of representative injector placement within the posterior portion of the nucleus accumbens shell. **E,** Schematic representation of select intra-anterior-NASh injector locations; ● = 100 ng THC group, ◆ = 100 ng THC + 1 µg CYP. **F,** Microphotograph of representative intra-anterior-NASh bilateral cannulae placements

3.2.2 Intra-NASh THC produces dose-dependent, anatomically dissociable reward or aversion effects via separate opioid receptor substrates

Given previous evidence demonstrating functional differences in anterior vs. posterior NASh region in reward vs. aversion processing (Skelly et al., 2010) and evidence demonstrating that cannabinoid signaling can modulate reward or aversion signals via MOR vs KOR receptor substrates (Ahmad et al., 2013), we first examined the effects of THC (10ng or 100ng/0.5µl), directly in the aNASh or pNASh alone, or in combination with selective MOR [CYP(0.5µg and 1µg/0.5µl)] vs. KOR [Nor-BNI (1µg/0.5µl)] antagonists using a CPP procedure (*see methods*). We hypothesized that aNASh THC would produce rewarding effects through selective MOR transmission, whereas pNASh THC would produce aversive behavioural effects through a KOR-dependent signaling mechanism. First, we examined the potential motivational effects of intra-aNASh THC. ANOVA comparing time spent in the drug-paired environment and the vehicle paired environment between groups revealed a main effect of environment ($F_{(1,110)} = 46.094$, $p < .001$) and a significant interaction of treatment and environment ($F_{(5,110)} = 9.802$, $p < .001$; **Figure 3.2A**) on times spent in drug vs. VEH-paired environments. *Post-hoc* analyses showed that rats microinfused with the following drugs spent significant more time in the drug-paired environment than the vehicle-paired side: THC (100 ng; n = 10; $p = .002$), THC (100 ng) + CYP (0.5 µg; n = 10; $p = .01$), or THC (100 ng) + nor-BNI (1 µg; n = 10; $p = .002$). In contrast, rats receiving vehicle ($n = 20$), a lower dose of THC (10 ng; $n = 10$), THC (100 ng) + CYP (1 µg; $n = 11$) displayed no significant difference between time spent in the drug-paired environment and the vehicle-paired environment ($p < .05$; **Figure 3.2A**). Intra-aNASh CYP (1µg) alone displayed no significant difference between time spent in the drug-paired environment and the vehicle-paired environment ($p < .05$; **Figure 3.2C**). These results indicate that bilateral THC in the anterior NASh produces dose-dependent conditioned place preference through a MOR dependent-substrate, independently of KOR transmission.

Next, we examined the motivational effects of intra-pNASh THC. Again, using two different doses of THC (10ng and 100ng/0.5µl), two doses of the selective KOR antagonist, Nor-BNI (0.5µg and 1µg/0.5µl) in combination with the higher dose of THC, Nor-BNI alone (1µg/0.5µl) or the higher dose of THC in combination with CYP (1µg/0.5µl). ANOVA comparing difference scores between groups reveled a main effect of environment ($F_{(1,116)} = 26.584$, $p < .001$) and a significant interaction of treatment and environment ($F_{(5,116)} = 7.950$, $p < .001$; **Figure 3.2B**). *Post-hoc* analyses showed that rats microinfused with the following drugs spent significant more time in the vehicle-paired environment than the time-paired side: THC (100 ng; $n = 11$; $p = .004$), THC + nor-BNI (0.5 µg; $n = 11$; $p = .009$), and THC (100 ng) + CYP (1 µg; n = 11; $p < .001$). In contrast, rats receiving vehicle ($n = 10$) a lower dose of THC (10 ng; $n = 10$), THC + a higher dose of nor-BNI (1 µg; $n = 11$), or nor-BNI alone (1 µg; $n = 10$) displayed no significant difference between time spent in the drug-paired environment and the vehicle-paired environment ($p < .05$; **Figure 3.2B**). Intra-pNASh nor-BNI (1µg) alone displayed no significant difference between time spent in the drug-paired environment and the vehicle-paired environment ($p < .05$; **Figure 3.2C**). Thus, bilateral THC in the posterior NASh produces dose-dependent conditioned place aversion (CPA). These aversive effects were dose-dependently blocked by a selective KOR antagonist, but not by a MOR antagonist.

3.3.3 Intra-aNASh THC potentiates sub-threshold morphine reward salience

Given our findings that aNASh THC produced robust rewarding effects through a MOR-dependent substrate and previous evidence showing that stimulation of µORs within the NASh can potentiate drug reward salience (Richard & Fields, 2016), we next examined how intra-NASh THC may modulate the motivational effects of an exogenous opioid, morphine, using a sub-reward threshold conditioning dose of morphine (0.05 mg/kg; i.p.; *see methods*). ANOVA comparing time spent in the drug-paired environment and vehicle-paired environment between rats receiving intra-aNASh vehicle or THC (100 ng) revealed a main effect of environment ($F_{(1, 26)} = 21.224$, $p < .001$) and a significant interaction of treatment and environment ($F_{(1, 26)} = 6.172$, $p = .02$). *Post-hoc* analysis revealed that rats receiving intra-aNASh vehicle ($n = 8$) displayed no significant difference between time spent in the drug-paired environment and the vehicle-paired environment but rats receiving intra-aNASh THC spent significantly more time in the drug-

paired environment than the vehicle-paired environment (n = 7; p = .02; **Figure 3.2D**). ANOVA comparing time spent in the drug-paired environment and vehicle-paired environment between rats receiving intra-pNASh vehicle (n = 7) or THC (100 ng; n = 6) demonstrated no main effects or interaction (p >.05; **Figure 3.2D**). Thus, consistent with the ability of aNASh THC to produce rewarding effects through a MOR-dependent substrate, aNASh THC potentiated the reward salience of normally sub-reward threshold CPP. In contrast, THC in the pNASh had no effect on sub-threshold morphine CPP behaviours.
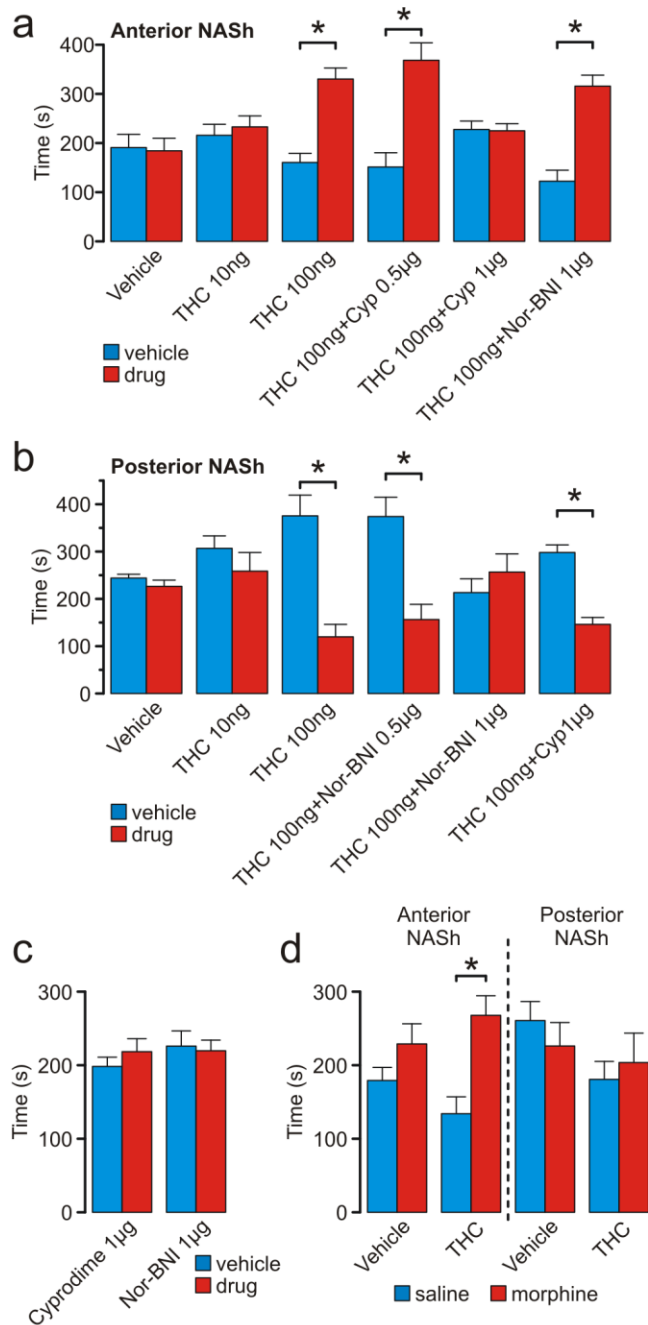
Figure 3.2 Effects of intra-NASh THC on conditioned place preference: interactions with the opioid system. A, Anterior NASh (+2.5mm from bregma) microinfusions of THC dose dependently increases preference for the drug paired side. Co-administration of cyprodime, but not Nor-BNI dose-dependently restores responding to baseline. **B,** Posterior NASH (+1.5mm from bregma) THC dose dependently decreases preference for the drug paired side. Co-administration of Nor-BNI, but not cyprodime, dose-dependently restores responding to baseline.

x

67

C, Infusions of THC (100 ng) into the anterior NASh, but not the posterior NASh, potentiate the reward to sub-threshold morphine (0.05 mg/kg) $* = p < .05$

3.3.4 Intra-NASh THC has no effect on sucrose reward processing

To determine if the effects of intra-NASh THC on affective processing may modulate non-drug-related motivational effects, we next examined the processing of natural, sucrose-related reward (*see methods*). Percent sucrose consumed was determined by dividing the amount of sucrose consumed by the amount of total liquid (water plus sucrose) consumed. Analyses showed no significant difference between rats receiving intra-aNASh vehicle ($n = 7$) and intra-aNASh THC ($n = 8$; $t_{(13)} = -.051$, $p > .05$) or intra-pNASh vehicle ($n = 8$) and intra-pNASh THC ($n = 8$, $t_{(14)} = .152$, $p > .05$; **Figure 3.3**), indicating that the effect of THC on reward and aversion in the NASh is drug-specific and does not generalize to natural reward.
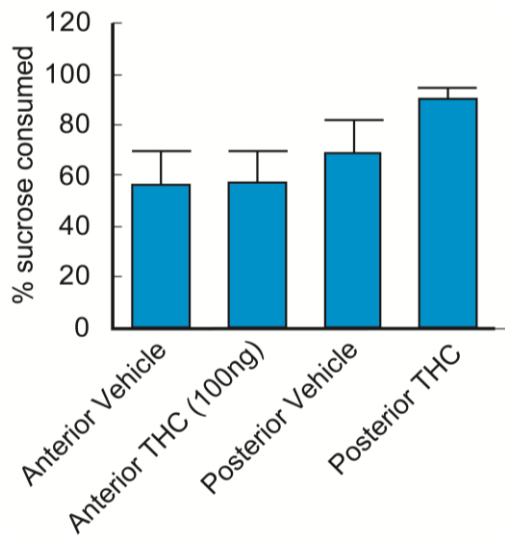
Figure 3.3 Effects of intra-NASh THC on a sucrose preference test. THC has no significant effect on the percent consumption of sucrose vs total liquid consumed.

3.3.5 THC modulates social interaction and cognition selectively in the posterior NASh

Previous studies have demonstrated that cannabinoid signaling can strongly modulate social behavioural phenomena through actions in the NASh (Skelly et al., 2010). Therefore, we examined the potential effects of intra-NASh THC on social motivation behaviours and cognition (social memory). A simplified diagram of the experimental procedure is presented in **Figure 3.4A-B**. In phase 1, sociability scores (measuring motivation to interact with a novel rat) were calculated by measuring times spent interacting with a novel rat and subtracting times spent interacting with an empty box. ANOVA showed a main effect of treatment on Phase 1 sociability scores ($F_{(3, 26)} = 3.156$, $p = .042$). *Post-hoc* analyses revealed a significant difference between intra-pNASh THC ($n = 8$) and intra-pNASh vehicle ($n = 8$; $p = .009$), intra-aNASh vehicle ($n = 7$; $p = .029$) and intra-aNASh THC ($n = 7$; $p = .038$; **Figure 3.4C**). No significant differences were observed between any other groups. In phase 2, social memory scores were calculated by taking times spent with a new, novel rat and subtracting times spent with the previously encountered, familiar rat. ANOVA showed a main effect of treatment for social memory scores ($F_{(3, 26)} = 3.516$, $p = .029$). *Post-hoc* testing revealed a significant difference between intra-pNASh THC ($n = 8$) and intra-pNASh vehicle ($n = 8$; $p = .013$), intra-aNASh vehicle ($n = 7$; $p = .009$) and intra-aNASh THC ($n = 7$; $p = .013$) (**Figure 3.4D**). Thus, intra-NASh THC selectively impairs natural social motivation and social memory cognition selectively in the posterior region of the NASh.
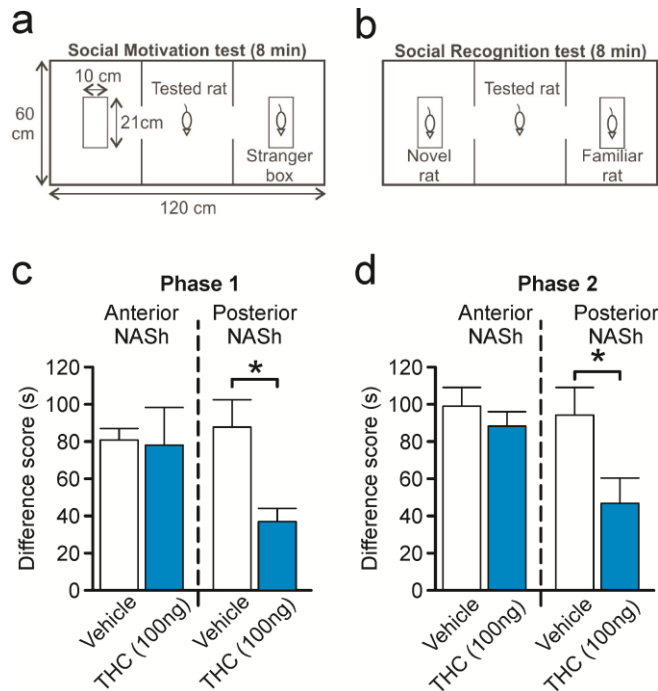
Figure 3.4 Effects of intra-NASh THC on social motivation and social recognition. A, B Apparatus and rats' placements for the sociability and social recognition test respectively. **C,** Microinfusions of THC in the pNASh, but not the aNASh, significantly reduced sociability $* = p$ <.05. **D,** Microinfusions of THC in the pNASh, but not the aNASh, significantly reduced social recognition $* = p < .05$.

3.3.6 THC differentially modulates medium spiny neuron activity in anterior vs. posterior NASh

The activity states of NASh MSN neurons are strongly correlated with reward vs. aversive motivational states (Carlezon & Thomas, 2009) and we have previously demonstrated that cannabinoid CB1 transmission can produce rewarding effects by inhibiting NASh MSN neurons or aversive effects by activating these neurons (Ahmad et al., 2017). To determine if the anatomically localized effects of THC on reward or aversion were correlated with MSN activity state modulation, we next performed *in vivo* single-unit recordings in the posterior and anterior NASh, combined with ICV infusions of THC. We used a dose 10x our highest behaviourally effective dose (1µg/µl) for these systemic studies to control for CSF diffusion (*see methods*). A total of n=15 MSNs were sampled in the aNASh (see **Figure 3.5A** for representative aNASh

recording location) and n=14 MSNs in pNASh (see **Figure 3.6A** for representative pNASh recording location). Population analysis of aNASh MSNs revealed that 66.6% showed decreased activity, 0% increased, and 33.3% were unchanged, relative to baseline, following THC administration (**Figure 3.5B**). An analysis of average firing frequency recorded 10 min pre vs. post ICV THC infusion revealed that THC significantly decreased firing rates in the aNASh ($t_{(14)}$ = 2.738, p = .016; **Figure 3.5C**). A representative rastergram showing a typical inhibitory response to THC in the aNASh is shown in **Figure 3.5D**.

For MSN neurons recorded in the pNASh, population analysis revealed that 14.3% of MSNs showed decreased activity, 50% increased and 35.7% showed no change, relative to baseline (**Figure 3.6B**). Analysis of average firing frequencies recorded 10 min pre vs. post ICV THC revealed that THC significantly increased firing rates relative to baseline ($t_{(13)}$ = -2.288, p = .04; **Figure 3.6C**). A representative rastergram showing a typical excitatory response pattern to THC administration in the pNASh is shown in **Figure 3.6D**. Thus, whereas THC strongly inhibits MSN activity in the aNASh, THC has a predominantly excitatory effect in the pNASh.
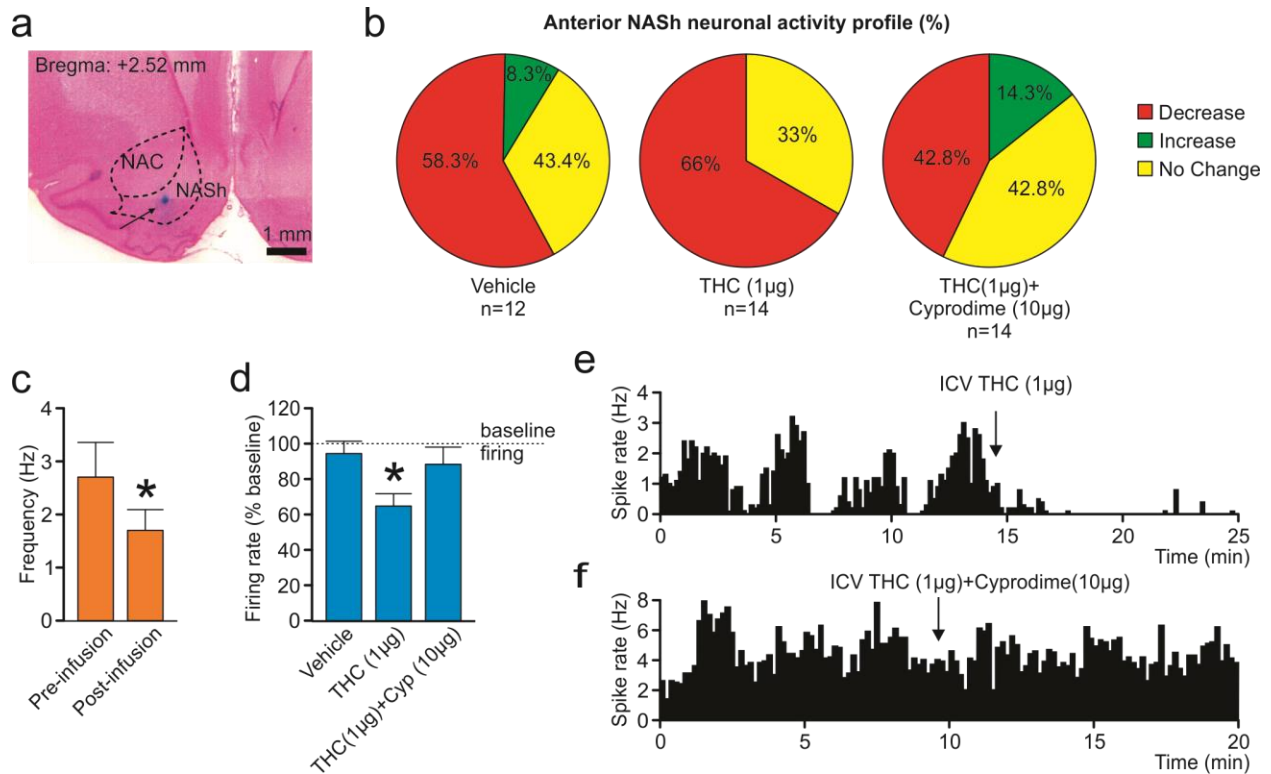
Figure 3.5 Effects of ICV THC and CYP on anterior NASh medium spiny neurons activity patterns. A, Representative microphotograph showing typical intra-aNASh in vivo MSN recording location **B,** Summary of experimental neuronal groups showing relative changes (no change, increase, or decrease) in firing frequencies following ICV pharmacological treatments **C,** ICV THC significantly decreased spontaneous aNASh MSN neuronal firing frequency. **D,** ICV THC alone (1µg/µl) caused a significant decrease in spontaneous aNASh MSN neuronal firing frequency rates vs baseline activity. This inhibitory effect was reversed by co-administration of the effective dose of CYP (10µg/µl). **E,** Sample rastergram showing typical aNASh MSN response pattern following ICV THC (1µg/µl) infusion. **F,** Sample rastergram showing typical aNASh MSN response pattern following ICV THC (1µg/µl) and CYP (10µg/µl) infusion.
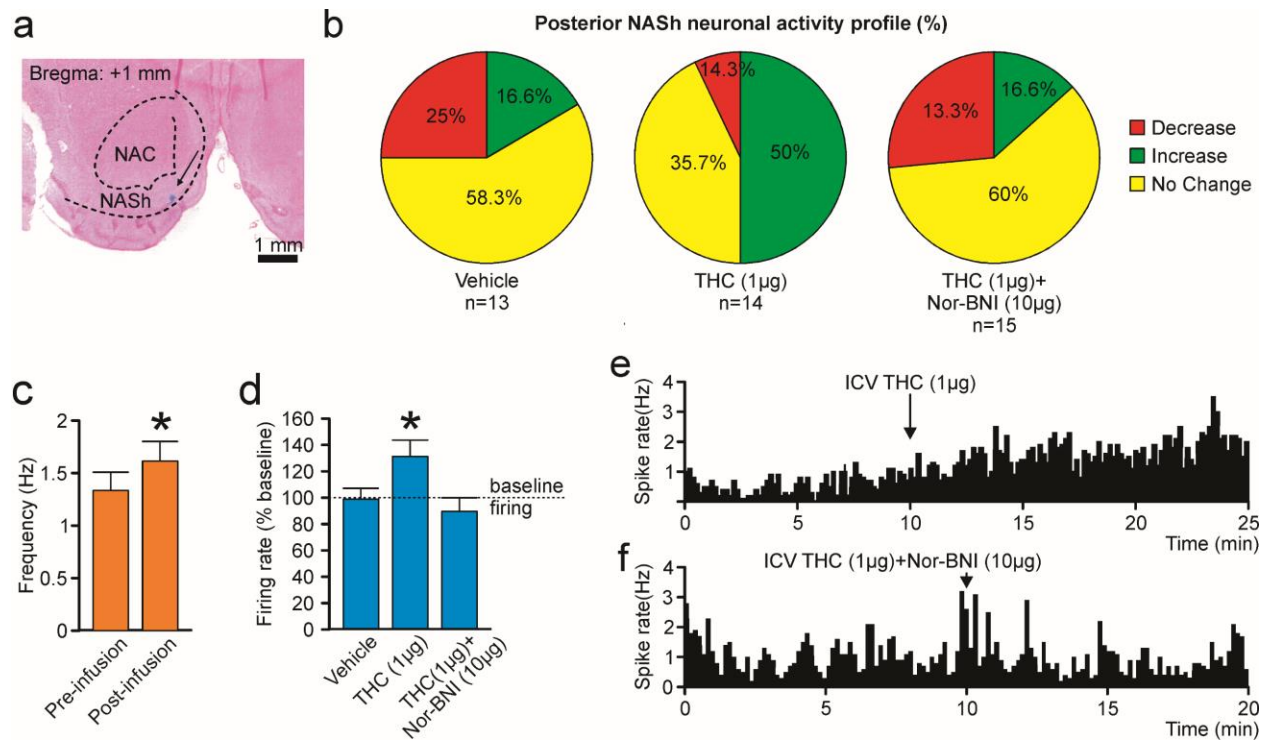
Figure 3.6 Effects of ICV THC and nor-BNI on MSN activity patterns in the pNASh. A, Representative microphotograph showing typical intra-pNASh in vivo MSN recording location. **B,** Summary of experimental neuronal groups showing relative changes (no change, increase, or decrease) in firing frequencies following ICV pharmacological treatments. **C,** ICV THC significantly increased spontaneous pNASh MSN neuronal firing frequency. **D,** ICV THC alone (1µg/µl) caused a significant increase in spontaneous aNASh MSN neuronal firing frequency rates vs baseline activity. This excitatory effect was reversed by co-administration of the effective dose of nor-BNI (10µg/µl). **E,** Sample rastergram showing typical pNASh MSN response pattern following ICV THC (1µg/µl) infusion. **F,** Sample rastergram showing typical aNASh MSN response pattern following ICV THC (1µg/µl) and nor-BNI (10µg/µl) infusion.

3.3.7 THC produces differential changes in the power of high-frequency γ-oscillations

In the above described studies, LFPs were recorded concurrently with single-unit activity. The signal was divided into bins of 2 seconds and 410 different frequency values. An analysis was performed to determine the power each frequency had on the signal. ANOVA tests were

conducted on delta, theta, alpha, beta, low-frequency gamma, and high-frequency gamma oscillations. Delta, theta, alpha showed no main effect of treatment. A sample spectrograph of an aNASh LFP of high-frequency γ-oscillations recording is shown in **Figure 3.7A**. ANOVA comparing the power of beta ($F_{(2,35)} = 7.857$, $p = .002$), low-frequency γ-oscillations ($F_{(2,35)} = 4.899$, $p = .013$; **Figure 3.7B**), high-frequency γ-oscillations ($F_{(2,35)} = 3.963$, $p = .028$; **Figure 3.7B**) between treatment groups revealed a significant main effect of treatment. *Post-hoc* analyses revealed that the change in power of beta and low-frequency γ-oscillations between rats treated with ICV THC and vehicle were not significantly different ($p > .05$). Rats receiving ICV THC + Cyprodime, however, showed significantly increased power of beta ($p = .001$) and low-frequency γ-oscillations ($p = .015$) compared to THC alone but not vehicle. Rats receiving ICV THC, however, showed significantly increased power of high-frequency γ-oscillations relative to VEH controls ($p = .010$; **Figure 3.7B**) or rats treated with ICV THC + CYP ($p = .045$; **Figure 3.7B**). The VEH group also did not differ significantly from the ICV THC + CYP group ($p > .05$; **Figure 3.7B**). THC, therefore, increased the power of high-frequency γ-oscillations in the aNASh and this effect was reversed by co-treatment with the MOR antagonist.

Next, we ran an analysis on pNASh LFPs. Delta, theta, alpha, and beta showed no main effect of treatment. A sample spectrograph of high-frequency gamma of pNASh LFP is shown in **Figure 3.7C**. ANOVA of low-frequency γ-oscillations ($F_{(2,38)} = 5.520$, $p = .008$) and high-frequency γ-oscillations ($F_{(2,38)} = 5.130$, $p = .011$; **Figure 3.7D**), however, revealed a main effect of treatment. *Post-hoc* analyses revealed that rats treated with THC+ NorBNI showed significantly increased power of low-frequency γ-oscillations versus THC alone but not vehicle. THC treatment significantly decreased the power of high-frequency γ-oscillations relative to VEH controls ($p = .004$; **Figure 3.6D**) or rats treated with ICV THC + Nor-BNI ($p = .019$; **Figure 3.7D**). Rats treated with ICV vehicle did not differ significantly from rats treated with ICV THC + Nor-BNI ($p > .05$; **Figure 3.6D**). Thus, in direct contrast to the anterior NASh, pNASh THC decreased the power of high-frequency γ-oscillations, through a KOR-dependent signaling mechanism.
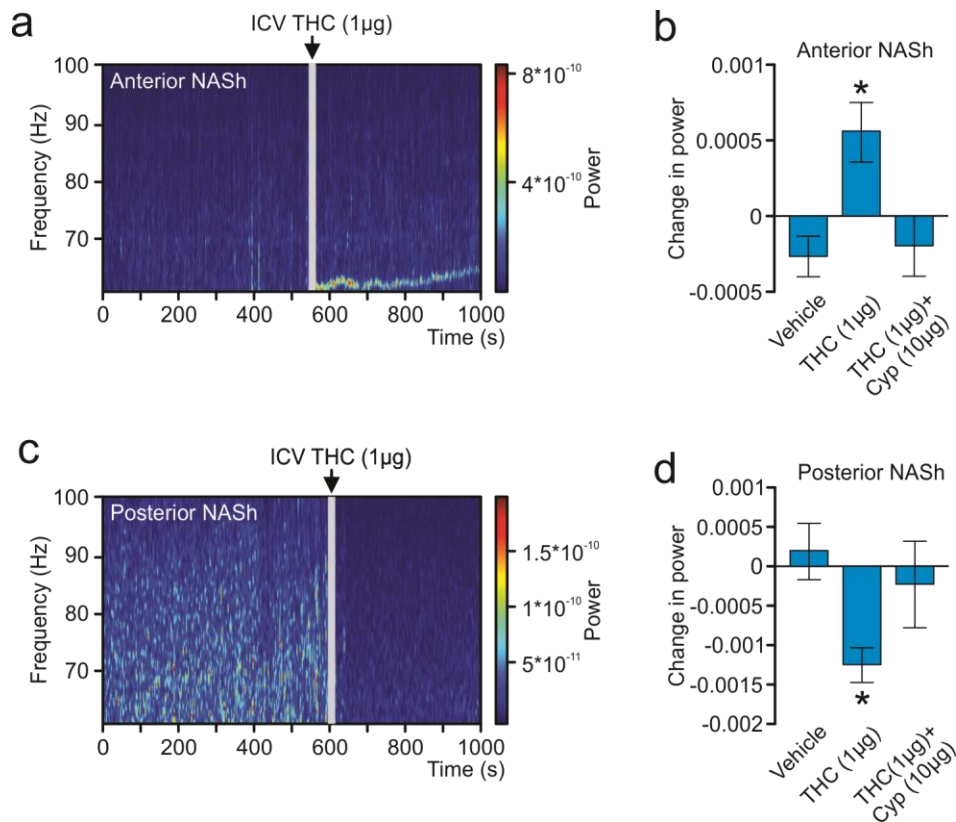
Figure 3.7 Effect of ICV THC on the power of high-frequency gamma oscillations in the local field potential signal in the NASh. A, Sample spectrograph showing typical high-frequency γ-oscillations in the pNASh following ICV infusion of THC **B,** ICV THC significantly decreases the power of high-frequency γ-oscillations in the pNASh. * = *p* < .05 from the other two groups. **C,** Sample spectrograph showing typical high-frequency γ-oscillations in the aNASh following ICV infusion of THC **D,** ICV THC significantly increases the power of high-frequency γ-oscillations in the aNASh

3.4 DISCUSSION

The nucleus accumbens serves as a critical neural nexus point for the integration of affective information from numerous extrinsic inputs, including the ventral tegmental area (VTA), amygdala, ventral hippocampus (vHIPP) and prefrontal cortex (PFC). In addition, THC has been shown to strongly modulate intra-NAc signaling from these regions, including DAergic signals from the VTA (Cheer, 2004; Morra, Glick, & Cheer, 2010; Morra, Glick, & Cheer, 2012; Oleson & Cheer, 2012) and glutamatergic signals from the PFC and vHIPP (Loureiro et al., 2016a; Pistis

et al., 2002; Rigucci et al., 2018). Previously, we and others have reported that THC directly within the NAc can strongly modulate mesolimbic DAergic activity and NAc neuronal activity states (Cheer, 2004; Fitoussi et al., 2018; Morra et al., 2012; Oleson & Cheer, 2012). In addition, THC has been shown to directly activate VTA DA neurons (French, 1997). Nevertheless, the mechanisms by which THC may produce bivalent, reward and aversion effects within the NAc has not previously been characterized.

In the present study, we demonstrate not only that THC produces bivalent effects on reward and aversion processing, but identify anatomically, pharmacologically and neuronally dissociable mechanisms, directly in the in the anterior vs. posterior NASh that are responsible for these effects. We report that intra-NASh infusions of THC produced robust μOR-dependent reward effects selectively in the aNASh but produced κOR-dependent aversive effects in pNASh. In addition, intra-aNASh THC selectively potentiated morphine-related reward salience but not natural, sucrose reward salience. In contrast, intra-pNASh THC reduced social motivation and social memory processing, without influencing morphine-related reward processing. Finally, we found that ICV infusions of THC produced a predominant decrease in MSN activity, consistent with an accumbal reward signature (Carlezon & Thomas, 2009). In direct contrast, THC in the pNASh induced a significant increase in MSN neuronal activity with a concomitant decrease in high-frequency γ-oscillations. Together, these data characterize the critical functional differences in the NASh related to THC modulation of affective processing and demonstrate that distinct OR substrates are responsible for these seemingly contradictory effects.

The difference in OR function between the aNASh and the pNASh is not fully understood but previous studies have similarly suggested differential effects of these OR subtypes on DA-mediated phenomena. For example, NASh activation of MORs stimulates DA release while KOR activation decreases DA release (Di Chiara & Imperato, 1988). Since DA transmission in the NASh is strongly linked to both motivational processing (Ikemoto & Panksepp, 1999) and addiction behaviours (Di Chiara et al., 2004; Oleson & Cheer, 2012), differential changes in DA release caused by stimulation of distinct NAc OR substrates could potentially underlie these bivalent affective phenomena. Previous studies have demonstrated the ability of cannabinoids to regulate DA release (Cheer, 2004; Fadda et al., 2006; Kuepper et al., 2010) and enhanced phasic

DA release by drugs of abuse is regulated by cannabinoid receptor signaling (Cheer et al., 2007). Furthermore, more recent studies have suggested unique circuitry between so-called hedonic hotspots in the NASh and a second hedonic hotspot localized in the ventral pallidum (VP). Simultaneous activation of both hotspots was necessary to produce "liking" reactions to sucrose (Smith & Berridge, 2007). This may suggest that the NASh and the VP cooperate to produce a unique, isolated hedonic circuit which can enhance the salience of reward. Future studies are required to examine if THC-induced changes in affective processing may similarly depend on NAc-VP functional interactions and potential DA signaling alterations.

Clinical and pre-clinical studies have demonstrated biphasic effects of THC on reward and aversion processing (Chen et al., 1991; Lepore et al., 1995; Parker & Gillies, 1995). For example, strain-dependent differences in sensitivity to the rewarding or aversive stimulus properties of THC has been demonstrated in rodents (Parker and Gillies, 1995). In humans, THC exposure produces differential rewarding or aversive effects via modulation of striatal activity states and can disrupt emotional processing (Bossong et al., 2013, 2015; Englund et al., 2013; Freeman et al., 2015). Like many drugs of abuse, THC elicits striatal DA release (Bossong et al., 2015) and can reduce limbic network activity when processing negative emotional stimuli (Bossong et al., 2013). THC, however, also causes paranoia, anxiety and negative mood (Englund et al., 2013; Freeman et al., 2015) which increases with dose (Childs et al., 2017). The present findings suggest that relative sensitivity to the motivational properties of THC may depend upon distinct effects of THC within anterior vs. posterior NAc regions via dissociable OR signaling mechanisms. However, future studies are required to determine if similar regional differences in the ventral striatum in humans may similarly underlie the bivalent affective properties of THC.

The cannabinoid and opioid receptor systems functionally interact during the processing of motivational behaviours (Ahmad et al., 2013; Pickel et al., 2004; Zimmer et al., 2001). For example, systemic THC administration has been shown to increase heroin self-administration in rats (Solinas et al., 2005) and intra-NAc CB_1 receptor blockade has been shown to attenuate morphine self-administration (Caillé & Parsons, 2006). Consistent with these reports, the present study identifies the anterior NASh region as a local accumbal zone critical for modulating opioid-related reward salience. Beyond the NAc, we have previously reported that intra-vHIPP CB1R

activation strongly increases the reward salience of morphine (Loureiro et al., 2016b). In addition, CB1R activation in the PFC was shown to switch the hedonic valence of systemic or intra-VTA morphine from rewarding to aversive, through dissociable μOR vs. κOR-dependent receptor mechanisms, directly in the VTA (Ahmad et al., 2013). Interestingly, intra-basolateral amygdala (BLA) activation or blockade of CB1R transmission has been shown to switch morphine reward behaviours into aversion effects and potentiate the reward salience of sub-threshold morphine, via functional modulation of MSN neuronal states in the NAc (Ahmad et al., 2017). Thus, inhibition of CB1R signaling in the BLA with an antagonist of CB1R, was shown to potentiate the rewarding effects of morphine by causing inhibition of NASh MSN activity states. The present study reveals a novel, THC-mediated mechanism directly in the aNASh, that similarly potentiates opioid reward salience via inhibition of MSN neuronal activity. Thus, the relative activity levels of NAc MSN neuronal populations appears to be a critical mechanism regulating not only the motivational valence of THC itself, but also in the interaction of CB1R signaling with the processing of opioid-related reward or aversion behaviours.

Mesolimbic LFP signals are closely linked to the activity of individual neurons and implicated in motivation, drug addiction and psychosis (Cohen et al., 2009; Ge et al., 2018; Uhlhaas, Haenschel, Nikolić, & Singer, 2008). More specifically, γ-oscillations have been implicated in sensory integration, associative learning and cognition (Uhlhaas et al., 2008). For example, previous studies have demonstrated that local firing activity of individual neurons is mediated by regionally distinct changes in γ-oscillations in the ventral striatum of rats during reward processing (Kalenscher et al., 2010) and there is increased striatal γ-oscillation activity immediately following associative reward delivery (van der Meer & Redish, 2009). The role of γ-oscillations in striatal reward processing is also demonstrated in humans during the processing of monetary rewards or losses, which are correlated with distinct γ-oscillation patterns in the NAc, suggesting that γ-oscillation patterns within the striatum may serve as a gating mechanism for the relative encoding of rewarding or aversive valences during motivated behaviours (Cohen et al., 2009). The present findings reveal for the first time that striatal γ-oscillation patterns are similarly linked to THC-dependent affective processing and suggest that THC-induced reward states are associated with potentiated γ-oscillations while aversive states are linked to inhibition of these oscillatory patterns, within anterior vs. posterior accumbal zones.

Beyond signaling reward or aversion states, changes in γ-oscillation states have been linked to the psychotomimetic effects of THC. For example, THC exposure induces strong dysregulation in γ-oscillation states similar to those observed in schizophrenia (Cortes-Briones et al., 2015; Nottage et al., 2015; Skosnik, Krishnan, Aydt, Kuhlenshmidt, & O'Donnell, 2006; Y. Sun et al., 2011). Due to the increasing evidence of links between THC exposure and neuropsychiatric side-effects (Kuepper et al., 2011; Radhakrishnan, Wilkinson, & D'Souza, 2014), the present findings have important implications for how THC may lead to disturbances in emotional regulation via γ-oscillation alterations. In terms of drug-related effects, previous studies have demonstrated that specific patterns of γ-oscillations within the NAc were present during aversive opioid withdrawal states (Dejean et al., 2017), suggesting a link between opioid-related aversion signals and striatal γ-oscillation disturbances. Thus, altered γ-oscillation states may be critical biomarkers for striatal-dependent processing of rewarding or aversive motivational states and THC modulation of cannabinoid receptor signaling may serve to differentially control reward or aversion processing in distinct anterior vs. posterior regions of the NAc.

In summary, the results of the present study reveal several novel mechanisms to account for how THC differentially modulates mesolimbic activity states and bivalent affective processing via interactions with the opioid receptor system. In addition, these findings have important implications for understanding how the effects of THC in anatomically distinct regions of the NASh may underlie the neuropsychiatric side-effects of cannabis, including its dependence-producing properties and psychotomimetic side-effects.

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CHAPTER 4

4   Δ^9-tetrahydrocannabinol (THC) Regulates Memory, Anxiety and Sensorimotor Gating via Dissociable Modulation of the Wnt and mTOR Signaling Pathways in the Nucleus Accumbens Shell

4.1 INTRODUCTION

Acute and neurodevelopmental exposure to Δ-9-tetrahydrocannabinol (THC), the primary psychoactive compound in cannabis, is associated with psychotomimetic side-effects and an increased risk of serious neuropsychiatric disorders, such as schizophrenia. Schizophrenia is a complex and devastating disorder characterized by disturbances in emotional salience attribution (Katthagen et al., 2016; Palaniyappan, Simmonite, White, Liddle, & Liddle, 2013), deficits in neurocognitive sensory filtering such as paired-pulse inhibition and facilitation deficits (Winton-Brown et al., 2015; Wynn et al., 2004), abnormalities in memory formation (Behrendt, 2016; Paz-Alonso et al., 2013) and heightened anxiety and paranoia (American Psychological Association, 2013; Buonocore et al., 2018; Malcolm, Picchioni, & Ellett, 2015; Morrison & Cohen, 2014; Tandon et al., 2013). Importantly, clinical and pre-clinical evidence has demonstrated that exposure to THC is capable of producing all of these psychiatric endophenotypes via its modulatory influence on mesocorticolimbic dopamine (DA) transmission and associated molecular signaling pathways (Bossong et al., 2015; Radhakrishnan et al., 2014; Renard et al., 2017). For example, acute or neurodevelopmental exposure to chronic THC has been shown to induce long-term hyperactivation of subcortical ventral tegmental area (VTA) mesolimbic DA activity and associated adaptations in cortical molecular signaling cascades linked to disorders like schizophrenia, including the glycogen-synthase-kinase-3 (GSK3), protein kinase B (Akt), mammalian target of rapamycin (mTOR) and p70-S6-Kinase (p70S6K) pathways (Fitoussi et al., 2018; Renard et al., 2017).

The ventral striatum (nucleus accumbens; NAc) is a brain region that is critically involved in the neuropathological phenotypes associated with schizophrenia and anxiety-related disorders (Bahi & Dreyer, 2018; Holt et al., 2005; Kim et al., 2008; McCollum & Roberts, 2015; Morris et al., 2012) and serves as an important target for the psychotropic effects of THC. For example, human imaging studies have shown that THC exposure strongly modulates activity states in the ventral striatum where it has been shown to disrupt cognitive performance (Bhattacharyya et al., 2012; Bhattacharyya et al., 2009; Fusar-Poli et al., 2009). In pre-clinical studies, acute THC has been shown to act within the NAc to induce hyperactivity in VTA DA neurons by reducing inhibitory GABAergic inputs to the DA system, and concomitantly potentiate the emotional salience of normally non-salient associative fear-memory cues (Fitoussi et al., 2018).

In terms of anxiety-related processing, previous research has established that THC produces seemingly contradictory effects. For example, cannabis users may report euphoria, feelings of well-being and joviality (Osborne & Fogel, 2008); and THC has been demonstrated to be anxiolytic at low doses (Berrendero & Maldonado, 2002; Rubino et al., 2007). Conversely, THC also has dependence-producing properties (Gardner, 2002; Tanda & Goldberg, 2003; Zangen et al., 2006), can modulate associative fear memory processing (Fitoussi et al., 2018; Klumpers et al., 2012) and produces anxiogenic effects at higher doses (Bhattacharyya et al., 2010; Crippa et al., 2009; Englund et al., 2013). Finally, THC has been previously demonstrated to impair recognition memory (Kasten, Zhang, & Boehm, 2017; Swartzwelder et al., 2012), similar to memory impairments observed in schizophrenia patients (Forbes, Carrick, McIntosh, & Lawrie, 2009; Grimes, Zanjani, & Zakzanis, 2017; Herbener, 2008; Herbener, Rosen, Khine, & Sweeney, 2007).

At the molecular level, substantial evidence implicates a role for the wingless/ integrated (Wnt) signaling cascade (i.e. protein kinase B [Akt], glycogen synthase kinase-3 [GSK-3] and β-catenin) and the mammalian target of rapamycin (mTOR) pathway (i.e. p70S6 kinase [p70S6K], protein kinase B [Akt] and mTORc) directly within the limbic system, during the acquisition, consolidation, retrieval, and reconsolidation of associative fear memory (Gafford, Parsons, & Helmstetter, 2013; Jarome, Perez, Hauser, Hatch, & Lubin, 2018; Jobim et al., 2012; Maguschak & Ressler, 2011; Xu et al., 2015). For example, disruption of Wnt signalling, specifically β-catenin, in the amygdala, prevented consolidation of fear-memory (Maguschak & Ressler, 2011). Additionally, inhibition of mTOR in the amygdala or hippocampus impaired the formation and reconsolidation of avoidance memory (Jobim et al., 2012). Furthermore, activation of CB1 receptors, the primary target for THC, can functionally modulate these signalling pathways and their phosphorylation states (Korem, Lange, Hillard, & Akirav, 2017; Puighermanal et al., 2013) suggesting that they may underlie the influence THC has on fear and anxiety-related behaviours.

In the present study, we used a combination of pre-clinical behavioural assays targeting cognitive and affective endophenotypes associated with schizophrenia-related deficits in rats, in combination with anatomically localized molecular signalling analyses to examine the effects of

direct microinfusions of THC on Wnt and mTOR signalling proteins, associative fear memory, prepulse inhibition (PPI) and prepulse facilitation (PPF), object recognition memory, and anxiety. We report that THC infused in the posterior region of the NAc shell (pNASh) potentiates the formation of associative fear memory to sub-threshold footshock, impairs PPI/PPF, produces anxiety and disrupts object recognition memory through a GSK-3 dependent signaling mechanism. Conversely, THC infused into the anterior NASh (aNASh), blocks the formation of associative fear memory to supra-threshold footshock and reduces anxiety. When co-administered with an Akt activator, the formation of associative fear memory returned, the formation of object recognition memory was disrupted, and anxiety was further reduced. These findings reveal several novel and dissociable mechanisms for the psychotropic effects of THC within the NASh, mediated through distinct anatomical and molecular substrates.

4.2 MATERIALS AND METHODS

4.2.1 Animals and Surgery

Male Sprague Dawley rats (300 to 350 g; Charles River, Quebec, Canada) were used in compliance with the Canadian Council for Animal Care and institutional guidelines. Rats were housed under controlled conditions (12-hour light/dark cycle and food/water access ad libitum). Rats were anesthetized with a mixture of ketamine (80 mg/ml) and xylazine (6 mg/ml) and placed in a stereotaxic device. Stainless steel guide cannula (22- gauge; PlasticsOne) were implanted bilaterally into either the aNASH at the following coordinates (Paxinos & Watson, 2005): AP: +2.5 mm from bregma, L: ±2.6 mm, DV: -7.4 mm from the dural surface, or the pNASH at the following coordinates: anteroposterior (AP): +1.5 mm from bregma, lateral (LAT): ±2.6 mm, dorsoventral (DV): -7.4 mm from the dural surface. Guide cannulae were held in place using jeweler's screws and dental acrylic cement. After completion of behavioural experiments, rats received an overdose of pentobarbital (240 mg/kg, i.p.) and were perfused with isotonic saline followed by 10% formalin. Brains were extracted and post-fixed 24 h before being placed in a 25% formalin-sucrose solution for one week. Brains were sliced (60 μm) using a cryostat and stained with Cresyl violet. Injector tips placements were localized using a light

microscope. Rats with cannula placements found outside the anatomical boundaries of the NASh were excluded from data analysis.

4.2.2 Drug Treatment and Administration

The following drugs were used during behavioural experiments or protein analysis: Δ^9-tetrahydrocannabinol (THC; 100 ng/0.5 µl; Tocris Bioscience), the selective GSK3 inhibitor SB216763 (300 ng/ 0.5µl; Tocris Bioscience) and the AKT phosphorylation promoter SC-79 (10nM/0.5µl; Tocris Bioscience). The dosage of SB216763 was based on a previously published intra-NA effective dose (Wickens, Quartarone, & Beninger, 2016). THC was shipped in ethanol then dissolved in cremaphor. The ethanol was evaporated with nitrogen gas. The solution was then diluted with saline to the desired concentration. SB216763 was dissolved in DMSO and then diluted in PBS for a final 1% DMSO in PBS. SC-79 was dissolved in DMSO and then diluted in PBS for a final 20% DMSO in PBS. Intra-NASh microinfusions were performed immediately prior to each conditioning session. A total volume of 0.5 µl per hemisphere was delivered via a 28-gauge microinfusion injector over a period of 1 min. Microinjectors were left in place for an additional 1 min to ensure adequate diffusion from the tip. The intra-cranial dose selections for the above compounds were based upon our previously published or piloted dose-response curves using these compounds (Ahmad et al., 2013; Norris et al., 2016) which demonstrated maximal behavioural efficacy and the absence of non-specific behavioural side effects.

4.2.3 Protein Extraction and Western Blots

To examine the potential localized effects of intra-NAc THC on select molecular signaling pathways (described below), a subset of experimental rats was given intra-aNASh infusions of THC (100 ng/ 0.5µl per side; $n= 8$) or vehicle ($n = 8$) or intra-pNASH infusions of THC (100 ng/ 0.5µl per side; $n= 8$) or vehicle ($n = 8$). Ten minutes later, the rats were anesthetized with pentobarbitol and their brains were removed and rapidly frozen. The section of the brains containing the NAc were cut using a cryostat to obtain coronal sections (60 µm), from which bilateral micropunches were taken from around the infusion site within the NASh. The Western blotting procedure was conducted as described previously (Lyons et al., 2013). Protein levels were measured using the bicinchoninic acid (BCA) assay. The sample solutions were there

diluted with buffer to make all concentration equals and all wells were loaded with 60µl. Run time and transfer time were adjusted based on molecular weight. Primary antibody dilutions were as follows: α-tubulin (1:120000; Sigma-Aldrich), phosphorylated GSK-3α/β ser21/9 (p-GSK-3α/β; 1:1000; Cell Signaling Technology), total GSK-3α/β ser21/9 (t-GSK-3α/β; 1:1000; Cell Signaling Technology), phosphorylated Akt Ser473 (p-Akt-Ser473; 1:1000; Cell Signaling Technology), phosphorylated Akt thr308 (p-Akt-thr308; 1:1000; Cell Signaling Technology), total Akt (t-Akt; 1:1000; Cell Signaling Technology), β-catenin (1:10000; Sigma-Aldrich), phosphorylated mTOR ser2448 (p-mTOR;1:2000; Cell Signaling Technology), total mTOR (t-mTOR; 1:2000, Cell Signaling Technology), phosphorylated p70S6K thr389 (p-p70S6K; 1:1000; Cell Signaling Technology), total p70S6K (t-p70S6K; 1:1000; Cell Signaling Technology). Species appropriate secondary antibodies for LICOR imaging were all used at a concentration of 1:20,000 (Thermo Scientific).

4.2.4 Olfactory Fear Conditioning

We used a previously described olfactory fear conditioning paradigm to measure fear memory (Draycott et al., 2014; Lauzon et al., 2009; see also Chapter 2). Two distinct environments were used. Environment A was a 30" x 30" Plexiglass box with black spots on a white background and environment B was a 30" x 30" Plexiglass box with black and white stripes. The designated shock environment had a metallic grid shock floor while the designated test environment had a smooth grey Plexiglas floor. The environments were assigned as "shock" and "test" in a counterbalanced manner such that all rats with "shock" environment A were tested in environment B and all rats with "shock" environment B were tested in environment A. On day 1 (habituation) rats were given sham microinfusions into the NASh and habituated to both conditioning environments for 30 min. Footshocks were delivered at an intensity of 0.8 mA, which we have previously reported to produce highly robust fear memories, or 0.4 mA, which does not produce fear memories (Draycott et al., 2014; Lauzon et al., 2009). On day 2 (conditioning phase) rats were returned to the room and, immediately prior to being placed in the previously assigned "shock" environment, rats received intra-NASh microinfusions of THC (100ng/0.5µl); the GSK3 inhibitor SB216763 (300ng /0.5µl) in combination with THC; the AKTser473 phosphorylation promoter SC-79 (10nM /0.5µl), in combination with THC. Two odours were delivered during conditioning, almond and peppermint. One odour was presented

with a footshock (CS+) and the other was presented in the absence of a footshock (CS-). After one minute in the "shock" environment, the CS- odour was presented for 20 sec. Two min later, the CS+ odour was presented for 19 sec followed by a 1 sec footshock delivered through the shock floor. This cycle was repeated 5 times. On day 3 (test phase) rats were placed in the previously assigned test environment. They were given 1 min to explore the environment before odour presentations began, during which time baseline levels of freezing behaviour were recorded. Both CS+ and CS- odours were presented in a fully counterbalanced order for 5 min and amounts of time rat's spent freezing (lack of movement except for respiration) was recorded and analyzed with ANY-MAZE™ video software for offline analysis. Fear scores were calculated by subtracting the time (s) spent freezing to the CS- from the percent of time (s) spent freezing to the CS+.

4.2.5 Pre-pulse Inhibition and Facilitation

Pre-pulse modulation of the Acoustic Startle Response (ASR) was conducted in sound-attenuating startle chambers (LE116, Panlab, Spain) using the StartFear system and STARTLE software modules (PACKWIN-CSST, PACKWIN V2.0, Spain), which enable the recording and analysis of animal movement and startle response through a high sensitivity Weight Transducer system. Rats were placed into large, perforated Plexiglass tubes, set on the motion-sensitive platform in the sound attenuating chamber. Rats acclimated to the startle chambers for 5 minutes/day with background noise (white noise; 68 dB) over 2 days. During the final acclimation session, rats underwent an input/output (I/O) function test consisting of 11 increasing startle pulses (from 70 to 120 dB, 5 dB increments) to determine the appropriate gain setting for each individual rat. The testing procedure consisted of the following phases: the acclimation phase, a habituation phase (Block 1), prepulse inhibition (PPI) measurement (Block 2), and prepulse facilitation (PPF) measurement (Block 3). White background noise (68 dB) was ubiquitously presented during all phases of the experiment. During Block 1, 10 pulse alone trials (110 dB white noise, 20 ms duration) were randomly delivered at 15–20 s inter-trial intervals (ITIs). Block 2 consisted of 10 different trials presented 10 times each in a randomized order at 15–20 s intervals: 10 pulse-alone trials, and 10 of each of the three different prepulse-pulse trial types (72, 76, 80 dB) with interstimulus intervals (ISI) of 30, 100 and 800 ms. Whether PPF or PPI is induced seems to be best predicted by ISI and prepulse type. Very short (<10-15 ms) or

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very long (>1000 ms) ISIs tend to be the most effective at inducing PPF. Block 3 consisted of 10 different trials presented 10 times each in a randomized order at 15–20 s intervals: 10 pulse-alone trials, and 10 of each of the three different prepulse-pulse trial types (72, 76, 80 dB) with interstimulus intervals of 1000, 1500 and 2000 ms. PPF intervals times were based on previous human testing on schizophrenic patients (Wynn et al., 2004). Pulse-alone trials consisted of a startle stimulus-only presentation, whereas prepulse-pulse trials consisted of the presentation of a weaker non-startling prepulse (white noise, 20 ms duration) before the interstimulus interval and startling stimulus. Blocks 2 and 3 were conducted on separate days and were each preceded by Block 1. PPI was calculated for each animal and each trial condition as PPI (%) = (1 − average startle amplitude to pulse with prepulse/average startle amplitude to pulse only) × 100. The final number of rats in each group was as follows: Intra-anterior NASh VEH group (anterior/VEH), $n = 8$; Intra-anterior NASh THC group (anterior/THC), $n = 9$; Intra-posterior NASh VEH group (posterior/VEH), $n = 9$; Intra-posterior NASh THC group (posterior/THC), $n = 9$.

4.2.6 Novel Object Recognition

Rats were tested using the object recognition task as described previously (Renard et al., 2017). This task evaluates the ability of the rat to discriminate between the familiarity of previously encountered objects; normal rats typically spend more time exploring a novel object than a familiar object. The test sessions consisted of two 3-min trials. During the first trial (T1 acquisition trial), each rat was placed in the center of an arena containing two identical objects placed in the far corners 15 cm from the side wall. After a delay of 60 min during which the rat was returned to its cage, and both objects were replaced (one by an identical copy, the other by a novel object in the same location), the rat was returned to the arena for the second trial (T2 test trial). Between rats, both the role (familiar or novel object) and the relative position of the two objects were randomly counterbalanced. Object exploration was considered when the head of the rat was facing the object, or the rat was touching or sniffing the object. Times spent in exploration were videotaped with a video-tracking system (ANY-maze; Stoelting) and analyzed by an experimenter. Exploration times were recorded and used to calculate discrimination index [time spent with novel object/total time exploring both objects] *100.

4.2.7 Elevated Plus Maze

This task seeks to evaluate the anxiety levels of the animal. The apparatus consists of four arms: two open and two enclosed with walls. The testing session consisted of 2 minutes of acquisition followed by 8 min of testing. The rats were infused with one of the testing drugs then placed in apparatus and allowed to freely explore. Time spent in the open and closed arms was recorded by with video-tracking software (ANY-Maze; Stoelting) and analysed by an experimenter.

4.3 RESULTS

4.3.1 Histological Analysis

Histological analysis revealed injector placements localized within the anatomical boundaries of the shell subdivision of the NASh. Sample NASh microinfusion locations and schematic illustration of several experimental groups across the anterior-posterior axis of the NASh are shown in **Figure 4.1**.
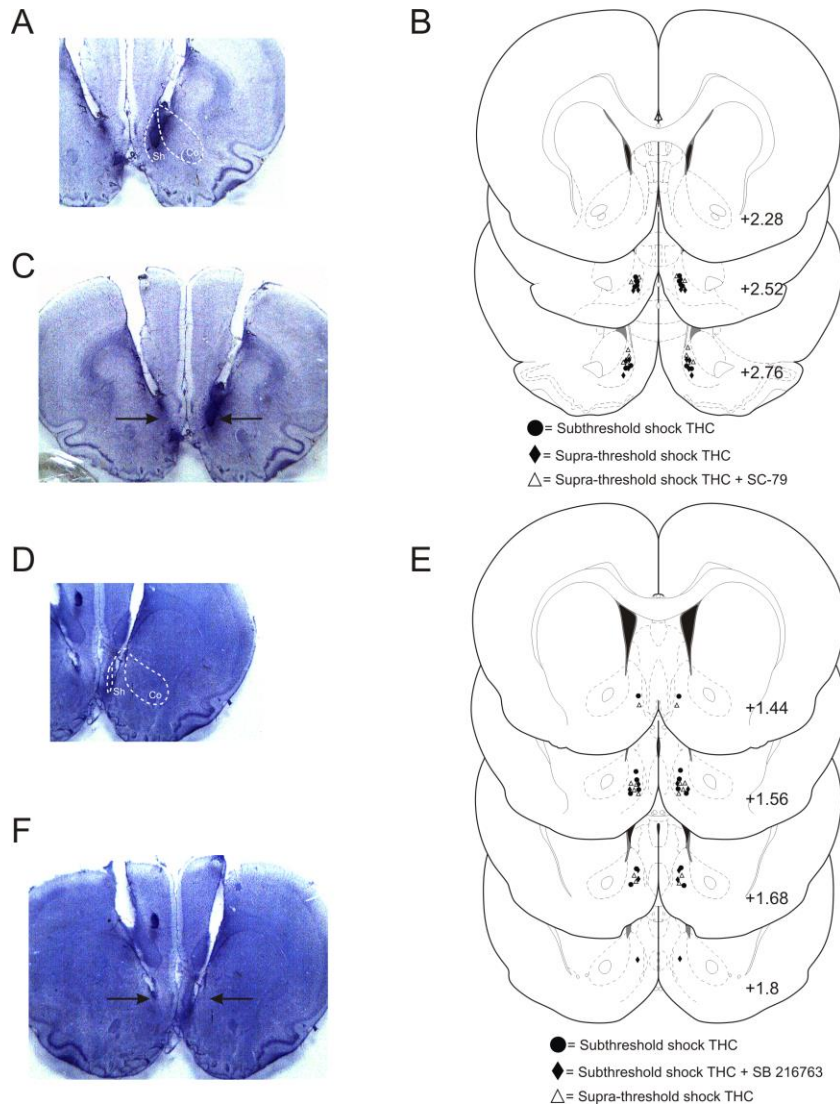
Figure 4.1 Histological analysis of intra-NASh microinjection sites. A, Microphotograph of representative injector placement within the anterior portion of the nucleus accumbens shell. **B,** Schematic representation of select intra-anterior-NASh injector locations; ● = 100 ng THC group, ◆ = 100 ng THC + 10 nM SC-79. **C,** Microphotograph of representative intra-anterior-NASh bilateral cannulae placements. **D,** Microphotograph of representative injector placement within the posterior portion of the nucleus accumbens shell. **E,** Schematic representation of select intra-posterior-NASh injector locations; ● = 100 ng THC group, ◆ = 100 ng THC + 300 ng SB216763. **F,** Microphotograph of representative intra-posterior-NASh bilateral cannulae placements.

4.3.2 Intra-aNASh THC infusions produce decreases in phosphorylated protein levels of Akt Ser473 and mTOR ser2448

We first examined the potential localized effects of intra-aNASH THC (100 ng/ 0.5 µl) exposure on the expression levels of the Akt and mTOR signalling pathways (β-catenin, GSK-3α/β, Akt, p70S6K and mTOR) as previous studies from our lab have demonstrated that THC exposure can profoundly alter their activity and modulate THC-related affective and cognitive side-effects in neural regions such as the prefrontal cortex (Renard et al., 2017). In addition, previous studies have implicated these specific molecular pathways as being critical for striatal-mediated cognitive and affective processing phenomena (Bergeron, Chagniel, Bureau, Massicotte, & Cyr, 2014; Cuesta et al., 2017; Ding et al., 2013; Luo et al., 2016; Xu et al., 2015). In previously reported dose-response analyses, we have identified this dose of intra-NAc THC (100 ng) as being pharmacologically and behaviourally effective for localized intracranial infusions (Norris et al., 2016, Fitoussi et al., 2018). In the present series of studies, we separately analyzed posterior vs. anterior NAc regions to determine if anatomically distinct effects of THC may be identifiable between these regions. A simplified diagram of these molecular signaling pathways and their known mechanistic downstream neurophysiological and neuropsychiatric effects is presented in **Figure 4.2**.

THC in the Anterior NASh selectively decreases phosphorylation states of Akt and mTOR

First, we examined any potential changes in protein expression comparing rats who received intra-aNASh THC (100 ng/ 0.5µl) vs. vehicle. Western blot analysis revealed a significant reduction in phosphorylated expression levels of Akt-Ser473 (pAktSer473; $t_{(6)} = 2.789$, $p = .032$) and the ratio between pAktSer473 and total Akt (tAkt; $t_{(6)} = 2.545$, $p = .044$) but not tAkt, phosphorylated Akt-Thr308 (pAktThr308) or the ratio between pAktThr308 and tAkt ($p > .05$; **Figure 4.3A**). There was also a significant reduction in phosphorylated mTOR (pmTOR) vs. vehicle ($t_{(6)} = 2.718$, $p = .035$;) and the ratio between total tmTOR (tmTOR) and pmTOR ($t_{(6)} = 2.744$, $p = .034$) but no significant change in levels of tmTOR ($p > .05$; **Figure 4.3C**). There was no significant change in phosphorylated GSK-3 (pGSK3), total GSK-3 (tGSK3), the ratio between pGSK3 and tGSK3 (**Figure 4.3E**), phosphorylated p70S6K (p-p70S6K), total p70S6K

or the ratio between p-p70S6K and t- p70S6K (*data not shown*); or β-catenin (*p* > .05; **Figure 4.3G**).

THC in the Posterior NASh selectively decreases phosphorylation states of GSK3 and increases β-catenin levels

Next, we examined any potential changes in protein expression levels between rats who received intra-pNASh THC (100 ng/ 0.5µl) or vehicle. Western blot analysis revealed a significant reduction in pGSKα ($t_{(13)}$ = 2.933, *p* = .012) and the ratio between pGSKα and tGSKα ($t_{(13)}$ = 2.493, *p* = .027) but not tGSKα, pGSKβ, tGSKβ or the ratio between pGSKβ and tGSKβ (*p* > .05; **Figure 4.3F**). There was also a significant increase in β-catenin vs. vehicle ($t_{(5)}$ = -2.674, *p* = .044; **Figure 4.3H**). There was no significant change in pAktSer473, pAktThr308, tAKT, the ratio between pAktSer473 and tAkt, the ratio between pAktThr308 and tAkt (**Figure 4.3B**), phosphorylated p70S6K (p-p70S6K), total p70S6K (t- p70S6K) or the ratio between p-p70S6K and t- p70S6K (*data not shown*), pmTOR, tmTOR, or the ratio between pmTOR and tmTOR (*p* > .05; **Figure 4.3D**).

Thus, these findings demonstrate several critical dissociations in terms of THC-dependent modulation of the GSK3-Wnt vs. mTOR signaling pathways, via anatomically localized effects in the anterior vs. posterior NAc sub-regions. We next performed a battery of behavioural assays (see methods) to further explore the potential mechanistic roles for these effects on affective and cognitive processing phenomena.
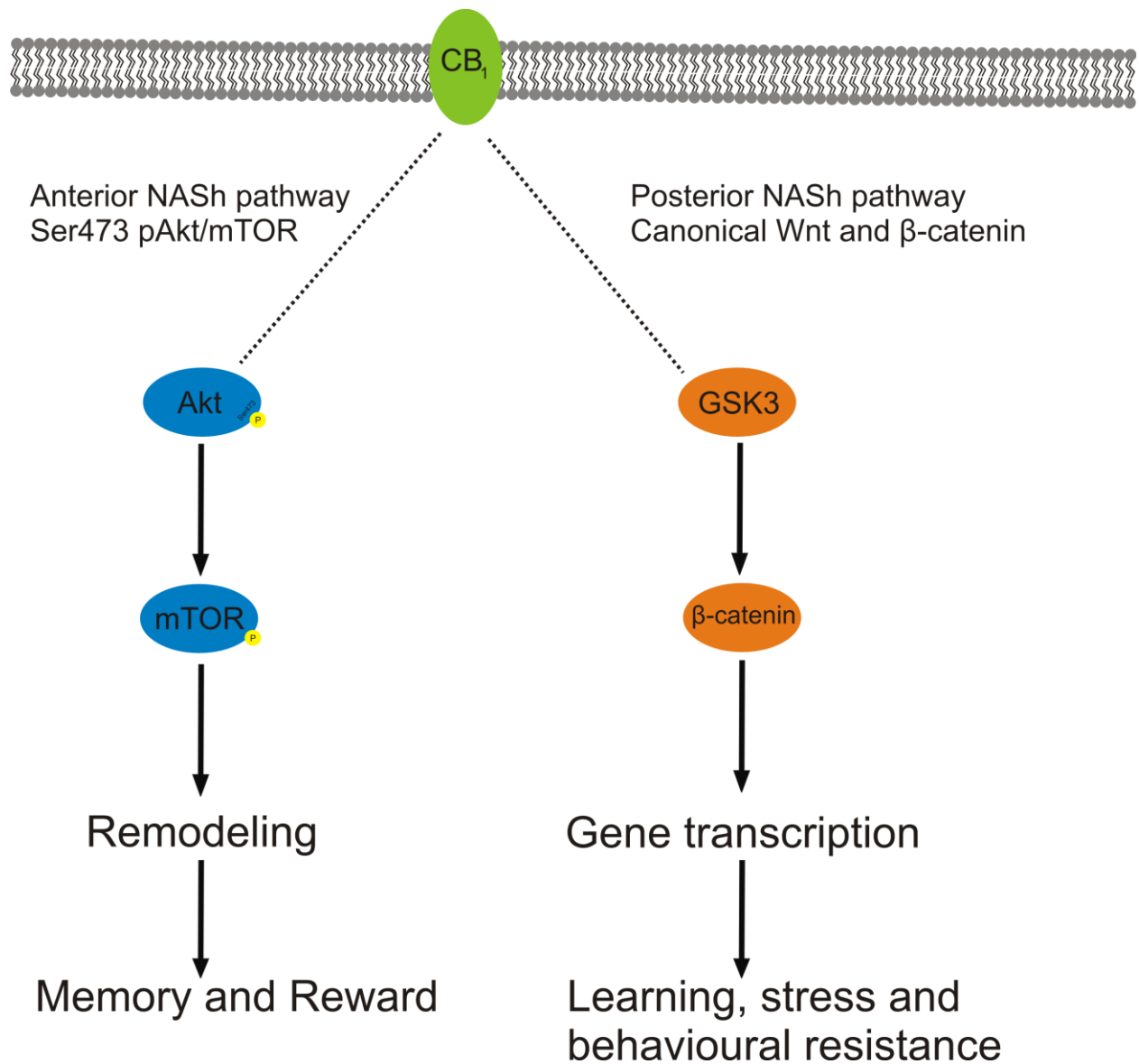
Figure 4.2 Proposed CB₁ signaling pathways. pAkt Ser473 to mTOR pathway, known to be involved in cellular remodeling and GSK3 to β-catenin Wnt pathway, involved in gene transcription
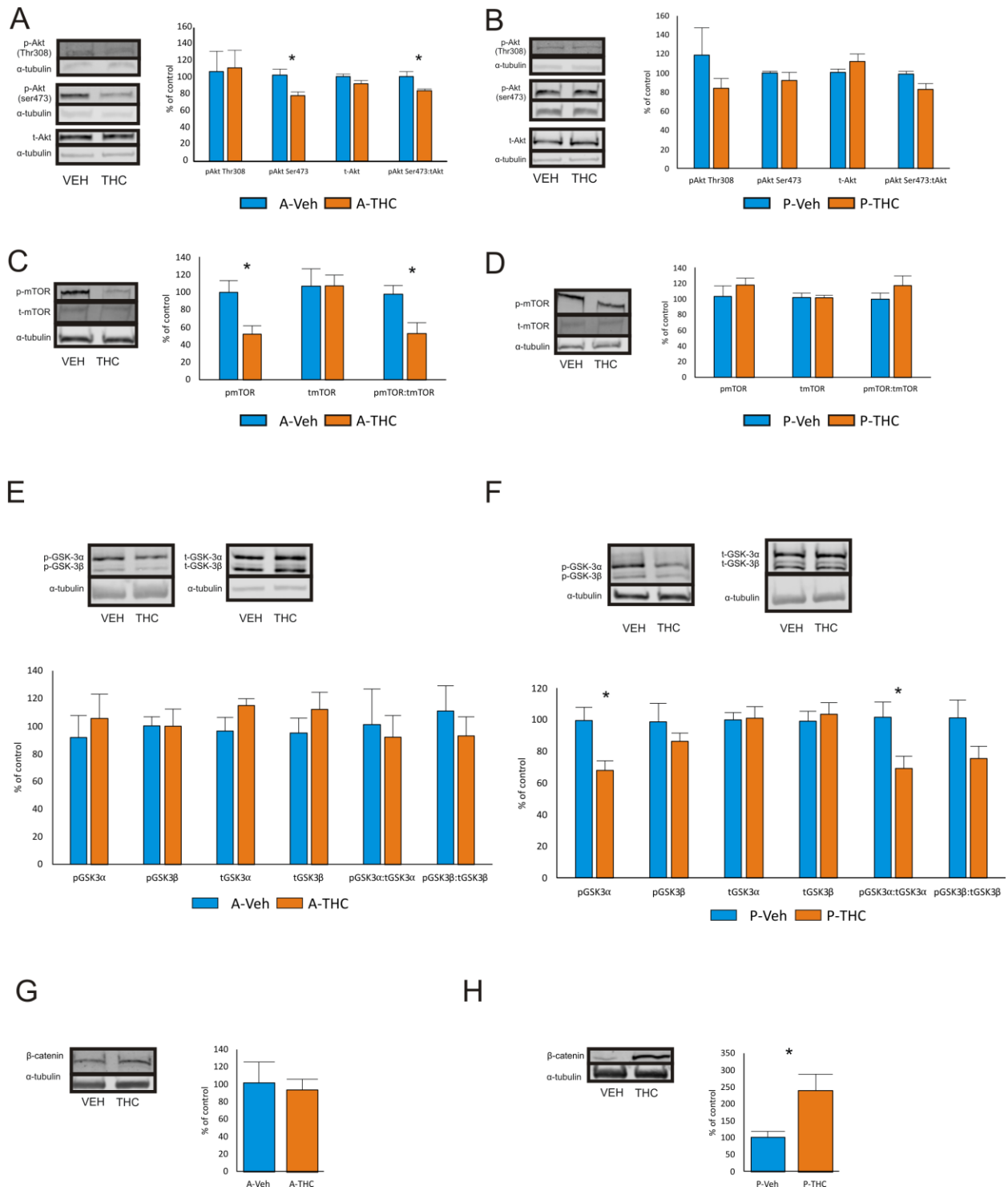
Figure 4.3 Effects of intra-NASh THC on signaling proteins. A, Intra-aNASh THC reduces

Ser473 pAkt and the ratio of Ser473 pAkt to tAkt * = p < .05 **B,** Intra-pNASh THC has no effect

on Akt levels **C,** Intra-aNASh THC reduces pmTOR and the ratio of pmTOR to tmTOR * = p <

.05. **D,** Intra-pNASh THC has no effect on mTOR levels **E,** Intra-aNASh THC has no effect on

GSK3 levels. **F,** Intra-pNASh THC reduces pGSK3 and the ratio of pGSK3 to tGSK3 * = p <

.05. **G,** Intra-aNASh THC has no effect on GSK3 levels. **H,** Intra-pNASH THC increases β-

catenin * = p < .05. **I,** Intra-aNASh THC has no effect on β-catenin levels.

4.3.3 Intra-NASh THC produces anatomically dissociable effects on associative fear memory formation through distinct GSK3 vs. Akt signaling mechanisms

Given previous evidence demonstrating functional differences in fear processing in the anterior
vs. posterior NASh regions (Castro, Terry, & Berridge, 2016) and the ability of THC to modulate
the affective salience of fear memory-related conditioning cues (Fitoussi et al., 2018; Klumpers
et al., 2012), we next examined the potential effects of THC (100ng/ 0.5 μg) in the aNASh vs.
pNASH during the encoding of associative olfactory fear memory [*see methods*]. In these
studies, we examined the potential effects of aNASh vs. pNASh exposure on modulating the
salience of fear-related memory encoding using both a sub-threshold footshock cue or a supra-
threshold conditioning cue, as previously described (Fitoussi et al., 2018). Given our previous
findings (**Figure 4.3**) showing selective effects on GSK3 vs. Akt phosphorylation states in the
posterior vs. anterior NASh, we further explored the potential roles of these signaling pathways
by challenging the effects of aNASh or pNASh THC with co-administration of a selective Akt
phosphorylation activator (SC-79) or a GSK3 inhibitor (SB216763: effective at inhibiting both
GSK-3α and GSK-3β isoforms), respectively. Importantly, since intracellular GSK3 levels are
negatively regulated by phosphorylation state (i.e. GSK3 phosphorylation results in lower *active*
amounts of GSK3), our finding of reduced pGSK3α (**Figure 4.3F**) would predict higher GSK3
activity levels. Thus, we chose a selective inhibitor of active GSK3 to challenge this effect
behaviourally. We selected doses of SC-79 or SB216763 based on dose-response pilot studies in
our laboratory and on previously published work reporting effective doses within this range for
in vivo behavioural intracranial infusions in the NAc (Wickens et al., 2017).

First, we examined the potential effects of intra-pNASh THC on the formation of a sub or supra-threshold associative fear memory. For supra-threshold fear conditioning, intra-pNASh THC ($n = 7$) had no effect on freezing behaviours relative to VEH control rats ($n = 7$; $t_{(12)} = -.323$, $p > .05$). In contrast, for sub-threshold fear conditioning, One-way ANOVA comparing groups revealed a main effect of treatment on freezing scores ($F_{(2,18)} = 16.519$, $p < .001$; **Figure 4.4A**). *Post-hoc* tests revealed that rats receiving THC (100 ng/ 0.5 µl; $n = 7$) demonstrated significantly greater freezing relative to VEH controls ($n = 6$; $p = .001$). In addition, co-administration with the selective GSK3 inhibitor, SB216763 (300 ng/ 0.5 µl) reversed the effects of THC on potentiation of sub-threshold fear memory formation, as this group did not show increased freezing relative to VEH controls ($p > .05$; **Figure 4.4A**). Thus, THC in the pNASh was able to potentiate the salience of normally non-salient fear conditioning memories through a GSK3-dependent mechanism, while having no apparent effect on the encoding of supra-threshold associative fear memory.

Next, we examined the potential effects of intra-aNASh THC on the formation of sub or supra-threshold associative fear memory. For sub-threshold fear conditioning, intra-aNASh THC ($n = 6$) had no effect on freezing behaviours relative to VEH control rats ($n = 7$; $t_{(11)} = .235$, $p > .05$). In contrast, for supra-threshold fear conditioning, One-way ANOVA comparing groups revealed a main effect of treatment on freezing scores ($F_{(2,18)} = 11.111$, $p = .001$; **Figure 4.4A**). *Post-hoc* tests revealed that rats receiving THC (100 ng/ 0.5 µl; $n = 7$) demonstrated significantly less freezing relative to VEH controls ($n = 8$; $p = .004$). In addition, co-administration with the Akt promotor, SC-79 (10 µM/ 0.5 µl; $n = 8$) reversed the effects of THC on potentiation of supra-threshold fear memory formation, as this group did not show decreased freezing relative to VEH controls ($p > .05$; **Figure 4.4B**). Thus, THC in the aNASh was able to block the salience of normally salient fear conditioning memories by reducing Akt activity, while having no apparent effect on the encoding of sub-threshold associative fear memory.

In summary, THC in the anterior vs. posterior NASh differentially regulates the encoding of associative fear memories via dissociable GSK3 vs Akt-dependent signaling mechanisms. Intra-pNASh THC amplifies the emotional salience of normally non-salient fear memory through a GSK3-dependent mechanism while having no effect on the encoding of normally supra-

threshold fear memories. In direct contrast, intra-aNASh THC blocks the formation of normally salient associative fear memories by suppressing Akt activity, while having no effect on the salience encoding of normally non-salient fear conditioning cues.
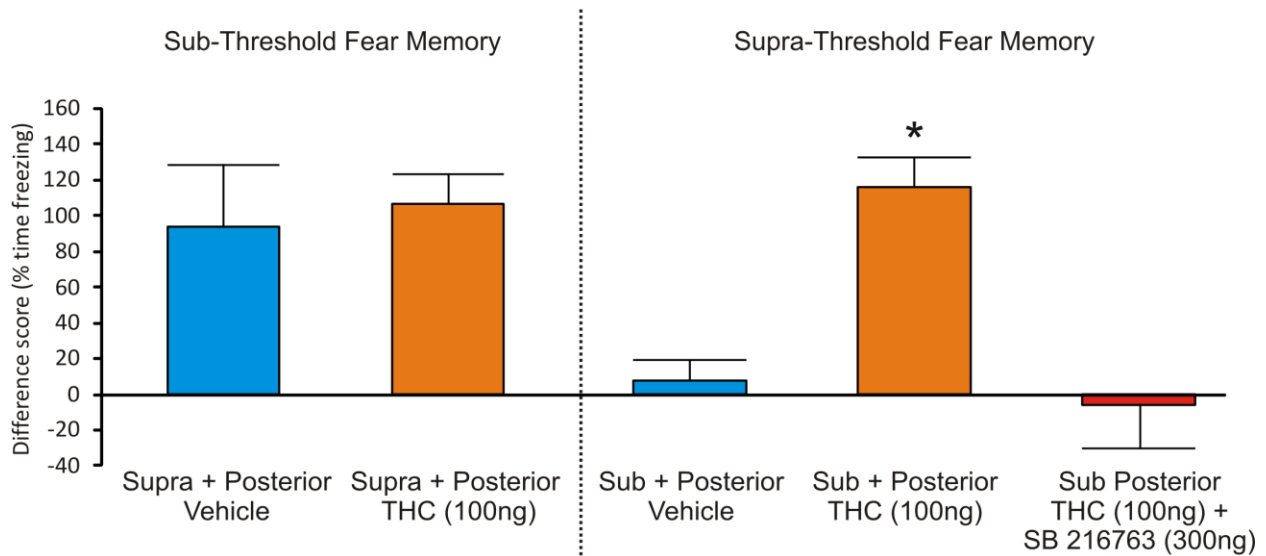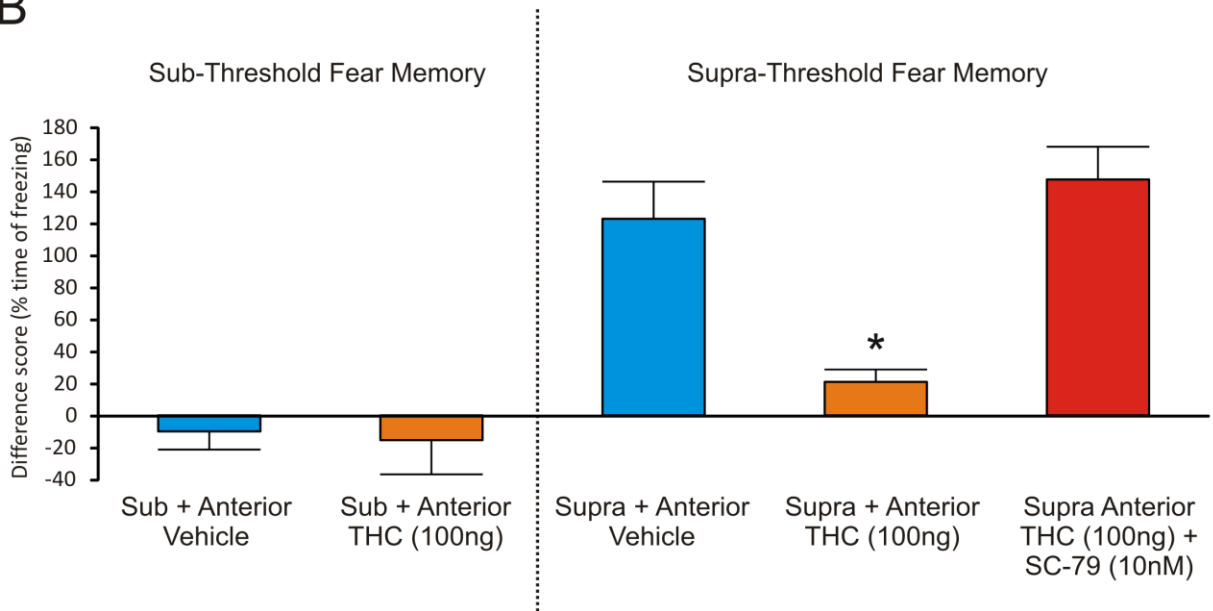
Figure 4.4 Effects of intra-NASh THC olfactory fear conditioning. A, Olfactory fear

conditioning difference scores (the difference between the percent of time spent freezing to the

CS+ and the CS-) for microinfusions into the posterior NASh (+1.5mm from bregma). $* = p <$

.05 **B**, Olfactory fear conditioning difference scores (the difference between the percent of time

spent freezing to the CS+ and the CS-) for microinfusions into the anterior NASh (+2.5mm from bregma). * = p < .05. Sub = shock of 0.4mA, Supra = shock of 0.8mA.

4.3.4 Intra-NASh THC selectively impairs prepulse inhibition and facilitation

Given the known ability of THC exposure to disrupt sensorimotor and cognitive filtering (Bhattacharyya et al., 2012; Bhattacharyya et al., 2010; Morgan et al., 2012, Renard et al., 2016) and the previous studies that have implicated the nucleus accumbens in the acoustic startle response (Goto, Ueki, Iso, & Morita, 2004; Wan & Swerdlow, 1996), we next examined the effects of aNASh or pNASh THC on prepulse inhibition and facilitation behaviours. First to ensure that drug treatment had no effect on baseline startle, ANOVA comparing responses to the startle pulse alone was conducted and showed no significant difference between groups ($F_{(5,41)} =$ 1.952, $p > .05$). Repeated measures ANOVA for intra-pNASH infusions revealed a main effect of treatment ($F_{(2,19)} = 138.031$, $p < .001$), a main effect of startle condition (interstimulus interval and decibel level; $F_{(8,152)} = 43.788$, $p < .001$) but no treatment x startle condition interaction ($F_{(24,216)} = .949$, $p > .05$). *Post-hoc* tests using Tukey's HSD to control for multiple comparisons revealed that PPI in rats receiving intra-pNASh THC ($n = 6$) was significantly lower than rats receiving intra-pNASh vehicle ($n = 8$) in the following startle conditions: 76db/ 30ms ($p = .001$), 76db/100ms ($p = .023$), 76db/ 800ms ($p = .024$), and 80db/100ms ($p = .004$; **Figure 4.5A**) and was significant lower that rats receiving intra pNASh THC plus SB216763 ($n = 8$) for the following startle conditions: 76db/ 30ms ($p = .019$), and 80db/100ms ($p = .002$). Repeated measures ANOVA for intra aNASh infusions, testing if there was any difference in PPI between vehicle ($n = 8$), THC ($n = 9$), and THC + SC-79 ($n = 8$), revealed a main effect of startle condition ($F_{(8,176)} = 27.290$, $p < .001$) but no main effect of treatment ($F_{(2,22)} = .927$, $p > .05$) or a startle x treatment interaction ($F_{(16,176)} = .775$, $p > .05$; **Figure 4.5B**). These data indicate that THC in the pNASh but not the aNASh impairs PPI.

Next, repeated measures ANOVA for intra pNASh infusions on PPF data revealed a main effect of startle condition ($F_{(8,136)} = 12.943$, $p < .001$), treatment ($F_{(5,35)} = 3.763$, $p = .044$) and a treatment x startle condition interaction ($F_{(16,136)} = 3.549$, $p < .001$; **Figure 4.5C**). *Post-hoc* tests revealed that rats receiving THC ($n = 7$) differed significantly from rats receiving vehicle ($n = 7$) during the 72db/2000ms ($p = .008$) and 76db/2000ms conditions ($p = .033$). Rats receiving THC + SB216763 ($n = 6$) did not differ significantly from THC treated rats at either 72db/2000ms ($p > .05$) or 76db/2000ms ($p > .05$), nor VEH controls ($p > .05$), indicating SB216763 attenuated the

effect of THC. Repeated measures ANOVA for intra-aNASh THC revealed a significant main effect of startle condition ($F_{(8,144)} = 21.720$, $p < .001$), startle x treatment interaction ($F_{(16,144)} = 12.943$, $p < .001$) but no main effect of treatment ($F_{(2,18)} = .204$, $p > .05$). *Post-hoc* tests revealed that rats receiving THC ($n = 8$) did not differ significantly from vehicle ($n = 8$) during any startle condition, rats receiving THC + SC-79 ($n = 8$) did differ significantly from vehicle during the 72db/2000ms ($p = .038$) and 82db/1000ms ($p = .001$). These data indicate that intra-pNASh THC exposure selectively impairs PPF and PPI behaviours through a GSK3 dependent mechanism. In contrast, intra-aNASh THC has no effect on PPI measures or PPF in and of itself. However, co-administering intra-aNASh THC with the Akt activator, SC-79, induces effects similar to those of intra-pNASh THC exposure.
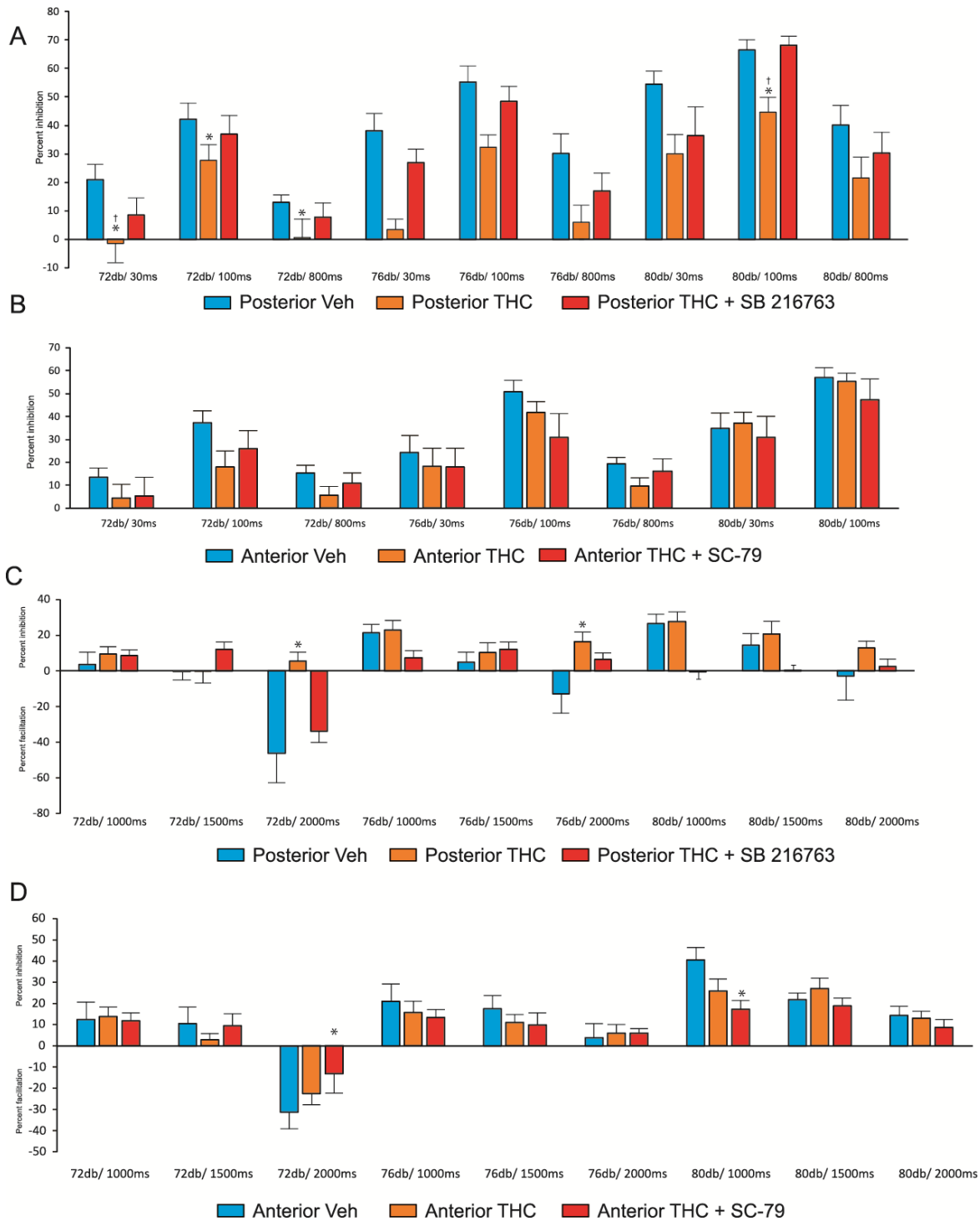
Figure 4.5 Effects of intra-NASh THC on prepulse inhibition and facilitation. A, Intra-pNASh THC reduces overall PPI p < .05. **B,** Intra-aNASh THC has no effect on PPI at any tested range. **C,** Intra-pNASh THC significantly reduces PPF behaviours, which is restored by co-administration of the GSK3 blocker SB216763. **D,** Intra-aNASh THC has no effect on PPF in

and of itself, but co-administration with the Akt activator (SC-79) induces impairments similar to those observed following pNASh THC exposure ($p < .05$)

4.3.5 Intra-NASh THC produces opposing effects on object recognition memory by altering GSK3 and Akt signalling

Given previous studies demonstrating that systemically administered THC impairs object recognition memory (Kasten et al., 2017; Quinn et al., 2008; Swartzwelder et al., 2012) and the established role of the NAc in the processing of object recognition memory (Asif-Malik, Dautan, Young, & Gerdjikov, 2017), we next examined the potential effects of intra-NASh THC on a novel object recognition task, again comparing anterior vs. posterior regions combined with pharmacological targeting of GSK3 or Akt. First, examining THC effects in the pNASh, ANOVA comparing the object recognition index between groups revealed a main effect of treatment ($F_{(2,18)} = 19.011$, $p < .001$). *Post-hoc* tests revealed that rats receiving intra-pNASh THC had significantly impaired object recognition scores relative to VEH controls (100 ng/ 0.5µg; $n's = 7$; $p = .038$). However, co-administration with the GSK3 inhibitor, SB216763 (300 ng/ 0.5 µg; $n = 7$) reversed this effect relative to THC treated rats ($p < .001$) and resulted in significantly greater object recognition scores relative to vehicle controls ($p = .008$; **Figure 6A**).

Next, examining THC effects in the aNASh, ANOVA comparing the object recognition index between groups, revealed a main effect of treatment ($F_{(2,16)} = 9.191$, $p = .002$). *Post-hoc* tests revealed that rats receiving a co-administration of intra-aNASh THC and the Akt promoter SC-79 ($n = 7$) had significantly impaired object recognition scores relative to VEH controls (100 ng/ 0.5µg; $n = 6$; $p = .008$) and THC alone ($n = 6$; $p = .004$; **Figure 6B**). Taken together, these data suggest that intra-pNASh THC impairs novel object memory through a GSK3 dependent mechanism and while intra-aNASh THC has no effect, simultaneously activating AKT produces deficits on object memory similar to intra-pNASh THC, consistent with our previously observed effects with PPF behaviours. Interestingly, co-administration of THC with a GSK3 inhibitor in the pNASh produced a pro-cognitive effect, relative to VEH controls.
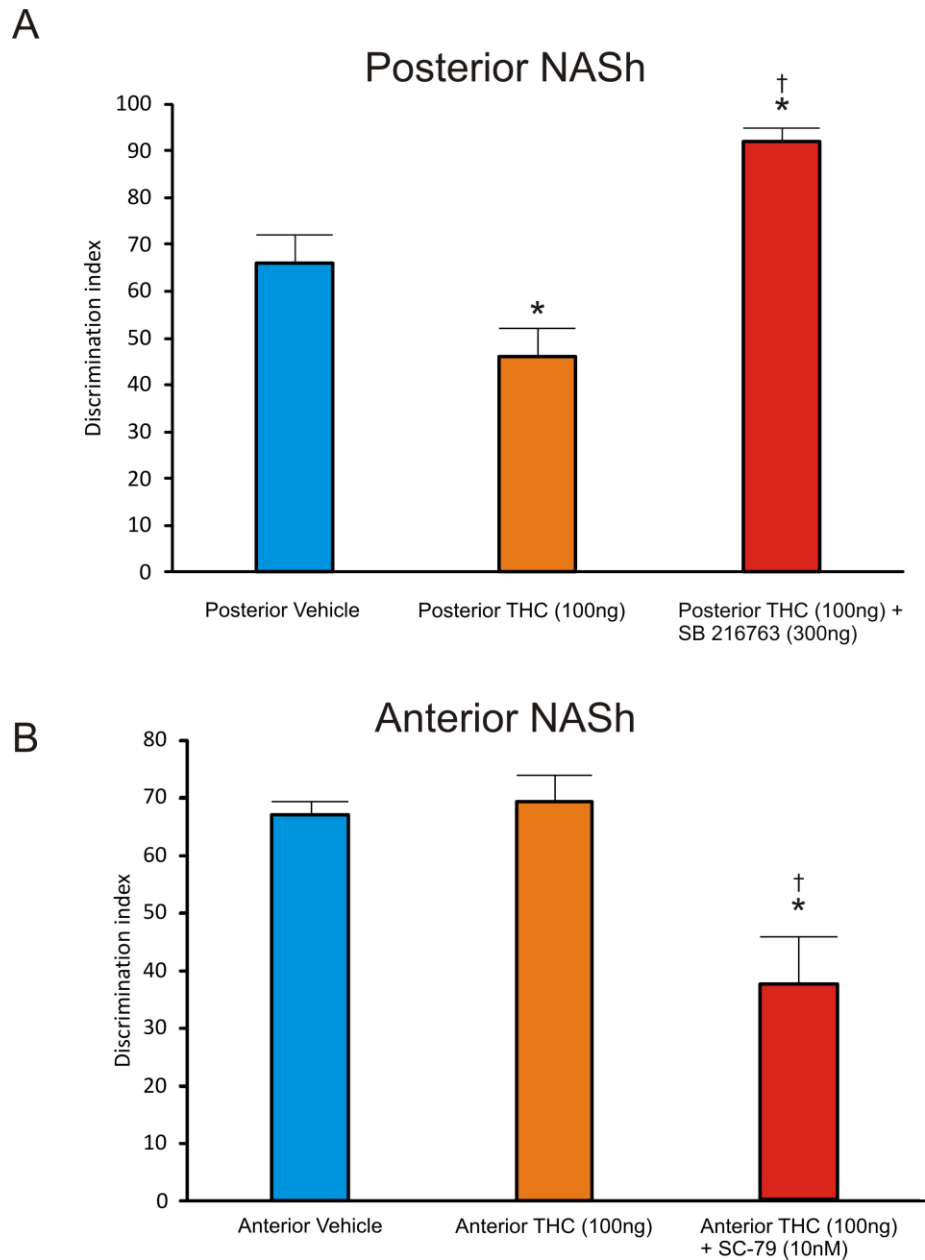
Figure 4.6 Effects of intra-NASh THC on object recognition memory. A, Intra-pNASh THC reduces the discrimination index (the amount of time spent exploring the novel object divided by the total exploration time), which is restored above vehicle by co-administration of the GSK3 blocker SB216763 * = $p < .05$ compared to Posterior Vehicle, † = $p < .05$ compared to Posterior THC. **B,** Intra-aNASh THC has no effect on the discrimination index (the amount of time spent exploring the novel object divided by the total exploration time) but when THC is co-administered with the Akt phosphorylation promoter SC-79, the discrimination index is reduced * = $p < .05$ compared to Anterior Vehicle, † = $p < .05$ compared to Anterior THC.

4.3.6 GSK3 blockade reverses the anxiogenic effects pNASh THC while AKT activation in the aNASh potentiates THC-induced anxiolysis

The elevated plus maze is a commonly used test of anxiety and systemically administered THC is known to be anxiogenic in this task (Onaivi, Green, & Martin, 1990). We therefore evaluated the potential effects of intra-NASh THC on this task, comparing the effects of anterior vs. posterior THC and related GSK3 and Akt signaling mechanisms. First, examining the effects of intra-pNASh THC, ANOVA on time (s) spent in the closed arms revealed a main effect of treatment ($F_{(5,40)} = 23.299$, $p < .001$). *Post-hoc* tests revealed that rats receiving intra-pNASh THC (100 ng/ 0.5 µg; $n = 8$) spent significantly more time in the closed arms compared to vehicle ($n = 7$; $p = .012$) but rats receiving intra-pNASh THC (100 ng/ 0.5 µg) + SB216763 (300 ng/ 0.5 µg) spent significantly less time in the closed arms than vehicle ($n = 7$; $p = .005$). Additionally, rats receiving intra-pNASH THC spent significantly more time in the closed arms than rats receiving intra-pNASh THC + SB216763 ($p < .001$; **Figure 4.7A**). Next, ANOVA testing on time (s) spent in the open arms following infusions into the pNASH, revealed a main effect of treatment ($F_{(5,40)} = 23.249$, $p < .001$). *Post-hoc* tests revealed that rats receiving intra-pNASh THC (100 ng/ 0.5 µg; $n = 8$) spent significantly less time in the open arms compared to vehicle ($n = 7$; $p = .003$) but rats receiving intra-pNASh THC (100 ng/ 0.5 µg) + SB216763 (300 ng/ 0.5 µg) spent significantly more time in the open arms than vehicle ($n = 7$; $p = .029$). Additionally, rats receiving intra-pNASH THC spent significantly less time in the open arms than rats receiving intra-pNASh THC + SB216763 ($p < .001$; **Figure 4.7A**).

Subsequently, examining the effects of intra-aNASh THC, ANOVA on time (s) spent in the closed arms revealed a main effect of treatment ($F_{(5,40)} = 12.535$, $p < .001$). *Post-hoc* tests revealed that rats receiving intra-aNASh THC (100 ng/ 0.5 µg; $n = 8$) spent significantly less time in the closed arms compared to vehicle ($n = 7$; $p = .002$) and rats receiving intra-pNASh THC (100 ng/ 0.5 µg) + SC-79 (10 µM/ 0.5 µg; $n = 7$; $p < .001$; **Figure 4.7B**). Next, ANOVA testing on time (s) spent in the open arms following infusions into the aNASH, revealed a main effect of treatment ($F_{(5,40)} = 6.535$, $p = .006$). *Post-hoc* tests revealed that rats receiving intra-aNASh THC (100 ng/ 0.5 µg) + SB216763 (300 ng/ 0.5 µg; $n = 8$) spent significantly more time in the open arms compared to vehicle ($n = 7$; $p = .004$; **Figure 4.7B**). Taken together, these data indicate that intra-pNASh THC is anxiogenic on the EPM and the anxiogenic effect can be

reversed by GSKII blockade. Additionally, intra-aNASh THC is anxiolytic, an effect that is enhanced by AKT activation.
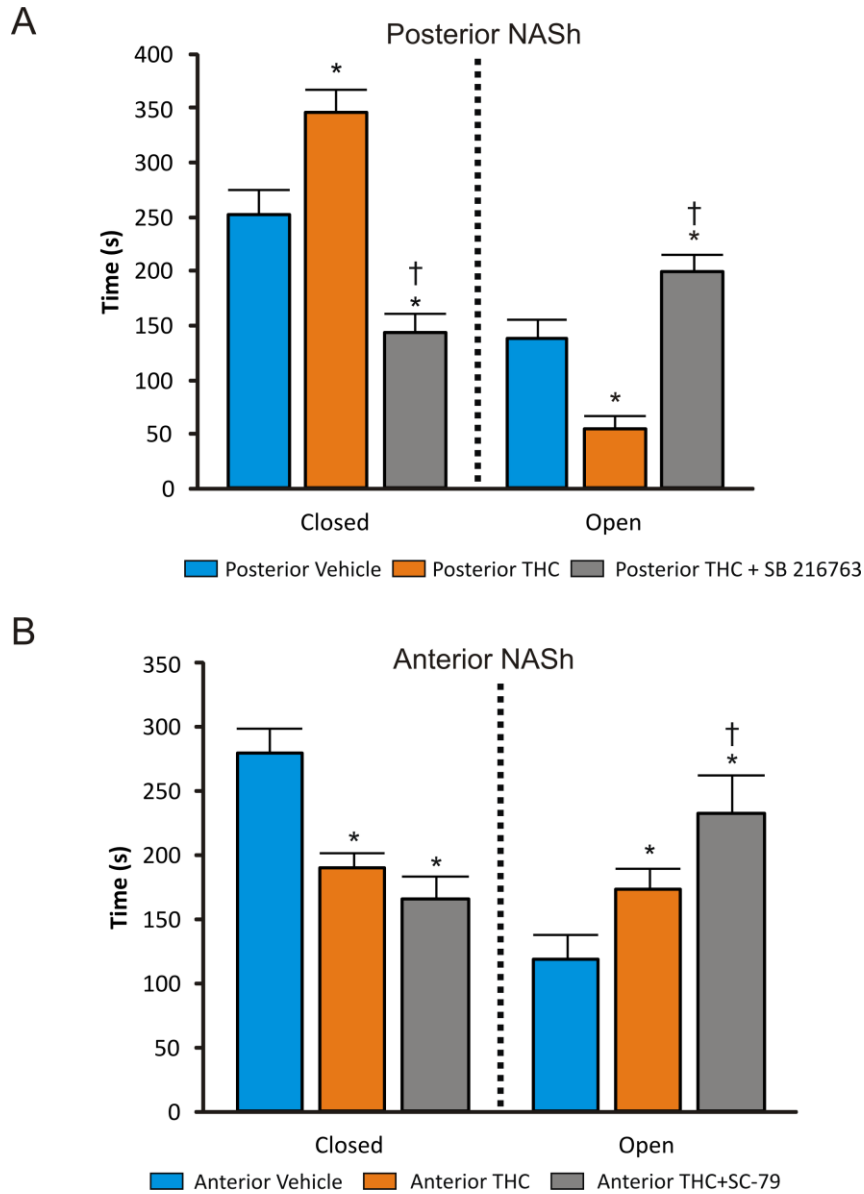
Figure 4.7 Effects of intra-NASh THC on anxiety in the elevated plus maze. A, Intra-pNASh THC decreases the amount of open arm time and increases the amount of closed arm time, which are both restored by co-administration of the GSK3 blocker SB216763 * = $p < .05$ compared to vehicle, † = $p < .05$ compared to THC. **B,** Intra-aNASh THC increases the amount of open arm and decreases the amount of closed arm time, when co-administered with the Akt phosphorylation promoter SC-79, the anxiolytic effect is increased further * = $p < .05$ compared to vehicle, † = $p < .05$ compared to THC.

4.4 DISCUSSION

The nucleus accumbens serves as a critical point of integration in the mesolimbic circuitry for associative memory formation, cognitive and affective information processing. Previous evidence has demonstrated that THC strongly modulates signalling from regions that input the NAc, most notably the VTA (Cheer, 2004; Morra, Glick, & Cheer, 2010; Morra, Glick, & Cheer, 2012; Oleson & Cheer, 2012), and that intra-NAc THC modulates mesolimbic signalling and NAc neuronal activity states (Cheer, 2004; Fitoussi et al., 2018; Morra et al., 2012; Oleson & Cheer, 2012). The NAc is also critically involved in the processing of fear-related associative memory and sensorimotor cognitive filtering (PPI) (Fadok et al., 2010; Iordanova, Westbrook, & Killcross, 2006). Nevertheless, the precise molecular and neuroanatomical mechanisms by which THC can produce dissociable effects within the ventral striatum on emotional memory formation and sensory integration have not been previously identified.

In the present study, we demonstrate that THC produces anatomically dissociable, bivalent effects on associative memory processing, anxiety, object memory, and sensorimotor gating via dissociable molecular signalling pathways. First, targeting a battery of molecular biomarkers in the anterior vs. posterior NASh sub-regions, we found that THC in the pNASh selectively decreases phosphorylation of GSK3 and decreases expression levels of β-catenin. In contrast, infusions of THC into the aNASh selectively decreases phosphorylated AktSer473 and phosphorylated mTOR expression levels. Together, these findings are the first demonstration for regionally selective modulation of these specific pathways within discrete NASh sub-regions and reveal new insights into how THC can produce localized effects on these signaling pathways within a common neural region.

Behaviourally, we report that intra-pNASh infusions of THC produced GSK3 dependent potentiation of fear memory salience; induced anxiogenic effects and impaired PPI and PPF. In addition, pNASh THC exposure induced object recognition memory deficits. In contrast, intra-aNASh THC blocked the encoding of normally supra-threshold associative fear memory and produced anxiolytic effects. When THC was co-administered with an Akt activator, the formation of associative fear memory was restored, time in the open arms during EPM increased above the levels of THC alone, PPF was impaired and object recognition memory was reduced.

Together, these data demonstrate the functional importance of the Wnt and mTOR pathways underlying regionally selective affective and cognitive impairments induced by THC in the anterior vs. posterior NASh subregions.

At the molecular level, we and others have previously identified the roles of the Wnt and mTOR pathways in the functional effects of THC. For example, chronic or acute THC exposure profoundly alters phosphorylation and expression levels of GSK3, Akt, mTOR, p70S6K, in the prefrontal cortex (Renard et al., 2016; Renard, Rosen, et al., 2017; Renard, Szkudlarek, et al., 2017). In addition, mesolimbic Wnt signalling is critical for the processing of fear, anxiety and memory processing (Alimohamad, Rajakumar, Seah, & Rushlow, 2005; Cuesta et al., 2017; Korem et al., 2017; Maguschak & Ressler, 2011; Proto et al., 2017; Xu et al., 2011; Xu et al., 2015). More specifically, Wnt was previously demonstrated to be necessary for the consolidation of fear memory in the hippocampus (Xu et al., 2015). Moreover, downregulating β-catenin in the NAc was previously shown to impair extinction learning (Korem et al., 2017), indicating a critical role for this protein in striatal-mediated learning and memory processing. Additionally, a previous study demonstrated that infusions of atypical antipsychotics, cause increased GSK3 phosphorylation and therefore a decrease in GSK3 activity (Alimohamad et al., 2005; Li, Rosborough, Friedman, Zhu, & Roth, 2007). In the present study, we found that infusions of THC into the pNASh produced several pathological effects, including impairments in PPI/PPF and increased anxiety, concomitant with decreased phosphorylation of GSK3. Together, these findings suggest that both the psychotomimetic effects and potentiation of fear memory formation induced by THC are regulated by local changes in canonical Wnt and β-catenin signalling in the pNASh.

The mTOR pathway has similarly been implicated in the processing of associative fear memory (Gafford et al., 2013; Jarome et al., 2018; Jobim et al., 2012; Puighermanal et al., 2013). For example, central administration of THC increased phosphorylation of Akt through a CB_1 receptor-dependent mechanism (Ozaita, Puighermanal, & Maldonado, 2007) and the effects of THC on memory formation in the hippocampus are modulated by mTOR signalling. While THC changes the activity of many distinct signalling molecules, the results of the present study provide a novel dissociation of separate localized signalling cascades within the NASh that show

clear and distinct patterns of activity following localized THC exposure. Future studies are required to further characterize the effects of intra-NASh THC on other signaling pathways in the Wnt interactome in order to fully elucidate these localized, THC-mediated signaling sequelae.

Given the established effects of THC on disrupting sensorimotor gating and cognitive filtering (Renard et al., 2017), we next evaluated how intra-NASh infusions might influence PPI and PPF. Many previous studies have demonstrated that patients with schizophrenia have impairments in sensorimotor gating and therefore decreased PPF and PPI (Swerdlow et al., 2018; Wynn et al., 2004). Indeed, deficits in PPI and PPF are established endophenotypes found in individuals considered to be at high risk of full-onset psychosis, and this risk is increased with exposure to cannabis (Winton-Brown et al., 2015). Whereas PPI abnormalities are considered to reflect deficits in sensory filtering, PPF deficits likely reflect deficits in sustained attention, both of which are cardinal cognitive endophenotypes associated with schizophrenia and other neuropsychiatric disorders. Thus, PPI deficits can be restored to normal levels by treatment with atypical anti-psychotics (Wynn et al., 2007). PPI is also impaired by THC in animal models (Malone & Taylor, 2006), and can be restored by co-administration of anti-psychotics (Nagai et al., 2006). Consistent with the present study, GSK3 has been implicated as an important molecular substrate necessary for normal PPI behaviours (Kapfhamer et al., 2010). Additionally, previous research has identified the NAc as an important neural region for PPI processing (Mohr, von Ameln-Mayerhofer, & Fendt, 2009). The results of the present study demonstrate that by increasing the phosphorylation state of the Wnt signalling pathway, consistent with a pro-psychotic mechanism, intra-pNASh THC can impair both PPI and PPF, selectively in the posterior NASh.

Consistent with the present study, previous research has demonstrated the importance of the NA for object memory (Nelson, Thur, Marsden, & Cassaday, 2010; Sargolini, Roullet, Oliverio, & Mele, 2003). For example, microinfusions of a NMDA antagonist into the NAc impaired object recognition memory (Sargolini et al., 2003). NMDA receptors are also known to regulate GSK3 phosphorylation (De Sarno, Bijur, Zmijewska, Li, & Jope, 2006) which suggests a potential common mechanism for impairing object recognition memory with intra-pNASh microinfusions

of THC. Interestingly, we showed that activating the phosphorylation of Akt caused intra-aNASh to more closely resemble the effects of intra-pNASh, which is consistent with what we know about the substantial interactions between the GSK3 and mTOR pathways (Shi et al., 2014) within the Wnt interactome. Previous studies have also demonstrated that i.p. injections of THC impairs object recognition memory (Kasten et al., 2017) but none have examined the exact role the NASh plays in the process. The present study demonstrated that GSK3 signalling in the pNASh is involved in THC mediated impairment of object recognition memory.

Considerable controversy exists, however, in the literature surrounding CB_1 agonists and anxiety. While some studies have demonstrated anxiogenic effects (Genn, Tucci, Marco, Viveros, & File, 2004; Schramm-Sapyta et al., 2007), others have shown anxiolytic effects (Berrendero & Maldonado, 2002). More recent studies, however, have suggested that the division between the seemingly opposing effects of THC on anxiety is primarily dose-dependent (Childs et al., 2017; Tiziana Rubino et al., 2007) including within the mesolimbic system (Rubino, Guidali, et al., 2008). As THC dose increases, the effect of THC generally switches from anxiolytic to anxiogenic. The present study is the first to suggest that there are anatomically distinct processes within the anterior vs. posterior NASh regions mediated by separate molecular pathways that can influence the behavioural effects of THC in this affective domain.

Consistent with our results showing selective down-regulation of GSK3 phosphorylation states in the posterior NASh (and hence, increased local levels of active GSK3), previous research has indicated that hyperactive GSK3 activity levels can produce anxiety (Mines, Yuskaitis, King, Beurel, & Jope, 2010; Qiao et al., 2018). Interestingly, the anxiolytic effect of intra-aNASh THC was not sensitive to an increase in Akt activity but this is consistent with some previous research indicating that the anxiolytic effect of THC is not sensitive to mTOR signalling (Puighermanal et al., 2013). Future research should attempt to elucidate the mechanism behind intra-aNASh THC anxiolysis and examine if increasing the dose of centrally administered THC increases the likelihood of stimulating the pNASh vs the aNASh.

In summary, the results of the present study provide several novel mechanisms for how THC differentially modulates CB_1 signalling pathways and bivalent effects on fear memory, object

recognition memory, anxiety, and sensorimotor gating. The present findings provide crucial new information for understanding the underlying molecular mechanisms of THC in anatomically distinct regions of the NASh and how they may underlie both the affective and cognitive side-effects of cannabis exposure.

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CHAPTER 5

5   General Discussion

Characterizing both the effects of cannabis exposure represents one of the most complex challenges facing the neuropsychiatric research community. Given the overwhelming phytochemical complexity of cannabis, there is an urgent need to fully characterize the unique and sometimes opposing neurophysiological properties of these independent plant constituents. Currently, THC and CBD represent the best characterized of these phytocannabinoids in terms of their underlying pharmacological properties as well as their distinct and dissociable effects on various neuropsychiatric phenomena.

The objectives of my thesis project were to characterize the neuronal, molecular, behavioural and pharmacological mechanisms responsible for the effects of THC and CBD, directly within the mammalian nucleus accumbens and associated mesolimbic regions such as the VTA. To achieve these research objectives, I have used an integrative combination of molecular protein signal analyses, behavioural pharmacological assays and *in vivo* neuronal electrophysiology targeting anatomically distinct sub-regions of the mammalian nucleus accumbens, with a special focus on the shell division of this structure. The combined results of my thesis projects have yielded a series of new insights and discoveries into the underlying neuronal, molecular and pharmacological effects of both THC and CBD, within distinct neuroanatomical sub-regions of the mammalian ventral striatum.

5.1 Cannabidiol modulates fear memory formation through interactions with serotonergic transmission in the nucleus accumbens shell and modulation of ventral tegmental area neuronal activity

In light of previous evidence of the therapeutic potential of CBD to treat anxiety (Bergamaschi et al., 2011; Crippa et al., 2011), schizophrenia (Leweke et al., 2012; Schubart et al., 2014; Zuardi, Crippa, Hallak, Moreira, & Guimaraes, 2006) and PTSD (Blessing, Steenkamp, Manzanares, & Marmar, 2015) and the role the mesolimbic circuit plays in psychiatric conditions, we first sought to examine the influence of CBD on mesolimbic neuronal function (Chapter 2). Previous research has also indicated that systemic administration of CBD enhanced fear memory extinction in humans (Das et al., 2013) and blocks the reconsolidation of fear memory in an

animal model (Stern, Gazarini, Takahashi, Guimarães, & Bertoglio, 2012). Given previous evidence demonstrating the importance of the NAc in fear memory processing (Fadok, Darvas, Dickerson, & Palmiter, 2010), we selected this brain region for our first studies.

We found that microinfusions of CBD into the NASh during fear memory formation reduced subsequent freezing behaviour when presented with an odour that had been previously paired with a footshock. The formation of an associative fear memory was restored when CBD was co-administered with the 5-HT$_{1A}$ antagonist NAD 299 but not the broad-spectrum dopamine antagonist α-flu or the CB$_1$R antagonist rimonabant. Furthermore, we tested the sensitivity of the rats to footshock following intra-NASh infusions of CBD compared to vehicle and found no difference. Taken together, these data indicate that CBD reduces the formation of associative fear memory formation directly in the NAc through activity on the 5-HT$_{1A}$ receptor, but independently of DAergic receptor transmission via the D$_1$/D$_2$ subtypes. Next, we sought to determine the underlying cellular activity responsible for these behavioural effects. Using *in vivo* electrophysiological techniques to record VTA neurons, we determined that intra-NASh CBD infusions decreased both firing and bursting rate of VTA DA neurons and concomitantly increased the activity of a subgroup of non-DA, presumptive GABAergic neurons. Finally, to ensure the change in activity of the VTA neurons and the behaviour were not epiphenomena, we performed a pharmacological disconnection study by simultaneously infusing CBD into the NASh and GABA$_A$/GABA$_B$ blockers into the VTA in contralateral brain hemispheres. We found that while the GABA blockers had no effect on their own, they restored the formation of associative fear memory when co-administered with intra-NASh CBD. Thus, this evidence demonstrated the importance of NASh→VTA projections targeting GABAergic receptor substrates directly in the VTA, for the functional effects of intra-NASh CBD. Therefore, we demonstrated for the first time that CBD blocks the formation of associative fear memory by modulating the NASh/VTA circuitry through a 5-HT$_{1A}$ dependent mechanism directly in the NASh.

Our results suggested that the neuroanatomical substrate responsible for the effects of CBD on the consolidation of fear memory are likely due to a direct modulation of the NASh/VTA circuit. More importantly, our findings provided further evidence for the effects of CBD on serotonergic

5-HT$_{1A}$ receptor signalling and indicate that despite producing its actions within the NASh, CBD can modulate fear-related behaviours independently of actions directly on DA D$_1$/D$_2$R receptor substrates. Together, the findings reported in Chapter 2 identify CBD as a potential therapeutic option for anxiety-related disorders. Indeed, there is now growing evidence that CBD possesses anxiolytic properties (Almeida et al., 2013; Blessing et al., 2015; Crippa et al., 2011). Our findings characterize a highly localized neuroanatomical locus wherein CBD might serve as an anxiolytic compound and provides a specific functional mechanism (reduction of DAergic activity in the mesolimbic pathway) by which CBD may produce these effects. In addition, given our findings that CBD strongly dampens the activity of mesolimbic DAergic states (consistent with previous studies in our lab (Renard et al., 2016)), these findings have important implications for CBD as a potential treatment for addiction-related behaviours. Indeed, virtually all drugs of abuse directly or indirectly activate the mesolimbic DA system (Volkow, Fowler, Wang, Swanson, & Telang, 2007; Young, Gobrogge, & Wang, 2011). The ability of CBD to inhibit DAergic activity states in the VTA suggests potential anti-addictive effects of CBD. Indeed, there is already existing evidence suggesting that CBD may serve as an effective inhibitor of opioid-related addiction phenomena (Hurd, 2017).

5.2   THC Regulates Reward and Aversion Processing via Dissociable Opioid Receptor Substrates and Neuronal and Oscillatory Modulation in Distinct Striatal Sub-Regions

In Chapter 3, I described a series of integrative experiments aimed at precisely characterizing the neuroanatomical loci of THC's bivalent effects on reward and aversion-related behavioural processing. While it is well-established that THC can produce both reward and aversion effects, until now, the neurobiological mechanisms behind these bivalent effects have not been characterized. The NAc can be subdivided into the core (NACo) and shell (NASh) sub-regions. In terms of neuropsychiatric pathology, considerable evidence implicates a more critical role for the NASh, particularly in terms of functionally important connections with VTA-related DAergic transmission (Yang et al., 2018). However, within the NASh itself, emerging evidence points to important differences between the anterior and posterior poles of this sub-region. For example,

there are functional differences in terms of affective processing between the anterior and posterior NASh regions. The anterior region being critical for the processing of hedonic, reward-related information, both for drug-related and natural associative reward stimuli; and the posterior NASh being critical for the processing of aversion-related affective information (Castro *et al*, 2016; Castro and Berridge, 2014; Mahler *et al*, 2007; Reynolds and Berridge, 2002; Smith and Berridge, 2007). Nevertheless, the underlying molecular mechanisms within the NASh responsible for modulating aversive, fear-related associative memory formation and cognitive function have not been characterized, particularly in relation to the potential involvement of the endocannabinoid system and the potential influence of THC within these accumbal sub-regions.

Substantial amounts of clinical and pre-clinical research have demonstrated that functional anatomical divisions exist within the NAc. For example, beyond the divisions between the NASh and the NACo sub-regions, the anterior and posterior NASh are anatomically and functionally distinct. Previous studies have demonstrated the existence of a "hedonic hotspot" at the anterior pole of the NASh associated with a localized concentration of MORs and a corresponding paucity of KORs (Arvidsson et al., 1995; Castro & Berridge, 2014; Peciña & Berridge, 2000). Furthermore, previous evidence indicates a strong interaction between the endocannabinoid system and the opioid system (Ahmad & Laviolette, 2017; Mascia et al., 1999; Pickel et al., 2004; Skelly et al., 2010). For example, rewarding effects associated with low doses of THC were absent in MOR knockout mice and aversion to high doses of THC was absent in KOR knockout mice (Ghozland et al., 2002). Therefore, we hypothesized that the rewarding effects of THC were due to activation of MORs in the aNASh and that the aversive effects of THC were due to activation of KORs in the pNASh.

Consistent with this general hypothesis, we found that intra-aNASh microinfusions of THC produced strong conditioned reward effects in a CPP procedure through a MOR dependent mechanism. In contrast, intra-pNASh microinfusions of THC produced aversion through a KOR dependent mechanism. Additionally, intra-aNASh THC, but not intra-pNASh THC, potentiated reward to a sub-threshold dose of morphine. We also found that intra-pNASh, but not intra-aNASH THC, reduced social motivation and social recognition. Previous studies have demonstrated the importance of the anterior/ posterior division in the NASh in reward processes

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through stimulation of the endocannabinoid system and endogenous opioid receptors (Castro et al., 2016; Mahler et al., 2007). The data presented in chapter 3, however, demonstrates for the first time the significance of the division in reward from drug of abuse.

The powerful motivational properties of drugs of abuse are what encourages repeated and compulsive use. Understanding the mechanisms behind drug-related reward effects is important for developing effective treatments for addiction. The changing attitudes towards the recreational use of cannabis also necessitates the investigation of THC reward specifically to insure proper policies of harm reduction. Like all drugs of abuse, THC effects DA release (Bossong et al., 2015; Fitoussi, Zunder, Han, & Laviolette, 2018; Oleson & Cheer, 2012), and cannabinoid signaling plays a vital role in controlling DA release following the abuse of other rewarding substances (Cheer et al., 2007). Fully elucidating the role of cannabinoid signaling in producing reward, will further our understanding of the general mechanisms of addiction as well as the effects specific to cannabis abuse and dependence. Additionally, in the wake of legalization, cannabis is increasingly viewed as a harmless drug, but it can still produce numerous negative acute and long-term effects. Rates of cannabis use are likely to continue to increase and a proper understanding of the mechanisms behind some of the negative effects will help to minimize them.

For example, many cannabis users report feelings of panic, paranoia and dysphoria following THC exposure (Freeman et al., 2015; Murray, Morrison, Henquet, & Di Forti, 2007). Our results suggest that the source of many of the negative effects of cannabis involve stimulation of the pNASH by THC. The respective sensitivities of the aNASh and pNASh to THC administration could be the source of the substantial variation in experience between individuals following cannabis consumption. The data presented in Chapter 3 could direct a new area of inquiry in human research to understand the biphasic effects of cannabis use on these psychotropic side-effects.

Next, to examine the functional mechanisms underlying these behaviours, we performed *in vivo* electrophysiological recordings within these anatomically distinct NASh sub-regions. We simultaneously recorded single-unit activity of MSNs and LFP. We discovered that ICV

infusions of THC strongly reduced the activity of aNASh MSNs but increased their activity states within the pNASh. The activity of striatal MSNs has been demonstrated to be vital for mediating motivation and learning in both animal and human models (Soares-Cunha, Coimbra, Sousa, & Rodrigues, 2016). Previous studies have suggested that the activity of MSNs can be subdivided into the direct pathway, which controls reward, mediated by D_1R expressing neurons that input directly into the basal ganglia, and the indirect pathways which controls aversion and is mediated by D_2R expressing neurons that input into the ventral pallidum. Recently, however, this model has been disputed as overly-simplistic and that the distinct pathways do not solely mediate opposing processes (Kupchik et al., 2015; Soares-Cunha, Coimbra, David-Pereira, et al., 2016; Soares-Cunha, Coimbra, Sousa, & Rodrigues, 2016). The electrophysiological results presented in Chapter 3 provide further evidence that the role the NA plays in both reward and aversion cannot be distinctly parceled into the direct and indirect pathways. Further study is needed to fully elucidate the complex interplay of mechanisms that underlie the role of the NAc in motivation.

Finally, we found that ICV infusions of THC caused an increase in the power of high-frequency gamma oscillations when recording in the aNASh but a decrease in the power of high-frequency gamma oscillations when recording in the pNASh. Gamma oscillations are altered by THC administration in human models (Cortes-Briones et al., 2015) and are associated with the psychotomimetic effects of THC (Nottage et al., 2015). Deficits in gamma oscillations are also common in schizophrenia and associated with many of the cognitive problems present in the disorder (McNally, McCarley, & Brown, 2013; Sun et al., 2011; Uhlhaas & Singer, 2010; Woo, Spencer, & McCarley, 2010). The association with schizophrenia and some of the negative effects of THC suggest that the changes we observed to gamma oscillations in the pNASh, may be responsible for some of the pro-psychotic effects of THC. Additionally, changes in gamma oscillations have been observed during reward processing in both rat (van der Meer & Redish, 2009) and human models (Cohen et al., 2009). Methamphetamine induced increases in high-frequency gamma oscillations in the nucleus accumbens are cannabinoid-receptor dependent (Morra et al., 2012), thus implicating gamma activity in the NAc in the rewarding states induced by drugs of abuse. In light of the previous research, our results demonstrating that THC increases the power of high-frequency gamma oscillations in the aNASh further demonstrate the

importance of both gamma oscillations of cannabinoid signaling to rewarding states created by drugs of abuse. Our results, however, suggest that the positive motivational states associated by the induction of high-frequency gamma oscillations can be attributed specifically to the aNASH. Overall, our results suggest that the seemingly contradictory effects of THC on appetitive processing are due to differential stimulation of distinct areas within the NASh. We have demonstrated that, for the first time, the effects of a drug of abuse on reward is rooted in the functional difference within the NASh.

5.3 Δ^9-tetrahydrocannabinol (THC) Regulates Memory, Anxiety and Sensorimotor Gating via Dissociable Modulation of the Wnt and mTOR Signaling Pathways in Anatomically Distinct Nucleus Accumbens Shell Sub-Regions

THC is well known to cause both relaxation and paranoia (Freeman et al., 2015; Rubino et al., 2007). However, the anatomical and molecular mechanisms underlying these seemingly contradictory psychotropic effects remain unclear. Given our results detailed in Chapter 2, demonstrating that CBD could block the consolidation of fear memory formation by acting in the NASh and our results detailed in Chapter 3, demonstrating that the biphasic effects of THC on appetitive processing is due to dissociable and anatomically distinct processes within the NASh, we next sought to examine if the effects of THC on fear, memory, anxiety and sensorimotor gating are also rooted in localized differences with the NASh, via differential modulation of neuropsychiatric-related molecular signaling pathways.

The cannabinoid system has been heavily implicated in the processing of emotionally salient stimuli and memory, including fear and anxiety. CB_1 receptors, are expressed ubiquitously throughout the NAc and previous studies have demonstrated that CB_1 signalling in the NAc is involved in a variety of affective and motivated behaviours, including food intake, addiction, stress and fear processing (Deshmukh & Sharma, 2012; Kuhnert, Meyer, & Koch, 2013; Li et al., 2018; Papilloud, Guillot de Suduiraut, Zanoletti, Grosse, & Sandi, 2018; Pedroza-Llinás et al., 2013; Wang et al., 2010). For example, peripubertal stress increased gene expression for CB_1

within the NASh, suggesting the involvement of the ECS in regulating stress response (Papilloud et al., 2018). Anandamide (AEA), one of the primary endocannabinoids and a CB_1 agonist, has been demonstrated to have biphasic effects on anxiety in the prefrontal cortex, producing both anxiogenic and anxiolytic effects (Rubino et al., 2008), suggesting that the eCB system plays a role in the balance of anxiety. Additionally, direct infusion of a selective CB_1 agonist directly into the pNASH induced anxiogenesis in the EPM (Kochenborger et al., 2014). Thus, the apparent balancing role that the NAc eCB system plays in the modulation of affective information processing may suggest that exogenous cannabinoids, such as THC, may similarly produce both anxiogenic and anxiolytic effects. The exact molecular mechanisms behind how THC modulates anxiety and memory, however, have never been fully investigated.

Previous work in our lab has established that THC alters the levels of many molecular signaling proteins associated with psychosis and mood disorders (Renard et al., 2017). Previous evidence has also implicated Wnt signaling pathways within the mesolimbic system in the consolidation of fear memory (Maguschak & Ressler, 2011) and extinction (Korem, Lange, Hillard, & Akirav, 2017). Additionally, mTOR signaling has been implicated in the formation and reconsolidation of fear memory (Jobim et al., 2012), cannabinoid-mediated modulation of memory (Puighermanal et al., 2009), and the anxiogenic, but not anxiolytic effects, of THC (Puighermanal et al., 2013). Therefore, targeting these specific molecular biomarkers in the accumbens, we sought to examine if the effects of THC on fear-related memory formation, anxiety and cognition, were mediated by differential modulation of these molecular signaling pathways in anatomically localized NASh subregions.

First, we found that, infusions of THC into the aNASh selectively decreased the phosphorylation of Akt at ser473, but not total Akt or phosphorylated Thr308 Akt, and decreased phosphorylated mTOR, but not total mTOR expression levels. In contrast, we found that infusions of THC into the pNASh decreased phosphorylated, but not total, GSK3 expression levels and increased expression levels of β-catenin. Thus, these findings revealed novel and double-dissociable effects of THC within localized NASh sub-regions on several well-established molecular biomarkers associated with neuropsychiatric disorders. At the behavioural level, we found that infusions of THC into the aNASh blocked the formation of associative fear memory to a supra-

threshold footshock conditioning stimulus. However, this effect was reversed through pharmacological activation of Akt with SC-79. Conversely, we found that infusions of THC into the pNASh, potentiated the formation of associative fear formation to a sub-threshold footshock through a GSK3-dependent mechanism. Next, given previous evidence implicating THC and Wnt signaling in psychosis (Alimohamad, Rajakumar, Seah, & Rushlow, 2005; Renard et al., 2017), and previous research demonstrating that a major behavioural hallmark of schizophrenia is a deficit in sensorimotor gating as assessed by a disruption in PPI (Braff & Geyer, 1990; Takahashi et al., 2008), we tested the effects of intra-NASh infusions of THC on PPI and PPF. Intra-pNASh, but not aNASh THC administration impaired both PPI and PPF. However, PPF was restored by blocking GSK3 activity. Subsequently, we tested the effects of intra-NASh THC on object recognition memory. We found that intra-pNASh THC decreased discrimination between the novel and familiar objects through a GSK3 dependent mechanism. Intra-aNASh THC, however, had no effect on discrimination unless it was co-administered with the Akt activator SC-79. Finally, we examined if the effects of THC on anxiety in the EPM were mediated by the same processes. We found that intra-pNASh infusions of THC decreased open-arm time and increased closed-arm time through a GSK3 dependent mechanism. Intra-aNASh THC increased open-arm time and decreased closed-arm time, which was consistent with previous evidence (Puighermanal et al., 2013). However, the anxiolytic effect of THC was not sensitive to pharmacological modulation of Akt signaling by SC-79 co-administration.

Striatal mTOR signaling is known to be importantly involved in learning and memory processes. For example, increases in phosphorylated mTOR levels were observed following skill learning in the striatum (Bergeron, Chagniel, Bureau, Massicotte, & Cyr, 2014). The decreased activity in the mTOR pathway following aNASh THC infusion is therefore the likely source of the fear memory deficits. Previous studies have demonstrated specific genetic factors in the mTOR pathway that link vulnerability to psychotic disorders following cannabis exposure. For example, the genetic risk of schizophrenia and psychosis in families can in part be determined by the sensitivity to the psychotomimetic effects of cannabis (Genetic Risk and Outcome of Psychosis (GROUP) Investigators, 2011). Interestingly, a specific single nucleotide polymorphism (SNP) on the AKT1 gene, which encodes the Akt protein, was associated with both psychotic disorder and sensitivity to the psychotomimetic effects of cannabis (Genetic Risk and Outcome of

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Psychosis (GROUP) Investigators, 2011). Furthermore, a subsequent study also linked the same SNP to cannabis-induced long-term cognitive alterations in psychosis (Van Winkel & Genetic Risk and Outcome of Psychosis (GROUP) Investigators, 2011). Therefore, our results demonstrate remarkable translational consistency with the human genetics literature in terms of THC-induced modulation of the Akt signaling pathway.

Previous studies have also demonstrated that mTOR is involved in the reward from drugs of abuse through the regulation of dopamine signaling (Liu, Li, Yu, Vickstrom, & Liu, 2017). mTOR signalling in the NASh is specifically involved in the regulation of dopamine concentration (Luo et al., 2016). These studies suggest that mTOR may therefore also be involved in the rewarding effects of aNASh THC infusions (Chapter 3). An important direction for future investigations would be to examine if THC may differentially affect the functional activity states of the anterior vs. posterior ventral striatum in patients with a family history of psychosis and/or how cannabis exposure might differentially modulate these subregions in the context of specific psychotropic side-effect profiles.

The Wnt signaling pathway, and more specifically canonical Wnt, is heavily involved in the regulation of transcriptional factors and regulates stress and behavioural resilience (Dias et al., 2014). The interaction between Wnt signaling and endocannabinoids in the NAc has also been demonstrated to regulate extinction learning (Korem et al., 2017). Dysregulation of this pathway is therefore likely to cause acute stress and is potentially responsible for the effects of THC infusion into the pNASh. Additionally, *in vitro* cell line studies have been conducted comparing neurons derived from human-induced pluripotent stem cells (hiPSC) from both schizophrenia and control lines. They determined that THC can induce similar changes to Wnt signaling as seen in the schizophrenia derived cells (Guennewig et al., 2018). Additionally, Min et al., (2011) identified a novel mechanism by which D_2 receptors can recruit β-catenin directly from the cytosol, bypassing GSK3 entirely, which may explain why both GSK3 and β-catenin increased following pNASh infusions of THC. Given the predominance of the D_2 indirect pathways in aversion processing and the data presented in chapter 3 demonstrating the role the pNASh plays in aversion, the changes in β-catenin may be involved more in motivational states than memory or anxiety. Taken together with the previous genetic research, the data presented in chapter 4 indicates that changes in molecular signaling in the NASh following THC infusions may also

underlie THC-induced short-term psychosis symptoms and increased risk for long-term psychotic disorders. Future studies should investigate if changes in pNASh activity induced by the effects of THC on Wnt signaling underlie the pro-psychotic effects of long-term cannabis use.

Taken together, our results indicate the effects of intra-NASh THC on fear memory, anxiety, object recognition, and sensorimotor gating are mediated by dissociable changes in Wnt and mTOR signaling. Our results suggest that in addition to the biphasic effects of THC on appetitive processing being routed in functional differences between the pNASh and aNASh as demonstrated in Chapter 3, these functional differences are also the putative underlying mechanism associated with the multifaceted, biphasic effects of THC.

5.4   Limitations

The brain is a complex system of interacting networks, but the nature of any reductionist approach must be to examine neural regions and sub-divisions individually, before one can understand overall systems-level functions. As discussed in Chapter 1, even the mesolimbic pathway consists of multiple brain areas that establish a very complex circuit. The experiments presented here are focused on a subdivision of one area within that circuit, the nucleus accumbens shell. However, the NASh does not exist in a vacuum. To fully understand exactly how compounds in cannabis may alter this pathway, the other areas in this circuit must also be fully examined. For example, the BLA and mPFC have both been shown to be very important for the role that cannabinoid signaling has on fear and anxiety (Aliczki et al., 2016; Phan et al., 2008; Ratano, Everitt, & Milton, 2014; Tan et al., 2011). Additionally, as discussed in Chapter 2, the endocannabinoid system interacts with many other neurotransmitter systems. While most of the present studies addressed the interactions with some other neurotransmitter systems, the endocannabinoid system heavily interacts with serotonin (Best & Regehr, 2008; Haj-Dahmane & Shen, 2011) and glutamate (Varma, Carlson, Ledent, & Alger, 2001) both of which have been heavily implicated in the normal function of the mesolimbic circuit. For example, glutamatergic signaling in the NASh is involved in both fear and desire and THC has been conclusively demonstrated to alter glutamate transmission (Colizzi, McGuire, Pertwee, & Bhattacharyya, 2016). A complete view of the effects of THC on the mesolimbic reward pathway necessitates a

thorough investigation into the dynamics between different areas and between different neurotransmitter systems.

Similarly, we only examined a fraction of the signaling proteins involved in the Wnt interactome. As discussed in Chapter 1, JNK is an important part of Wnt signaling and THC has previously been demonstrated to activate JNK (Rueda, Galve-Roperh, Haro, & Guzman, 2000). Other signaling proteins have also been implicated in the effects of THC on cognition. For example, extracellular signal related kinase (ERK) has been implicated in the effects of THC on memory (Derkinderen et al., 2003; Ruhl et al., 2014) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has been implicated in the anxiolytic effects of low doses of THC (Rubino et al., 2007). Additionally, we did not investigate the role of any signaling proteins on the effects of CBD, which might play a significant impact in its therapeutic potential (Renard et al., 2016).

Cannabis use remains widespread and will likely increase with changing societal attitudes towards the safety and the normalization of cannabis use for recreational and therapeutic purposes (Pacula, Powell, Heaton, & Sevigny, 2015; Wen, Hockenberry, & Cummings, 2015). Undoubtedly, the specific phytochemical compounds within cannabis have tremendous potential, both for therapeutic applications and also in terms of increasing the risk of neuropsychiatric disorders. Of the phytocannabinoids described in this thesis, THC has primarily been implicated for its potential negative effects on mental health, whereas CBD appears to show tremendous promise as a treatment for schizophrenia and potentially anxiety-related disorders. The findings described in this thesis characterize the effects of THC and CBD within the NASh and have revealed numerous neurobiological mechanisms that appear to underlie many of the psychotropic effects of cannabis use. These data contribute to a growing body of both clinical and pre-clinical research on the diverse pharmacological effects of cannabinoids. In addition, this evidence highlights the important, yet divergent properties of these phytochemicals in terms of their potential positive and negative effects on mental health.

5.5 Future Directions

As previously discussed the mesocorticolimbic circuitry is highly interconnected. Extending the work outlined in this thesis to examine potential interactions with other limbic regions such as

the mPFC and BLA, would help further characterize the effects of cannabis compounds on cognition, memory and emotion. Indeed, considerable evidence from our laboratory has extensively characterized how cannabinoid CB_1 receptor signaling within both the BLA and PFC can control emotional processing and memory formation (Ahmad & Laviolette, 2016; Ahmad et al., 2017; Tan et al., 2011; Tan, Ahmad, Loureiro, Zunder, & Laviolette, 2014). Examining specifically how THC and CBD may act within these neural regions would greatly expand our knowledge of how phytocannabinoid signaling across and within the mesocorticolimbic circuitry may differentially influence mental health related phenomena.

As discussed previously in Chapter 4, due to time limitations, we were only able to test a subset of potentially dozens of specific signaling proteins involved in the effects of THC within the NASh. While we were able to reveal several novel and functional roles for GSK3, mTOR and Wnt signaling within these THC effect parameters, examining the potential roles of other signaling pathways on the effects of THC in the mesolimbic circuitry would provide a more detailed and precise characterization of the molecular mechanisms underlying the effects of THC. For example, JNK is involved in non-canonical Wnt signaling and previous research has demonstrated that THC activates JNK through stimulation of CB_1Rs (Rueda et al., 2000). JNK activation in the NAc is also necessary for the consolidation of cocaine-associated reward memories (Ding et al., 2013) and cocaine-induced NAc plasticity (Dietz et al., 2012), which suggests that JNK activation might also be required for associative memories involving other drugs of abuse like THC. ERK has also been demonstrated to be substantially involved in the processing of reward in the NAc. Intra-NAc amphetamine CPP is dependent on ERK activation (Gerdjikov, Ross, & Beninger, 2004) and decreases in phosphorylated ERK in the NAc are associated with morphine administration (Rosas et al., 2016) and heroin seeking following cue presentation (Sun et al., 2015). CaMKII in the NA also plays an important role in changes associated with drugs of abuse. Inhibition of CaMKII in the NA blocks increased amphetamine consumption following sensitization (Loweth, Baker, Guptaa, Guillory, & Vezina, 2008) and blocks the reinstatement of morphine-seeking behavior (Liu, Zhang, Liu, & Yu, 2012). Additionally, as we did not evaluate the effects of CBD on any specific signaling proteins, future studies should examine if CBD may have similar and/or opposing effects on these related molecular signaling pathways.

Furthermore, in each of these studies, we only examined the effects of THC and CBD individually. Substantial amounts of previous research have indicated that THC and CBD alter the effects of one another. For example, THC/CBD co-administration demonstrates synergistic effects on behavior and pharmacokinetics (Hložek et al., 2017; Jacobs et al., 2016; Todd et al., 2017). Given the increasing amounts of THC and decreasing amounts of CBD in many of the cannabis products currently consumed, it is important to understand how CBD may potentially alter the effects of THC and/or act synergistically, when they are administered together.

An additional limitation to the present experiments concerns the complexity of memory formation and learning. For example, when we evaluated the effects of CBD and THC on fear memory formation, our drug administration procedures selectively targeted the memory acquisition (encoding) phase of the process. Thus, while our results show the powerful effects of CBD and THC on the acquisition of associative fear memory, memory formation and maintenance is a complex and multifaceted process that includes phases of consolidation, retrieval, reconsolidation and extinction. In order to fully characterize the potential risks of THC and/or the potential therapeutic effects of CBD, a more complete examination of memory processing is needed. Previous research has been dedicated to examining the effects of these compounds on other aspects of memory, such as recall and extinction, but the exact mechanisms behind those effects have not been fully explored. For example, studies examining how THC or CBD might modulate the consolidation, recall or extinction of associative fear memory or addiction-related memories, may provide important and useful insights into how they may more effectively be administered to clinical populations to target these neuropsychiatric symptom profiles.

Lastly, to more directly compare the neurophysiological effects of THC and CBD on neuronal activity dynamics, recording cells in the VTA following intra-NASh THC infusions and conversely, recording the activity of MSNs in the NASh following CBD infusions could provide new insights into the differential effects of THC and CBD on mesolimbic neuronal function.

5.6 CONCLUSIONS

Cannabis use remains widespread and will likely increase with changing societal attitudes towards the safety and the normalization of cannabis use for recreational and therapeutic purposes. Undoubtedly, the specific phytochemical compounds within cannabis have tremendous potential, both positive and negative, for therapeutic applications and in terms of increasing the risk of certain neuropsychiatric disorders. Of the phytocannabinoids examined in this thesis, THC has primarily been implicated for its potential negative effects on mental health, whereas CBD appears to demonstrate promise as a treatment for schizophrenia and potentially anxiety disorders and PTSD. Nevertheless, critical questions remain regarding the specific brain regions and precise molecular signaling mechanisms underlying these diverse effects. Remarkably, the data reported in this thesis underscores the incredible divergence of neurobiological and neuropsychiatric effects induced by two distinct phytochemicals located within the same plant.

The findings described in this thesis have extensively characterized the effects of THC and CBD specifically within the NASh and distinct anatomical sub-regions therein. Using integrative molecular, neuronal and behavioural testing, these studies have revealed numerous novel neurobiological mechanisms that appear to underlie many of the psychotropic effects of cannabis use. The data contained in this thesis will contributes to a growing body of both clinical and pre-clinical research on the diverse pharmacological effects of cannabinoids. In addition, this evidence highlights the important, yet divergent properties of these phytochemicals in terms of their potential positive and negative effects on mental health and will hopefully help lay the foundation for future development and innovations in cannabinoid-based mental health pharmacotherapies.

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Publications

Szkudlarek, H. J., Desai, S., Renard, J., Pereira, B., **Norris, C.**, Jobson, C., Rajakumar, N., Allman, B. Δ^9-tetrahydrocannabinol and Cannabidiol Produce Dissociable Effects on Prefrontal Cortical Executive Function and Regulation of Affective Behaviours. *Neuropsychopharmacology* IN PRESS.

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Presentations and Posters

Norris, C., Szkudlarek, H. J., Pereira, B., Laviolette, S.R., Δ^9-tetrahydrocannabinol (THC) Produces Bi-Phasic Rewarding and Aversive Effects in the Anterior vs. Posterior Nucleus Accumbens Shell Through Dissociable Mu vs. Kappa Opiate Receptor Mechanisms and Differential Modulation of Medium Spiny Neuron Activity States (2018) Oral presentation at ICRS2018

Norris, C., Szkudlarek, H. J., Laviolette, S.R., Δ^9-tetrahydrocannabinol (THC) Produces Bi-Phasic Rewarding and Aversive Effects in the Anterior vs. Posterior Nucleus Accumbens Shell Through Dissociable Mu vs. Kappa Opiate Receptor Mechanisms and Differential Modulation of Medium Spiny Neuron Activity States (November, 2017) Poster Presented at Neuroscience 2017

Szkudlarek, H. J., Desai, S, Renard, J. **Norris, C.**, Rajakumar, N., Allman, B., Laviolette, S.R. Intra-PFC Infusion of Cannabidiol Induces Impairments in Cognitive Flexibility Measured in a

Set-Shifting Task Through Serotonergic 5-HT1a Receptor Transmission (November, 2017) Poster Presented at Neuroscience 2017.

Norris, C., Loureiro, M., Kramar, C., Zunder, J., Renard, J., Rushlow, W., & Laviolette, S. R. Cannabidiol Blocks the Formation of Traumatic Memories Through a Serotonergic 5-HT$_{1A}$ Receptor Mechanism in the Nucleus Accumbens and Blunts Dopamine Activity (July, 2016), Oral presentation at Psychiatry Research Day

Norris, C., Loureiro, M., Kramar, C., Zunder, J., Renard, J., Rushlow, W., & Laviolette, S. R. (May, 2016) Characterizing the Effects of Cannabidiol in the
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Norris, C., Loureiro, M., Kramar, C., Zunder, J., Renard, J., Rushlow, W., & Laviolette, S. R. (March, 2016) Characterizing the Effects of Cannabidiol in the
Mesolimbic Dopamine Pathway. Poster presented at London Health Research Day

Norris, C., Mallet, P.E., Endocannabinoid system involvement in impulsivity and decision-making (November, 2013). Poster presented at Neuroscience 2013

Norris, C., Mallet, P.E. (May, 2013). Endocannabinoid system involvement in impulsivity and decision-making. Poster presented at 2013 SONA Conference.

Norris, C., Mallet, P.E. (April, 2012) *Genetic Influence of the Behavioural Effects of Δ^9-Tetrahydocannabinol*. Oral presentation at the 42nd Annual Ontario Psychology Undergraduate Thesis Conference, York University, Toronto.

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